Structural and functional analyses of IgE epitopes and their biological relevance

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„Ach was!"

Loriot

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Abstract

So far, the detailed knowledge of complex interactions of antibodies with their corresponding epitopes and the essential requirements for triggering complex mechanisms, especially in pathophysiological backgrounds like allergies, is only restricted. One reason for this deficit is the lack of monoclonal human antibodies, especially of the allergy relevant isotype IgE, occurring only with very low concentrations in the human serum and therefore turning the identification, characterization and interaction analysis of epitopes into a major challenge. Furthermore the availability of allergenic target structures and a precise knowledge about their properties on a molecular level are essential to understand allergic reactions in more detail. At present, available recombinant technologies open up a wide range of possibilities to obtain detailed molecular insights into complex binding patterns of antibodies to their antigens.

The aim of this work was the evaluation of B cell IgE epitopes and their biological relevance by investigations of antibody interactions with their antigens, using recombinant methodologies on different molecular levels in the context of allergy.

One of the major reasons for IgE-mediated anaphylaxis are hypersensitivities due to hymenoptera venoms, which represent an ideal clinical condition for studying type I allergic reactions. The hymenoptera venom allergens Api m 1 and Api m 10 were recombinantly produced in different cell lines resulting in proteins with a varying degree of cross-reactive carbohydrate determinants (CCDs). They were used as tools for precise analyses of specific patients IgE reactivities against CCDs or peptidic epitopes and for a detailed reactivity dissection recombinant approaches could be established. Additionally the evaluation of the significance of the new venom component Api m 10 and the assessment of its allergenicity was possible. The low molecular weight honeybee venom allergen and putative protease inhibitor Api m 6 was immunochemically characterized and its function could be underlined by molecular modeling. Interestingly, despite its small size the protein exhibited an explicit IgE-sensitizing potential.

An authentic B cell epitope of the clinically relevant major timothy grass pollen allergen Phl p 5 was characterized using a specific fully human IgE antibody. Therefore Phl p 5 fusion proteins and recombinant IgE were generated and evaluated in immunoblotting and mediator release assays. The epitope could be described as a defined loop region exclusively present in the isoform Phl p 5a, contradicting the hypothesis that IgE epitopes are preferably defined by large surface areas as shown in only two crystal structures so far. Furthermore the first full set of Phl p 5-specific allergy-related antibody isotypes was generated, representing valuable tools for investigations of fundamental mechanisms and structure/function relationships in allergy.

For the first time direct molecular insights into the interaction of the clinically relevant structure α-Gal with a recombinant IgE antibody as well as human serum immunoglobulins were obtained. Mapping the detailed footprint by STD-NMR the recognition patterns of the antibodies and the carbohydrate-epitope could be shown on atomic level, but in cellular degranulation assays no activation was achieved using the IgE. Against the background of hapten-specific antibodies, monoclonal TNP-specific IgE constructs were generated. By characterization of functional features of the antibodies performing SPR-analyses and cellular degranulation assays a suitable model system for detailed analyses of hapten/antibody interactions with defined experimental conditions could be established. This can give new insights in the molecular interplay of antibodies or proteins with low molecular target structures, such as CCDs.

The results of this work can contribute to a better understanding of complex interactions of antibodies with their antigens and help elucidating the nature of B cell epitopes.

Zusammenfassung

Das Wissen über komplexe Wechselwirkungen von Antikörpern mit ihren spezifischen Epitopen, sowie über die notwendigen Voraussetzungen, die insbesondere vor pathophysiologischen Hintergründen wie Allergien zur Induktion komplexer Mechanismen führen, ist nach wie vor begrenzt. Ein Grund dieses bestehenden Defizits ist die stark eingeschränkte Verfügbarkeit monoklonaler Antikörper, speziell des allergierelevanten Isotyps IgE, der im humanen Serum in nur sehr geringen Konzentrationen vorkommt und daher die Identifikation, Charakterisierung und Interaktionsanalyse von Epitopen zu einer großen Herausforderung macht. Des Weiteren sind das ausreichende Vorhandensein allergener Zielstrukturen und umfassende Kenntnis über deren Eigenschaften auf molekularer Ebene essentiell, um allergische Reaktionen im Detail zu verstehen. Eine Anzahl derzeit zugänglicher rekombinanter Technologien eröffnet umfangreiche Möglichkeiten um detaillierte Einblicke in komplexe Bindungsmuster von Antikörpern an ihre Antigene zu erhalten.

Ziel dieser Arbeit war die Evaluierung von B Zell IgE Epitopen und ihrer biologischen Relevanz durch ausführliche Untersuchungen von Antikörper/Antigen Interaktionen im Kontext der Allergie und mittels rekombinanter Methodiken auf unterschiedlichen molekularen Ebenen.

Einer der Hauptgründe für IgE-vermittelte Anaphylaxien sind Hypersensitivitäten auf Grund von Hymenopterengiften, welche diesbezüglich ein geeignetes Krankheitsbild für Untersuchungen der Typ-I Allergie darstellen. Die Hymenopterengiftallergene Api m 1 und Api m 10 wurden rekombinant in unterschiedlichen Zelllinien produziert, woraus Proteine mit einem unterschiedlich hohem Grad angefügter kreuzreaktiver Kohlenhydrat-Determinanten (CCDs) hervorgingen. Diese wurden für eingehende Untersuchungen zur Differenzierung der IgE Reaktivitäten von Patientenseren mit CCDs und peptidischen Epitopen eingesetzt und es konnten rekombinante Ansätze für umfassende Reaktivitätsanalysen etabliert werden. Außerdem war es möglich die Signifikanz der kürzlich beschriebenen Bienengiftkomponente Api m 10 zu evaluieren und die Allergenität des Antigens zu bewerten. Das Minorallergen Api m 6, eine Bienengiftkomponente mit niedrigem Molekulargewicht und einer putativen Funktion als Proteaseinhibitor, wurde immunchemisch charakterisiert und mittels molekularem Modeling konnten Struktur-Funktions-Beziehung beschrieben werden. Interessanterweise zeigte das Protein trotz seiner geringen Größe ein eindeutiges Potenzial zur IgE Sensibilisierung.

Unter Einsatz eines spezifischen, vollständig humanen IgE Antikörpers wurde ein authentisches B Zell Epitop des klinisch relevanten Majorallergens Phl p 5 des Wiesenlieschgrases charakterisiert. Dazu wurden zunächst Phl p 5 Fusionsproteine sowie

rekombinante IgE Antikörper generiert und im Immunoblot und zellulären Degranulations-assays evaluiert. Das Epitop konnte auf eine exklusiv in der Isoform Phl p 5a vorkommende Schleifenregion eingegrenzt werden, was der Hypothese widerspricht, wonach IgE Epitope vor allem durch ausgedehnte Bereiche an Proteinoberflächen definiert sind, wie bislang anhand zweier Kristallstrukturen gezeigt werden konnte. In diesem Zusammenhang wurde ein vollständiges Set allergierelevanter Antikörperisotypen mit Spezifität für Phl p 5a generiert, womit sehr nützliche Hilfsmittel für weitere Untersuchungen fundamentaler Mechanismen der Allergie sowie von Struktur-Funktions-Beziehungen zur Verfügung stehen. Erstmalig konnten molekulare Einblicke in die Interaktion der klinisch relevanten CCD Struktur α-Gal mit einem rekombinanten IgE Antikörper, sowie mit humanen Serum-Immunglobulinen erhalten werden. Durch detailliertes Epitop-Mapping mittels STD-NMR wurde das Bindungsmuster der Antikörper an das Kohlenhydrat-Epitop auf atomarer Ebene erhalten. Der monoklonale IgE zeigte im Degranulations-assay jedoch keine inhärente Fähigkeit zur zellulären Aktivierung.

Vor dem Hintergrund hapten-spezifischer Antikörper wurden TNP-bindende IgE-Konstrukte dargestellt. Die funktionellen Eigenschaften der Antikörper konnten in SPR-Analysen und in zellulären Degranulations-assays charakterisiert werden.

Im Rahmen dieser Arbeit konnten passende Modellsysteme für die detaillierte Analyse von Hapten/Antikörper Interaktionen unter definierten experimentellen Bedingungen etabliert werden, wodurch neue Einblicke in die molekularen Wechselwirkungen von Antikörpern oder Proteinen mit niedermolekularen Zielstrukturen, wie z.B. CCDs, erhalten werden können. Die dadurch erhaltenen Ergebnisse stellen die Grundlage für ein besseres Verständnis komplexer Interaktionen von Antikörpern mit ihren Antigenen dar und können zur Aufklärung der Beschaffenheit von B Zell Epitopen beitragen.

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1 Introduction

1.1 Allergy

An allergy is an abnormal, pathogenic reaction of the adaptive immune system that occurs in response to otherwise normally harmless, non-infectious substances in the environment, which are called allergens. In the developed world around 25 % of people are affected by allergic disorders, such as hay fever, eczema, asthma and anaphylaxis [1]. The diversity of the symptoms reflects the diversity of allergenic sources, ranging from certain foods, pollen, or latex to mites and insect venoms.

The term "allergy" was first introduced by Clemens von Pirquet in 1906 to distinguish a physiological state of beneficial, protective immunity from a harmful immune response [2]. In 1963 Coombs and Gell expounded the four-group classification of hypersensitivity reactions on the basis of the underlying mechanisms by which specific antigens can induce cellular and tissue injury [3]. According to this, reactions are either type I (anaphylactic or immediate), type II (cytolytic or cytotoxic), type III (immune complex, antibody-mediated) or type IV (cell-mediated or delayed).

The classical IgE dependent form of allergy is described by the type I reaction, in which IgE antibodies attached to mast cells or basophils are involved. The cross-linking of cellular receptors by an antigen causes cell activation and leads to the release of certain mediators, which excite the clinical symptoms within minutes. Often mentioned symptoms are urticaria, asthma and anaphylaxis. The type II reaction is independent from IgE, but mediated by circulating IgM or IgG antibodies and appears due to an *antibody dependent cellular cytotoxicity* (ADCC) caused by complement-mediated lysis or cytotoxic action by killer cells. The antibodies react with autoantigens, e.g. portions of cells such as erythrocyte membranes or with unrelated antigens, such as drugs bound to cell surfaces. Symptoms typically emerge within a few minutes to several hours after antibody-antigen binding. Type II reactions are e.g. blood transfusion reactions and many drug allergies, notably against penicillin.

Type III reactions are also mediated by IgG antibodies, which interact with antigens and form immune complexes that are deposited in the basal membrane or along small vessels. The complexes can mediate either complement activation or effector cell infiltration into the affected tissue, causing local damage [4]. The Arthus reaction and the serum sickness are type III hypersensitivity reactions, leading to effects like vasculitis or arthritis, respectively. Type IV reactions are not antibody dependent but triggered by antigen specific T lymphocytes. The Type IV reaction can be subdivided into three groups in which T cells react with cell bound or associated as well as soluble antigens. In the first

group tissue damage is caused by macrophages, which are activated by *T helper cells of the subtype 1* (Th1), leading to inflammation. In the second group eosinophils activated by Th1 are predominant, and in the third group the damage is caused directly by cytotoxic T cells (CTLs). Mentioned disorders are contact dermatitis, chronic asthma, chronic allergic rhinitis or tissue rejection.

In clinical practice the discrimination between the hypersensitivity reactions classified by Coombs and Gell sometimes is difficult and additionally humoral and cellular immune responses may overlap or occur simultaneously [5]. This classification is not consistent with the present knowledge of the dynamic immune response, with interacting dendritic and T helper cells, the mediation of effector cells of several types, chemokines and cytokines. Therefore a further nomenclature was proposed by Johansson *et al.*, which distinguishes between allergic and nonallergic hypersensitivity, and also between IgE-mediated and non-IgE-mediated allergy (Fig. 1.1) [6]. According to this nomenclature "hypersensitivity" should be used as an umbrella term and an "allergy" describes clinical reactions initiated by immunologic mechanisms. In the majority of cases, where the patients suffer from IgE-mediated allergy the term is equalized with the type I hypersensitivity reaction [6].
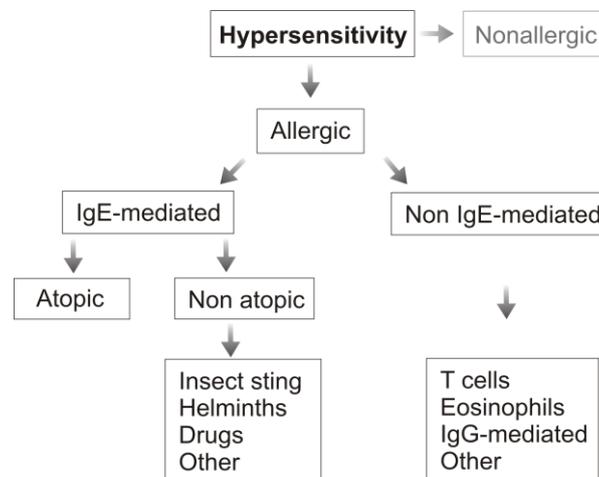
Fig. 1.1: Clinical classification of hypersensitivity reactions, mod. after Johansson *et al*. [6].

On the other hand an allergy can also be non IgE-mediated, then the reaction is initiated predominantly by mechanisms associated with allergen-specific antibodies other than IgE, or it is cell-mediated [6]. Nonallergic hypersensitivity is existent when immunologic mechanisms can be excluded, like in the case of intolerance or "pseudoallergies".

1.1.1 Immediate hypersensitivity reaction

The immediate hypersensitivity reaction, also known as anaphylactic hypersensitivity, is a generalized IgE-mediated allergic reaction, which can cause a range of symptoms from minor inconvenience to death. Currently more is known about the pathophysiology of IgE-mediated reactions than about their normal physiological function, perhaps because the immediate hypersensitivity reaction is the most frequently diagnosed allergy at present concerning more than 500 million people worldwide and about 25 % of the population in industrialized countries [1]. In allergic individuals, the IgE level, which is generated in response to an allergen, may be thousands of times higher than in those without allergies. The personal or familial tendency to produce IgE antibodies is called atopy, but also environmental factors can influence the ability to produce increased IgE levels. The much discussed "hygiene hypothesis" [7] indicates that the lack of early childhood exposure to infectious agents like viruses, bacteria or parasites due to improved hygiene, vaccinations and antibiotics in industrialized nations increases the susceptibility to allergic diseases caused by a disturbed equilibrium of the T helper cell subtypes [8].

An established hypersensitivity type I reaction can only occur after a primary response to an allergen, which is released from an allergen-carrying particle on the mucosal surface or in the case of an insect sting directly injected into the skin. This first stage called sensitization is the decisive event in the development of an allergy. During this phase *antigen-presenting cells* (APCs), especially *dendritic cells* (DCs) but also mast cells and basophils, take up allergens, process them while migrating to regional lymph nodes or to sites in the local mucosa and present the resulting peptides via *major histocompatibility complex II* (MHC II) to *naïve T cells* (Th0). This results in the induction of the humoral immune response because Th0 cells can be activated by binding the peptide-MHC II complex with their *T cell receptor* (TCR) (Fig. 1.2).
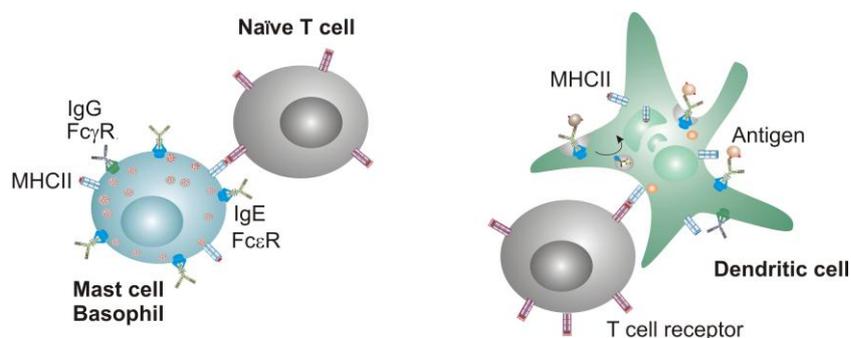
Fig. 1.2: Antigen presentation by APCs to naïve T cells. Mast cells, dendritic cells and also basophils take up allergens, process them and present resulting peptides via their MHC II to Th0 cells, which are interacting with their T cell receptor. MHC: major histocompatibility complex; FcεR: Fc receptor for IgE; FcγR: Fc receptor for IgG.

The differentiation of naïve Th0 cells into a Th1 or Th2 effector cell is then initiated and depends on the stimulation of coreceptors and environmental cytokines. For their differentiation T cells need at least three essential signals. First through their TCR, second an appropriate co-stimulation and third the activation of cytokine receptors.

The chemokines *Interleukin 12* (IL-12), IL-18, *Interferon-α* (IFN-α) and IFN-γ favour the development of Th1 cells but also inhibit the development of Th2 cells [9, 10]. The dominant factor in the development of Th1 is IL-12, which is produced by monocytes, macrophages and DCs. Through the activation of the *signal transducer and activator of transcription 1 and 4* (STAT1/4) and in the following of the transcription factor *T box expressed in T cells* (T-bet) [11] the development of Th1 cells is promoted.

The differentiation of the Th2 cells is promoted by "early IL-4" that is secreted by mast cells, basophils, natural killer cells and eosinophils [12]. IL-4 binds its receptor on the surface of CD4+ cells leading to a positive feedback, in which STAT6 together with other TCR-induced signals activates the transcription of IL-4 [13]. The transcription factor *GATA binding protein 3* (GATA3) is also activated, leading to the development of the Th2 cells and cytokine secretion [14].

Both cell types, Th1 and Th2, express their characteristic pattern of cytokines. Th1 cells secrete INF-γ, IL-2 and lymphotoxin-α [15], providing protective cell-mediated immunity against intracellular pathogens like bacteria, viruses or protozoans. In a Th2 response the cells produce cytokines necessary for stimulation and recruitment of eosinophils (IL-5, IL-9, *granulocyte/macrophage-colony stimulating factor* (GM-CSF), for different inflammatory tissue reactions (IL-9, IL-13) and for stimulation of B cells to IgE class switching (IL-4, IL-13). Subsequently the B cells secrete *specific IgE antibodies* (sIgE), which first diffuse locally and then are distributed systemically by the lymph- and blood-system to the mucosa, where they bind via their Fc portion tightly to high-affinity IgE receptors on tissue-resident mast cells. If an allergen-specific Th2 cell is activated by binding of the TCR to a suitable peptide-MHC II complex of a B cell, the clonal expansion of the Th2 as well of the activated B cell is started. Thereby allergen-specific memory B cells as well as a pool of long-lived memory T cells is formed and the sensitization phase is completed.

Sensitization itself does not provoke any symptoms in contrast to the effector phase that starts upon renewed contact to the allergen. The effector phase can be subdivided into an early and a late phase, which are classified by the participation of specific inflammatory cells. The early phase is initiated by reexposition to the allergen and its recognition by sIgE bound to their surface Fcϵ receptors on mast cells and basophils. Consequently, sIgE molecules are cross-linked by bivalent or multivalent allergens and subsequent aggregation of the receptor occurs by which the effector cells are activated via intracellular

signaling processes. Within the cytoplasmic tail of the receptors, the *immunoreceptor tyrosine-based activation motifs* (ITAMs) are phosphorylated resulting in the release of different mediators, such as histamine [16], platelet-activating factor [17], prostaglandins and leukotriens [18], proteoglycans and serine proteases [19, 20], and further other mediators, contributing to local and/or systemic symptoms within minutes after exposure [17]. Typical acute symptoms are vasodilation, increased vascular permeability, vascular leakage, contraction of smooth muscles and oedema, increased mucus secretion, conjunctivitis and asthma [21, 22]. In the late phase of the allergic inflammatory response, typically 2-6 h after allergen-exposure, the recruitment of inflammatory cells, like macrophages, T cells, eosinophils and basophils takes place [12]. Late phase reactions reflect the action of these innate and adaptive immune cells, eliciting symptoms like erythema, bronchoconstriction, oedema, and pain.

The different CD4+ T effector cells provide antigen- and location-specific responses following TCR engagement and thereby regulate and control the host immune defense against different classes of pathogens. Their broad influence on the immune system has to be tightly regulated throughout the development from bone marrow, liver and thymus, until peripheral differentiation and resulting effector function [23]. To date at least seven subsets of the still growing CD4+ T cell family are described in literature, including Th1, Th2, Th17, *regulatory T* (Treg) cells, *T follicular helper* (Tfh) cells and additionally Th9 and Th22 cells [23-27] (Fig. 1.3). The different subsets are characterized by their cytokine expression profile and up-stream transcription factor usage. Treg cells are induced by the *transforming growth factor β* (TGF-β) that causes activation of STAT5, leading to the expression of the transcription repressor *forkhead box P3* (FOXP3), resulting in the secretion of IL-10 and TGF-β. The main feature of Tregs is their ability to suppress both adaptive and innate immune responses [28, 29], thereby maintaining tolerance to self-antigens. They also can suppress allergen-induced specific T cell activation, effector cells of allergic inflammation such as mast cells, basophils, and eosinophils as well as IgE production [30-32].

Beyond cytokine induction Tregs are also able to interact directly with DCs and e.g. compete with naïve T cells in a physical manner by creating aggregates around DCs and in this way inhibit their maturation. Notably in the context of allergy secreted IL-10 induces IgG4 production and in parallel suppresses allergen-specific IgE [33, 34].

Another subset, the Th17 cells, are also induced by TGF-β and IL-6 [35]. Through the activation of STAT3 the transcription factor *RAR-related orphan receptor γt* (RORγt) is expressed [36]. Th17 cells produce IL-17, but also other cytokines including IL-9, IL-10, IL-21, IL-22, and in humans additionally IL-26 and regulate host defense against extracellular bacteria and fungi [37].
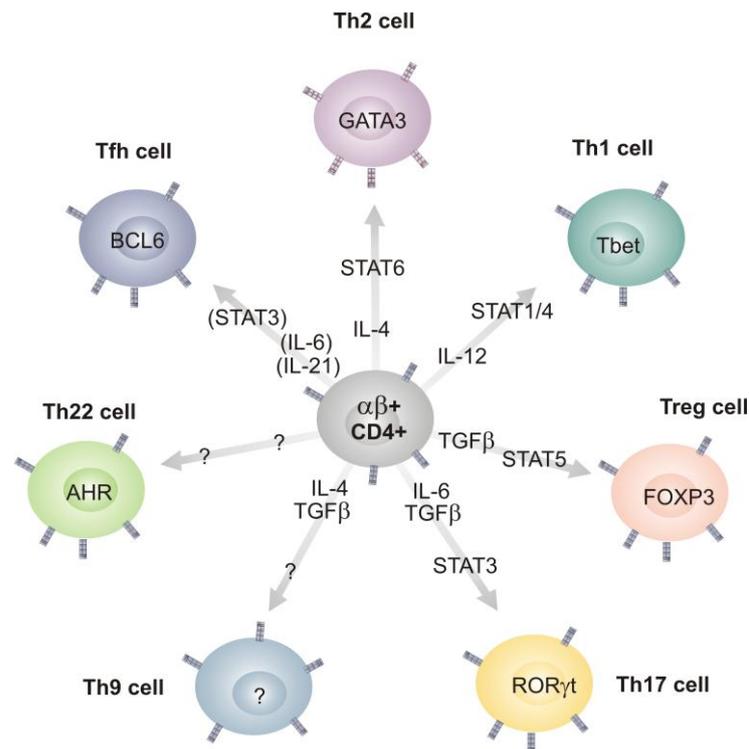
Fig. 1.3: T helper cell differentiation. Simplified schematic representation of different ways of Th cell differentiation. STAT: signal transducer and activator of transcription; IL: Interleukin; TGF-β: transforming growth factor β; GATA3: GATA binding protein 3; Tbet: T box expressed in T cells; FOXP3: forkhead box P3; RORγt: RAR-related orphan receptor γt; AHR: aryl hydrocarbon receptor; αβ: TCR type with α and β chain; CD: cluster of differentiation.

The *follicular helper T cells* (Tfh) cells have a specialized function in supporting B cells making antibody responses [38, 39]. They are important for the formation and maintenance of germinal centers and regulate through their interactions B cell differentiation into plasma and memory B cells. It is supposed that Tfh cells are induced by IL-6 and IL-21 that activate the expression of the transcription factor BCL-6 via STAT3 [40, 41]. Tfh cells express high amounts of the chemokine receptor CXCR5, which facilitates their localization to B cell follicles in which the ligand CXCL13 is expressed. They produce IL-21 and IL-6 leading to a positive feedback on their differentiation and expansion. Two relatively novel T cell populations of effector T helper cells have been described, Th9 and Th22 cells, which are partially not described in detail so far. Th9 cells are stimulated by IL-4 and TGF-β, are characterized by IL-9 and IL-10 secretion [30], and have been shown to induce tissue inflammation [42]. Th22 cells produce their namesake cytokine IL-22 but also IL-17 on a low level. The function of IL-22 depends on the activation of signal transduction and activators of transcription [43].

Their key transcription factor is the *aryl hydrocarbon receptor* (AHR), and the cells play an important and complicated role in autoimmune and inflammatory diseases [44].

Despite numerous checkpoints T helper cell dysfunctions can arise, leading to different types of immunopathologies like autoimmune diseases in the case of Th1 and Th17 or allergies in the case of Th2 responses [24, 30]. In allergic individuals regulatory response mechanisms seem to be compromised. Obviously, Treg cells, which maintain the functional tolerance, play an important role in the regulation of Th2 responses to allergens through the expression of IL-10 and TGF-β. An imbalance between these both subsets results in atopy [45].

1.2   Antigenic determinants

The interaction between antibodies and antigens depends on close contact between the surface structures of both participants. The area of contact of the antigen is called the epitope or antigenic determinant, which can further be subdivided into a peptidic and nonpeptidic epitope. The area on the surface of the paired VL-VH domains of an antibody that recognizes and interacts with the epitope is called paratope. In principle peptidic epitopes can be divided into linear and conformational epitopes, based on their structure. But their distinction is only of limited relevance, since all peptidic epitopes studied so far show some contributions of amino acids that are distant in the linear sequence, and strictly sequential epitopes are likely to be the exception [46].

As nonpeptidic epitopes haptens have to be mentioned, which are small molecules of usually less than 5 kDa. Because of their small size haptens are not able to elicit an immune response on their own, but when they are attached to an immunogenic carrier such as a protein. For efficient B cell activation and differentiation T cells are required. Haptens are not recognized by T cells, but their carrier part. The carrier-specific T cells then can stimulate not only carrier-specific B cells, but also hapten-specific B cells [46, 47]. Remarkably, a hapten specific antibody can react with the isolated haptenic molecule. In complexes of antibodies with haptens, the hapten is usually bound in a pocket at interface between the VH and VL domains [48].

So called hybrid epitopes are defined by antibodies that react primarily to a hapten attached to a specific carrier or site on the carrier, but not to the free carrier, nor to the hapten coupled to a different carrier [46].

1.2.1   IgE epitopes

In order to become IgE producing plasma cells naïve B cells need several different molecular signals. The B cell development from stem cells starts in the bone marrow and afterwards the immature, naïve B cells travel to the lymph nodes for activation. If B cells bind their specific antigen, they process it and present the peptides on their surface via MHC II. Th2 cells, which recognize their matching antigenic peptide via their TCR, produce not only IL-4 that leads to a positive feedback, but also increase the expression of CD40L that can stimulate CD40 on B cells. This leads to an upregulation of the costimulatory molecules CD80 and CD86 on B cells, which interact with CD28 on T cells, leading to a higher expression of CD40L. The production of IL-4 also stimulates the activation of B cells. Together with the CD40/CD40L interaction the immunoglobulin class-switch recombination in B cells [12, 49] is stimulated and allergen specific IgE antibodies are produced. The circulating IgE can bind then to FcεR on mast cells and basophils.

IgE antibodies have only a short plasma half-life in humans of about 2.5 days, compared to IgG with 21 days. The IgE concentration in sera is the lowest of all immunoglobulin isotypes and with 1-400 ng/ml in non-atopic humans very low, IgG concentrations are normally in the range of 8-16 mg/ml [50]. During parasitic infections, immunodeficiency diseases or also hematologic malignancies an inceased level of IgE is observed [51]. But for approaches like X-ray crystallography studies where monoclonal antibodies in milligram amounts are required the low concentration is the main limitation and therefore the identification of epitopes is currently a major challenge. The epitopes of T and B cells exhibit important differences. While T cell epitopes are only linear and distributed on the primary structure of the allergen, B cell epitopes recognized by IgE antibodies have been elucidated as either linear or conformational and are exposed on the antigen surface. These discontinuous epitopes depend on the 3 dimensional structure of the protein and changes in the protein folding may lead to changes in the number of epitopes.

So far linear B cell epitopes were defined by probing overlapping synthetic peptides of the allergens for the binding of IgE from the sera of allergic patients [52]. But epitopes to which IgE antibodies bind most tightly are most commonly formed by residues that become contiguous on the antigen surface after folding [52-55]. Experimentally, these conformational IgE epitopes can be investigated by examining crystal structures of complexes of the allergen and the binding fragments (Fab) of relevant antibodies [55, 56]. Only a few X-ray structures of complexes of allergens and Fab fragments have been determined up to now, for example the major allergen from Timothy grass pollen Phl p 2 [57] or the bovine milk β-lactoglobulin Bos d 5 [58] with recombinant Fab fragments of human IgE, but also monoclonal IgG antibodies in complex with their corresponding allergens were investigated [55, 56, 59]. Based on these limited data it was suggested,

that IgE epitopes have a tendency to be planar rather than convex [58]. It was also found that IgE epitopes may have a tendency to cluster [60, 61] what supports the hypothesis that for some allergens IgE epitopes are not randomly distributed over the allergen surface [62] keeping in mind that an allergen must have at least two nonoverlapping epitopes for cross-linking [63]. Furthermore it has been suggested that IgE antibodies tend to be more often cross-reactive than IgG antibodies [62].

In the context of the question if IgE epitopes are special, further studies have to be done, since there is not yet enough evidence for the statement that human IgE epitope structures differ dramatically from other B-cell epitope structures.

1.2.2 Glycosylation patterns and CCDs

Allergens are normally harmless, exogenic substances that can elicit harmful immune responses in line with an allergic reaction. Examples for allergens are cellular or secreted eukaryotic proteins, which show different forms of posttranslational modifications. The glycosylation is one characteristic modification with varying composition and extent depending on the tissue and the organism. Carbohydrate epitopes are currently a matter of debate in allergy research since up to now their role in diagnosis and treatment of allergic diseases has not been completely clarified [64, 65].

Since most glycans are common to all mammals it seemed to be that glycan-parts of mammalian glycoproteins are not immunogenic. That's in fact usually the case, but today some crucial exceptions are known. In the 1970s for the first time an oligosaccharide with unknown, strange features, which had not been found in mammalian glycoproteins before, was mentioned when a Japanese group elucidated the structure of the pineapple protease bromelain [66]. Later the glycans were described as core α1,3-fucose and β1,2-xylose [67].

In the beginning of the early 1980s Aalberse *et al.* showed that specific IgE from patients' sera with bee venom allergy cross-reacted with extracts from various allergenic foods as well as with insect venoms [68]. Observing that the binding of IgE to the honeybee venom phospholipase A2 could be inhibited by glycopeptides from bromelain, they related the N-glycans of plants for the first time to allergy and termed the responsible allergic structures, which are almost certainly the most frequently encountered individual epitope structures for IgE [64], as crossreactive carbohydrate determinants (CCDs). These small glycans are nonpeptidic epitopes that form self-contained antigen determinants that are poor immunogens on their own, but can cause strong crossreactivities independent of their carrier molecules. From the immunological point of view, glycoproteins may be classified as hapten–carrier complexes, with the glycan as hapten [46].

Glycans or rather CCDs are not able on their own to induce antibodies, but will do so when attached on a carrier molecule and thereby presented to the immune system. CCDs are widely distributed on nonmammalian glycoproteins from plants and lower animals and occur in natural rubber latex, pollen, vegetables, fruits, hymenoptera venoms, and in some pathogenic worms [69]. These special glycotopes are produced by glycosyl-transferases, which have been identified and characterized in different organisms [70-73]. At present their natural relevance is not described sufficiently, but there are some hints about implications in immunology and developmental biology [74]. Because these enzymes are not present in the human system and the resulting carbohydrate structures are of xenobiotic nature they exhibit a high immunogenicity. Carbohydrate specific antibodies are found in the context of different diseases, e.g. bacterial or viral infections, parasite infestations, malignant tumors and in transmission problems regarding blood group antigens or xenotransplantations. But so far the interactions between antibodies and their carbohydrate determinants are not characterized sufficiently. One possible reason for difficulties in function analyses of human antibodies could be the described low affinities of the carbohydrate/antibody interactions.

Only a few structures of antibodies in complex with their corresponding carbohydrate epitope are characterized, such as Lewis-X, Lewis Y or LPS [75-78]. But in these cases murine antibodies resulting from infections or immunizations were used and are therefore not completely comparable to the human system. In the following three key elements are discussed in detail, namely the α1,3-fucose and the β1,2-xylose, both attached to the core structure of N-linked carbohydrates, as well as the α1,3-galactose, which is also found on N-linked carbohydrate chains (Fig. 1.4).

Both the α1,3-fucose and the β1,2-xylose occur in plants and helminths [64, 79], xylosylation could also be detected in snails [80]. Core α1,3-fucose residues alone are found in insect glycoproteins, e.g. in honeybee venom phospholipase A2 (Api 1) [81, 82], or hyaluronidase [83, 84] and in the fruitfly *Drosophila melanogaster* [85]. The core α1,3-fucose is usually accompanied by a second α1,6-fucose.

Bromelain is the glycoprotein which was used for CCD reactivity investigations for the first time. It is widely used as simple marker for the presence of IgE specific for carbohydrate epitopes. But it has to be mentioned that this protease is rather a special case, since in plant glycoproteins more usually glycans with three mannose residues are found, e.g. on *horseradish peroxidase* (HRP) [86, 87]. In order to detect the presence of core β1,2-xylose and/or α1,3-fucose, anti-HRP is probably the most widely used antiserum. The diagnostic relevance of these both structures has been described several times, but their clinical relevance is still discussed [88].
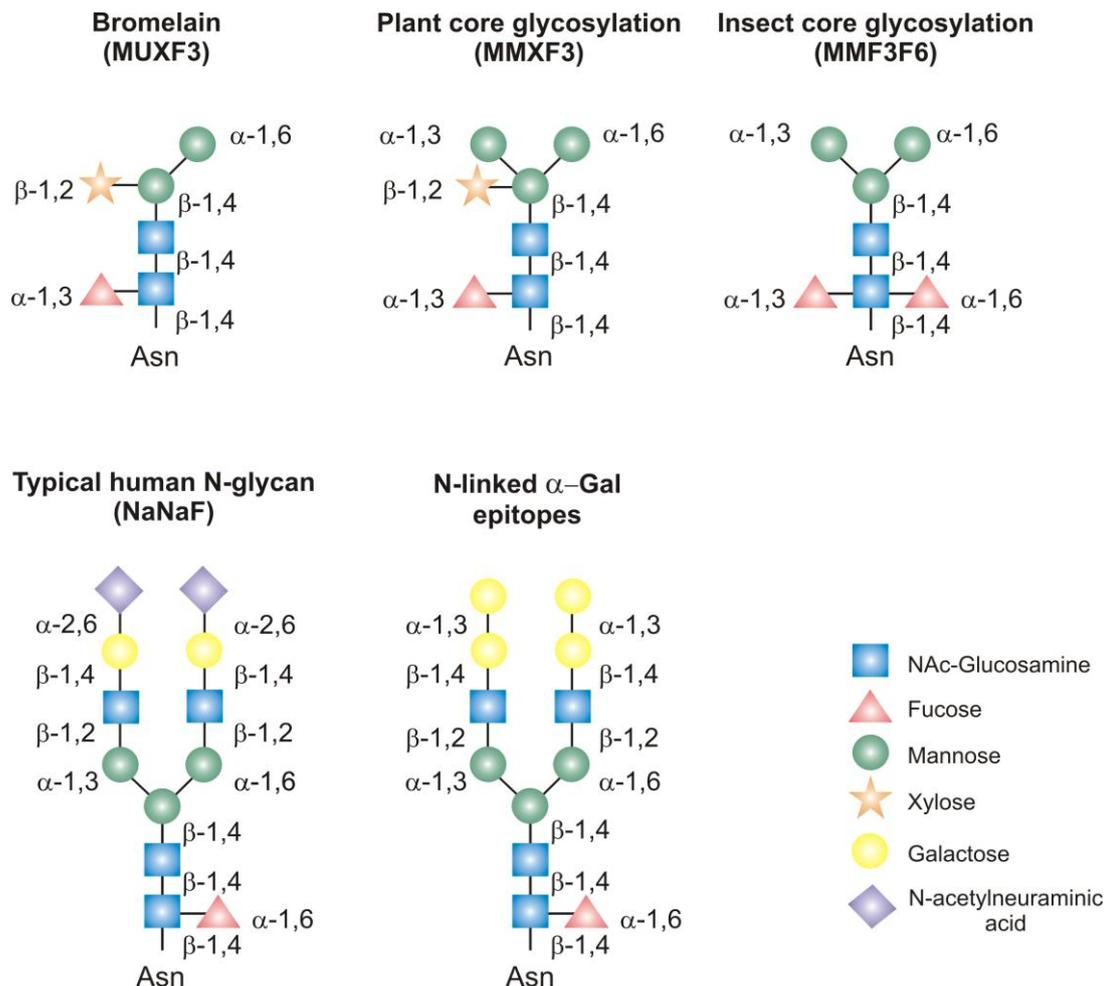
Fig. 1.4: Schematic representation of N-glycans with CCDs of different origins. The oligosaccharide structures are shown as symbols with the additional information about linkage positions. MUXF3 and MMXF3 represent typical complex-type plant N-glycans, MMF3F6 a typical insect core glycosylation. NaNaF constitutes a typical, diantennary mammalian complex-type N-glycan with sialic acids. The α-Gal epitope is found on N-linked carbohydrate chains of glycoproteins.

This is not the case for another CCD structure, the α1,3-galactose (Galα1,3Galβ1-4GlcNAc-R, α1,3-gal, α-Gal) which has been described in the context of the administration of the therapeutic monoclonal antibody cetuximab (Fig. 1.5) that induced different anaphylaxis syndromes [89]. The α1,3-galactose is found on glycolipids and glycoproteins of non-primate mammals, prosimians and New World monkeys, but not in apes, Old World monkeys and humans [90, 91]. The gene of the responsible glycosylation enzyme *α1,3galactosyltransferase* (α-1,3GT) was inactivated in ancestral Old World primates, probably in the context of the development of the immune system acting against α-Gal expressing pathogens [91, 92], leading to an elimination of the α-Gal epitope in these primates [93, 94].

1.2.3   Clinical relevance of CCDs

The diagnostic relevance of CCD specific antibodies, resulting from interference of protein-specific IgE and CCD reactive IgE, is given without any doubt, but the importance of these modifications in the context of allergy and therapy still has to be evaluated. It has been shown that CCD specific IgE can cause false-positive and clinically irrelevant results in *in vitro* tests [68, 95] like for example in diagnostic approaches such as the skin prick test (SPT), which is the basic principle to diagnose e.g. pollen allergy [95].

Antibodies against α1,3-fucose and β1,2-xylose exist with a relatively high prevalence of 30-40 % in allergic persons, which after an immune therapy can arise up to 50 % [96], so obviously these CCDs show immunogenic effects. Although it was demonstrated that induced anti-CCD IgE from atopic patients were capable to trigger mediator release from basophils [97, 98], they are considered to have low or no biological activity. At present the clinical relevance of antibodies against α1,3-fucose and β1,2-xylose in the human system remains a matter of debate and controversial discussions [99-101].

In contrast to these uncertain evaluations recently a clear clinical relevance of the α-Gal epitope was confirmed [89]. α-Gal represents an immunogenic glycan structure for humans, apes and Old World monkeys leading to the production of specific anti-gal antibodies. For the human system it is known that about 1 % of circulating immunoglobulins in the serum are α-Gal specific, in particular of the isotype IgG2 [92, 102], which is considered to act as blocking antibody. But also α-Gal specific IgM and IgA antibodies are present in humans [102]. The anti-gal antibodies, mainly provoked by gastrointestinal bacteria, are a substantial reason for problems in xenotransplantations of e.g. pig organs to humans, acting as immunological barrier. Demonstrating the harmful potential of glycans and underlining the importance of α-Gal dependent and IgE mediated allergic immune responses, it was found that α-Gal specific IgE antibodies were capable of eliciting anaphylactic reactions in patients, who were medicated with the monoclonal anti-EGFR antibody cetuximab for the first time (Fig. 1.5) [89].

Cetuximab is a chimeric mouse-human monoclonal antibody of the IgG1 isotype, developed against the *epidermal growth factor receptor* (EGFR) and approved for use in metastatic colorectal cancer and squamous-cell carcinoma of the head and neck [103-105]. The antibody is expressed in a mouse myeloma cell line, which is producing also α-1,3GT, leading to the presentation of α-Gal epitopes in the variable heavy chains.
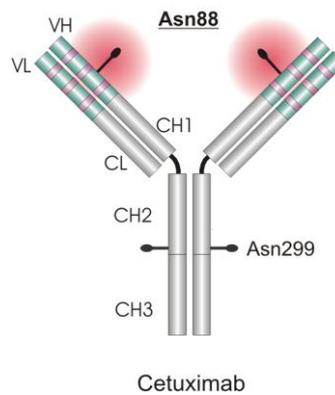
Fig. 1.5: Schematic representation of the therapeutic antibody cetuximab and its cross-reactive α-Gal epitopes. Cetuximab is a chimeric mouse-human monoclonal IgG1 antibody. The constant human chains are shown in gray, the murine VH and VL chains, containing the complementarity determining regions (CDRs) colored in pink, are shown in green. The two N-linked glycans containing the α-Gal epitopes located at Asn88 are highlighted in red.

In search for origins of sensitization the α-Gal epitope has also been identified as food allergen. A clear correlation between α-Gal and allergies against red meat was observed in patients who had α-Gal specific IgE and reported delayed anaphylaxis, angioedema, or urticaria occurring 3-6 hours after eating beef, pork, or lamb [106].

A connection between tick bites and allergy was observed in patients within a restricted area of the United States [107], indicating rather a local sensitization. It was additionally shown that α-Gal is responsible for the IgE binding to cat IgA, a newly identified cat allergen [108]. At present all described clinical appearances in the context of CCDs base just upon different phenomenological observations, but the underlying mechanisms are still unknown, since adequate monoclonal antibodies were not available so far. Structural and molecular data regarding the antibody/CCD interactions are still rare and require further investigation.

1.3 Hymenoptera venom allergy and venom allergens

Beside foods and drugs, stings by social hymenoptera insects, like honeybees, wasps or ants, are one of the 3 major causes of anaphylaxis, which is one of the most severe hypersensitivity reactions in hymenoptera venom allergic patients [109]. Worldwide the order Hymenoptera includes more than 100 000 species [110]. The species that are a main cause of allergic reactions belong to the suborder *Apocrita* and the infraorder *Aculeata*, which are made up of the superfamilies *Apoidea* (*Apinae* and *Bombinae* subfamilies) and *Vespoidea* (*Vespinae* and *Polistinae* subfamilies) [111].

Over the last decade epidemiological population-based studies revealed a prevalence of systemic reactions to hymenoptera stings ranging from 0.3 % to 8.9 % with anaphylaxis in 0.3–42.8 % of cases [111, 112]. In Central Europe, most anaphylactic reactions are elicited by honeybees (*Apis mellifera*) and certain types of yellow jacket (*Vespula vulgaris, Vespula gemanica*) [110], in adults wasp stings are the most common cause of anaphylaxis [113]. Hymenoptera venom is a complex mixture of various allergens that play important roles in the induction of allergic reactions after stings, as well as of several non-allergenic low molecular weight substances [114]. The composition of the venom can furthermore vary depending on the living conditions of the insect or its developmental stage. In principle all components of the venom may contribute to sensitization, symptoms, and success of a therapy. The best known allergens of honeybee and yellow jacket venom are listed in Tab. 1.1.

Tab. 1.1: Overview of characterized honeybee and yellow jacket venom allergens. CRP: Carbohydrate-rich protein; DPP IV: Dipeptidylpeptidase IV; MRJP: Major Royal Jelly Protein.

Allergen	Name / function	MW [kDa]	% dry weight	N-glyco-sylation sites
Honeybee venom allergens				
Api m 1	Phospholipase A2	17	12	1
Api m 2	Hyaluronidase	45	2	2
Api m 3	Acid Phosphatase	49	1 - 2	2 - 3
Api m 4	Melittin	3	50	-
Api m 5	Allergen C / DPP IV	100	< 1	5 - 7
Api m 6	Protease Inhibitor	8	1 - 2	-
Api m 7	Protease	39	?	2 - 4
Api m 8	Carboxylesterase	70	?	4
Api m 9	Carboxypeptidase	60	?	4 - 5
Api m 10	CRP / Icarapin	55	< 1	4
Api m 11	MRJP8 / MRJP9	55 / 60	?	6 / 3
Api m 12	Vitellogenin	200	?	3
Yellow jacket venom allergens				
Ves v 1	Phospholipase A1	35	6 - 14	-
Ves v 2a	Hyaluronidase	45	1 - 3	2 - 3
Ves v 2b	Hyaluronidase (inactive)	45	?	2
Ves v 3	DPP IV	100	?	3 - 6
Ves v 5	Antigen 5	25	5 - 10	-
Ves v 6	Vitellogenin	200	?	4

The *venom immunotherapy* (VIT) at present is the most effective treatment, reducing the risk of systemic reactions in hymenoptera venom allergic patients [115]. During therapy the peripheral tolerance is induced by development of allergen-specific regulatory T cells, which suppress proliferative and cytokine responses against the venom allergens [115]. In the following a decrease of T-cell proliferation and a shift of the cytokine secretion pattern from Th2 to Th1 or Th0 is observed [116, 117]. In order to improve efficacy and safety of VIT different approaches have been studied.

Since it is known that T cells, which bear histamine receptors [118], could influence immunotherapy, premedication with antihistamine during the initial phase of treatment has been recommended [119]. The concept of allergen modifications postulates that allergens can be modified by chemical or recombinant diversifications to destroy conformational IgE binding B cell epitopes but not linear T cell epitopes, in order to reduce allergic side effects and simultaneously preserve protective immunity [120]. The duration of VIT has great influence on the success of treatment and it has been shown that for long-term protection at least 3 years of treatment are necessary [121, 122].

Diagnostic performance and therapeutic approaches of hymenoptera venom allergies in principle base upon venom extracts of honeybees or wasps. The composition and quality of the venom preparations differ depending on natural variations and also on processing of the extracts. In the recent years significant progress has been made on the identification, characterization and recombinant production of venom allergens, but still only a restricted number of recombinant allergens is available so far.

For a better understanding of allergic reactions and in order to develop new strategies for immune therapies the availability of recombinant allergens and a precise knowledge about them on a molecular level is essential.

1.4   Objective

In the context of allergy, especially hymenoptera venom and CCD associated anaphylactic reactions, the aim of this work was a detailed investigation and evaluation of antibody/antigen interactions combining different recombinant methodologies. Detailed molecular insights can contribute in understanding complicate interactions of antibodies with their antigens and can help elucidating the nature of IgE B cell epitopes.

In this regard the immunoreactivities and interactions of IgE immunoglobulins with their corresponding epitopes should be dissected.

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2 Material and Methods

2.1   The putative serine protease inhibitor allergen Api m 6 from *A. mellifera* venom: recombinant and structural evaluation

2.1.1   Materials

Monoclonal anti-V5 antibody was purchased from Invitrogen (Karlsruhe, Germany). Polyclonal rabbit anti-HRP serum, anti-rabbit-IgG alkaline phosphatase (AP) conjugate and anti-mouse-IgG AP conjugate were obtained from Sigma (Taufkirchen, Germany). Monoclonal AP conjugated anti-IgE antibody was purchased from BD Pharmingen (Heidelberg, Germany).

2.1.2   Sera

Sera from honeybee venom-sensitized patients with HBV-specific IgE and/or positive intradermal skin test results were selected at random from the institutional serum bank. All patients had given their informed written consent to draw an additional serum sample and all experiments applying human sera were approved by the local ethics committee.

2.1.3   Cloning of cDNA

The separated stinger with attached venom sack and glands of honeybee (*A. mellifera*) was used for total RNA isolation using peqGoldTriFastTM (Peqlab Biotechnologie, Erlangen, Germany). The gene-specific primer 5'-TCATCCTGGGAGGCATTTAGA TCGCGG-3' and SuperScript III RT (Invitrogen) were used to synthesize cDNA from the isolated total RNA. The cDNA of mature of Api m 6 was amplified using Pfu DNA polymerase (Fermentas, St. Leon-Roth, Germany) and the primers 5'-TTTGGAGGATTTGGAGGATTTGGAGGAC-3' and 5'-TCATCCTGGGAGGCATTTA GATCGCGG-3'. Subcloning for sequencing was done using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen).

2.1.4   Cloning and recombinant bacterial expression of Api m 6

For expression in *E. coli* the Api m 6 coding region was amplified in two consecutive PCR reactions adding a C-terminal V5 epitope using the primers 5'-GATCCATATGTTTGGAGGATTTGGAGGATTTGGAGGAC-3', 5'-GACCGAGGAG AG GGTTAGGGATAGGCTTACCGGCTGGGAGGCATTTAGATCG-3' and 5'-GGTGGTTGC TCTTCCGCACGTAGAATCGAGACCGAGGAGAGGGTTAGGG 3'. The PCR product was

subcloned into the vector pTXB1 (New England Biolabs, Bad Schwalbach, Germany) via NdeI and SapI. The vector was further modified by addition of a second chitin binding domain (CBD). Additionally the Api m 6 coding region was amplified using the primers 5'-GATCGATATCTTTGGAGGATTTGGAGGATTTGGAGGAC-3' and 5' GATCCCGCGGT CCTGGGAGGCATTTAGATCGCGG-3' and the PCR product was cloned via EcoRI and SacII into the vector pMalc2x (New England Biolabs) which was modified by addition of a C-terminal V5 epitope. Expression and purification of the fusion proteins were performed according to the recommendations of the manufacturer.

2.1.5 Cloning and recombinant insect cell expression of Api m 6

For insect cell expression the Api m 6 coding sequence was amplified using the primers 5'-GATCGATATCTTTGGAGGATTTGGAGGATTTGGAGGAC-3' and 5'-GATCCCGC GGTCCTGGGAGGCATTTAGATCGCGG-3'. The PCR product was subcloned via EcoRV and SacII into the vector pIB/V5-His (Invitrogen) which was modified by addition of a melittin signal sequence and a N-terminal 10-fold His-Tag followed by a SacII restriction site [123]. The Api m 6 containing vector was used to transfect *Spodoptera frugiperda* (Sf9) cells (Invitrogen) applying Cellfectin transfection reagent (Invitrogen) according to the recommendations of the manufacturer. Cells were selected for stable integration of the vector by addition of 80 mg/ml Blasticidin S (Invitrogen) to the medium. Medium of confluent stably transfected insect cell cultures was collected, concentrated, and used for Western blotting.

2.1.6 Western blotting

For immunoblot procedures the recombinant allergens were separated by SDS-PAGE and immobilized onto nitrocellulose membranes. Anti-V5 epitope mAb was applied according to the recommendations of the manufacturer and bound antibodies visualized via corresponding secondary antibodies conjugated to AP and nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate.

2.1.7 Immunoreactivity of patient sera with recombinant proteins

For assessment of specific IgE immunoreactivity of human sera in ELISA, 384 well microtiter plates (Greiner, Frickenhausen, Germany) were coated with purified recombinant proteins (20 µg/ml) at 4 °C overnight and blocked with 40 mg/ml milkpowder in PBS. Thereafter, human sera were diluted 1:2 with PBS and incubated in a final volume of 20 µl for 4 hours at room temperature. After washing 4 times with PBS, bound IgE were detected with a monoclonal AP-conjugated anti-human IgE antibody. After washing 4 times with PBS, 50 µl of substrate solution (5 mg/ml 4-nitrophenylphosphate, AppliChem,

Darmstadt, Germany) per well were added. The plates were read at 405 nm. The lower end functional cut-off, indicated as line, was calculated as the mean of the negative controls plus 2 SDs. Reactivities only slightly higher than the cut-off were excluded. For ELISA procedures with anti-V5 epitope mAb and HRP antiserum the antibodies were applied according to the recommendations of the manufacturer and bound antibodies visualized via corresponding secondary antibodies conjugated to AP as described above.

2.1.8 Construction of a homology model of Api m 6

A homology model of Api m 6 was built applying the Local Meta-Threading-Server (LOMETS) for protein structure prediction [124] and the *Apis mellifera* chymotrypsin/cathepsin G inhibitor-1 (AMCI-1) as a template. Antigenic determinants/conformational epitopes of Api m 6 were predicted using the Conformational Epitope Prediction Server (CEP) [125].

2.1.9 Other methods

Molecular biology standard procedures were performed according to established protocols [126].

2.2 Close-up of the immunogenic alpha-1,3-Gal epitope as defined by a monoclonal chimeric IgE and human serum using saturation transfer difference (STD) NMR

2.2.1 Production of Recombinant Antibodies

For establishing chimeric mouse/human antibodies, the VH and VL sequences of the $\alpha-$Gal-specific antibody M86 was used as template for gene synthesis [127]. Variable regions were assembled in the form of a single chain Fv (scFv) and introduced into phagemid vectors, allowing prokaryotic production as both soluble fragment and scFv-displaying phage. Homodimeric IgG1 and IgE and heterotetrameric IgE immunoglobulins were produced using recently established vector systems [128]. The variable regions VH and VL were amplified using oligonucleotides containing restriction sites at the 5 and 3 termini of the VH (gatcatttaaatgtgtccagtgtgaggtgaaactggag and gatcgtcgaccccgaga cagtgacagaagttcc) and VL (gatccctgcagggtgccagatgtgatgtggtgatgacac and gatcggcgcg cccacagtccgtttgatttcgag) by PCR, respectively, in such a way that a 4xHis tag is generated at the C-terminal end of the heavy chain. Subsequently, the DNA was introduced into the different expression vectors. Human embryonic kidney cells (HEK-293, ATCC number CRL-1573) were cultivated in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 100 ml/liter fetal calf serum, 10 kallikrein-inactivating unit/liter penicillin, and 100 mg/liter streptomycin. Tissue culture reagents were obtained from Life Technologies. HEK-293 cells were transfected by using 3 µg of the particular expression vector DNA complexed with polyethylene imine (Sigma-Aldrich). The secreted immunoglobulins were purified from the culture medium by affinity chromatography using Ni^{2+}-NTA-agarose (Qiagen) according to the manufacturer's recommendations.

2.2.2 Amplification and Cloning of FcεRI-IgY Fc and CD64-IgY Fc

The cloning and expression of the soluble IgE Fc receptor FcεRI-IgY Fc has been described elsewhere [129]. The human CD64 extracellular domains were amplified without the original signal sequence using one PCR primer containing a Pfl23 II site (gatccgtacgtgtgggcaagtggacaccacaaaggc) and another primer containing an Sgs I site (gatcggcgcgccatgaaaccagacaggagttgg) and introduced into pcDNA3.1/zeo providing a rat immuno-globulin leader sequence and avian Fc regions [130].

2.2.3 Assessment of Immunoreactivity in ELISA

For assessment of immunoreactivity in direct ELISA the particular proteins (10 µg/ml) were applied to microtiter plates, incubated at 4 °C overnight and blocked with 4 % MPBS at RT for 1 h. The recombinant immunoglobulins (1 µg/ml diluted with 2 % MPBS) were added to the wells and incubated for 1 h at RT. The ELISA was performed according to established protocols and detected with human Ig isotype specific antibody conjugates and para-nitrophenyl phosphate (pNPP) as a substrate at 405 nm.

The immunoreactivity of immunoglobulins with their particular Fc receptors was demonstrated by Sandwich ELISA. Therefore, bovine thyroglobulin (50 µg/ml) was applied to microtiter plates, incubated at 4 °C overnight and blocked with 4 % MPBS at RT for 1 h. Thereafter, hIgE, cIgE and hIgG1 (1 µg/ml diluted with 2 % MPBS) were added to the wells, incubated at RT for 1 h and subsequently incubated with FcεRI-IgY Fc or CD64-IgY Fc (1 µg/ml diluted with 2 % MPBS) according to established protocols and detected with a chicken IgG specific antibody conjugate and pNPP as a substrate at 405 nm.

For immunoblot procedures, the particular recombinant antibodies were separated by SDS-PAGE. Visualization was then performed with human Ig isotype specific antibodies conjugated to alkaline phosphatase and nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP).

2.2.4   *In vitro* mediator release assay with humanized rat basophilic leukemia cells (RBL-SX38)

In vitro degranulation was analyzed as described previously [131]. Soluble alpha-Gal proteins were biotinylated using TFPA-PEG3-Biotin (Tetrafluorophenylazide-(triethylene-glycol)-Biotin (Pierce) according to the recommendations of the manufacturer and incubated with streptavidin-coated Roti-MagBeads (Roth). After sensitization of RBL-SX38 cells with IgE and washing with incomplete Tyrode´s buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$), alpha-Gal-carrying proteins or alpha-Gal-coated beads were added to the wells and incubated for 60 min at 37 °C. As reference, cross-linking was achieved by addition of polyclonal anti-human IgE serum (1 µg/ml from goat, Bethyl). β-hexosaminidase release of viable versus lysed cells was assessed with p nitrophenyl N-acetyl-glucosaminide (Sigma Aldrich) as a substrate.

2.2.5   Surface Plasmon Resonance Analysis

The interaction affinity of cetuximab, alpha-Gal-HSA, thyroglobulin and immobilized IgE as well as the affinity of a TNP-specific mouse IgE (clone C38-2) and immobilized TNP-BSA have been determined by surface plasmon resonance (SPR) measurements using the SPR-2 affinity sensor from Sierra Sensors, Hamburg, Germany. The IgE was covalently coupled to a total of 2400 resonance units on a carboxymethylated sensor chip surface (SPR-2 affinity sensor) using standard NHS/EDC coupling procedure via primary amines and capping by ethanolamine. The uncoupled surface served as reference. Measurements were performed at 20 °C in buffer containing 10 mM monosodium phosphate, 40 mM disodium phosphate and 100 mM NaCl, pH 7.5 with 0,01 % Tween-20. For the kinetic analyses, increasing concentrations of the alpha-Gal carrying antigens (7 – 167 nM for cetuximab, 121 – 564 nM for alpha-Gal-HSA, 15-120 nM for thyroglobulin) were injected at a flow rate of 25 µl/min. The association phase was monitored for 120 seconds, the dissociation phase for 90 seconds. Sensor surfaces were regenerated after each binding cycle by two subsequent injections of 50 mM Tris buffer, pH 10. After subtracting reference cell signals, resulting binding data were fitted to a Langmuir 1:1 binding model by using global fit analysis (evaluation software, Sierra Sensors) and the dissociation constant at equilibrium KD was calculated.

2.2.6   STD NMR

Buffer exchange against deuterated PBS and concentrating of affinity-purified, anti-Gal-specific antibodies to 450 µg/ml was performed by using AMICON Ultra-4 10K centrifugal filter devices. Oligosaccharides were obtained from Dextra (Reading). The Gal-Gal

disaccharide was purchased as methylglycoside and as biotinylated carbohydrate. Saturation transfer difference (STD) NMR experiments were performed at 298 K on a Bruker 500 MHz spectrometer equipped with a 5 mm inverse triple resonance probe head and a Bruker 700 MHz spectrometer equipped with a 5 mm inverse triple resonance probe head with cryo technology. The PBS NMR buffer contained 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 176 mM KH_2PO_4 in D_2O and was adjusted to pH 7.4. The on resonance pulse for antibody saturation was set to 0 Hz (500 MHz) or -500 Hz (700 MHz), respectively, and the off resonance pulse to 28500 Hz (500 MHz) or 28000 Hz (700 MHz). Saturation was achieved by a train of 90° Gaussian-shaped pulses of 50 ms yielding a total saturation time of 2 s with an attenuation of 45 dB (500 MHz) or 40 dB (700 MHz). The spectra were acquired with a spectral width of 8000 Hz, 64k time domain data points and 2 transients using a pseudo-2D Bruker standard pulse sequence (stddiff.3, 500 MHz). A relaxation delay of 4 s was applied. For suppression of protein background a T1ρ-filter was used applying a field strength of 11.5 kHz and a duration of 15 ms. Each experiment was performed with a total of 1024 scans. STD experiments on serum were performed using a pseudo-2D Bruker standard pulse sequence (stddiffesgp2d) that contained the excitation sculpting sequence for the suppression of residual HDO (700 MHz). The spectra were recorded with a spectral width of 7000 Hz, 32k time domain data points and 2 transients. The FIDs of the on and off resonance spectrum were stored and processed separately. Subtraction of the on and off resonance spectrum resulted in the STD NMR spectrum. Protein:ligand ratios of 1:260 (4.81 µM of the M86 antibody with 1.25 mM of the Gal-Gal-OMe disaccharide) and 1:200 (5.84 µM of the purified serum antibodies with 1.17 mM of the Gal-Gal-OMe disaccharide) were used.

2.2.7   Affinity purification of alpha-Gal-specific immunoglobulins

For purification of anti-alpha-Gal-specific antibodies from human serum an alpha-Gal-specific affinity resin was generated. Therefore, 1 mL Affi-Gel 10 (BIO-RAD) activated matrix was rinsed with 3 volumes of ddH_2O for 20 minutes followed by an incubation step with 4 volumes of 100 mM HEPES buffer, pH 7.5, containing bovine thyroglobulin (70 mg/mL) for 4 h at 4 °C on a roller-mixer. Remaining active esters were quenched subsequently by the addition of 100 µL ethanolamine (1 M). Prior to first use the affinity resin was washed with 60 volumes PBS, pH 7.4. To isolate alpha-Gal-specific antibodies, 15 mL human serum of an alpha-Gal-positive donor was subjected to the forecited anti-Gal specific affinity matrix. Unbound proteins were removed by washing with 100 volumes of PBS, pH 8.0, 500 mM NaCl, and alpha-Gal-specific antibodies were subsequently eluated into 300 µL of 1 M Tris-HCl, pH 7.5, for immediately neutralization using 700 µL of 0.1 M glycine buffer, pH 2.0. Immunoreactivity was subsequently assessed by ELISA.

2.2.8 Other Methods

SDS-PAGE, immunoblotting, and ELISA as well as standard procedures in molecular biology were performed according to established protocols [126].

2.3 Evaluation of Different Glycoforms of Honeybee Venom Major Allergen Phospholipase A2 (Api m 1) Produced in Insect Cells

2.3.1 Materials

Crude HBV collected by electrostimulation and native purified PLA2 were purchased from Latoxan (Valence, France). Anti-V5 antibody was purchased from Invitrogen (Karlsruhe, Germany). Polyclonal rabbit anti-HRP serum as well as anti-rabbit-IgG AP conjugate, anti-mouse IgG AP conjugate and bromelain from pineapple stem was obtained from Sigma (Taufkirchen, Germany). The monoclonal AP conjugated anti-IgE antibody was purchased from BD Pharmingen (Heidelberg, Germany). The MUXF-HSA conjugate was kindly provided by Siemens Healthcare Diagnostics (Los Angeles, CA, USA). The rat basophilic leukemia cell line RBL-SX38 was kindly provided by Prof. J.-P. Kinet (Harvard University, Boston, MA, USA). Sera with a positive test to HBV and/or YJV (sIgE >0.35 kU/L) were obtained from the institutional serum bank. All patients had given their informed written consent to draw an additional serum sample.

2.3.2 Cloning of cDNA

Total RNA was isolated from honeybee (*Apis mellifera carnica*) venom sacks using peqGold TriFast™ (Peqlab Biotechnologie, Erlangen, Germany). SuperScript III Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) was used to synthesize cDNA. The Api m 1 mature peptide coding region was amplified using Pfu DNA polymerase (Fermentas, St. Leon-Rot, Germany) and the primers 5'-ATA ATA TAT CCA GGA ACG TTA TGG TG-3' and 5'-ATA CTT GCG AAG ATC GAA CCA TTG-3' and subcloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) with the pCR-Blunt II-TOPO vector. A C-terminal V5 epitope and a 10-fold His-tag as well as 5' BamHI and 3' NotI restriction sites were added by PCR and the PCR product was subcloned into the pAcGP67-B baculovirus transfer vector (BD Pharmingen, Heidelberg, Germany) after restriction digest with BamHI and NotI.

2.3.3 Site directed mutagenesis

For generation of the inactive Api m 1 H34Q, histidine 34 was substituted by glutamine by using the QuikChange site directed mutagenesis Kit (Stratagene, La Jolla, USA) according to the manufacturers' recommendations and employing the primers 5'-GCA TGC TGT CGA ACC CAA GAC ATG TGC CCG GAC G-3' and 5'-CGT CCG GGC ACA TGT CTT GGG TTC GAC AGC ATG C-3'. For the non-glycosylated form of Api m 1 H34Q N13Q, asparagin 13 was changed to glutamine by employing the primers 5'-GGT GCG GGC ATG GTC AAA AGT CGT CCG GCC C-3' and 5'-GGG CCG GAC GAC TTT TGA CCA TGC CCG CAC C-3'.

2.3.4 Recombinant baculovirus production and expression

Recombinant baculovirus was generated by cotransfection of *Spodoptera frugiperda* (Sf9) cells (Invitrogen) with BaculoGold bright DNA (BD Pharmingen) and the baculovirus transfer vector pAcGP67-B Api m 1 H34Q or Api m 1 H34Q N13Q, respectively, according to recommendations of the manufacturer. High titer stocks were produced by three rounds of virus amplification. Optimal multiplicity of infection (MOI) for subsequent protein expression was determined empirically by infection of Sf9 cells with serial dilutions of virus stocks.

2.3.5 Expression in baculovirus-infected Sf9 cells and protein purification

High titer stocks of recombinant baculovirus containing the Api m 1 coding DNA were used to infect Sf9 or HighFive cells (Invitrogen) (1.5-2.0×10^6 cells per ml) in a 2000 ml suspension flask (400 ml suspension culture). For protein production the cells were incubated at 27 °C and 110 rpm for 72 h. Cellular supernatants were then applied to a nickel-chelating affinity matrix (Ni-NTA-agarose, Qiagen, Hilden, Germany). After washing with NTA-binding buffer (50 mM sodium phosphate, pH 7.6, 500 mM NaCl) the protein was eluted with NTA-binding buffer containing 300 mM imidazole.

2.3.6 Immunoreactivity of human sera

For assessment of specific IgE immunoreactivity, 384 well microtiter plates (Greiner, Frickenhausen, Germany) were coated with recombinant allergens, nApi m 1, HBV and the CCD marker MUXF-HSA (10 µg/ml) at 4 °C overnight and blocked with 40 mg/ml skimmed milk powder in PBS at room temperature. Human sera were diluted 1:2 in PBS and applied for 4 hours at room temperature. Wells were rinsed 4 times with PBS and incubated with an alkaline phosphatase conjugated mouse anti-human IgE antibody (BD

Pharmingen, clone G7-26) diluted 1:1000 in 20 mg/ml skimmed milk powder in PBS. Wells were rinsed 4 times with PBS and substrate solution (5 mg/ml 4-nitrophenylphosphate, AppliChem, Darmstadt, Germany) was added. After 30 minutes absorption was measured at 405 nm. The lower end functional cut-off indicated as lines was calculated as the mean of the negative controls plus 2 SDs.

2.3.7 *In vitro* degranulation assay with rat basophil leukemia cells (RBL-SX38)

In vitro degranulation was analyzed essentially as described [131]. In brief, RBL-SX38 cells were cultivated in 96-well plates (1×10^5 cells per well) for 24 h at 37 °C in 5 % CO_2. After washing with Tyrode´s buffer (137 mM NaCl, 2.8 mM KCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 0.4 mM NaH_2PO_4 and 10 mM Hepes, pH 7.4, BSA 0,1 % w/v) cells were incubated with native PLA2 or PLA2 H34Q (0.01-100 µg/ml in Tyrode´s buffer) at 37 °C for 1 h and then washed twice with Tyrode´s buffer. β-hexosaminidase release was assessed using 4-nitrophenyl N-acetyl-glucosaminide as a substrate. The supernatant of stimulated cells was incubated for 1 h at 37 °C and the enzymatic reaction was stopped by adding carbonate buffer (0,1 M, pH 10.0). Absorbance was measured at 405 nm. Cells were washed twice as described before and total cell lysate was obtained by addition of 0,1 % Triton-X-100 in Tyrode´s buffer and incubation for 1 h at 37 °C. β-hexosaminidase release was assessed as the percentage of total cell content which was ascertained by processing the supernatant of the cell lysate in the same way as the supernatant of the activated cells. All measurements were performed as triplicates and subjected to statistical analysis via student's t-test.

2.3.8 Other methods

SDS-PAGE, Western blotting, and standard procedures in molecular biology were performed according to established protocols [126]. Lectin blots (DIG Glycan Differentiation Kit, Roche Diagnostics, Mannheim, Germany) were performed according to the recommendations of the manufacturer.

2.4 Api m 10, a genuine *A. mellifera* venom allergen, is clinically relevant but underrepresented in therapeutic extracts

2.4.1 Materials

Crude HBV as well honeybee phospholipase A2 were purchased from Latoxan (Valence, France). Therapeutic grade HBV preparations were obtained from three different manufacturers. Anti-V5 antibody was purchased from Invitrogen (Karlsruhe, Germany). Polyclonal rabbit anti-HRP serum as well as anti-rabbit-IgG AP conjugate and anti-mouse IgG AP conjugate were obtained from Sigma (Taufkirchen, Germany). The monoclonal AP conjugated anti-IgE antibody was purchased from BD Pharmingen (Heidelberg, Germany). Monoclonal AP conjugated monoclonal anti-IgG1 antibody was from Acris Antibodies (Herford, Germany) and AP conjugated monoclonal anti-IgG4 was from Siemens Healthcare Diagnostics (Los Angeles, CA).

2.4.2 Sera

Three groups of sera from hymenoptera venom-sensitized patients were selected: (i) sera with negative sIgE test to vespid venom (i3 <0.35 kU/L) but positive test to HBV (i1 >0.35 kU/L) (n=17); (ii) sera with negative sIgE test to HBV (i1 <0.35 kU/L) but positive test to vespid venom (i3 >0.35 kU/L) (n=16); (iii) sera with positive sIgE test to both (i1 and i3 >0.35 kU/L) (n=51). Beekeepers were recruited during daily clinical practice. All patients had given their informed written consent to draw an additional serum sample.

2.4.3 Protein biochemistry

400 µg of *A. mellifera* venom dissolved in 30 µl 5x PAGE loading dye were subjected to SDS-PAGE. Bands in the range of 35 kDa were excised, the proteins digested in-gel by trypsin (Roche Diagnostics, Penzberg, Germany) and resulting peptide fragments were sequenced on a Waters Micromass QToF2 mass spectrometer (Waters, Milford, MA, USA) by tandem mass spectrometry according to the manufacturer's instructions.

2.4.4 Cloning of cDNA

Total RNA was isolated from the separated stinger with attached venom sack and glands of honeybee (*A. mellifera carnica*) using peqGold TriFast™ (Peqlab Biotechnologie, Erlangen, Germany). SuperScript III RT (Invitrogen, Karlsruhe, Germany) and the gene-specific primer 5'-TCAAGCAGTTAATACATCTCCTTGG-3' were used to synthesize cDNA from the isolated total RNA.

Api m 10 cDNA was amplified using Pfu DNA polymerase (Fermentas, St. Leon-Roth, Germany) and the primers 5'-TTCCCTGGTGCA-CACGATGAGG-3' and 5'-TCAAGCAGTTAATACATCTCCTTGG-3'.

2.4.5 Cloning and expression of Api m 10 in insect cells

Subcloned Api m 10 cDNA was used for secondary amplification of the coding region using Pfu DNA polymerase in two consecutive PCR reactions adding a N-terminal 10-fold His-tag and V5 epitope using the primers 5'-AAG CCT ATC CCT AAC CCT CTC CTC GGT CTC GAT TCT ACG TGG CTC GAG TCT AGA TTC CCT GGT GCA CAC GAT GAG G-3', 5'-GAT CGG ATC CCA TCA CCA CCA CCA TCA TCA CCA CCA CCA TTC TTC TGG TGG TAA GCC TAT CCC TAA CCC TCT CCT CGG-3'and 5'-GAT CGC GGC CGC TCA AGC AGT TAA TAC ATC TCC TTG G-3'. The PCR product was subcloned via BamHI and NotI into the digested baculovirus transfer vector pAcGP67-B (BD Pharmingen, Heidelberg, Germany).

2.4.6 Recombinant baculovirus production

Spodoptera frugiperda cells (Sf9) (Invitrogen, Karlsruhe, Germany) were grown at 27 °C in serum-free medium (Express Five SFM; Lonza, Verviers, Belgium containing 10 µg/ml gentamycin; Invitrogen, Karlsruhe, Germany). Cell density was determined by haemocytometer counts, cell viability was evaluated by staining with Trypan Blue. Recombinant baculovirus was generated by cotransfection of Sf9 cells with BaculoGold bright DNA (BD Pharmingen, Heidelberg, Germany) and the baculovirus transfer vector pAC-GP67-B containing Api m 10. High titer stocks were produced by three rounds of virus amplification and optimal MOI for protein expression was determined empirically by infection of Sf9 cells in 100 ml suspension flasks (1.5-2 x 10^6 cells/ml in 20 ml suspension culture) with serial dilutions of high titer virus stock.

2.4.7 Expression in baculovirus-infected insect cells

High titer stock of recombinant baculovirus was used to infect 400 ml suspension culture of Sf9 or HighFive cells (Invitrogen, Karlsruhe, Germany) (1.5-2.0x10^6 cells per ml) in 2000 ml flasks. For protein production the cells were incubated at 27 °C and 110 rpm for 72 h.

2.4.8 Protein purification

The supernatant of baculovirus-infected cells was collected, adjusted to pH 8, centrifuged at 4000 x g for 5 minutes, and applied to a nickel-chelating affinity matrix (NTA-agarose, Qiagen, Hilden, Germany). The column was washed with binding buffer (50 mM sodium

phosphate, pH 7.6, 500 mM NaCl) and pre-eluted with NTA binding buffer containing 20 mM imidazole. The recombinant protein was eluted from the matrix using NTA-binding buffer containing 300 mM imidazole. Purification was confirmed by SDS-PAGE.

2.4.9 Recombinant bacterial expression and purification of Api m 10

For expression in *E. coli* the Api m 10 coding region was amplified using the primers 5'-GAT CCA TAT GTT CCC TGG TGC ACA CGA TG-3' and 5'-GGT GGT TGC TCT TCC GCA AGC AGT TAA TAC ATC TCC TTG G-3' and inserted into the digested vector pTXB1 (New England Biolabs, Bad Schwalbach, Germany) via NdeI and SapI. The vector was further modified by addition of a second chitin binding domain (CBD). Expression and purification of the fusion protein was performed according to the recommendations of the manufacturer.

2.4.10 Western blotting

For immunoblot procedures the purified recombinant allergens, the crude venom, as well as the therapeutic venom preparations were separated by SDS-PAGE and immobilized onto nitrocellulose membranes. Human sera were diluted 1:10 with 5 mg/ml BSA in PBS and applied to the corresponding Western blots. Visualization of bound IgE was then performed with anti-human IgE mAb conjugated to alkaline phosphatase and nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate according to the recommendations of the manufacturer. The recombinant monoclonal IgE antibodies were diluted using DMEM supplemented with 100 ml/l heat-inactivated fetal calf serum and applied to the corresponding Western blots and visualized as described above. For Western blot procedures with anti-V5 epitope mAb and anti-HRP antiserum the antibodies were applied according to the recommendations of the manufacturer and bound antibodies visualized via corresponding secondary antibodies conjugated to alkaline phosphatase as described above.

2.4.11 Immunoreactivity of patient sera with recombinant proteins

For assessment of specific IgE, IgG, IgG1 and IgG4 immunoreactivity of human sera in ELISA, 384 well microtiter plates (Greiner, Frickenhausen, Germany) were coated with purified recombinant proteins (20 µg/ml) at 4 °C overnight and blocked with 40 mg/ml milkpowder in PBS. Thereafter, human sera were diluted 1:2 with PBS and incubated in a final volume of 20 µl for 4 hours at room temperature. After washing 4 times with PBS bound IgE, IgG, IgG1 or IgG4 were detected with a monoclonal alkaline phosphatase-conjugated anti-human IgE, IgG, IgG1 or IgG4 antibody (BD Pharmingen, Heidelberg, Germany) diluted 1:1000. After washing 4 times with PBS 50 µl of substrate solution

(5 mg/ml 4-nitrophenylphosphate, AppliChem, Darmstadt, Germany) per well were added. The plates were read at 405 nm. The lower end functional cut-off indicated as lines was calculated as the mean of the negative controls plus 2 SDs. Reactivities only slightly higher than the cut-off were excluded.

2.4.12 Basophil activation test

The basophil activation test was performed as described previously [132, 133] with modifications as recommended by the manufacturer (Flow-CAST; Bühlmann Laboratories, Basel, Switzerland). In brief, within 3 h after sampling of patient blood, aliquots of 50 µl whole blood were preincubated for 10 min at 37 °C with stimulation buffer containing IL-3. Subsequently, basophils were activated for 40 min at 37 °C in a water bath with either native or recombinant allergens at a volume of 100 µl. Thereby, HBV (in a concentration of 50 ng/ml) as well as the allergens (Api m 10 produced in either insect cells or *E. coli* and the established major allergen Api m 1, in a concentration range of 0.08–1000 ng/ml) were applied in concentrations according to those reported in the literature [134-136]. A murine mAb against the human high-affinity FcεR served as a positive stimulation control. Plain stimulation buffer was used as a negative stimulation control. To quantify activated basophils, cells were stained with 20 µl reagent containing a mixture of mAbs to human CD63 labeled with PE (anti-CD63–PE) and human IgE labeled with FITC (anti-IgE–FITC) for 30 min on ice. RBCs were lysed, and WBCs were fixed (BD FACS Lysing Solution; BD Biosciences, San Jose, CA) for 5 min at room temperature. After centrifugation (5 min at 1200 x g), cells were resuspended in 500 µl of stop solution. Flow cytometric analysis of basophil activation was performed on a FACScan flow cytometer (BD Biosciences). IgE-staining and side scatter were employed to gate on at least 500 basophils that expressed a high density of surface IgE. Subsequently, within this gate, the percentage of activated basophils (i.e., coexpressing CD63) was measured.

2.4.13 Characterization of patients

The sIgE levels for honeybee venom (HBV) (i1) and yellow jacket venom (YJV) (i3) were determined with the Immulite 2000 (Siemens Healthcare Diagnostics, Los Angeles, Ca.) or ImmunoCap 250 (Phadia, Uppsala, Sweden) as described in detail elsewhere [137], and for Api m 10, Api m 1, and the MUXF-HSA conjugate as described in the materials and methods section. For intradermal testing of patients with suspected insect venom allergies serial 10-fold dilutions of venom extracts with concentrations ranging from 0.0001 to 0.1 mg/L were performed. Histamine hydrochloride and physiologic saline were used as positive and negative control solutions, respectively. Intradermal tests were rated positive when the wheal size was >5 mm in diameter with a surrounding erythema.

2.4.14 Immunoreactivity of patient sera with recombinant proteins

Specific IgE, IgG, IgG1 and IgG4 immunoreactivity of human sera from hymenoptera venom allergic patients as well as from beekeepers with purified recombinant proteins (20 µg/ml) was assessed in ELISA. The lower end functional cut-off indicated as lines was calculated as the mean of the negative controls plus 2 SDs. Reactivities only slightly higher than the cut-off were excluded.

2.4.15 Statistical analysis

Statistical analysis was performed with Prism 3.0 software (GraphPad Software, San Digo, CA). Correlation coefficients were calculated using Pearson correlation analyses.

2.4.16 Other methods

Molecular biology standard procedures such as PCR, DNA-restriction, ligation, transformation, and plasmid-isolation were performed according to established protocols [126]. The chimeric human monoclonal IgE antibodies were generated essentially as described recently [128].

2.5 Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a

2.5.1 Expression of recombinant Phl p 5a and Phl p 5b constructs

For expression of recombinant Phl p 5 in *E. coli*, the pMal system (New England Biolabs) was used. Expression clones containing the coding sequence of Phl p 5a and b were kindly provided by Arnd Petersen (Research Center Borstel, Borstel, Germany). Both isoforms were purified from *E. coli* lysates by affinity chromatography using an amylase column and 10 mM maltose in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.5) for elution. Phl p 5 fragments were obtained by introducing particular PCR fragments into the vector pTXB1 (New England Biolabs) and expressed as N-terminal fusion proteins with an intein sequence and a chitin binding domain (CBD). Purification from the *E. coli* cell lysates was performed using chitin beads and elution from the column by addition of 50 mM dithiotreitol (DTT, SigmaAldrich) according to the recommendations of the manufacturer. The oligonucleotides used for generation of the fragments were as follows: F1 a: gatccatatggcaggtaaggcgacgaccgag (Nde I for) and gatcgctcttccgcagccggcgatgat gcggag (Sap I back); F2 a: gatccatatgaagtacaggacgttcgtcgcaacc (Nde I for) and gatcgctctt ccgcagccggcgatgatgcggag (SapI back); F3 a: gatccatatggcaggtaaggcgacgaccgag (Nde I

for) and gatcgctcttccgcacttgtaggcggcgtcgagcttg (Sap I back); F4 a: gatccatatgaagtacagg acgttcgtcgcaacc (Nde I for) and gatcgctcttccgcacttgtaggcggcgtcgagcttg (Sap I back); F5 a: gatccatatgaagtacaggacgttcgtcgcaacc (Nde I for) and gatcgctcttccgcactcggctgtcttgtag cgagc (Sap I back); chimeric F1: gatcccatggcctgttccaacaaggccttcgcggagg (Nco I for) and gatcgcgatcgcacaggaggtgagcgcggccttg (AsiS I back).

2.5.2   Production of recombinant antibodies

Heterotetrameric IgG and IgE immunoglobulins were produced using recently established vector systems [138]. The variable regions vH and vL were amplified using oligonucleotides containing restriction sites at the 5´- and 3´-termini of the vH (gatcatttaa atgtgtccagtgtgaggtgcagctggtgg and gatcctcgagacggtgaccaggt) and vL (gatccctgcagggtg ccagatgtgagctcacccagtctccatc and gatcgcgatcgcacgtttgatttccacc) by PCR, respectively. IgA2 heavy chain constant regions were amplified from total cDNA of human PBMCs restriction sites were introduced at the 5´- and 3´-termini of the cH (gatcctcgagc gcatccccgaccagcc and gatcggcccagccggcctcaatggtggtgatggtagcaggtgccgtccacc) and lambda cL (gatcgcgatcgcacagcccaaggctgccc and gatctctagactatgaacattctgtaggggc) by PCR, respectively, in a way that a 4 x His-Tag is generated at the C-terminal end of the heavy chain. Subsequently, the DNA was introduced into the different expression vectors. Human embryonic kidney cells (HEK-293, ATCC number CRL-1573) were cultivated in Dulbecco´s modified Eagle medium (DMEM) supplemented with 100 mL/L fetal calf serum, 10 kIU/L penicillin, and 100 mg/L streptomycin. Tissue culture reagents were obtained from Life technologies. HEK-293 cells were transfected by using 2 µg of the particular expression vector DNA complexed with polyethylene imine (Sigma Aldrich). The secreted immunoglobulins were purified from the culture medium by affinity chromatography using a protein A-medium (SureMAb columns, GE Healthcare) or Ni-NTA-agarose (Qiagen) according to the manufacturers' recommendations.

2.5.3   Amplification and cloning of FcεRI-IgY Fc and CD64-IgY Fc

The cloning and expression of the soluble IgE Fc receptor FcεRI-IgY Fc has been described elsewhere [129]. The human CD64 extracellular domains were amplified without the original signal sequence using one PCR primer containing a Pfl23 II site (gatccgtacgtgtgggcaagtggacaccacaaggc) and another primer containing an Sgs I site (gatcggcgcgccatgaaaccagacaggagttgg) and introduced into pcDNA3.1/zeo providing a rat immunoglobulin leader sequence and avian Fc regions [130].

2.5.4   Assessment of immunoreactivity in ELISA and immunoblot

For assessment of immunoreactivity in direct ELISA the particular proteins (10 µg/ml diluted with 2 % MPBS) were applied to microtiter plates, incubated at 4 °C overnight and blocked with 5 % MPBS at RT for 1 h. Thereafter, the recombinant immunoglobulins (1 µg/ml diluted with 2 % MPBS) were added to the wells and incubated for 1 h at RT. The ELISA was performed according to established protocols and detected with human Ig isotype specific antibodies conjugates and para-nitrophenyl phosphate (pNPP) as a substrate at 405 nm. The immunoreactivity of immunoglobulins with their particular Fc receptors was demonstrated by Sandwich ELISA. Therefore, FcεRI-IgY Fc or CD64-IgY Fc (1 µg/ml diluted with 2 % MPBS) were applied to microtiter plates, incubated at 4 °C overnight and blocked with 5 % MPBS at RT for 1 h.

Thereafter, hIgE, cIgE and hIgG1 (1 µg/ml diluted with 2 % MPBS) were added to the wells, incubated at RT for 1 h according to established protocols and detected with a chicken IgG specific antibody antibody conjugate and pNPP as a substrate at 405 nm. For immunoblot procedures, the particular recombinant antibodies were separated by SDS-PAGE. Visualization was then performed with human Ig isotype specific antibodies conjugated to alkaline phosphatase and nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP). Reactivity with Phl p 5 in immunoblot was performed after separation of aqueus timothy grass pollen extract (Siemens Healthcare Diagnostics) by SDS-PAGE. Recombinant IgE antibodies were applied at 1 µg/ml in 2 % MPBS and visualised human Ig isotype specific antibodies conjugated to alkaline phosphatase and (NBT/BCIP).

2.5.5   *In vitro* mediator release assay with rat basophil leukemia cells (RBL-SX38)

In vitro degranulation was analysed as described previously [131]. Soluble MBP-Phl p 5a was biotinylated using TFPA-PEG3-Biotin (Tetrafluorophenylazide-(triethyleneglycol)-Biotin (Pierce) according to the recommendations of the manufacturer and incubated with streptavidin-coated Roti-MagBeads (Roth). After sensitization of RBL-SX38 cells with IgE and washing with incomplete Tyrode´s buffer (10 mM HEPES, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$), Phl p 5a-coated beads were added to the wells and incubated for 60 min at 37 °C. As a positive control, cross-linking was achieved by addition of polyclonal anti-human IgE serum (1 µg/ml from goat, Bethyl). β−hexosaminidase release of viable versus lysed cells was assessed with p-nitrophenyl N-acetyl-glucosaminide (Sigma Aldrich) as a substrate.

2.5.6   Hybridoma generation

The establishment of the Phl p 5-specific hybridoma line was achieved by immunisation with Phl p 5a (Biomay, Vienna, Austria) according to standard protocols by Biogenes GmbH (Berlin, Germany).

2.5.7   Other Methods

SDS-PAGE, immunoblotting, and ELISA as well as standard procedures in molecular biology were performed according to established protocols [126]. Monoclonal anti-human IgE (BD Biosciences), polyclonal anti-human IgG (Fc-specific, Sigma Aldrich), polyclonal anti-human IgA (Sigma Aldrich) or polyclonal anti-chicken IgG (Rockland Immunotech) conjugated to alkaline phosphatase were used for specific detection in ELISA and immunoblot. Quantitation of allergen specific IgE was performed using an ImmunoCAP 250 analyser (Phadia, Uppsala, Sweden) and an Immulite 2000 analyser (Siemens Healthcare Diagnostics) according to the recommendations of the manufacturers.

2.6   Establishment of a model system for hapten specific IgE antibodies

2.6.1   Conversion of TNP-specific scFv into scFv-IgE antibodies

In a previous work of Deckers *et al.* [139] the phage display technology was used to select a synthetic *single-chain antibody fragment* (scFv) library against the *2,4,6-trinitrotoluene* (TNT)-surrogate *2,4,6-trinitrophenol* (TNP) that was conjugated to *bovine serum albumin* (BSA) (Fig. 2.1). In the selection process different elution strategies were performed, resulting in the anti-TNP scFv clone 2.18 when a pH shift was used and the anti-TNP scFv clone 4.8 when free TNT was used for competitive elution. Additionally a selection with an avian TNP specific library, designed by Dirk Heinrich, against TNP-BSA and a non-competitive elution resulted in the anti-TNP scFv clone 5. The scFv sequences 2.18 and 5 were available in the phagemid vector pHen2 (provided by G. Winter, *MRC Centre of Protein Engineering,* Cambridge, UK). They were converted into bivalent scFv based homodimeric IgE constructs by cloning the scFv sequences into the mammalian expression vector pcDNA3.1/Zeo (Invitrogen Life Technologies, Karlsruhe, Germany) which is regulated by the CMV-promoter. The vector contained the constant domains 2-4 of the human IgE heavy chain, as well as a rodent signal sequence that ensures the secretion of expressed constructs into the cellular supernatant, and a C-terminal 4xHis-tag. The scFv clone 4.8 was already available as scFv-IgE.

2,4,6-trinitrotoluene (TNT)   2,4,6-trinitrophenol (TNP)   2,4,6-trinitrobenzenesulfonic acid (TNBS)   2,4,6-trinitrophenyl (TNP) -conjugate
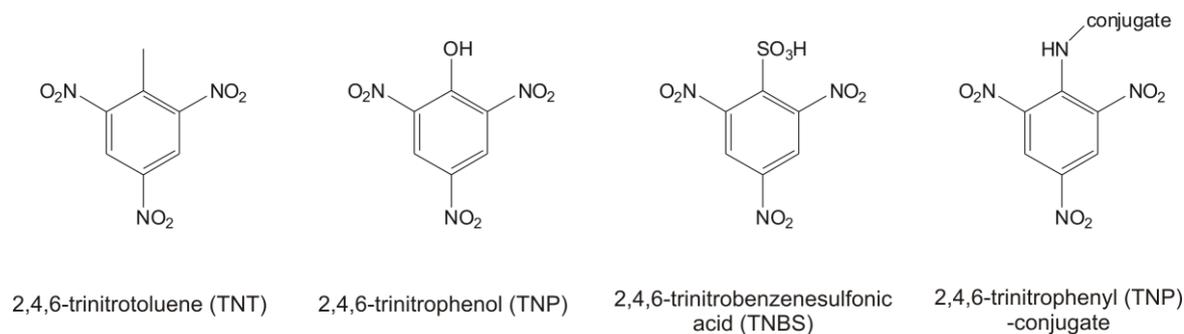
Fig. 2.1: Representation of the structures of TNT, TNP, TNBS and a TNP-conjugate. For coupling reactions the TNT substitute TNBS is used, containing a reactive sulfonic acid group that reacts with primary amines resulting in TNP-conjugates, such as TNP-BSA.

For the amplification of clone 2.18 by PCR the specific primers 5'-gatcatttaaatgtgtccagtgtcaggtgcagctggtggag-3' and 5'-gatcggcgcgccacctaggacggtcagcttg-3' and for clone 5 the specific primers 5'- gatcatttaaatgtctccagtgtgccgtgacgttggacgag-3' and 5'- gatcggcgcgcctaggacggtcagggttgtc-3' were used and the resulting sequences were inserted via SgsI and SmiI into the vector.

2.6.2 Production of recombinant scFv-IgE antibodies

Human embryonic kidney cells (HEK-293, ATCC number CRL-1573) were cultivated in Dulbecco´s modified Eagle medium (DMEM) supplemented with 100 ml/l fetal calf serum, 10 kIU/l penicillin, and 100 mg/l streptomycin. Tissue culture reagents were obtained from Invitrogen, life technologies, Karlsruhe, Germany. HEK-293 cells were transfected by using 2 µg of the particular expression vector DNA complexed with polyethylene imine (Sigma Aldrich, Taufkirchen, Germany). The expression and immunoreactivity of the resulting stable expressed antibodies in the supernatant was assessed in immunoblot and ELISA.

2.6.3 Detection of the scFv-IgE constructs in immunoblot

The antibody expression was analyzed by immunoblot. Therefore the particular recombinant antibodies were separated by SDS-PAGE. Visualization was then performed with polyclonal goat anti-human IgE serum (Bethyl Lab. Inc., Montgomery, US) and anti-goat IgG conjugated to alkaline phosphatase (Sigma Aldrich, Taufkirchen, Germany), as well as nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate (NBT/BCIP).

2.6.4 Assessment of immunoreactivity in ELISA

For assessment of immunoreactivity in direct ELISA the particular proteins (20 µg/ml) were applied to microtiter plates, incubated at 4 °C overnight and blocked with 4 % FKS at RT for 1 h. Thereafter, the supernatants of the recombinant immunoglobulins were added to the wells and incubated for 1 h at RT. The ELISA was performed according to established protocols and detected with monoclonal anti-human-IgE antibody (BD Biosciences, Heidelberg, Germany) conjugated to alkaline phosphatase and para-nitrophenyl phosphate (pNPP) as a substrate at 405 nm. For further investigations the antibody concentrations in the reactive cellular supernatants were determined using the "human IgE ELISA Quantitation Kit" (Bethyl Lab. Inc., Montgomery, US) according to the recommendations of the manufacturer.

2.6.5 Surface plasmon resonance analysis

The interaction affinity of the anti-TNP scFv-IgE antibodies 2.18, 4.8 and 5 and immobilized TNP-BSA were determined by *surface plasmon resonance* (SPR) measurements using the Biacore T100 affinity sensor (GE Healthcare, Freiburg, Germany). Biotinylated TNP-BSA was obtained by biotinylation of BSA in phosphate buffer pH 7.4 using photoactivatable tetrafluorophenyl azide-PEG3-biotin (TFPA-Biotin, Pierce, Thermo Scientific, Bonn, Germany). The biotinylated BSA was dialyzed and used for TNP-labeling by coupling *2,4,6-trinitrobenzenesulfonic acid* (TNBS, Sigma Aldrich, Taufkirchen, Germany) to BSA as a carrier protein, using 1000 fold excess of TNBS. The solution was purified by gelfiltration, using a PD-10 column (GE Healthcare, München, Germany). For the SPR analysis first streptavidin was covalently coupled to a total of 1000 *resonance units* (RU) on a carboxylated dextran sensor chip surface (CM5, Biacore, GE Healthcare, München, Germany) using standard coupling procedure with 0.4 M N-(3-Dimethylaminopropyl)-N`-ethylcarbodiimid (EDC) and 0.1 M N-Hydroxysuccinimid (NHS) via primary amines with 10 mM acetate-buffer pH 4.0 and capping by 1 M ethanolamine pH 8.5.

After that, biotinylated TNP-BSA was injected to only one flow cell to a total of 250 RU. The surface coupled with only streptavidin served as reference. Measurements were performed at 20 °C in buffer containing 50 mM sodium phosphate, and 100 mM NaCl, pH 7.5. For the kinetic analyses, antibody containing cellular supernatants were dialyzed against the running buffer and increasing antibody concentrations (~5–158 nM for 2.18-scFv-IgE, ~0,4–37,1 nM for 4.8-scFv-IgE, ~14–888 nM for 5-scFv-IgE) were injected at a flow rate of 10 µl/min. The association phase was monitored for 180 sec, the dissociation phase for 320 sec. Sensor surfaces were regenerated after each binding cycle by two subsequent injections of 50 mM Tris buffer, pH 12 and 50 mM citrate buffer pH 2. As

reference the interaction of the TNP-specific *purified mouse IgE κ Isotype Control* (clone C38-2, BD Biosciences, Heidelberg, Germany) with immobilized TNP-BSA was also investigated using the SPR-2 affinity sensor from Sierra Sensors, Hamburg, Germany.

Biotinylated TNP-BSA was covalently coupled to the chip surface as described above to a total of 100 resonance units on a carboxymethylated sensor chip surface (SPR-2 affinity sensor) using standard NHS/EDC coupling procedure via primary amines and capping by ethanolamine. The surface coupled with only streptavidin served as reference. Measurements were performed at 20 °C in buffer containing 10 mM monosodium phosphate, 40 mM disodium phosphate and 100 mM NaCl, pH 7.5 with 0,01 % Tween-20. For the kinetic analyses, increasing concentrations of the C38-2-muIgE (~4–100 nM) were injected at a flow rate of 25 µl/min. The association phase was monitored for 120 seconds, the dissociation phase for 90 seconds.

Sensor surfaces were regenerated after each binding cycle by two subsequent injections of 50 mM Tris buffer, pH 10. After subtracting reference cell signals, resulting binding data were fitted to a Langmuir 1:1 binding model by using global fit analysis (OriginPro 8.5G) and the dissociation constant at equilibrium KD was calculated.

2.6.6   *In vitro* mediator release assay with rat basophil leukemia cells (RBL-SX38)

The *rat basophil leukemia* (RBL-SX38) cells were cultured in Eagle's MEM supplemented with Earle's salts, L-glutamine, penicillin/streptavidin, carbonate and 15 % fetal calf serum at 37 °C and 5 % CO_2. For the *in vitro* mediator release assay the RBL cells were seeded in a 96-well culture plate 1 x 10^5 cells per well in 200 µl medium and incubated overnight at 37 °C and 5 % CO_2. After washing with Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES and 0.1 % bovine serum albumin) cells were incubated with 100 µl/well of the supernatants of the anti-TNP scFv-IgE antibodies in different dilutions in Tyrode's buffer at 37 °C for 1 h and then washed twice with Tyrode's buffer. TNP-BSA was also diluted in Tyrode's buffer and added to the wells. Negative controls were incubated with Tyrode's buffer without TNP-BSA to calculate spontaneous release.

As reference, cross-linking was achieved by addition of polyclonal anti-human IgE serum (1 µg/ml from goat, Bethyl Lab. Inc., Montgomery, US).

After 1 h at 37 °C, 30 µl of the supernatants were removed and transferred to another 96-well reading plate. In each well 10 µl *p-nitrophenyl N-acetyl-glucosaminide* (pNAG, Sigma Aldrich, Taufkirchen, Germany) as substrate were added and the supernatants of the stimulated cells were incubated for 1 h at 37 °C. The enzymatic reaction was stopped by adding 50 µl/well carbonate buffer (0.1 M, pH 10.0) and absorbance was measured at

405 nm. In parallel the cells were washed again 2 times with Tyrode's buffer and total cell lysate was obtained by addition of 0.1 % Triton-X-100 in Tyrode's buffer, 100 µl per well, and incubation for 1 h at 37 °C. After that again 30 µl of the supernatants were removed and incubated with pNAG as described above. The enzymatic reaction was stopped by adding 50 µl/well carbonate buffer and absorbance was measured at 405 nm.

The β-hexosaminidase release was assessed as the percentage of total cell content. All measurements were performed as triplicates.

2.6.7   Other methods

SDS-PAGE, immunoblotting, and ELISA as well as standard procedures in molecular biology were performed according to established protocols [126].

3 Results

The results presented in this thesis were obtained in collaboration with different members of different research groups as evident from the published and submitted manuscripts listed in the appendix section.

3.1 The putative serine protease inhibitor allergen Api m 6 from *A. mellifera* venom: recombinant and structural evaluation

Anaphylaxis mediated by IgE antibodies in hymenoptera venom allergic patients is one of the most severe hypersensitivity reactions. Over the last decade epidemiological population-based studies revealed a prevalence of systemic reactions to hymenoptera stings ranging from 0.3 % to 8.9 %, with anaphylaxis in 0.3–42.8 % of cases [111, 112]. Honeybee venom (HBV) is a complex mixture of various allergens that play important roles in the induction of allergic reactions after honeybee stings, as well as of several non-allergenic low molecular weight substances [114]. Due to the fact that all of the compounds in the venom may contribute to sensitization, symptoms, and success of VIT, their detailed characterization is of considerable interest. The low abundance of several of these components makes their recombinant availability a prerequisite for detailed immunologic characterization as well as for an improved component-resolved diagnosis and the design of efficient and safer therapeutic approaches. Only a restricted number of recombinant HBV allergens was available so far, but in the recent years significant progress has been made on the identification and recombinant production of venom allergens. The best known allergens of HBV are Api m 1 (phospholipase A2), Api m 2 (hyaluronidase), and Api m 4 (melittin), which are found in medium to high amounts [114]. Additionally, in the last years allergens of lower abundance, such as Api m 3 (acid phosphatase) [123], Api m 5 (DPP IV enzyme) [133], Api m 10 [140, 141], and Api m 11 [140] were identified, recombinantly produced, and characterized.

Another HBV allergen is the low molecular weight putative protease inhibitor Api m 6, which exists as 4 isoforms [142]. The primary structure of these isoforms shows a common core of 67 residues and varying sequences at the amino and carboxy terminus of at most six amino acids [142]. The genetic mechanism for this variation is unknown, but it has been shown that the protein-level variation has a simple genome-level cause [143]. Named as Api m 6.01 to 6.04 the isoforms show MWs of 7190, 7400, 7598, and 7808 Da, respectively, and migrate as 8-kd band in SDS-PAGE [142]. Api m 6 comprises a trypsin inhibitor like cysteine-rich domain containing 10 cysteine residues, no putative N-glycosylation sites, and is most likely identical to a 9 kDa protease inhibitor of bee

venom described decades ago [144]. Kettner et al. purified Api m 6 from whole HBV and demonstrated specific IgE recognition of approximately 40 % of HBV-sensitized patients in immunoblotting as well as T cell proliferation [142].

In this study we report the cloning and recombinant expression of honeybee venom allergen Api m 6 in *E. coli* and insect cells, as well as the evaluation of the specific IgE reactivity of the prokaryotically expressed protein. The obtained data suggest a relevant role of Api m 6 as sensitizing venom component in a fraction of HBV allergic patients since approximately 26 % of patients show specific IgE reactivity with Api m 6. Moreover, we generated a three-dimensional model of the mature allergen which underlines its putative role as protease inhibitor and allows first insights in structure function relationships.

3.1.1 Recombinant expression and characterization of Api m 6

For recombinant expression of Api m 6 the cDNA of the longest variant Api m 6.04 [142] was amplified from venom gland cDNA. The mature protein consists of 73 amino acids and has a theoretical molecular mass of 7.8 kDa. The sequence is devoid of threonine, methionine, and histidine and contains no putative N-glycosylation sites. Expression in *E. coli* as MBP (maltose binding protein) fusion protein and purification via affinity chromatography yielded soluble protein with an apparent molecular weight of approximately 60 kDa (Fig. 3.1A).
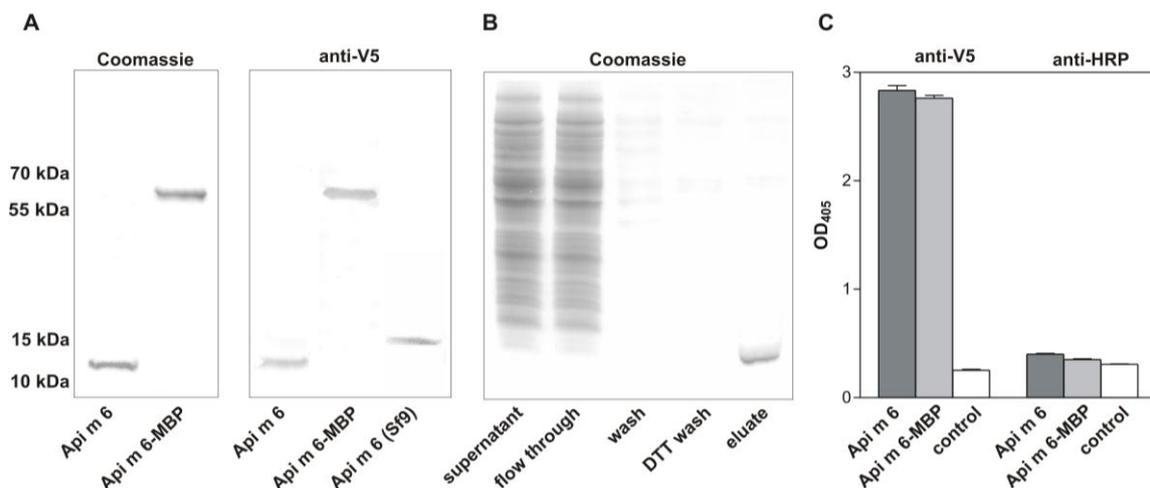
Fig. 3.1: Recombinant expression, purification and characterization of Api m 6. A: SDS-PAGE and immunoblot analyses of Api m 6 with and without MBP fusion partner recombinantly produced in *E. coli* or Sf9 insect cells visualized by either Coomassie blue staining or monoclonal anti-V5 epitope antibody. **B:** SDS-PAGE analysis of the purification of prokaryotically produced Api m 6 without fusion partner using the strategy of chitin binding domain (CBD) fusion followed by autocatalytic intein-mediated cleavage, resulting in pure target protein released from the Intein-CBD tag. **C:** Immunoreactivity of recombinant Api m 6 and Api m 6-MBP produced in *E. coli* in ELISA using monoclonal anti-V5 epitope antibody and polyclonal HRP antiserum. Results are presented as triplicates.

The recombinant allergen has an apparent molecular weight of approximately 12 kDa (Fig. 3.1A, B) taking into account the V5 epitope tag.

Both Api m 6 variants were reactive with a monoclonal anti-V5 epitope antibody in immunoblot (Fig 3.1A). ELISA analyses corroborated these data regarding protein identity (Fig. 3.1C). Additionally, missing reactivity of both proteins with a polyclonal HRP antiserum specific for α1,3-core fucosyl residues, the underlying principle of cross-reactive carbohydrate determinants (CCDs), demonstrates that expectedly the recombinant allergens are devoid of any CCD reactivity (Fig. 3.1C). Moreover, Api m 6 was produced by stable transfection of *Spodoptera frugiperda* (Sf9) insect cells showing an apparent molecular weight of approximately 15 kDa in immunoblot (Fig. 3.1A). However, the expression level in Sf9 cells proved to be very low, so that in consequence the both variants of prokaryotically produced Api m 6 were used for further specific IgE reactivity analyses.

Taken together these data demonstrate that honeybee venom allergen Api m 6 can be prokaryotically produced as soluble protein either with or without fusion partner. Since prokaryotically produced proteins are devoid of CCD reactivity the recombinant Api m 6 variants seem to be suitable target molecules to assess their relevance as proteinogenic allergens.

3.1.2   Screening of patient sera for IgE reactivity with Api m 6 variants

To evaluate the IgE immunoreactivity of Api m 6, individual sera of 31 randomly selected patients with a clinical history of insect venom allergy were assayed for Api m 6-specific IgE antibodies in ELISA applying Api m 6-MBP as well as non fused Api m 6 produced in *E. coli.* All patients were recruited during daily clinical practice and had sIgE for honeybee venom (i1) and/or showed a positive result in intradermal skin test with HBV. Of the 31 sera 8 (25,8 %) showed IgE reactivity with both Api m 6 variants (Fig. 3.2A). Although most Api m 6 reactive sera showed comparable reactivity with Api m 6-MBP and non fused Api m 6 (Fig. 3.2A, B), one serum exhibited a dramatically reduced reactivity to the non fused Api m 6.
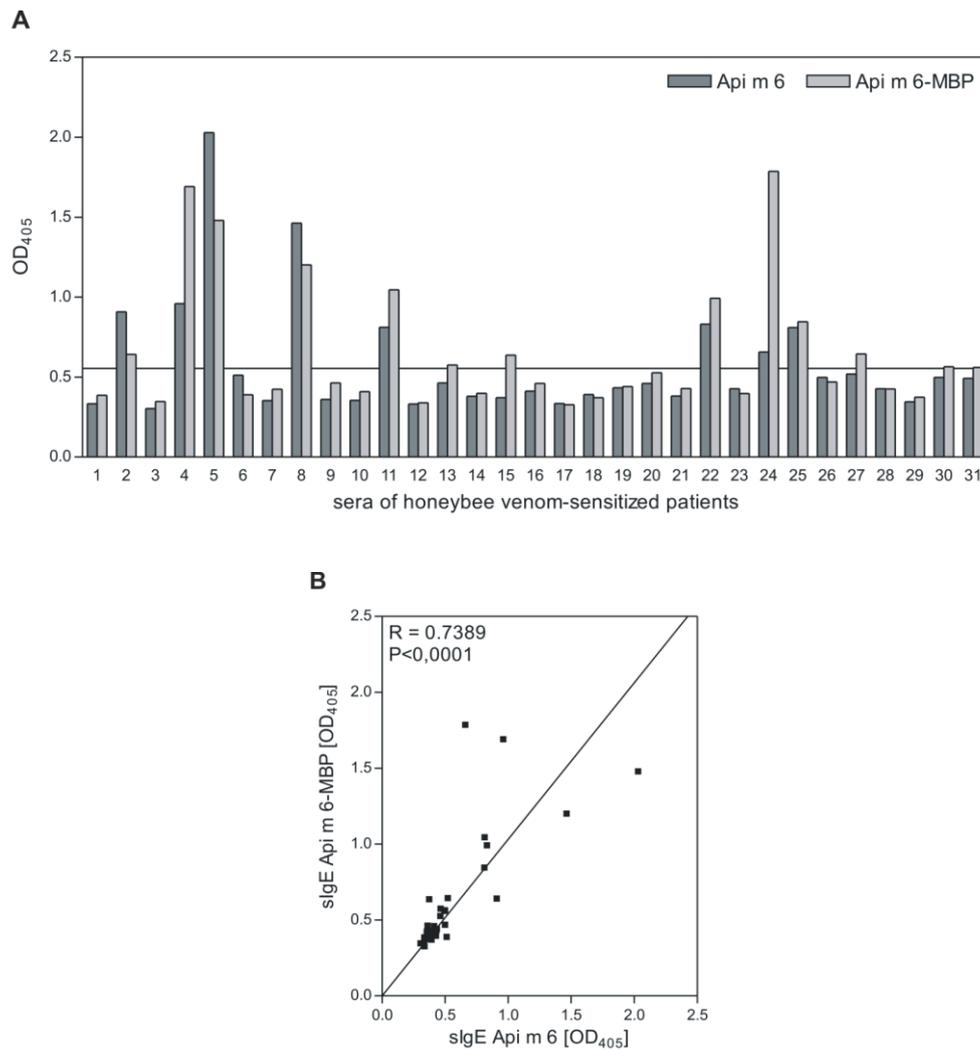
Fig. 3.2 IgE Immunoreactivity of individual patient sera with Api m 6 variants. A: The IgE reactivity with prokaryotically produced Api m 6 and Api m 6-MBP fusion protein was assessed by ELISA with 31 sera of honeybee venom-sensitized patients. The lower end functional cut-off of the ELISA is represented by a solid line. **B:** Correlation of the IgE reactivities obtained with the two prokaryotically Api m 6 variants was assessed by Pearson correlation analysis.

Since Api m 6 is devoid of any CCD-based cross reactivity, contains no putative N-glycosylation sites, its reactivity obviously includes specificity for the protein only.

3.1.3   Sequence alignment of Api m 6 with serine protease inhibitors

Api m 6 contains a trypsin inhibitor like (TIL) domain which suggests a role to act as a protease inhibitor in the venom. Interestingly, a small protease inhibitor was isolated from honeybee venom decades ago which corresponds to Api m 6 in terms of molecular weight and by the absence of the amino acids threonine, methionine, and histidine [145].

Figure 3.3 shows an alignment of the Api m 6 mature sequence with the *Apis mellifera* chymotrypsin/cathepsin G inhibitor-1 (AMCI-1), a protein of 56 amino acids isolated from honeybee hemolymph [146], as well as with the Ascaris suum chymotrypsin/elastase inhibitor (C/E-1), a protein consisting of 63 amino acids [141].


```
Apim6   FGGFGGFGGLGGRGKCPSNEIFSRCDGRCQRFCPNVVPKPLCIKICA-PGCVCRLG--YLRNKKKVCVPRSKCLPG 73
C/E-1   -----------GQESCGPNEVWTEC-TGCEMKCG-PDENTPCPLMCRRPSCECSPGRGMRRTNDGKCIPASQCP-- 61
AMCI-1  ------------EECGPNEVFNTCGSACAPTCA-QPKTRICTMQCR-IGCQCQEG--FLRNGEGACVLPENC--- 56
               .* .**::.  *    *     *        .   * *  .* *  *    *. . *:  .:*
                                                            T I L
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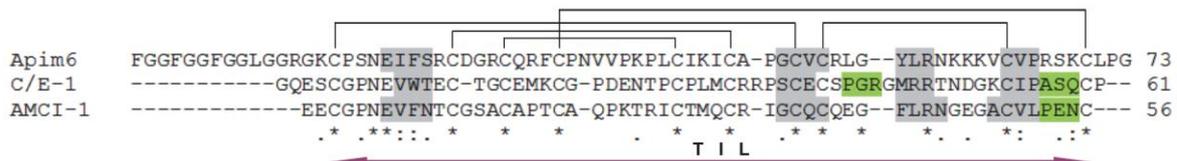
Fig. 3.3: Alignment of Api m 6 with other related proteins. Alignment of the amino acid sequences of Api m 6 (Genbank accession ABD51779), the *Ascaris suum* chymotrypsin/elastase inhibitor (C/E-1) (PDB ID 1EAI chain D) and the *Apis mellifera* chymotrypsin/cathepsin G inhibitor-1 (AMCI-1) (PDB ID 1CCV). Asterisks, colons, and periods indicate fully conserved, strongly similar, and weakly similar residues, respectively. Sequences forming beta strands are highlighted in grey and helical regions in green. The pairing cysteine residues 1-7, 2-6, 3-5, 4-10, and 8-9 are connected through black brackets and the TIL domain is illustrated by a purple arrow.

The sequence identity of Api m 6 with AMCI-1 and C/E-1 constitutes 33 % and 29 %, respectively. Basically, the sequence similarity is due to the presence of 10 cysteines in the TIL domain, which form five disulfide bonds pairing the cysteine residues 1-7, 2-6, 3-5, 4-10, and 8-9. Moreover, the TIL domain of all three proteins contains four beta strands at identical positions. In contrast to Api m 6, C/E-1 and AMCI-1 contain two and one short alpha helices, respectively.

3.1.4   Generation of a three-dimensional model of Api m 6

To obtain further insights in the potential function of Api m 6 the known NMR structure of AMCI-1 [147] was used as a template to generate a structural model of Api m 6 applying the Local Meta-Threading-Server (LOMETS) for protein structure prediction [124]. Shown in Fig. 3.4 is the model of Api m 6 (Fig. 3.4A) in comparison with the template NMR structure of AMCI-1 (Fig. 3.4B) as well as with C/E-1 (Fig. 3.4C) the structure of which was solved by crystallization [148].
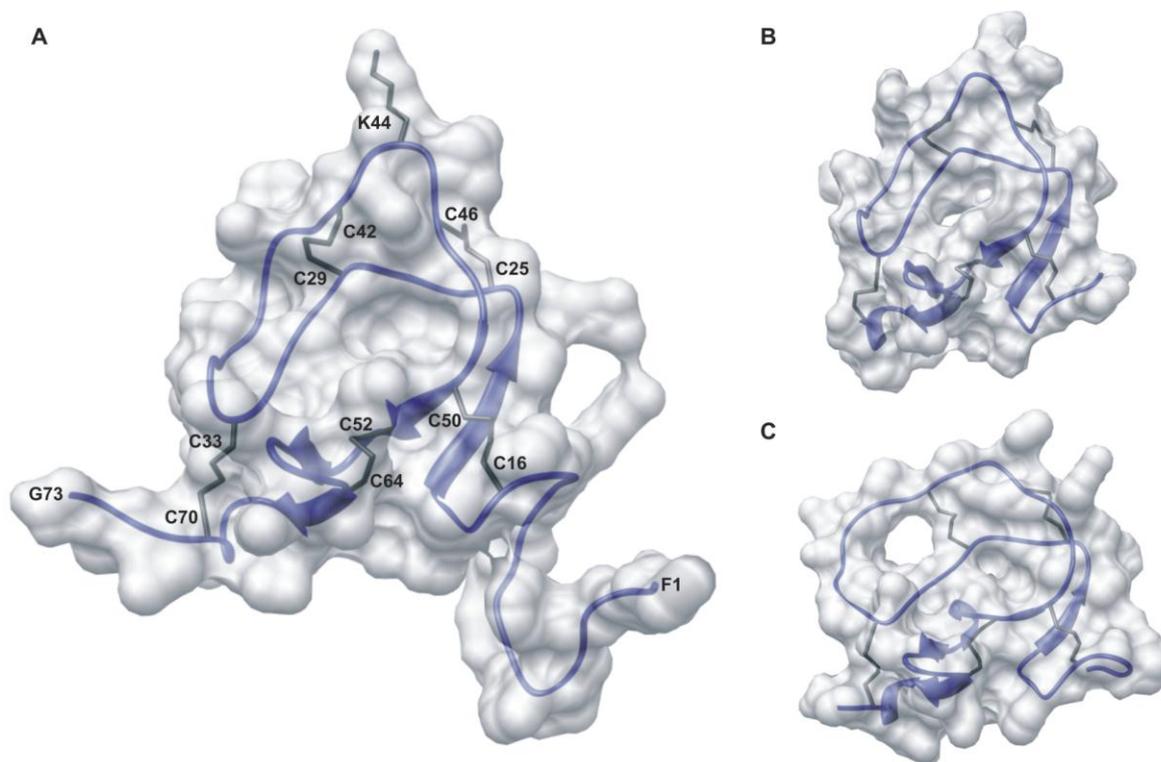
Fig. 3.4: Structure of Api m 6 and related proteins. A: Molecular model of Api m 6 in comparison with the solved structures of the *Apis mellifera* chymotrypsin/cathepsin G inhibitor-1 (AMCI-1) (**B**) and the *Ascaris suum* chymotrypsin/elastase inhibitor (C/E-1) (**C**). The modeling was performed using the structure of AMCI-1 (PDB ID 1CCV) as template. Lysine 44 of Api m 6 corresponds to the inhibitory important P1 position of the binding loop.

Obviously, Api m 6 and the both protease inhibitors exhibit a common fold which is dominated by an exposed binding loop including the protease binding site and showing a typical canonical conformation essential for the biologic activity of protease inhibitors. Further characteristic elements of all three structures are the antiparallel beta strands building the basis of the inhibitory loop and several turns. Due to the lack of extensive secondary structure elements the binding loop as well as the scaffold built by the beta sheets is stabilized by the presence of five disulfide bonds. Notably, the protein loop exhibits a strongly exposed lysine (K44) (Fig. 3.4A) constituting the P1 position which is important for enzyme binding and a potential recognition motif, together with the positions P3 as well as P3' which are occupied by cysteines (C42, C46). The sequential P3-P3' segment of the binding loop of canonical inhibitors represents the primary contact region and the flanking sequences form the so called secondary contact region which can also participate in enzyme binding [149]. The binding loop conformation of Api m 6 is similar to that found in several canonical serine protease inhibitors. In contrast to the two other shown protease inhibitors the coulomb coloring of the Api m 6 surface is strongly dominated by a positive charge (Fig. 3.5A).
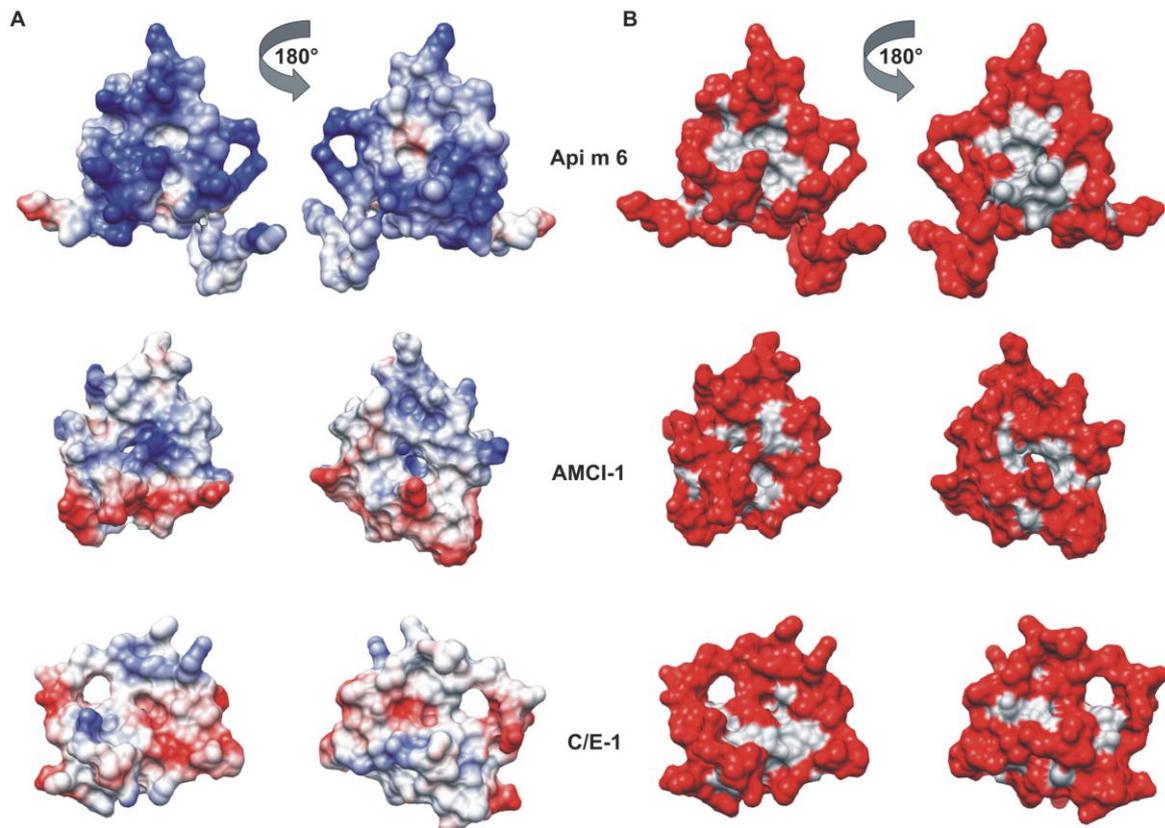
Fig. 3.5: Electrostatic potential and predicted epitopes of Api m 6 and related proteins. A: Coulombic surface coloring indicates the electrostatic potential ranging from basic (blue) to acidic (red) surface properties. **B:** Predicted conformational B cell epitopes of Api m 6, AMCI-1, and C/E-1 are highlighted in red. Epitope prediction was performed using the Conformational Epitope Prediction Server (CEP).

This observation is in line with the isoelectric point of Api m 6 of 9.7 which is relatively high when compared to C/E-1 (5.2) and AMCI-1 (4.8) and gives Api m 6 a strong basic character. As suggested by conformational epitope prediction, there is no impact of the differentially charged molecule surface on the density and distribution of epitopes that apparently cover the whole surface of all three molecules (Fig. 3.5B).

3.2 Close-up of the immunogenic alpha-1,3-Gal epitope as defined by a monoclonal chimeric IgE and human serum using saturation transfer difference (STD) NMR

Circulating concentrations of IgE, the antibody class responsible for allergic hyper-sensitivity, are linked to the development of several immune-mediated diseases [150]. IgE antibodies bound to their high affinity receptor (FcεRI) on mast cells and basophils mediate receptor cross-linking by allergens and trigger degranulation and release of proinflammatory mediators responsible for immediate-type hypersensitivity reactions. However, the exact interplay of different isotypes with their cognate allergens remains enigmatic.

Apart from the protein backbone, IgG and IgE may also be directed against xenobiotic and therefore immunogenic and cross-reactive carbohydrate determinants (CCDs) present on a plethora of proteins found in food, pollen and hymenoptera venom [151]. The hallmark of classical CCDs are alpha-1,3-linked core fucose residues found on insect venom allergens, and, additionally, beta-1,2-linked xylose on plant-derived CCDs. Antibody specificity for these spatially separated glycotopes represents the universal principle for reactivity of different proteins having CCDs [68]. The role of CCDs as a cause of allergic symptoms still is controversial [88]. IgE against classical CCDs has been shown clinically relevant [69, 152-154] but artificial or recombinant glycoproteins did not show clear cut effects in mediator release assays or skin prick tests [101, 155]. Recently, a novel type of CCD has entered the field and provided final evidence for the detrimental potential of glycans. Clearly IgE-mediated anaphylaxis via the well established Gal-alpha-1,3-Gal structure (alpha-Gal) as present on the chimeric therapeutic antibody cetuximab could be shown [89]. This epitope is also essential in meat-induced allergy [106] and for cross-reactivity to other mammalian allergens [156]. Strong induction of alpha-Gal-specific IgE very recently was correlated with bites of tick species present within a restricted area of the United States [107].

Anti-Gal IgG antibodies especially of the IgG2 subclass constitute up to 3 % of serum immunoglobulins in man, are induced by commensal bacteria and putatively exert a natural barrier function [102]. Their clinical relevance is well documented for xenotransplantation and blood group antigens providing alpha-1,3-linked galactose residues resulting in hyperacute xenograft rejection [157]. Scarce information however is available for alpha-Gal-specific IgE.

The interaction of polyclonal IgE with allergens has broadly been studied but detailed analyses of the particularities of IgE and its epitopes are hampered by two critical limitations, the low IgE levels in serum and the lack of specific human monoclonal

antibodies. Murine monoclonals often used as substitute are neither compatible with human cellular assays nor recognize authentic IgE epitopes and thus can provide indirect evidence only. This limitation would be obsolete if murine antibodies recognized B-cell epitopes identical to those of human antibodies, a scenario only true for small sized epitopes that obey identical immunological mechanisms in animals and man. Such a situation is given for IgE with specificity for CCDs, which are defined by their high immunogenicity in different species and their spatially extraordinarily well defined architecture. Structural and molecular data on the interaction of antibodies with carbohydrates [75-77, 158, 159] still are scarce and for the alpha-Gal epitope in particular not available at all. Molecular analyses of biomolecules with ligands of limited size such as carbohydrates can be obtained using saturation transfer difference (STD) NMR [160]. Thereby, saturation is transferred from a receptor protein to ligands and leads to specific attenuation of resonance signals of ligands that bind to the receptor. This attenuation is made visible by difference spectroscopy and allows identification and characterization of the ligands and their interaction.

Thus, the aim of our work was to gain access to alpha-Gal-specific human antibody isotypes allowing insights into the molecular and functional basis of their interaction. IgE and IgG were generated and employed for cellular activation tests and characterization of the IgE epitope by STD NMR. This work contributes to elucidation of the complex antibody carbohydrate interaction and molecular aspects in CCD-based anaphylaxis.

3.2.1   Generation of alpha-Gal-specific human antibody isotypes

To establish chimeric mouse/human antibodies, the VH and VL sequences of the alpha-Gal-specific antibody M86 were used as templates for gene synthesis [161]. The variable regions were assembled in form of a single chain Fv allowing prokaryotic production (Fig. 3.6).
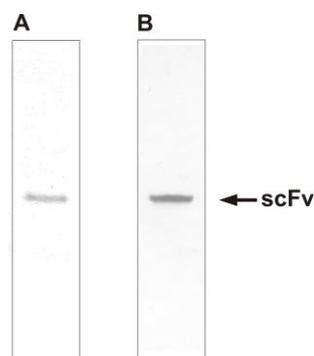
Fig. 3.6: Expression of soluble scFv. Purified proteins were assessed under by Coomassie staining (**A**), or immunoblot (**B**); the scFv was visualized using anti-myc antibodies conjugated to alkaline phosphatase.

The antibody fragments were subsequently converted to human IgG1 and IgE isotypes by fusion with signal sequences and the particular heavy and light chain constant regions.
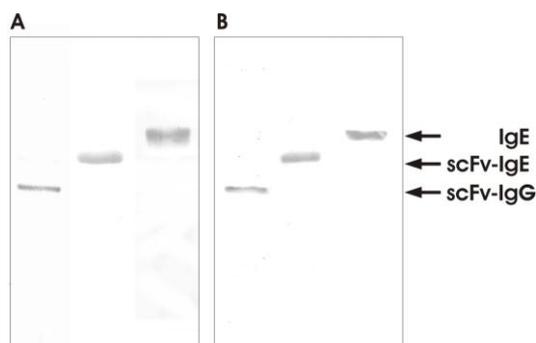
Fig. 3.7: SDS-PAGE and immunoblot analysis of IgG and IgE antibodies. Purified proteins were assessed under non-reducing conditions by Coomassie staining (**A**), or immunoblot (**B**); the different isotypes were visualized using anti-human IgG and IgE antibodies conjugated to alkaline phosphatase.

SDS-PAGE and immunoblotting of proteins isolated from culture supernatants of stably transfected HEK-293 cells showed apparent molecular masses in the expected range of 120 kDa, 150 kDa and 200 kDa for the homodimeric and heterotetrameric IgG and IgE antibodies suggesting that the secreted antibodies are properly folded and glycosylated, in particular the extensively glycosylated IgE (Fig. 3.7).

3.2.2 Characterization of the carbohydrate-specific antibody isotypes

Characterization of the recombinant proteins was pursued by different approaches to demonstrate both antigen and receptor binding. All antibodies detected alpha-Gal carrying thyroglobulin, cetuximab (n.d. for IgG), and HSA-conjugate in ELISA (Fig. 3.8A). Notably, the biotinylated Gal-alpha-1,3-Gal disaccharide exhibited no interaction, a fact that might reflect the specific sterical requirements of antibody binding (data not shown).

To confirm proper folding and glycosylation of the Fc domains, Ig Fc receptor extracellular domains of the ligand-binding alpha-chains of the human high affinity receptors FcεRI and FcγRI (CD64) fused with chicken IgG (IgY) Fc domains were used [129]. Recombinant IgE as well as IgG specifically bound to their soluble Fc receptors (Fig. 3.8B).
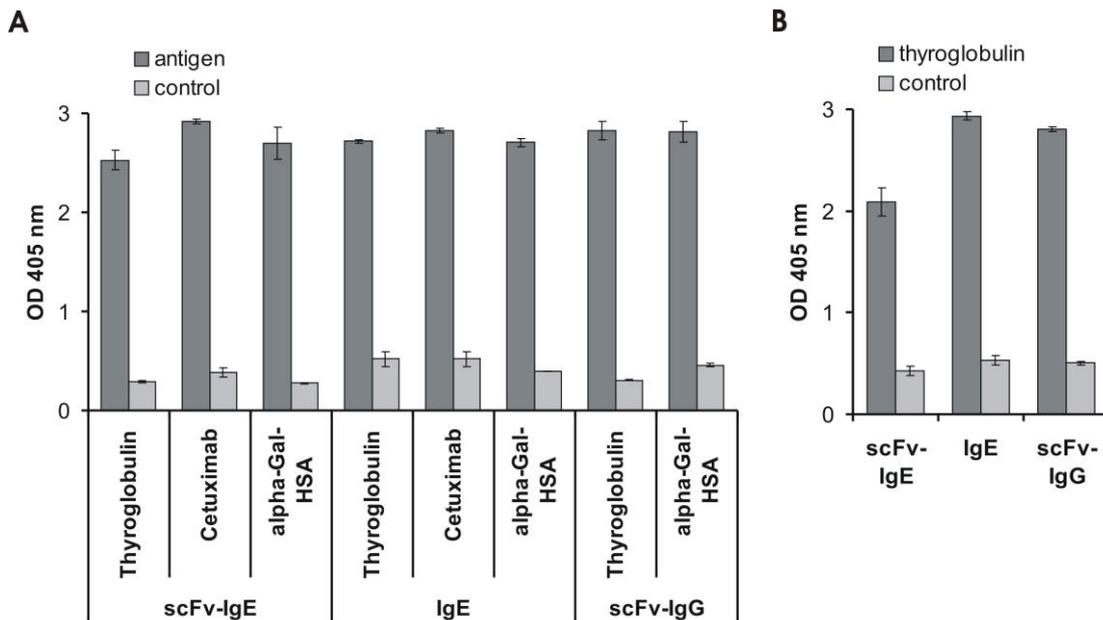
Fig. 3.8: Immunoreactivity with allergens and Fc receptor molecules. A: The immunoreactivity of the recombinant human antibodies was assessed in ELISA using thyroglobulin, cetuximab and alpha-Gal-HSA for the scFv-IgE and IgE and thyroglobulin and alpha-Gal-HSA for the scFv-IgG, and isotype specific antibodies conjugated to alkaline phosphatase. **B:** Simultaneous binding to the allergen and Fc receptors was performed as in A, but using the particular high affinity Fc receptors and anti-chicken IgG conjugated to alkaline phosphatase for detection.

These data underline that the recombinant isotypes reflect the natural interaction with both the antigen and the cellular receptors and, therefore, should mediate comparable effects *in vivo*.

SPR-Analyses using immobilized IgE and cetuximab, alpha-Gal-HSA, and thyroglobulin as analytes resulted in dissociation constants in the nanomolar range (Fig. 3.9) which is in the range for both natural anti-Gal antibodies and carbohydrate-specific binders [162, 163].

SPR analyses of the TNP-specific murine IgE C38-2 used below demonstrated affinities in a similar range. The HSA conjugate however showed a higher dissociation constant than the other alpha-Gal carrying proteins, a finding that might reflect the reduced sterical accessibility also seen for the biotinylated disaccharide and an impact of the missing third unit.
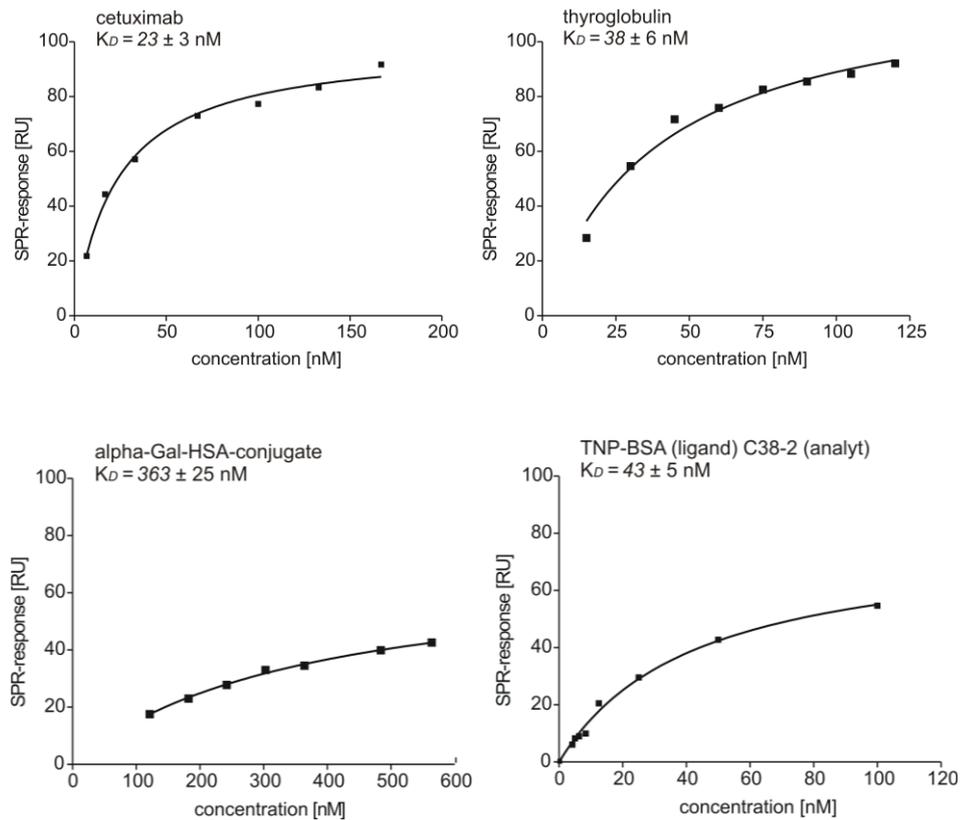
Fig. 3.9: SPR analyses of different alpha-Gal carriers. The dissociation constant KD was determined for alpha-Gal-HSA, thyroglobulin and cetuximab as analytes and the scFv-IgE as immobilized ligand as well as for the TNP-specific C38-2 as analyte and TNP-BSA as immobilized ligand. The concentration dependent curves were analyzed by the one-site-binding-model.

This finding also suggests that the valency of the alpha-Gal (cetuximab<alpha-Gal-HSA <thyroglobulin) and the molecular architecture of bivalent cetuximab does not significantly favour interaction.

3.2.3   Assessment of the potential for cellular activation

IgE-mediated cross-linking of the FcεRI and degranulation of RBL-SX38 cells was assessed by determination of β-hexosaminidase release (Fig. 3.10). Thereby, the medium affinity IgE C38-2 was used as activation control. Both anti-Gal IgE formats bound to the FcεRI and induced mediator release in an antigen-independent manner using anti-IgE antibodies to an extent comparable to the murine reference (Fig. 3.10A). Allergen-dependent cellular activation for monoclonal IgE usually is difficult to achieve, since in most cases only one epitope per allergen is available. Here, a multivalent molecule bearing at least two independent alpha-Gal epitopes is needed to bridge two identical IgE paratopes. We addressed this approach for the anti-Gal IgE using a broad panel of different alpha-Gal carrying proteins including alpha-Gal-HSA conjugate, thyroglobulin, and cetuximab and in parallel alpha-Gal microspheres generated by biotinylated

thyroglobulin clustered on streptavidin-coated particles (Fig. 3.10B). In contrast to the reference which also was cross-linked using a multimeric TNP-BSA-conjugate, neither the different alpha-Gal proteins nor alpha-Gal microspheres were able to induce significant mediator release (Fig. 3.10).
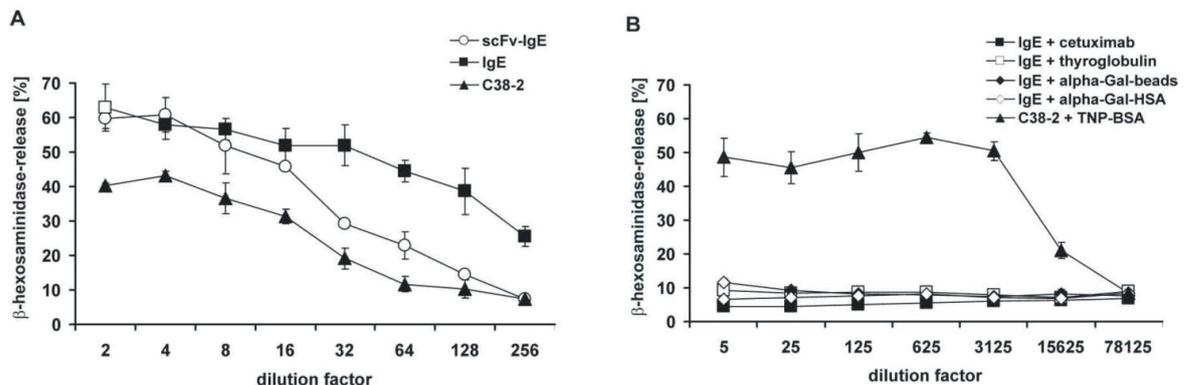
Fig. 3.10: Mediator release of humanized RBL-SX38 by alpha-Gal. RBL-SX38 cells providing the human FcεRI were sensitized with the scFv-IgE, IgE and the TNP-specific mouse IgE C38-2 as control (**A**). Antigen-independent degranulation was induced by the addition of anti-huIgE and anti-muIgE, respectively (**A**). Antigen-dependent degranulation was induced by the addition of different alpha-Gal carrying proteins, including biotinylated thyroglobulin immobilized to streptavidin coated beads, and by the addition of TNP-labeled BSA for the control (**B**). Degranulation was monitored by β-hexosaminidase activity released into culture supernatants. Data are mean +/- SD of triplicate measurements.

These data suggest that the alpha-Gal IgE exhibits intrinsic potential to cross-link the FcεRI and activate effector cells, which is not deployed in an antigen-dependent manner suggesting an impact of affinity or spatial organization on alpha-Gal-mediated anaphylaxis.

3.2.4 Epitope analysis

Variant specificities for alpha-Gal and distinct modes of binding are reported for different lectins and antibodies [164], but structural data remain scarce. Here, we aimed for a direct monitoring of the interplay of anti-Gal antibodies with the cognate antigen on a molecular level and comparability of the monoclonal IgE to polyclonal serum antibodies.

Interaction of the monoclonal chimeric IgE with the disaccharide Gal-1,3-Gal-OMe was followed by STD NMR (Fig. 3.11).
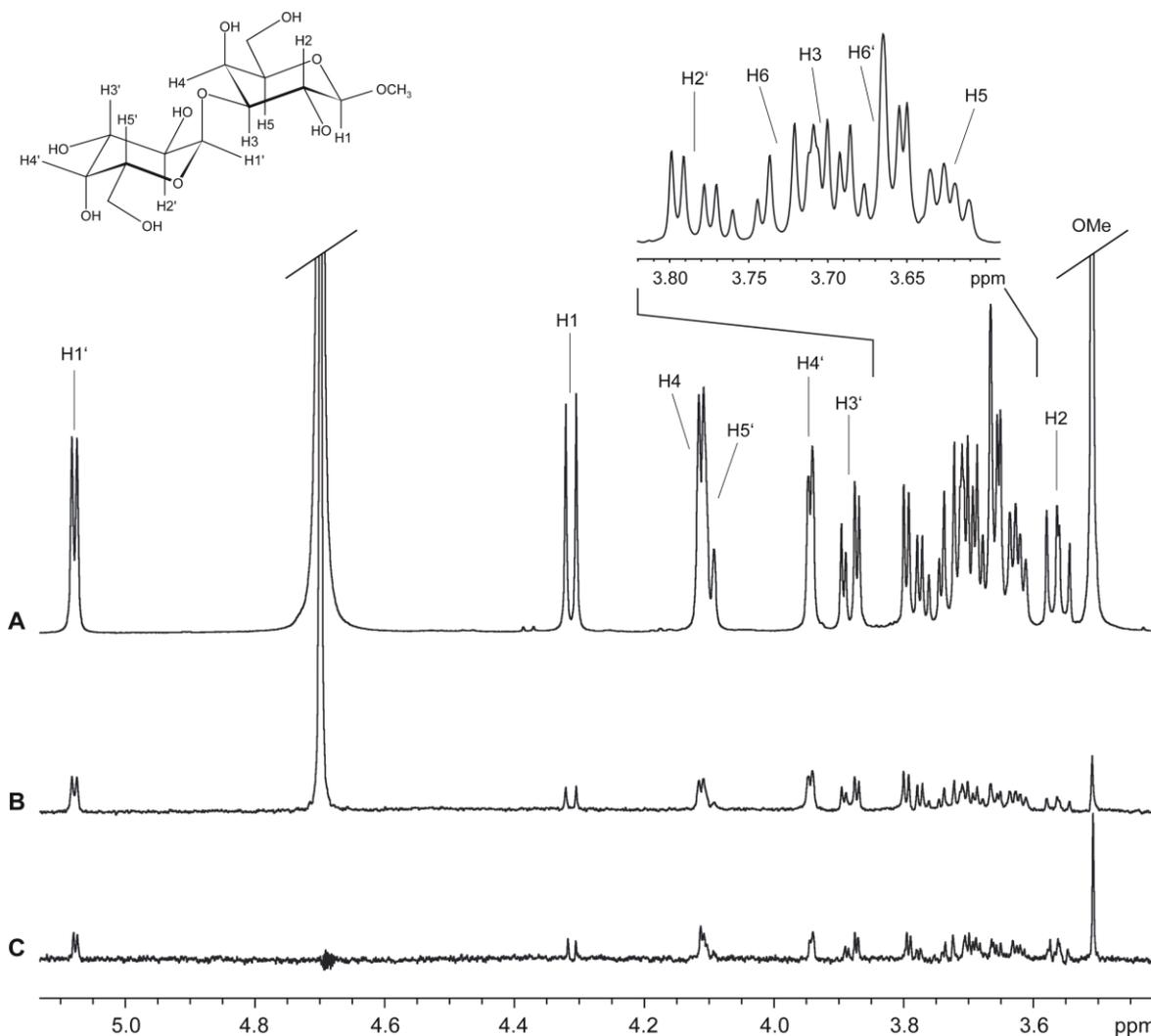
Fig. 3.11: Observation of STD signals for the alpha-Gal disaccharide. (**A**) Reference 1H NMR spectrum and (**B**) STD NMR spectrum of Galα1,3GalβOMe in the presence of the M86-based IgE (molecular ratio = 260:1). (**C**) STD NMR spectrum of Galα1,3GalβOMe in the presence of polyclonal antibodies purified from human serum (molecular ratio = 200:1). Spectrum B has been scaled by factor 5 of its original intensity. All spectra were recorded at a temperature of 298 K and at 500 MHz with 1024 scans (A and B), and at 700 MHz with 2048 scans, respectively (**C**). STD spectra in the figure are not artifact referenced.

Clear STD effects could be obtained for the carbohydrate ligand. The interaction foot-print of the M86-based IgE on Gal-1,3-Gal-OMe reveals strong interaction with both galactose residues (Fig. 3.12A). Major contacts of the terminal galactose are defined by the C3 proton having the highest STD signal and to a high extent the C2, and C4 protons. The C5, C1 and C6 protons also show medium STD effects. Although evident for nearly protons effects lower than 50 % were not included for reasons of reliability.
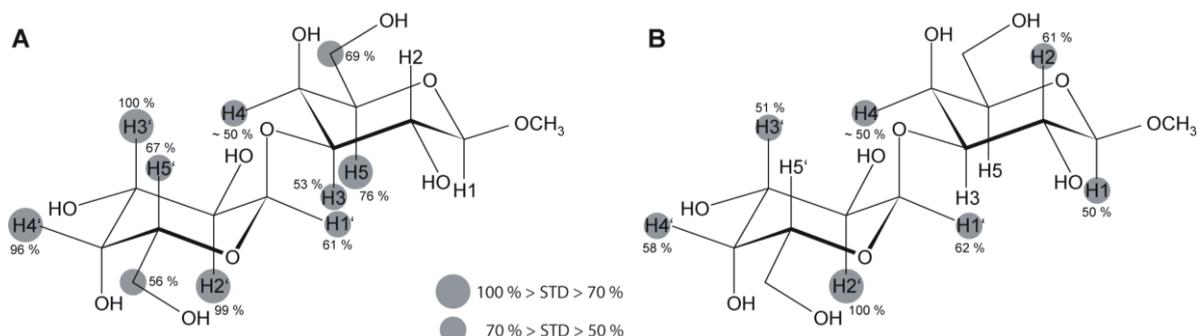
Fig. 3.12: Interaction footprint as assessed by STD NMR. Interaction footprint of Galα1,3GalαOMe binding to M86-based IgE (**A**) and polyclonal antibodies purified from human serum (**B**). Circles represent the relative size of the saturation transfer and reflect the vicinity of individual protons to protons in the binding pocket of the immunoglobulin as macromolecular binding partner. The closer the ligand protons are to protons of the binding pocket, the larger is the saturation transfer. Proton H4 could not be determined with high accuracy due to resonance overlap.

For the adjacent galactose the major contacts are the C5 proton and the C6, C3, and C4 with all other protons showing medium STD effects. The methyl glycoside contributes weakly only (Fig. 3.12A).

In parallel, polyclonal immunoglobulins were purified from serum of a donor having elevated serum IgE and IgG with specificity for alpha-Gal using thyroglobulin affinity chromatography. Enrichment of immunoglobulins specific for thyroglobulin was documented by ELISA (Fig. 3.13).
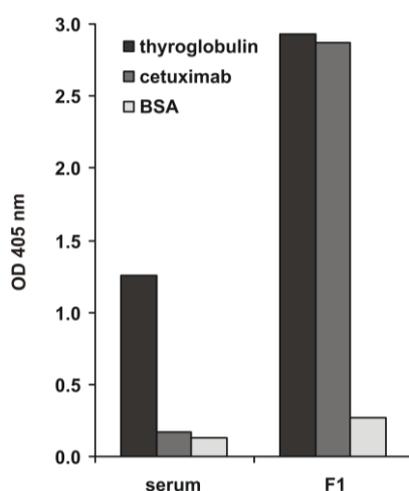
Fig. 3.13: Anti-Gal purification by affinity chromatography. The anti-Gal immunoreactivity of human serum and immunoglobulins purified by thyroglobulin affinity chromatography was assessed by ELISA using thyroglobulin or cetuximab and anti-human IgE antibodies conjugated to alkaline phosphatase.

Observed enrichment of anti-Gal antibodies with specificity for cetuximab suggests that the recognized moiety in the immunoglobulin preparation corresponds to those responsable for IgE-based alpha-Gal interaction. These antibodies then were subjected to STD NMR using the disaccharide Gal-1,3-Gal-OMe (Fig. 3.11).

Intriguingly, the interaction foot print of the polyclonal immunoglobulins reveals strong interaction with both galactose residues in a manner highly similar to that of the monoclonal antibody (Fig. 3.12A, B). Major contacts of the terminal galactose are defined by the C2 proton having the highest STD signal and the C3 and C4 protons. For the adjacent galactose major contact is the C2 proton followed by C1 showing medium STD effects. As above, weaker effects seen for most protons but lower than 50 % were not included for reasons of reliability. As for the monoclonal antibody the methyl glycoside contributes to a slightly increased extent only.

These findings verify that the Gal-Gal disaccharide block is crucial and sufficient for the epitope of the monoclonal M86 based IgE as well as human polyclonal antibodies present in the circulation. The glycosidic methyl group representing the downstream residue (GlcNAc or Glc) seems to contribute weakly.

3.3 Evaluation of Different Glycoforms of Honeybee Venom Major Allergen Phospholipase A2 (Api m 1) Produced in Insect Cells

Anaphylaxis due to honeybee (*Apis mellifera*) venom is one of the most severe clinical outcomes of IgE-mediated hypersensitivity reactions. Although venom immunotherapy is effective in the majority of patients the occurrence of systemic side effects in 20-40 % of treated individuals [165] and the failure of therapy in 10-20 % of cases [166] demands a component-resolved approach to HBV allergy. Since the use of native allergens is often hampered by means of quantity and purity recombinant allergens are increasingly introduced into diagnostic and therapeutic applications [167]. Moreover, the recombinant availability is a prerequisite for the rational design of hypoallergenic variants and molecules with defined characteristics such as proper folding and glycosylation and concurrent lack of cross-reactive carbohydrate determinants which still represent a challenge for adequate allergy diagnosis. Defining glycosylation of allergens can have intriguing consequences for our understanding of IgE reactivities reported previously for the hyaluronidases [168].

HBV is a complex mixture of biogenic amines, peptides, toxins and proteins, with most of them being enzymes. Many of the proteins and polypeptides in HBV have been reported to be allergens including phospholipase A2 (Api m 1) [169], hyaluronidase (Api m 2) [170],

acid phosphatase (Api m 3) [171], melittin (Api m 4) [172], dipeptidylpeptidase IV (Api m 5) [133], Api m 6 polypeptides (4 isoforms) [142], a CUB-serine protease (Api m 7) [173], a carboxylesterase (Api m 8), a carboxypeptidase (Api m 9) [174], and the Carbohydrate-rich protein (Api m 10), a component of unknown function [143].

HBV phospholipase A2 (Api m 1) was early identified as the major venom allergen [169]. Based on the fact that 97 % of HBV allergic patients have Api m 1 specific IgE antibodies Api m 1 was extensively studied as model allergen for genetic engineering and specific immunotherapy [120]. Moreover, Api m 1 is considered an ideal marker allergen since it is structurally unrelated to the phospholipase A1 (Ves v 1) in vespid venom [175].

In general, phospholipases constitute a major component of various venoms, e.g. from snakes, bees, wasps and spiders. The PLA2 of *A. mellifera* has been sequenced from a venom gland cDNA library [175] and encodes a signal peptide of 18 amino acids, a propeptide of 15 residues, and a major peptide of 134 residues [176], the catalytic activity and crystal structure of which is well documented [177-179]. The mature peptide contains 10 cysteine residues forming 5 disulfide bonds [180] and an oligosaccharide attached to asparagin 13 [81].

PLA2 (EC 3.1.1.4) from HBV is a typical group III secreted PLA2 [181], representing about 12 % of the dry weight of the venom [182]. The cytotoxic effects of PLA2 rely on its catalytic activity which leads to cleavage of phospholipids at the SN2 acyl chain and damage of structural membranes [182]. The enzyme in HBV is structurally not related to the phospholipases A1 in vespid venoms, and therefore, not cross-reactive. In contrast to unglycosylated vespid PLA1, PLA2 of honeybees exhibits CCD (cross-reactive carbohydrate determinant)-reactivity [183] which is essentially based on the presence of α-1,3-core fucose and can lead to results with no or only low clinical relevance in diagnostic tests and therefore represents a challenge for diagnostic approaches [154].

The aim of the present study was an improved production of soluble HBV PLA2 in glycosylation-competent insect cells. Therefore, we generated an inactive mutant form of PLA2 by site-directed mutagenesis, which apart from an improved expression behavior should exhibit a reduced cytotoxicity in cellular assays. We were able to produce both allergens as soluble and secreted proteins in high yields by baculovirus-based expression in Sf9 (*S. frugiperda*) as well as HighFive (*T. ni*) insect cells. By using cell lines with a varying capacity for α-1,3-core fucosylation, and, additionally, by generation of a non-glycosylated mutant form, we provided the evidence for reduced IgE cross-reactivity and improved performance of the resulting PLA2 molecules in diagnostic approaches to hymenoptera venom allergy.

3.3.1   cDNA cloning and recombinant expression in insect cells

For recombinant production the cDNA of the HBV allergen Api m 1 was amplified from whole *A. mellifera* venom gland cDNA. To avoid potentially detrimental effects of phospholipase activity on expression yields or on mediator release in cellular assays the inactive mutant Api m 1 H34Q was generated by site directed mutagenesis. To address the impact of glycosylation of the recombinant allergen on the IgE reactivity of sera derived from hymenoptera venom allergic patients an additional mutant version of Api m 1, Api m 1 H34Q N13Q, devoid of the singular glycosylation site was generated. Subsequently, both epitope tagged proteins were produced by baculovirus-based infection of insect cells and secretion of the proteins into the cellular supernatant. The glycosylated version Api m 1 H34Q was expressed in Sf9 (*S. frugiperda*) cells and additionally in HighFive (*T. ni*) cells, which in contrast to Sf9 cells provide α-1,3-core-fucosylation of N-glycans, the structure responsible for CCD-based cross-reactivity. The mutant version Api m 1 H34Q N13Q lacking the relevant asparagine and, therefore, any glycosylation was produced in HighFive cells.

The rApi m 1 versions were obtained with yields of approx. 5-10 µg/ml of culture supernatant without further optimization of culture conditions. SDS-PAGE analysis and Coomassie staining (Fig. 3.14A) of purified rApi m 1 H34Q produced in Sf9 as well as in HighFive insect cells showed three distinct bands in the range of approx. 18 to 22 kDa, a molecular mass to which the C-terminally fused V5 epitope and 6 fold His tag contribute 3 kDa. The Sf9-expressed purified Api m 1 H34Q showed a double band of comparable intensity and a third, less intense band of lower molecular weight. In contrast, for the HighFive-produced Api m 1 H34Q the protein with the highest molecular weight represents the predominant product, while the product with the lowest molecular weight is only barely visible. Applying an antibody directed against the V5 epitope (Fig. 3.14B) all bands of the Sf9-produced rApi m 1 H34Q were detected, a finding which is in good accordance with the distribution observed in Coomassie staining. The lowest molecular weight band of the HighFive-expressed rApi m 1 was not visible, most likely due to its reduced quantity. Native purified Api m 1, however, shows a similar distribution in Coomassie staining, whereas in HBV only the two higher molecular weight bands were visible. Thereby, the highest molecular weight versions represent the predominating forms. Non-glycosylated rApi m 1 H34Q N13Q appears as a single band of approx. 19 kDa (Fig. 3.14A). Application of the sensitive V5 epitope antibody, however, enabled detection of a faint double-band (Fig. 3.14B).
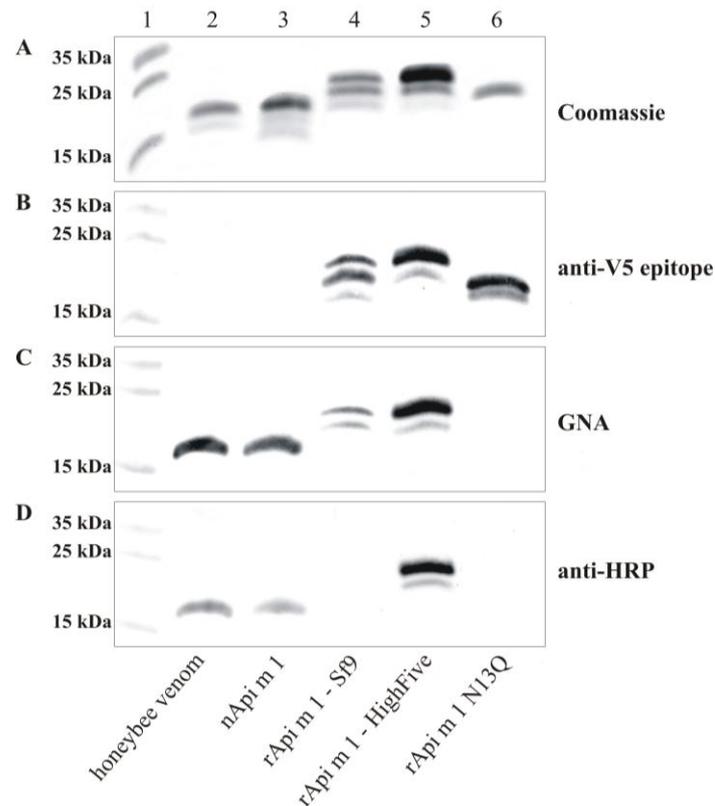
Fig. 3.14: Characterization of native and recombinant HBV phospholipase A2. Honeybee venom, native purified PLA2, recombinant PLA2 H34Q from Sf9 cells, recombinant PLA2 H34Q from HighFive cells, and recombinant PLA2 H34Q N13Q from HighFive cells were subjected to SDS-PAGE (10 % acrylamide) and analyzed by Coomassie staining and Western blot. **A:** Coomassie brilliant blue staining, B-D: Western blot analysis applying a monoclonal antibody directed against the V5 epitope C-terminally fused to the recombinant PLA2 versions (**B**), Galanthus nivalis agglutinin recognizing terminal mannose, 1,2-, 1,3- and 1,6-linked to mannose (**C**), and polyclonal HRP-antiserum produced in rabbit with specificity for α-1,3-core fucose (**D**).

Galanthus nivalis agglutinin (GNA) recognizes terminal mannose, 1,2-, 1,3- or 1,6-linked to mannose, a structure which is present in native Api m 1 as well as in glycans derived from lepidopteran insect cells. Thus, GNA reactivity indicates the presence of N-linked glycans. In immunoblot analysis of rApi m 1 H34Q (Fig. 3.14C) GNA reacted with the two higher molecular weight bands proving the lowest molecular weight form to be non-glycosylated, whereas in HBV and nApi m 1 only the highest molecular weight product is recognized. Thereby, the missing reactivity with rApi m 1 H34Q N13Q verified the successful substitution of the N-glycosylation site.

Apart from proteinic epitopes anti-HRP rabbit serum (Fig. 3.14D) is specific for plant-derived glycostructures including α-1,3-core fucose and β-1,2-xylose, the causative structures for CCD-based cross-reactivities. Its use in immunoblot revealed pronounced α-1,3-core fucosylation for rApi m 1 H34Q produced in HighFive cells as well as for native Api m 1. In contrast, rApi m 1 H34Q produced in Sf9 cells did not exhibit any CCD-based reactivity.

3.3.2 Immunoreactivity of rApi m 1

Reactivity of sera from patients with a clinical history of insect venom allergy was assessed by ELISA for specific IgE antibodies to the recombinant and native versions of Api m 1 (Fig. 3.15). MUXF-HSA, a conjugate that provides the core-fucosylated glycotope from pineapple stem bromelain was included as a marker for sIgE directed against CCDs. To illustrate the distinct recognition patterns observed in insect venom sensitized patients the sIgE-reactivity of selected sera is shown.
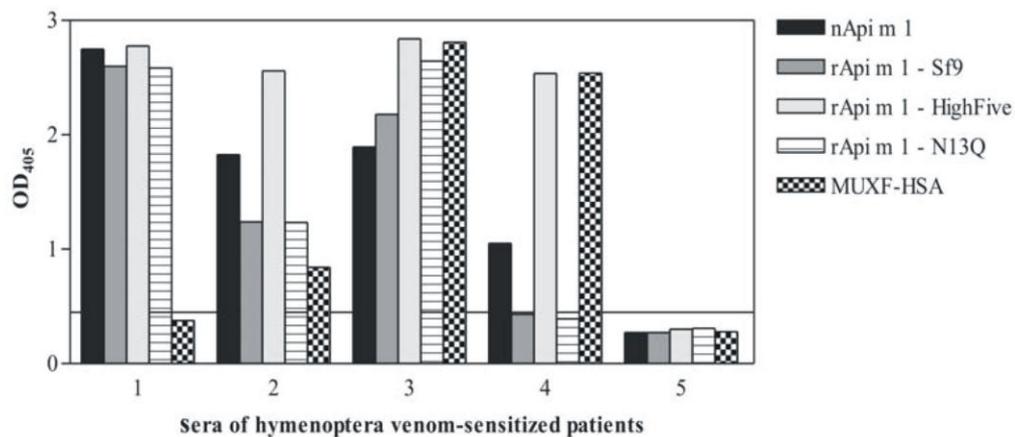
Fig. 3.15: IgE reactivity of individual patient sera with native and recombinant PLA2. The IgE reactivity of individual sera from hymenoptera venom-sensitized patients with native and recombinant PLA2 versions, as well as with the MUXF-HSA conjugate was assessed in ELISA as described in the Methods section. The lower end functional cut-off is represented by a solid line.

Serum 1 specifically recognized proteinic epitopes of Api m 1 as indicated by the missing reactivity with the CCD marker MUXF-HSA. All recombinant versions of Api m 1 and native Api m 1 showed comparable reactivity. In contrast, sera 2 and 3 exhibit reactivity with Api m 1 but apparently CCDs contribute to this binding as suggested by the reactivity observed with MUXF-HSA, and this contribution is at varying degrees according to the more pronounced signal with the fucosylated allergen in the case of serum 2. Serum 4 was reactive with native Api m 1 und HighFive-derived Api m 1 H34Q, but neither with the counterpart produced in Sf9 cells nor with non-glycosylated Api m 1, indicating exclusive CCD-reactivity, which is corroborated by the pronounced MUXF reactivity. Finally, serum 5 from a patient with a clinical history of YJV allergy neither recognizes proteinic epitopes of Api m 1 nor CCDs.

These data demonstrate that Sf9-derived glycosylated Api m 1 as well as non-glycosylated Api m 1 lack detectable α-1,3-core fucosylation which contributes to or is the exclusive reason for reactivity with sera having CCD-specific IgE antibodies.

3.3.3   Degranulation of rat basophilic leukemia (RBL) cells

Since native Api m 1 is described to induce mediator release from rodent and human basophils in an IgE-independent manner as a result of its catalytic activity, we tested the ability of nApi m 1 and Api m 1 H34Q to mediate degranulation of RBL cells, as a model system for effector cell degranulation (Fig. 3.16).
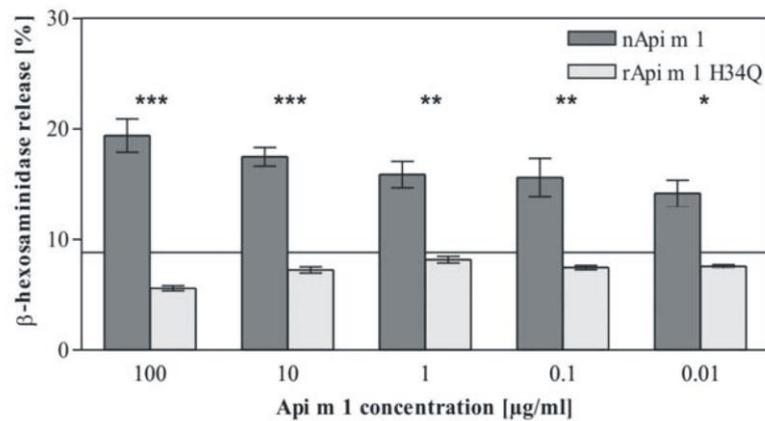
Fig. 3.16: IgE-independent activation of rodent basophils by active and inactive PLA2. Release of β-hexosaminidase from RBL-SX38 cells in response to native purified and recombinant inactive PLA2. The percent release is presented relative to total β-hexosaminidase content. All results were measured as triplicates and subjected to statistical analysis via student's t-test.

Upon incubation with nApi m 1 RBL cells showed significant β-hexosaminidase release over the entire concentration range from 0.01 to 100 µg/ml in a dose-dependent manner. In contrast, RBL cells incubated with inactive Api m 1 H34Q showed no degranulation when compared to negative controls.

These results confirm that the enzymatic activity of Api m 1 promotes IgE-independent effector cell activation.

3.4 Api m 10, a genuine *A. mellifera* venom allergen, is clinically relevant but underrepresented in therapeutic extracts

Although venom immunotherapy (VIT) is an effective treatment in the majority of patients, 10-20 % of patients were not protected by honeybee venom (HBV) immunotherapy [165, 166]. Thus, there is considerable interest in improving diagnosis as well as design, safety, and efficacy of therapy.

The most prominent HBV allergens include phospholipase A2 (Api m 1), hyaluronidase (Api m 2), and the basic peptide melittin (Api m 4) [172], all constituting medium to high abundance proteins of HBV [114]. Nevertheless, hymenoptera venoms comprise a more complex cocktail of different compounds all of which may contribute to sensitization, symptoms and success of VIT. Significant progress however has been made in the recent years to identify additional compounds of lower abundance such as the acid phosphatase Api m 3 [123], and the DPPIV enzyme Api m 5 [133].

Recombinant approaches are imperative for assessment of allergenicity and clinical relevance of such venom compounds, whereby expression should meet the requirements of proper folding and correct posttranslational modifications being potentially important for the establishment of conformational epitopes [184]. In particular glycan structures can contribute significantly to characteristics of venom proteins and constitute the underlying principle of cross-reactive carbohydrate determinants (CCD), a peculiarity of hymenoptera and plant allergens interfering with diagnosis and design of therapeutic strategies [151, 185]. A venom protein of considerable interest within this context is Api m 10, the carbohydrate-rich protein. Peptides of this protein were identified by two independent groups in 2005 [140, 186]. Insoluble, non-glycosylated protein obtained from *E. coli* exhibited IgE reactivity [143] and an inherent molecular lability. However, without established recombinant expression of soluble Api m 10, its relative abundance in whole HBV as well as its relevance in the context of sensitization and VIT remained elusive.

In this study we comparatively analyzed differentially glycosylated Api m 10 proteins for their IgE reactivity and basophil activation. The obtained data suggest a pivotal role of this protein as sensitizing agent in HBV allergy, thus supporting its status as a major allergen of clinical relevance in HBV. Furthermore, a monoclonal human IgE antibody allowed quantification of Api m 10 in *A. mellifera* venom, and also demonstrated the absence of this putatively essential component in widely used therapeutic preparations.

3.4.1   Recombinant expression and characterization of Api m 10

For recombinant expression of Api m 10, a putative venom allergen with a theoretical mass of 22 kDa [140, 186], the cDNA of splicing variant 2 was amplified from venom gland cDNA (Fig. 3.17).

```
Api m 10 V1   MKTLGVLFIAAWFIACTHSFPGAHDEDSKEERKNVDTVLVLPSIERDQMMAATFDFPSLS  60
Api m 10 V2   MKTLGVLFIAAWFIACTHSFPGAHDEDSKEERKNVDTVLVLPSIERDQMMAATFDFPSLS  60
              ************************************************************

Api m 10 V1   FEDSDEGSNWNWNTLLRPNFLDGWYQTLQSAISAHMKKVREQMAGILSRIPEQGVVNWNK  120
Api m 10 V2   FEDSDEGSNWNWNTLLRPNFLDGWYQTLQT----HMKKVREQMAGILSRIPEQGVVNWNK  116
              *****************************:      ************************

Api m 10 V1   IPEGANTTSTTKIIDGHVVTINETTYTDGSDDYSTLIRVRVIDVRPQNETILTTVSSEAD  180
Api m 10 V2   IPEGANTTSTTKIIDGHVVTINETTYTDGSDDYSTLIRVRVIDVRPQNETILTTVSSEAD  176
              ************************************************************

Api m 10 V1   SDVTTLPTLIGKNETSTQSSRSVESVEDFDNEIPKNQGDVLTA 223
Api m 10 V2   SDVTTLPTLIGKNETSTQSSRSVESVEDFDNEIPKNQGDVLTA 219
              ******************************************
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Fig. 3.17: Alignment of Api m 10 variants. Shown are Api m 10 splice variants 1 and 2. Peptides identified by mass spectrometry are highlighted in light grey. Signal sequences are italicized and putative glycosylation sites are represented in dark grey.

Expression in *E. coli* as aglycosylated protein [187] yielded soluble Api m 10 with an apparent molecular weight of approx. 35 kDa (Fig. 3.18A, B) suggesting a modified migration behavior due to its low pI. Glycosylated Api m 10 with or devoid of CCDs was produced by baculovirus-infection of *Trichoplusia ni* (HighFive) or *Spodoptera frugiperda* (Sf9) insect cells. Purification yielded recombinant Api m 10 with an apparent molecular mass of approx. 50 to 55 kDa (Fig. 3.18A, B) underlining the contribution of the carbohydrates to the overall characteristics.

In immunoblot, all three proteins were reactive with a monoclonal anti-Api m 10 IgE and a serum pool of HBV-sensitized patients (Fig. 3.18C, E). The use of an anti-HRP rabbit serum specific for α-1,3-core fucosyl residues verified pronounced CCD-based cross-reactivity for Api m 10 produced in HighFive cells. In contrast, glycosylated, Sf9-produced as well as *E. coli*-derived Api m 10 did not exhibit any CCD-reactivity (Fig. 3.18D). Comparable results were obtained with serum of a CCD-reactive but not HBV-allergic patient (Fig. 3.18F).
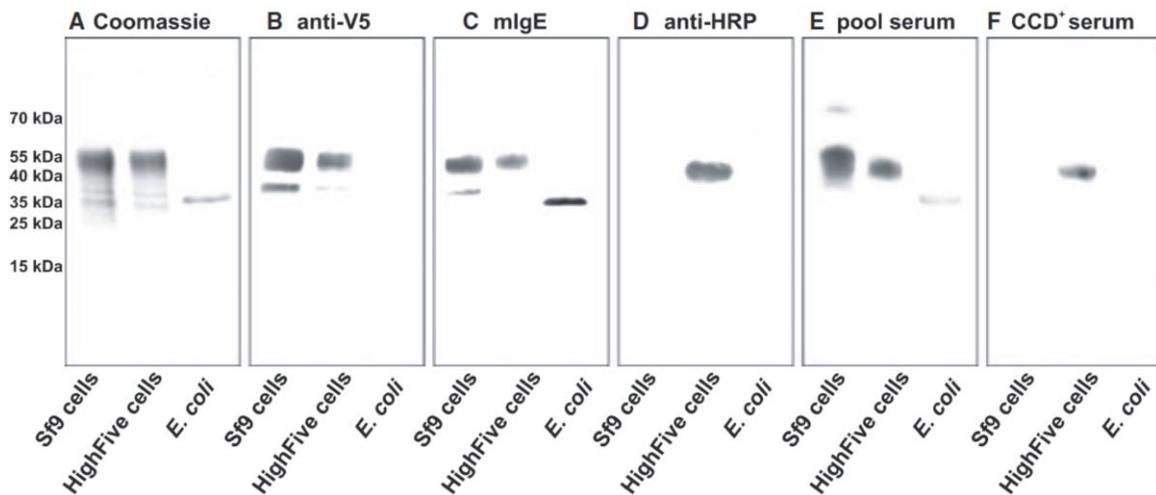
Fig. 3.18: Immunoreactivity of recombinant Api m 10 in Western blot. SDS-PAGE and immunoblot analysis of Api m 10 recombinantly produced in Sf9 and HighFive insect cells as well as in *E. coli* visualized by either Coomassie Blue staining or anti-V5 epitope antibody, monoclonal human anti-Api m 10 IgE antibody, anti-HRP antiserum, pooled sera of 5 HBV allergic patients and a CCD-positive serum.

ELISA analyses corroborated the data obtained in immunoblotting regarding protein identity and presence of CCDs (Fig. 3.19).
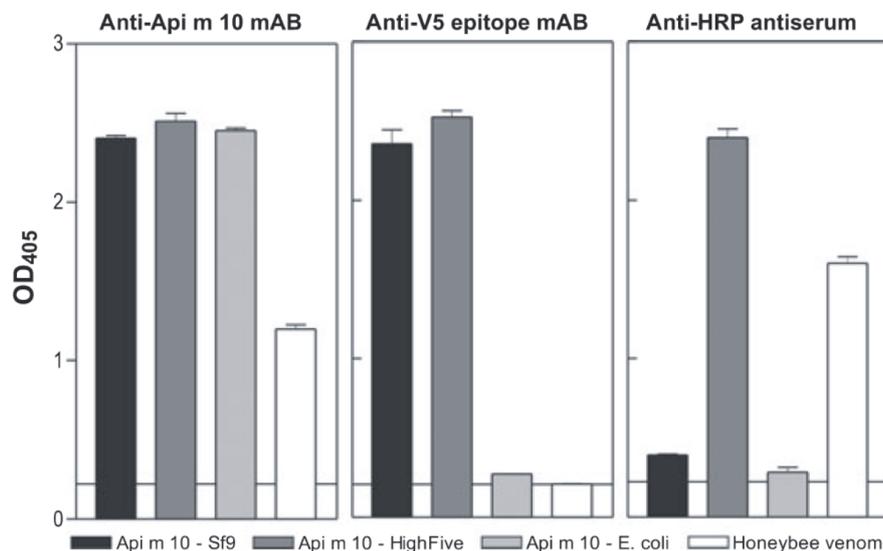
Fig. 3.19: Immunoreactivity of recombinant Api m 10 in ELISA. ELISA analysis of Api m 10 and HBV using the monoclonal anti-Api m 10 IgE, the anti-V5 epitope antibody, and the anti-HRP antiserum. Results are presented as triplicates.

These data demonstrate that the host defines the state of glycosylation [168] and, thereby, strongly influences the characteristics of the allergens.

3.4.2 Screening of patient sera for IgE reactivity with Api m 10 variants

Sera of 84 randomly selected patients with a clinical history of insect venom allergy were separated into three groups and assayed by ELISA for specific IgE antibodies to Api m 10 produced in Sf9 insect cells.

In group I of 51 sera double positive for HBV and YJV, thus, predominantly cross-reactive, 27 sera (52 %) exhibited pronounced reactivity with Sf9-derived Api m 10 (Fig. 3.20A). From group II of 17 sera with negative sIgE to yellow jacket venom (YJV) implying sensitization to HBV only without CCD reactivity, 8 sera (47 %) reacted with Api m 10 (Fig. 3.20B). In group III of 16 sera with negative sIgE to HBV but positive sIgE to YJV, none of the sera recognized Api m 10, suggesting the absence of a cross-reactive molecule in YJV (Fig. 3.20C). As anticipated, the presence of CCDs upon production of Api m 10 in HighFive insect cells resulted in augmented IgE reactivities in group I (data not shown).
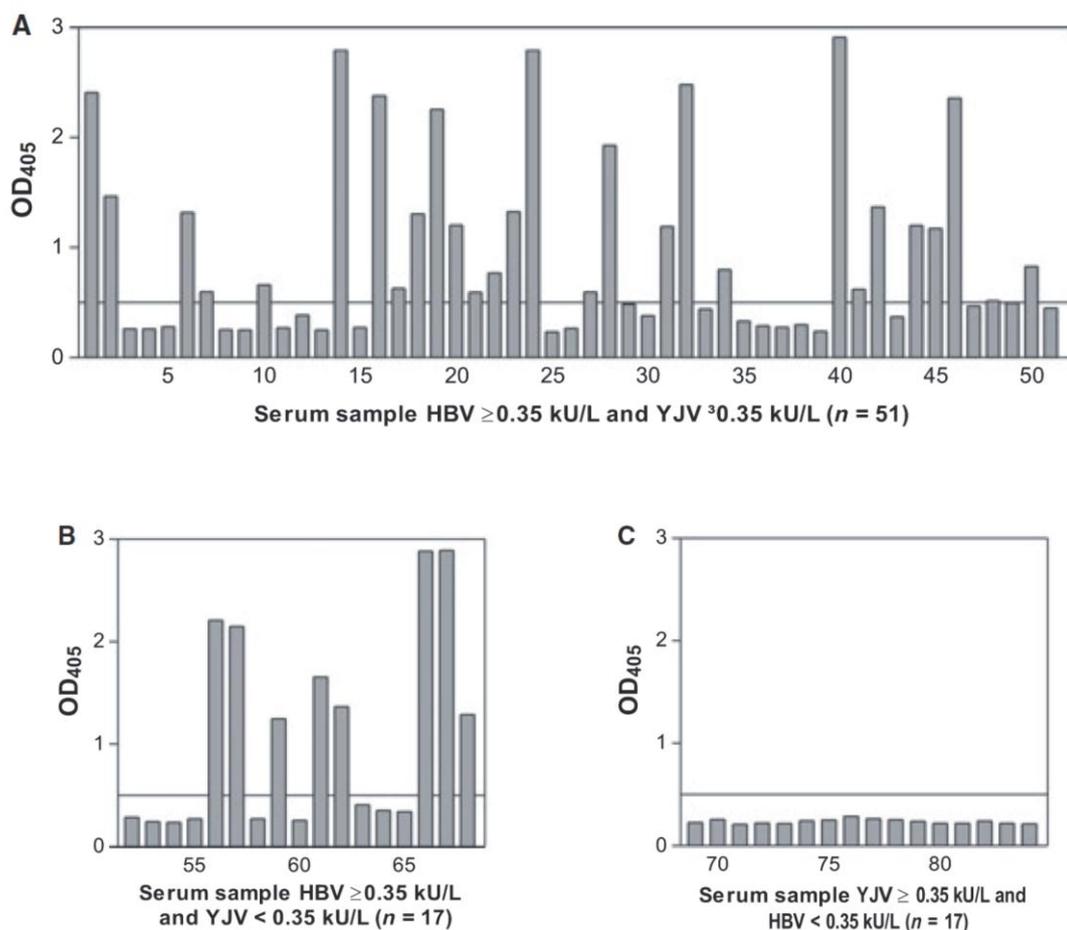
Fig. 3.20: IgE immunoreactivity of individual patient sera with recombinant Api m 10 produced in Sf9 insect cells. The IgE reactivity was assessed by ELISA with 17 sera of venom-sensitized patients with negative IgE to vespid venom (**A**), 16 sera of venom-sensitized patients with negative IgE to *A. mellifera* venom (**B**) and 51 double positive sera (**C**).

In comparative assessment of sIgE binding to Sf9- and *E. coli*-derived Api m 10 18/38 in group I (47 %) and 17/38 (44 %), respectively, exhibited pronounced reactivity with the protein variants (Fig. 3.21A), and in group II 5/11 sera (45 %) (Fig. 3.21B). Group III (Fig. 3.21C) exhibited no reactivity at all. Reactivities of selected sera found positive in ELISA (patient 16, 19, and 24, patient 69 as control) were further verified in immunoblot (Fig. 3.21E).
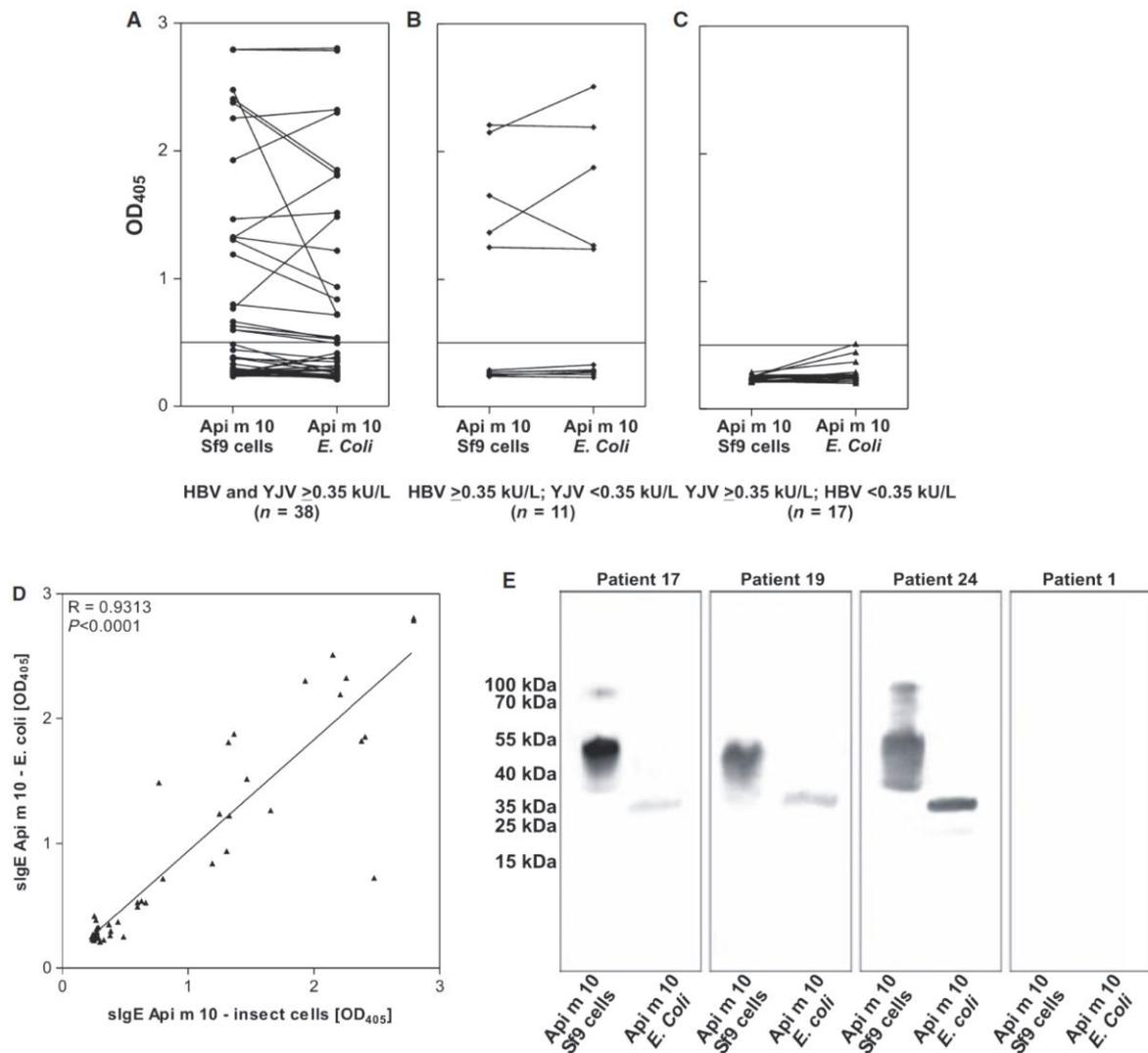
Fig. 3.21: IgE immunoreactivity of individual patient sera with Api m 10 produced in Sf9 insect cells and *E. coli*. The IgE reactivity was assessed with 38 double positive sera (**A**), 11 sera with negative IgE to vespid venom (**B**) and 16 sera with negative IgE to *A. mellifera* venom (**C**). Correlation was assessed by Pearson correlation analysis (**D**). In parallel, the reactivity of 4 particular sera with recombinant Api m 10 was assessed in immunoblot (**E**).

Moreover, sera of beekeepers as well as of venom-allergic patients were investigated for Api m 10- and Api m 1-specific IgG1, IgG4, and IgE antibody responses (Fig. 3.22).
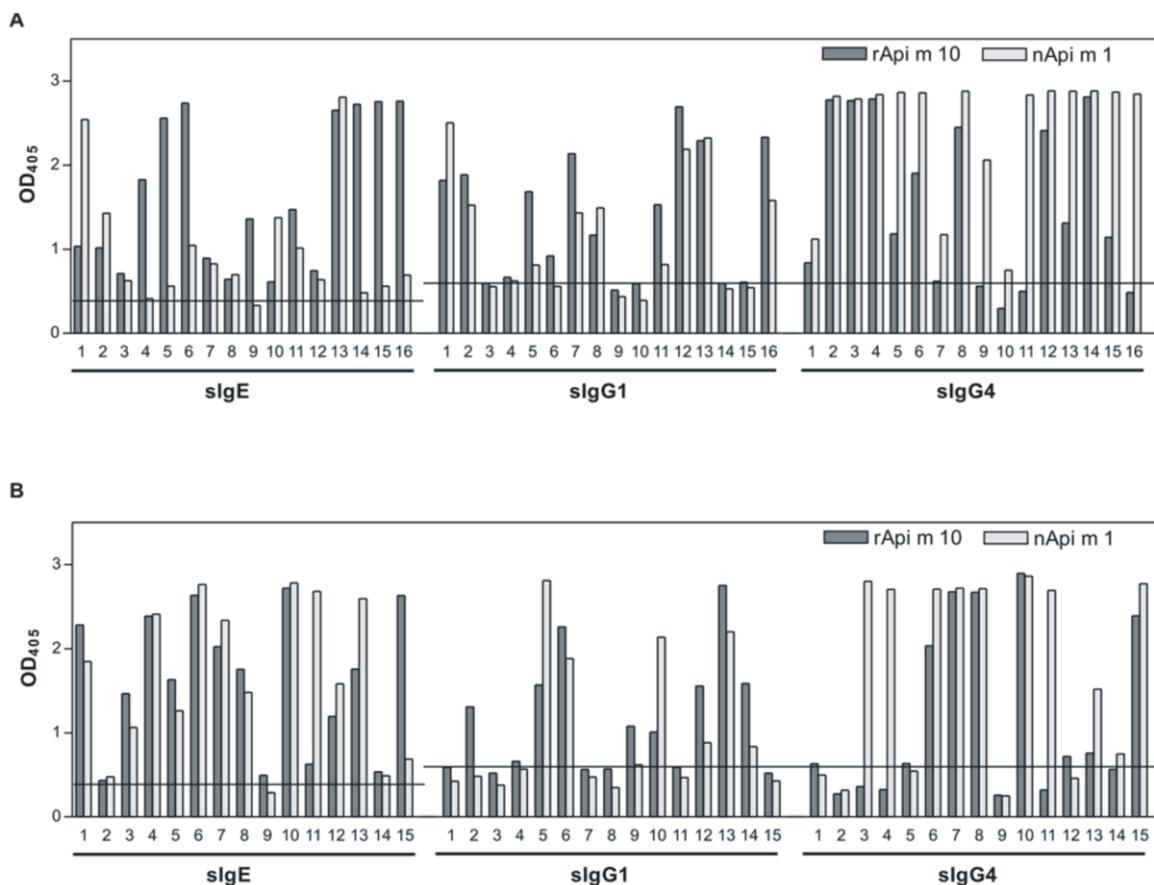
Fig. 3.22: Serological characterization of beekeepers and patients assessed in basophil activation test. The sIgE, sIgG1, and sIgG4 levels for rApi m 10 and nApi m 1 of beekeeper sera (**A**) as well as of sera from hymenoptera venom allergic patients assessed in basophil activation test (**B**) were determined by ELISA as described in the methods section. The lower end functional cut-off indicated as lines was calculated as the mean of the negative controls plus 2 SDs. Reactivities only slightly higher than the cut-off were excluded.

Thereby, over 50 % of beekeepers showed relevant IgG1 und IgG4 immune responses to Api m 10, a picture that although less pronounced was also evident for the venom-allergic patients.

3.4.3 Activation of basophils from venom-allergic patients

The capability of Api m 10 produced in HighFive insect cells or *E. coli* for activation of human basophils was assessed and related to that of established major allergens, Api m 1 (or Ves v 5). All blood samples were obtained from consecutively selected patients (n=15) with a clinical history of a severe reaction after a stinging event (13 with honeybee as relevant insect, 2 additional patients with yellow jacket as relevant insect). All patients had a positive intradermal skin test and sIgE test for HBV and/or YJV.

Overall, 8/13 patients with honeybee as relevant insect showed positive basophil activation with Api m 10. With the species-specific major allergen Api m 1 or Ves v 5, 14/15 patients had positive basophil activation. Fig. 3.23A-F depicts representative results for 6 exemplary patients.
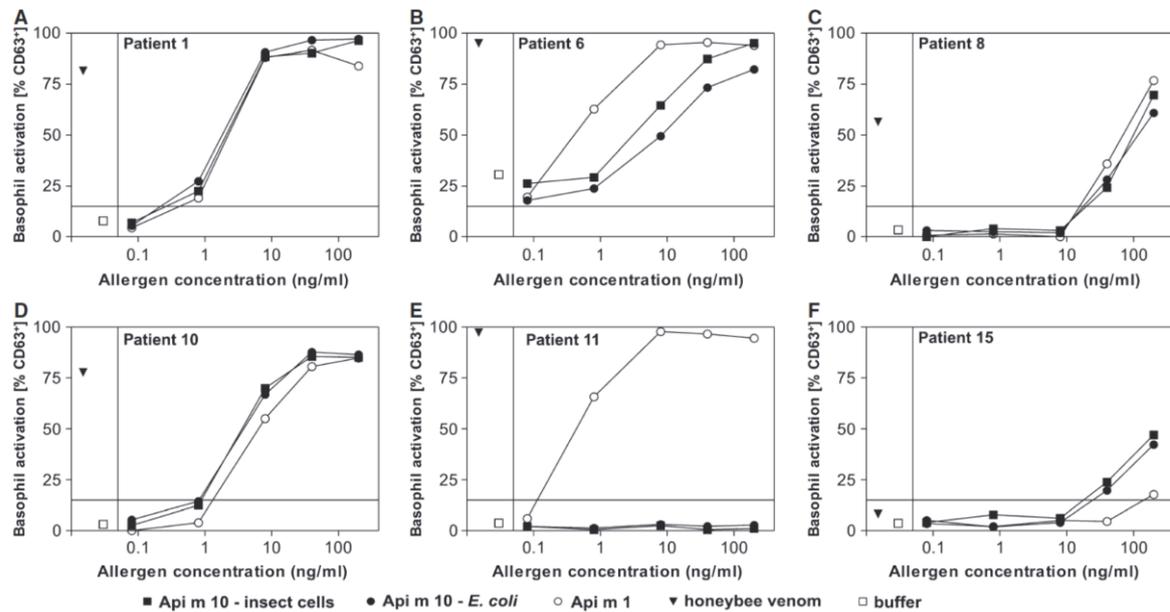
Fig. 3.23: Basophil activation tests with recombinant Api m 10. Human basophils from six exemplary HBV-sensitized patients were exposed to serial dilutions of Api m 10 produced in either insect cells (filled squares) or *E. coli* (filled circles) and Api m 1 as established reference allergen (open circles). Additionally stimulation with HBV (filled triangles) and plain stimulation buffer (open circles) is shown. Activation is shown as percentage of CD63$^+$ cells.

Patients 1, 8, and 10 showed comparable basophil activation (Fig. 3.23A, C, D) for Api m 10 and Api m 1, patient 6 (Fig. 3.23B) to a varying degree. Patient 11 exhibited activation by Api m 1 exclusively (Fig. 3.23E). Patient 15 (Fig. 3.23F) exhibited activation for Api m 10, and strongly reduced for Api m 1, a picture fully reflected in the sIgE titers. Interestingly, the basophil activation was comparable for the aglycosylated and the glycosylated protein.

Together these results show that Api m 10 is able to induce relevant effector cell activation and thus has to be considered as an important allergen in *Apis mellifera* venom. Moreover, carbohydrates appear to contribute to the overall IgE reactivity to a minor extent only.

3.4.4   Evaluation of native Api m 10 in *Apis mellifera* venom

To clarify the molecular integrity and concentration of Api m 10 in the native HBV and in preparations used for VIT a monoclonal human IgE antibody was generated (Fig. 3.24), the reactivity of which with Api m 10 was verified in ELISA and immunoblot (Fig. 3.18, Fig. 3.19).
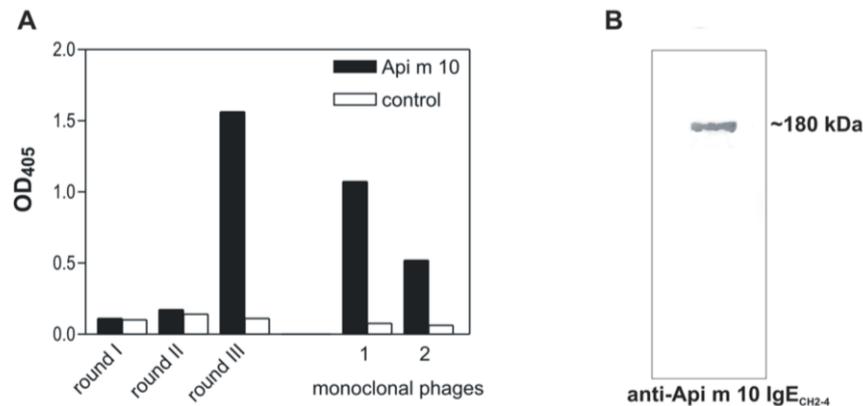
Fig. 3.24: Generation and expression of a monoclonal human IgE by selection of a combinatorial library. The immunoreactivity of phage-displayed polyclonal phages from three panning rounds and selected monoclonal phages against Api m 10 was analyzed by ELISA. Controls (white bars) were performed by omission of antigen (**A**). After conversion of the antibody gene into the format of a monoclonal human IgE antibody, clone 1 was produced in HEK293 cells and detected in cellular supernatant by using monoclonal anti-human IgE-AP conjugate as described in Material and Methods (**B**).

A human monoclonal IgE antibody with specificity for acid phosphatase (Api m 3) was applied analogously (Fig. 3.25A, lower panel).
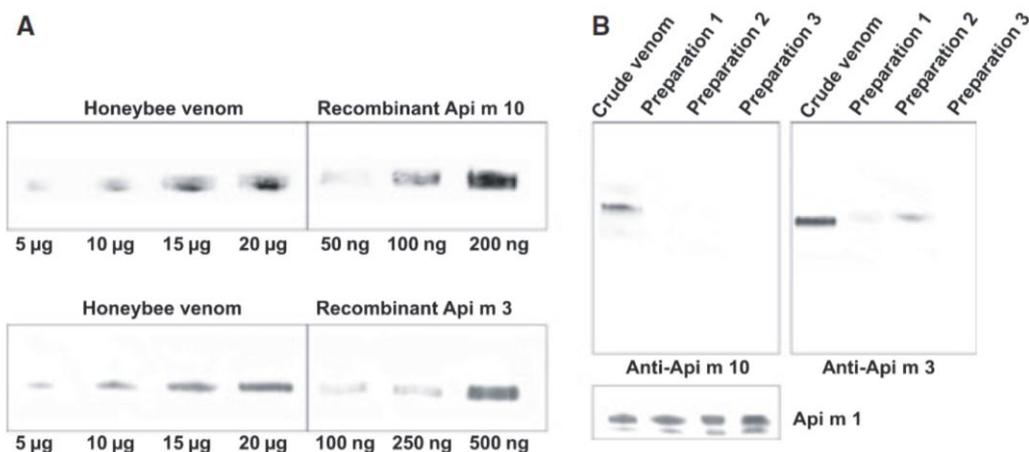
Fig. 3.25: Quantification of Api m 10 in HBV and therapeutic venom extracts. Concentration of Api m 10 in HBV was assessed by densitometric analyses of serial dilutions of recombinant Api m 10 and Api m 3 and in crude venom by immunoblotting using an anti-Api m 3 and an anti-Api m 10 IgE antibody (**A**). The presence of Api m 10 and Api m 3 in crude venom and venom preparations (each 25 µg per lane, Coomassie staining of Api m 1 was employed as additional loading control) was addressed analogously (**B**).

Applying this antibody in immunoblots of crude HBV a major band at 55 kDa was detected (Fig. 3.25A, upper panel).

To address the quantity of Api m 10 in HBV, the monoclonal IgE was applied to immunoblots providing serial dilutions of recombinant Sf9-derived Api m 10 and HBV (Fig. 3.25A, upper panel). Densitometric quantification suggested a concentration in the range of 8 ± 1 µg per g of crude HBV, corresponding to 0.8 % ± 0.1 % of dry weight compared with 1.7 % ± 0.4 % for Api m 3. Additionally, crude venom and three different HBV preparations for VIT from different allergen extract producers were analyzed (Fig. 3.25B, left panel). Api m 1 was used as a control for loading equal amounts (Fig. 3.25B, lower panel). In stark contrast to the crude venom, no reactivities at all were obtained for any of the three preparations. Api m 3 could be readily detected in whole HBV as well as in two of the preparations, although in significantly lesser amounts (Fig. 3.25B, right panel). These data demonstrate that the concentrations of the putatively labile HBV allergen Api m 10 and the already established and classical allergen Api m 3 are dramatically reduced in therapeutic venom preparations investigated in this study.

3.5   Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a

It is well established that the development of several immune-mediated diseases is linked to circulating concentrations of IgE, the antibody class responsible for allergic hypersensitivity [150]. IgE antibodies bound to their high affinity receptor (FcεRI) on mast cells and basophils mediate receptor cross-linking by allergens and trigger degranulation and release of proinflammatory mediators responsible for immediate-type hypersensitivity reactions. Long-term exposure to higher concentrations of allergens or therapeutic intervention by specific immunotherapy (SIT) results in a T-helper cell type-1 (Th1) shift in the immune response, leading to an increase in production of allergen-specific Ig, particularly of the IgG4 and IgA2 subclasses [188, 189]. These antibody isotypes are thought to exert their function by blocking the IgE/allergen interaction [190, 191], by recruitment of Fcγ receptors inhibiting FcεR mediated activation [192, 193], or by inhibition of IgE-facilitated allergen presentation [194]. Their activity may rely either on a molar excess or on affinity maturation during vaccination [195]. However, the exact interplay of antibodies with their cognate allergens still remains unclear.

50 % of patients suffering from type I allergy are sensitized to grass pollen proteins. These allergens are potent elicitors of clinical symptoms, such as rhinitis, conjunctivitis and asthma [196]. Phl p 5 represents one of the major pollen allergens of timothy grass

(*Phleum pratense*) and accounts for IgE binding in up to 60 % of patients [197]. Two isoforms, Phl p 5a and Phl p 5b exhibit high sequence similarity and differ only slightly in molecular masses and biochemical behaviour [198]. These isoproteins are composed of two alanine-rich (AR) repeats, which form four alpha-helices each and thereby, group 5 and 6 allergens represent the distinct protein class of 4-helix-bundle allergens [199].

Although the interaction of allergens with polyclonal serum-derived IgE has broadly been studied detailed analyses of the role of allergen-specific antibodies in pathophysiology as well as their interplay on a molecular level have been hampered by two critical limitations, the low IgE levels in serum and the lack of monoclonal allergen-specific antibodies of different isotypes. Approaches to generate human allergen-specific IgE-secreting hybridomas from immunized donors have not been successful so far [200]. Furthermore, conventional hybridoma technology is often limited by a low immunogenic potential of allergens [201] suggesting that allergens have less preferable binding sites for IgG antibodies compared to IgE antibodies. Additionally, resulting murine antibodies are neither compatible with established human specific assay formats nor recognize authentic IgE epitopes.

During the last decade sophisticated antibody technologies ranging from the generation of antibody fragment libraries to the design of tailor-made immunoglobulins have become tools for dissection of human immune responses. The establishment of combinatorial approaches has enabled the selection of monoclonal antibody fragments from synthetic library formats [202, 203] or immune repertoires against an almost unlimited panel of target molecules [204-206]. The former approach is particularly attractive for the generation of antibodies against the vast variety of allergenic molecules [138]. In contrast, the isolation of antibodies from libraries on the basis of lymphoid sources [207, 208] is high in effort due to the evanescent number of IgE-producing cells, but imperative for identifying allergy-related authentic antibodies. Hence, only scarce studies reported cloning of allergen-specific IgE antibody fragments [61, 161, 209].

The aim of our present work was to gain access to authentic allergen-specific human antibody isotypes allowing insights into the molecular basis of their interaction. Therefore, we employed antibody fragments of varying origin and produced recombinant IgE, IgA and IgG antibodies. On this basis the IgE epitope on the major timothy grass pollen allergen Phl p 5a was assigned and characterized by different techniques. The approach pursued here may facilitate the access to allergen-specific recombinant antibodies and contribute to the elucidation of the complex molecular interactions in allergy.

3.5.1   Generation of allergen-specific human antibody isotypes

For establishing fully human, authentic antibodies, the sequence of a Phl p 5a-specific Fab fragment selected from an immune library was used as template for gene synthesis [161]. In an alternative approach, a murine hybridoma line was generated using Phl p 5a as immunogen. Antibody purification and N-terminal sequencing yielded the information needed for cloning of the variable regions from hybridoma cDNA (data not shown).

For expression of antibody fragments as different Ig isotypes in mammalian cells, fusion proteins were generated by use of modular cassettes containing signal sequences and restriction sites for cloning of variable regions and heavy and light chain constant regions of the human allergy-related immunoglobulins IgG1, IgG4, IgE, IgA1, and IgA2.

SDS-PAGE and immunoblotting of proteins isolated from culture supernatants of stably transfected HEK-293 cells verified the secretion of all immunoglobulins. Identity of the immunoglobulins and the presence of the particular Ig chains was confirmed by SDS-PAGE and immunoblotting (Fig. 3.26).
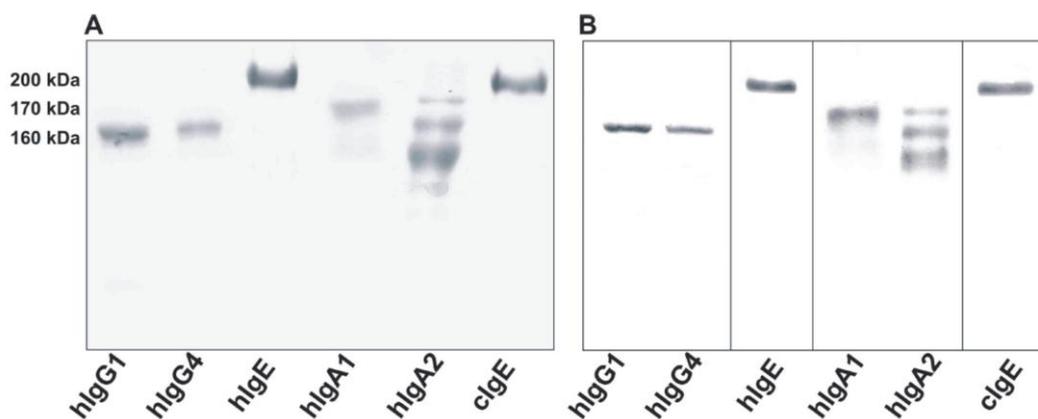
Fig. 3.26: SDS-PAGE and immunoblot analysis of IgG, IgE, and IgA antibodies. Purified proteins were assessed under non-reducing conditions by Coomassie staining (**A**), or immunoblot (**B**); the different isotypes were visualized using anti-human IgG, IgE, and IgA antibodies conjugated to alkaline phosphatase.

Molecular masses were found to be 160 kDa, 170 and 200 kDa for the heterotetrameric IgG, IgA and IgE antibodies, respectively. The expected molecular masses in SDS-PAGE suggest that the secreted antibodies are properly folded and glycosylated, in particular the extensively glycosylated IgE and IgA antibodies. The triple bands obtained for the IgA2 correlate with those reported previously for recombinant IgA2 m(1) and are considered to represent different associates of heavy and light chains [210].

3.5.2   Characterization of the human isotypes

Characterization of the recombinant proteins was pursued by different approaches to demonstrate both antigen and receptor binding. All recombinant antibody isotypes detected Phl p 5a in ELISA (Fig. 3.27A). Interestingly, Phl p 5b was neither bound by the human isotypes nor the chimeric 3-7 antibody, hinting at an epitope that is exclusively present in Phl p 5a.

Furthermore, soluble Ig Fc receptor constructs were produced to confirm proper folding and glycosylation of the Fc domains. Therefore, the extracellular domains of the ligand-binding α-chains of the human high affinity receptors FcεRI and FcγRI (CD64) were fused with chicken IgG (IgY) Fc domains and produced in mammalian cells [129]. Recombinant IgE as well as IgG specifically bound to their soluble Fc receptors (Fig. 3.27B).
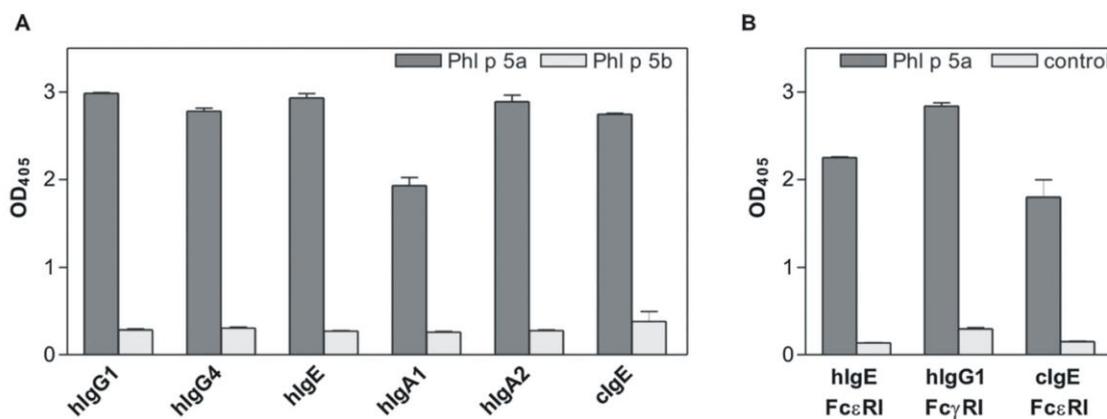
Fig. 3.27: Immunoreactivity with allergens and Fc receptor molecules. A: The immunoreactivity of the different recombinant human and chimeric antibodies was assessed in ELISA using MBP-Phl p 5a, and MBP-Phl p 5b, respectively, and isotype specific antibodies conjugated to alkaline phosphatase. **B:** Simultaneous binding to the allergen and Fc receptors was performed as in A, but using the particular high affinity Fc receptors and anti-chicken IgG conjugated to alkaline phosphatase for detection.

These data underline that the recombinant isotypes reflect the natural interaction with both the antigen and the cellular receptors and therefore should mediate comparable effects.

Applicability of the recombinant IgE antibodies in routine diagnostic approaches was demonstrated by detection of Phl p 5a using commercial AlaBLOT immunoblot strips that contain extracted timothy pollen antigens (Fig. 3.28A) and the laboratory analysers ImmunoCAP 250 and Immulite 2000 (Fig. 3.28B,C).
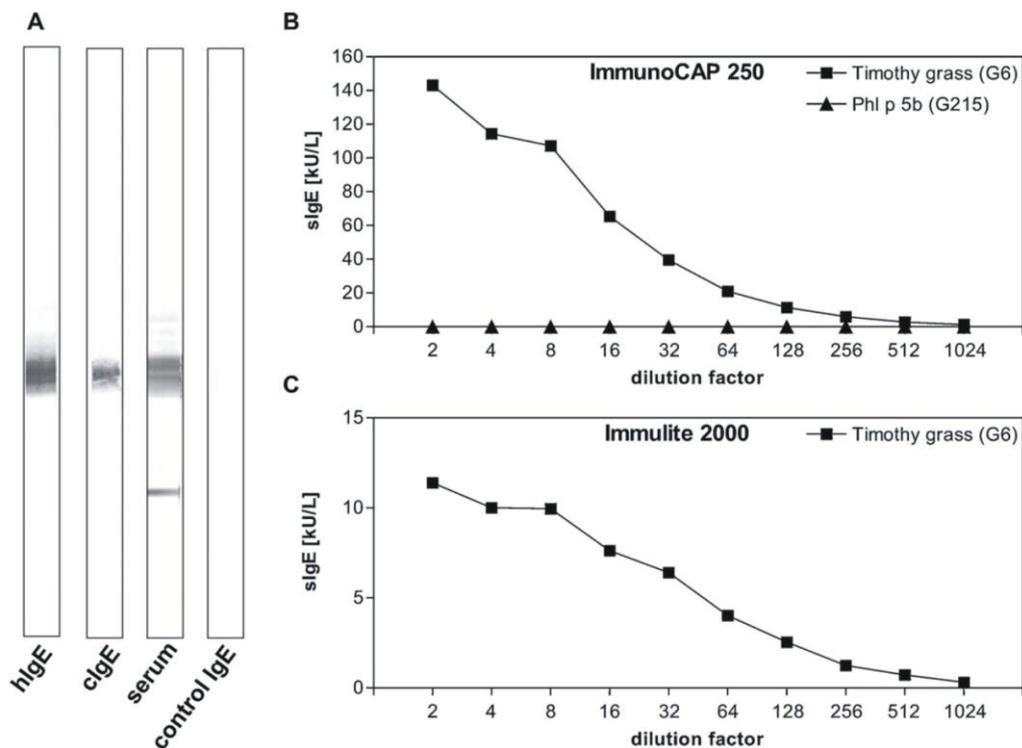
Fig. 3.28: Diagnostic applicability of recombinant IgE. A: Immunoreactivity in immunoblot-based allergen detection was assessed by applying the recombinant IgE and human serum from a grass pollen allergic donor to AlaBLOTs according to the manufacturer´s recommendations. Detection was performed with anti-human IgE conjugated to alkaline phosphatase. **B, C:** Immunoreactivity in diagnostic routine approaches using the laboratory analysers ImmunoCAP 250 and Immulite 2000 was assessed by applying the recombinant IgE according to established protocols to the particular allergen preparations.

Interestingly, serial dilutions of recombinant IgE yielded up to 143 kU/L corresponding to class 6 on the ImmunoCAP 250 and 11 kU/L, corresponding to class 3 on the Immulite 2000 equipment. The single Phl p 5 allergen (allergen code G215) provided by Phadia, however, was not recognized at all, implying either steric hindrance or an isoform bias in the preparations used by the manufacturers.

3.5.3 Assessment of the cellular activation by recombinant IgE

IgE-mediated cross-linking of the FcεRI and degranulation of RBL-SX38 cells was assessed by determination of β-hexosaminidase release (Fig. 3.29). Allergen-specific cellular activation is difficult to achieve since monoclonal IgE are not capable of cross-linking cellular receptors due to the availability of only one epitope per allergen. Thus, a multivalent molecule is needed to bridge two identical paratopes, an approach that has never been shown for recombinant monoclonal IgE. We addressed this issue by generating Phl p 5 microspheres using biotinylated allergen clustered with either soluble streptavidin (Fig. 3.29A,C) or immobilized on streptavidin-coated particles (Fig. 3.29B,D).

Monovalent allergen used as control was not able to activate RBL-SX38 cells as expected (data not shown). By contrast, using the soluble and the particle-based Phl p 5a microspheres efficient activation was demonstrated for hIgE and cIgE (Fig. 3.29C). Upon sensitization with hIgE, Phl p 5b microspheres did not induce activation, but cIgE and particle-based microspheres mediated degranulation.
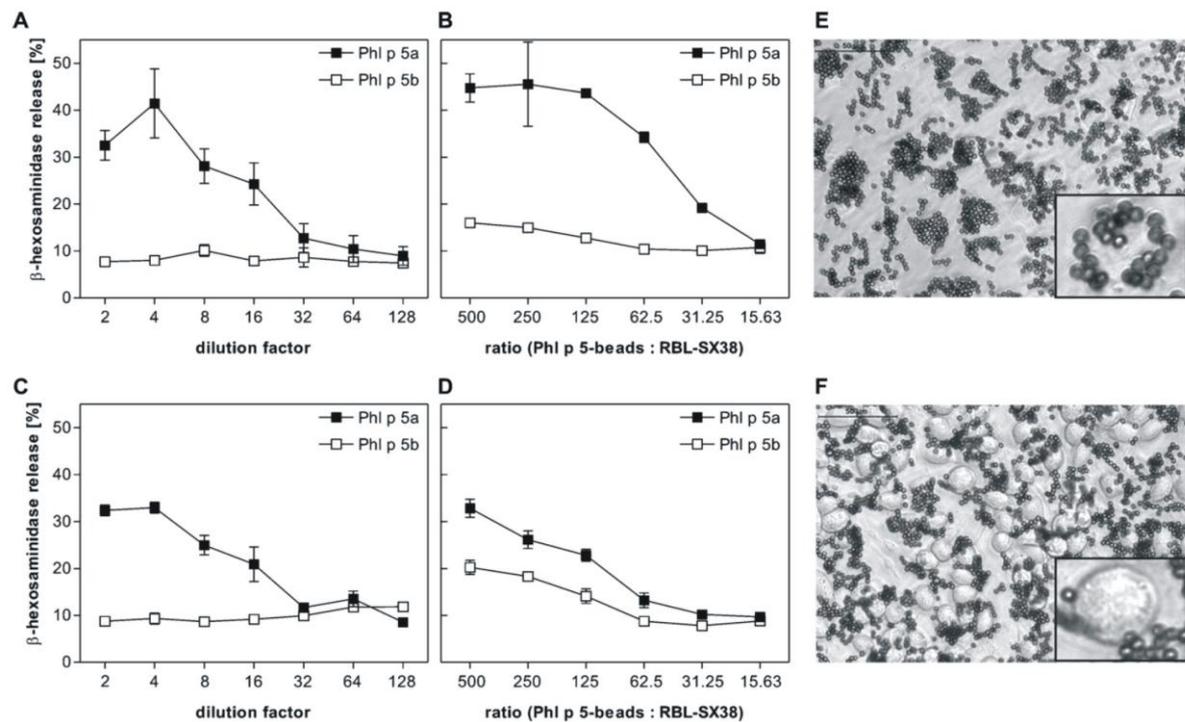
Fig. 3.29: Mediator release of humanised RBL-SX38 by Phl p 5 microspheres. RBL-SX38 cells providing the human FcεRI were sensitized with both hIgE (**A, B**) and cIgE (**C, D**). Degranulation was induced by addition of biotinylated Phl p 5a and b fusion proteins complexed with either soluble streptavidin (**A, C**), or immobilized to streptavidin coated beads (**B, D**). Degranulation was monitored by β-hexosaminidase activity released into culture supernatants. Data are mean +/- SD of triplicate measurements. **E, F:** Light microscopic images of RBL-SX38 cells sensitized with hIgE and beads coated with Phl p 5a and Phl p 5b, respectively (**E, F**).

Mediator release, however, was observed to a reduced extent suggesting an avidity effect overcoming a putatively reduced affinity. These data suggest that both IgE exhibit an intrinsic potential to cross-link the FcεRI and activate effector cells.

Given the recognition of non-overlapping, sterically available epitopes, however, monovalent soluble allergens are sufficient for receptor cross-linking. Sandwich ELISA using the hIgE and the cIgE as capture antibodies and the complementary antibody in form of another isotype for detection readily pointed to epitopes with an overall architecture enabling simultaneous binding of the hIgE and cIgE (Fig. 3.30A). Consequently, simultaneous use of both the fully human IgE and the chimeric IgE in mediator release assays also resulted in robust activation of RBL-SX38 cells using Phl p

5a in soluble, spatially non-clustered form (Fig. 3.30B). By contrast, Phl p 5b did not induce any activation.
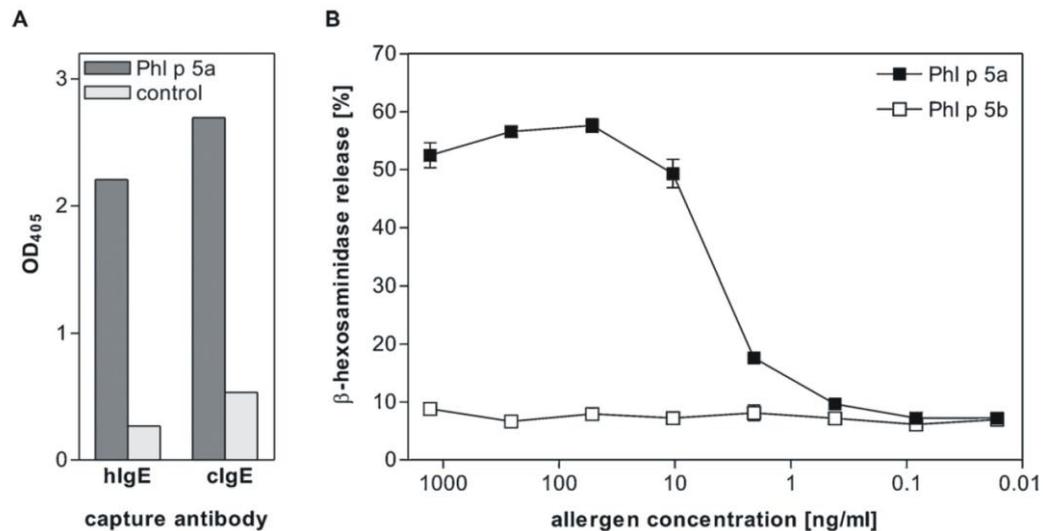
Fig. 3.30: Analysis of epitope distribution by ELISA and mediator release of RBL-SX38 cells. A: The epitope architecture was assessed in sandwich ELISA using the hIgE and the cIgE as capture antibody, and the complementary human and murine IgG isotypes for detection. **B:** Functional availability of the epitopes was verified by using RBL-SX38 cells sensitized simultaneously with hIgE and cIgE. Degranulation was induced by addition of an anti-human IgE serum. Degranulation was monitored by β-hexosaminidase activity released into culture supernatants.

These data show that the two IgE antibodies not only mimic the characteristics of natural IgE antibodies but also allow comparison with the natural situation in tissues using antibodies of different affinities.

3.5.4   Epitope analysis

As evident from the ELISA and mediator release assays both antibodies apparently exhibit specificity for Phl p 5a. Having this pattern in mind, Phl p 5a and b fragments and chimeras were generated to dissect the interplay of allergens with antibodies on a molecular level (Fig. 3.31A). These fragments represent variations of different elements of the N-terminal 4 helix bundle. Prokaryotic expression of allergens tends to intracellular aggregation yielding insoluble protein fractions, therefore, these proteins were fused with MBP (full length Phl p 5a and b) or CBD (all fragments including chimeric F1) to avoid detrimental effects on folding and solubility, and purified by affinity chromatography. Subsequent immunoblot analyses demonstrated hIgE reactivities with the fragments F1, F2, and F3, but not with F4 and F5 (Fig. 3.31B). These findings suggested that the unique insertion present in Phl p 5a functions as an IgE epitope.

In order to verify this observation we grafted the particular amino acids of Phl p 5a into the non-reactive Phl p 5b framework which resulted in de novo establishment of pronounced

IgE reactivity. This conversion includes the entire loop region according to the secondary structures observed in NMR analyses [211] and suggests that this stretch is sufficient and essential for epitope formation and IgE reactivity. Most prominent amino acid variations are located in a stretch of 20 amino acids at the N-terminus of the loop. This finding is consistent with the reactivities of the different allergen fragments and mutants. In contrast to hIgE, the cIgE recognized the Phl p 5 full length proteins only (Fig. 3.31C,D).

Fig. 3.31: Epitope analyses using fragments and mutants of Phl p 5 in immunoblot. A: Alignment of the N-terminal region of Phl p 5a and Phl p 5b (A, top) in comparison with generated fragments (F1 a - F5 a, and F1 b, chimeric F1) (A, bottom) was developed using ClustalW2. Total and partial conservation is indicated with stars, colons, and dots. Sequences forming predicted helix bundles are highlighted with a grey background. Residues of the Phl p 5a stretch grafted into the framework of Phl p 5b are depicted in red. **B, C, D:** Immunoreactivities of the fusion proteins shown in A in immunoblotting using hIgE (B), cIgE (C), and anti-fusion protein antibodies as loading controls. Detection was performed using the particular secondary antibodies conjugated to alkaline phosphatase.

The pattern revealing a reduced reactivity with Phl p 5b suggests an epitope within the C-terminal domain that is conserved in Phl p 5b to a certain degree only.

The hIgE epitope itself is defined by the second loop of the N-terminal first 4 helix bundle domain as evident after modelling both isoforms by using the structure of Phl p 6 as template (Fig. 3.32). Although not fully in accordance with the NMR data, this model is helpful to exemplify the structural basis of the epitope. The loop forming stretch significantly protrudes from the protein and represents a unique feature of Phl p 5a (Fig. 3.32A,C,E). Insertion of additional amino acids results in an elongation of the helical element in Phl p 5b (Fig. 3.32B,D,F) which is accompanied by a drastically increased exposure of the loop region. This region contains an elevated number of polar residues including lysine, serine, glutamate and asparagine residues.

Fig. 3.32: Molecular modelling and IgE epitope mapping on the surface of F1 a (A, C, E) and F1 b (B, D, F). The modelling was performed using the structure of Phl p 6 as template (front view in the A, B; top view in C, D; close up in E, F). The assignment of helical elements shown here does not coincide with that deduced from the NMR structure of the first AR repeat of Phl p 5a. Amino acids that correspond to the identified stretch are highlighted in red. The secondary structures and the surface representations visualize the variations of the molecular topology.

Although a continuous stretch within the primary structure, its secondary structure appears to be crucial for reactivity. As suggested by the IgE reactivities of F1-F5, interference with the folding of the two flanking helices into antiparallel strands abolishes the IgE reactivity.

3.6   Establishment of a model system for hapten specific IgE antibodies

The antibody cloning technology enables the engineering and genetic manipulation of antibody fragments that retain the targeting specificity of whole monoclonal antibodies, but exhibit other unique and superior properties for a range of diagnostic and therapeutic applications and can be produced more economically [212-214]. By joining the VH and VL domains with a flexible polypeptide linker *single chain variable fragments* (scFvs) can be generated preserving specific antigen-binding properties of the parental antibodies (Fig. 3.33). Using phage display technology [205, 206] with both immune and synthetic library formats, up to now antibody fragments have been successfully selected against a number of target structures ranging from small haptenic structures [215] to proteins [204] and also more complex sources [216-218].

Since TNT and its surrogate TNP exhibit only a small size and haptenic nature, the structures served as model system for the molecular recognition of minimal epitopes by antibodies. In order to obtain monoclonal IgE-based antibody constructs with defined immunoreactivities against TNT/TNP, a combinatorial library selection by phage display with the human semi-synthetic non-immune scFv library Griffin-I [204] was employed and the sequence of the TNP-specific scFv 2.18 was available when starting with this work. The scFv-IgE construct of clone 4.8 was also already available. The scFv of clone 5 stems from a selection of an avian TNP-specific library and was available as sequence in the phagemid vector pHen2. It has been shown that recombinant antibody constructs expressed in mammalian cells show a higher yield in relation to a lower molecular weight [128]. In order to generate bivalent antibody constructs with reduced molecular weight but retained effector functions the TNP-specific scFv-sequences were fused to the Fc part of an IgE antibody with deleted CL and CH1 domains, resulting in homodimeric scFv-based antibodies (Fig. 3.33). The generated human monoclonal scFv-IgE antibodies were analyzed for their immunoreactivity and specificity, as well as their interaction with the minimal epitope TNP in surface plasmon resonance experiments. The characterized antibodies exhibited varying affinities for their identical epitope and were further employed for cellular degranulation assays.

3.6.1   Generation of TNP-specific scFv-IgE antibodies

To establish TNP specific scFv-IgE antibody constructs, the phage-display derived scFv sequences 2.18 and 5 available in the phagemid vector pHen2 were used as templates. The scFv clone 4.8 was already available as scFv-IgE. The fragments were converted into

bivalent homodimeric scFv based IgE formats by cloning them in a one-step reaction into the mammalian expression vector pcDNA3.1/Zeo.

A schematic representation of the available fragments and generated antibody formats as well as of the underlying vectors is shown in Fig. 3.33.
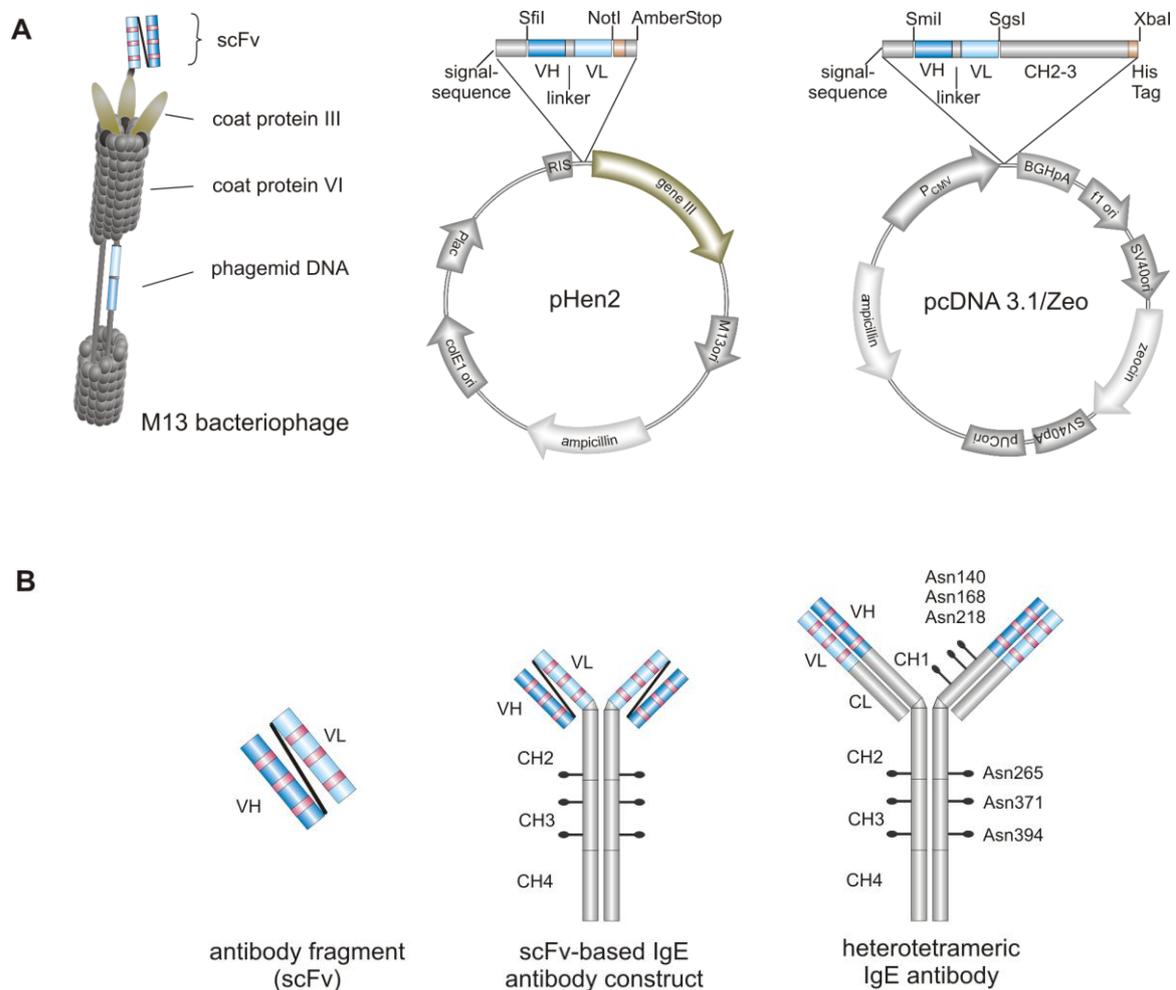
Fig. 3.33: Simplified representation of the used expression vectors and resulting antibody constructs. A: Structure of the bacteriophage M13 and the underlying vectors with expression cassettes. **B:** For the experiments artificial, truncated, homodimeric, scFv-based IgE constructs were used. For comparison also the heterotetrameric antibody format is shown. VH: variable heavy chain, highlighted in dark blue; VL: variable light chain, highlighted in light blue; CH: constant heavy chain; CL: constant light chain; Asn: N-glycosylation site; the CDRs are highlighted in red.

As expression system immortalized *human embryonic kidney* cells (HEK-293) that perform posttranslational modifications were used. It has been shown that artificial scFv based constructs assemble properly after expression resulting in bivalent antibodies that show a proper folding and glycosylation. Furthermore the truncated constructs show a higher expression level in mammalia HEK-293 cells compared to the full heterotetrameric isotypes [128].

The scFv-IgE constructs 2.18, 4.8 and 5 were expressed in stably transfected HEK-293 cells that secrete the antibodies into the cellular supernatant. The expression of the human IgE antibodies was verified in immunoblot as shown in Fig. 3.34. For specific detection a monoclonal goat anti-human IgE and an anti-goat-AP conjugate were used.
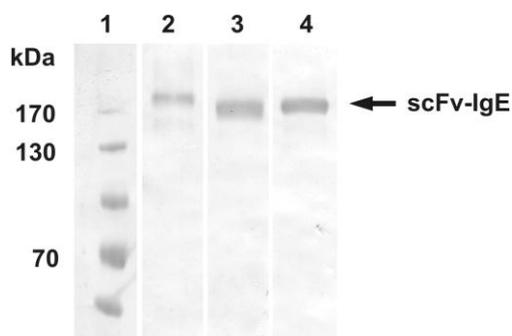
Fig. 3.34: Verification of the expression of TNP-specific monoclonal human scFv-IgE antibodies. The expression in cellular supernatant was verified in immunoblot by using monoclonal goat anti-human IgE (1:20,000) and anti-goat-AP conjugate (1:20,000). Lane 1: *PageRuler*^{TM} *Prestained Protein Ladder*; Lane 2: supernatant 2.18-scFv-IgE; Lane 3: supernatant 4.8-scFv-IgE; Lane 4: supernatant 5-scFv-IgE.

The SDS-PAGE with following immunoblot and specific detection of the constructs in the cellular supernatants of stably transfected HEK-293 cells showed apparent molecular masses in the expected range of 180 kDa for every homodimeric scFv-IgE. All three antibodies could be produced as secreted scFv-based IgE constructs that were properly folded and glycosylated.

The immunoreactivity of the antibodies with their target structure TNP was assessed in ELISA, as shown in Fig. 3.35. The supernatants were incubated with a TNP-KLH conjugate and for detection an anti-human IgE-AP conjugate was used.

All recombinant constructs detected their target structure TNP in ELISA. Therefore the generation of TNP-specific, reactive scFv-IgE antibodies could successfully be verified and the constructs were available for following approaches.
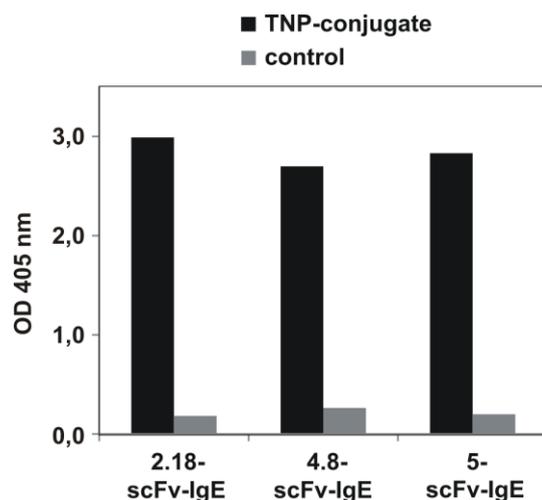
Fig. 3.35: Immunoreactivity of TNP-specific monoclonal human scFv-IgE antibodies. The immunoreactivity of different TNP-specific scFv-IgE antibodies was assessed by ELISA. A TNP-KLH conjugate was used as antigen, controls were performed by omission of antigen. For detection an anti-human IgE-AP conjugate was used (1:1000).

For further investigations the antibody concentrations in the cellular supernatants were determined using the *human IgE ELISA Quantitation Kit.*

3.6.2 Surface plasmon resonance analysis

In order to investigate the affinities of the TNP-specific antibodies, they were analyzed by surface plasmon resonance measurements. The antibody supernatants were injected as analytes in different concentrations over biotinylated TNP-BSA that was immobilized on a streptavidin coated biosensor surface. As reference the purified TNP-specific murine IgE C38-2 was applied.

The antibody concentrations and their corresponding SPR responses are presented in Tab. 3.1, and the resulting concentration dependent curves are shown in Fig. 3.36.

Tab. 3.1: Antibody concentrations of TNP-specific antibodies used for SPR measurements and their corresponding maximal SPR responses. The constructs 2.18-scFv-IgE, 4.8-scFv-IgE, 5-scFv-IgE and C38-2-muIgE were investigated. RU: resonance units.

2.18-scFv-IgE		4.8-scFv-IgE		5-scFv-IgE		C38-2-muIgE	
nM	RU	nM	RU	nM	RU	nM	RU
4.93	0.62	0.37	3.20	13.88	3.63	4.15	6.09
9.85	16.12	0.74	7.43	27.76	9.66	5.00	8.27
19.71	37.05	1.85	17.70	55.52	34.31	6.25	8.99
39.41	64.94	3.71	30.89	111.03	76.47	8.35	9.93
78.82	97.60	7.41	45.99	222.06	127.45	12.50	20.48
157.65	127.71	12.35	54.84	444.12	204.70	25.00	29.55
		37.06	77.54	888.24	303.82	50.00	42.73
						100.00	54.64

The SPR-responses were plotted against the concentrations, the curves were analyzed by the one-site-binding-model and the KD values were calculated.
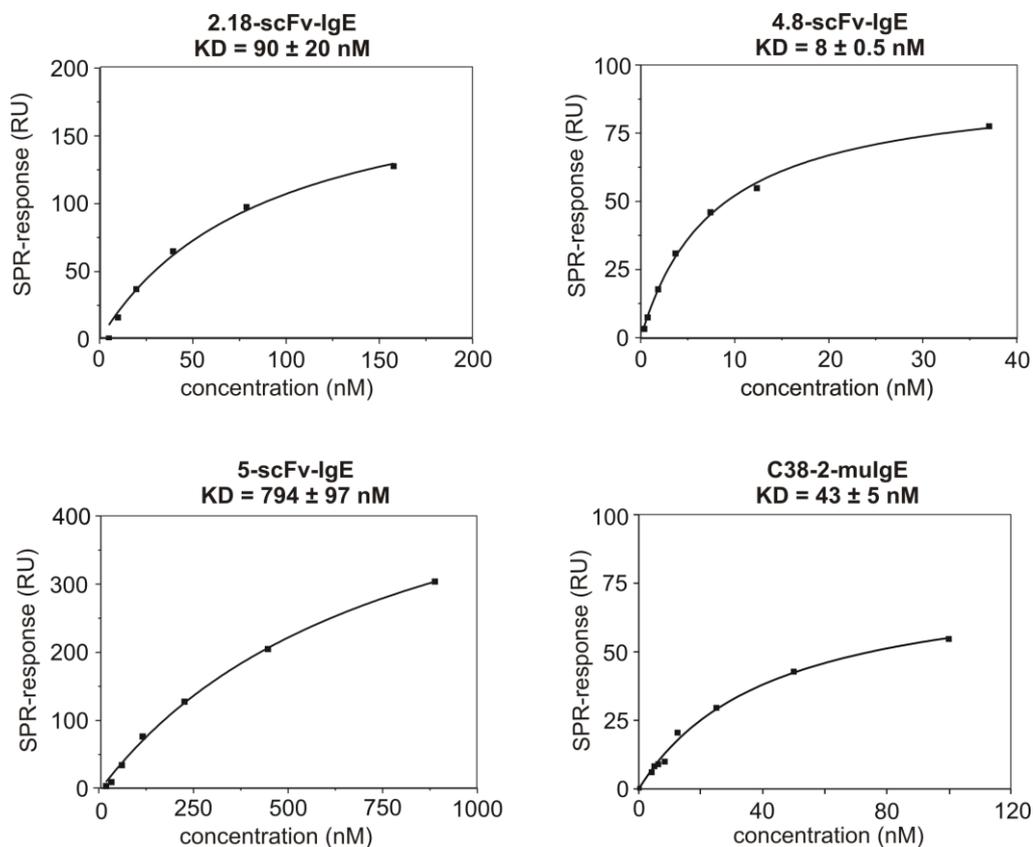
Fig. 3.36: Biosensor affinity measurements of 2.18-scFv-IgE, 4.8-scFv-IgE, 5-scFv-IgE and C38-2-muIgE with immobilized TNP-BSA. A kinetic analysis of the interaction of immobilized biotinylated TNP-BSA on a streptavidin coated surface with 2.18-scFv-IgE, 4.8-scFv-IgE, 5-scFv-IgE and C38-2-muIgE was performed by injecting varying concentrations of the analytes. A streptavidin surface served as control. The apparent KD values were calculated from the fits.

The SPR measurements led to calculated apparent KD values of 90 nM for 2.18-scFv-IgE, 8 nM for 4.8-scFv-IgE, 794 nM for 5-scFv-IgE and 43 nM for C38-2-muIgE, corroborating the functionality of the antibody constructs.

The TNP-specific 5-scFv-IgE showed the highest dissociation constant at equilibrium, the 4.8-scFv-IgE the lowest KD and thereby the highest affinity. The antibodies 2.18-scFv-IgE and C38-2-muIgE showed a medium affinity.

3.6.3 *In vitro* mediator release assay with rat basophil leukemia cells (RBL-SX38)

In order to evaluate the potential of the TNP-specific antibodies for cellular activation the IgE-mediated cross-linking of the FcεRI and degranulation of RBL-SX38 cells was assessed by determination of β-hexosaminidase release. The medium affinity of the murine C38-2 antibody was used as reference.

The TNP-specific scFv-IgE constructs were used for sensitization of RBL-SX38 cells. The supernatants exhibited the antibody concentrations of 56 µg/ml for the 2.18-scFv-IgE, 28 µg/ml for the 4.8-scFv-IgE and 90 µg/ml for the 5-scFv-IgE. The murine C38-2-IgE was used with a starting concentration of 800 ng/ml.

As shown in Fig. 3.37 all antibodies bound to the FcεRI and induced mediator release in an antigen-independent manner using anti-huIgE and anti-muIgE antibodies.
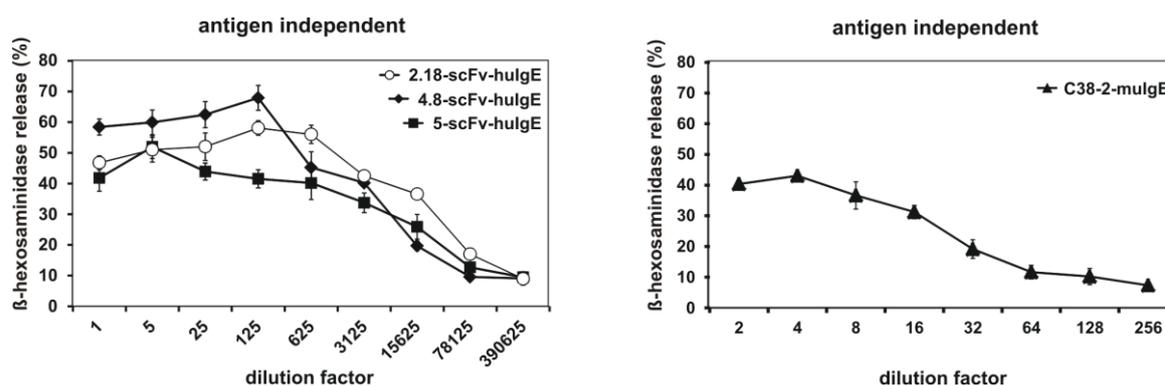
Fig. 3.37: Mediator release of humanized RBL-SX38 cells by antigen independent crosslinking. RBL-SX38 cells were sensitized with different concentrations of the scFv-IgE constructs 2.18 (56 µg/ml), 4.8 (28 µg/ml), and 5 (90 µg/ml) in cellular supernatants, as well as the purified murine C38-2-IgE (800 ng/ml). Degranulation was induced by addition of anti-huIgE and anti-muIgE (1 µg/ml) and was monitored by β-hexosaminidase activity released into culture supernatants. Data are mean +/- SD of triplicate measurements.

In order to ensure equal and comparable sensitization of the RBL-SX38 cells with the different scFv-IgE constructs, the supernatants were all used in 1:20 dilutions in the following experiments. With these concentrations all scFv-IgE constructs elicit a maximal β-hexosaminidase release when crosslinked with anti-IgE. The starting concentration of the murine C38-2-IgE was 1 µg/ml.

Since in most cases only one epitope per antigen is available, an antigen-dependent cellular activation for monoclonal IgE antibodies usually is difficult to achieve. For an efficient crosslinking of Fcε receptors a multivalent molecule bearing at least two independent epitopes is needed to bridge two identical IgE paratopes.

Thereby in the following release assay oligomeric TNP-BSA was used for an antigen dependent crosslinking as shown in Fig. 3.38.
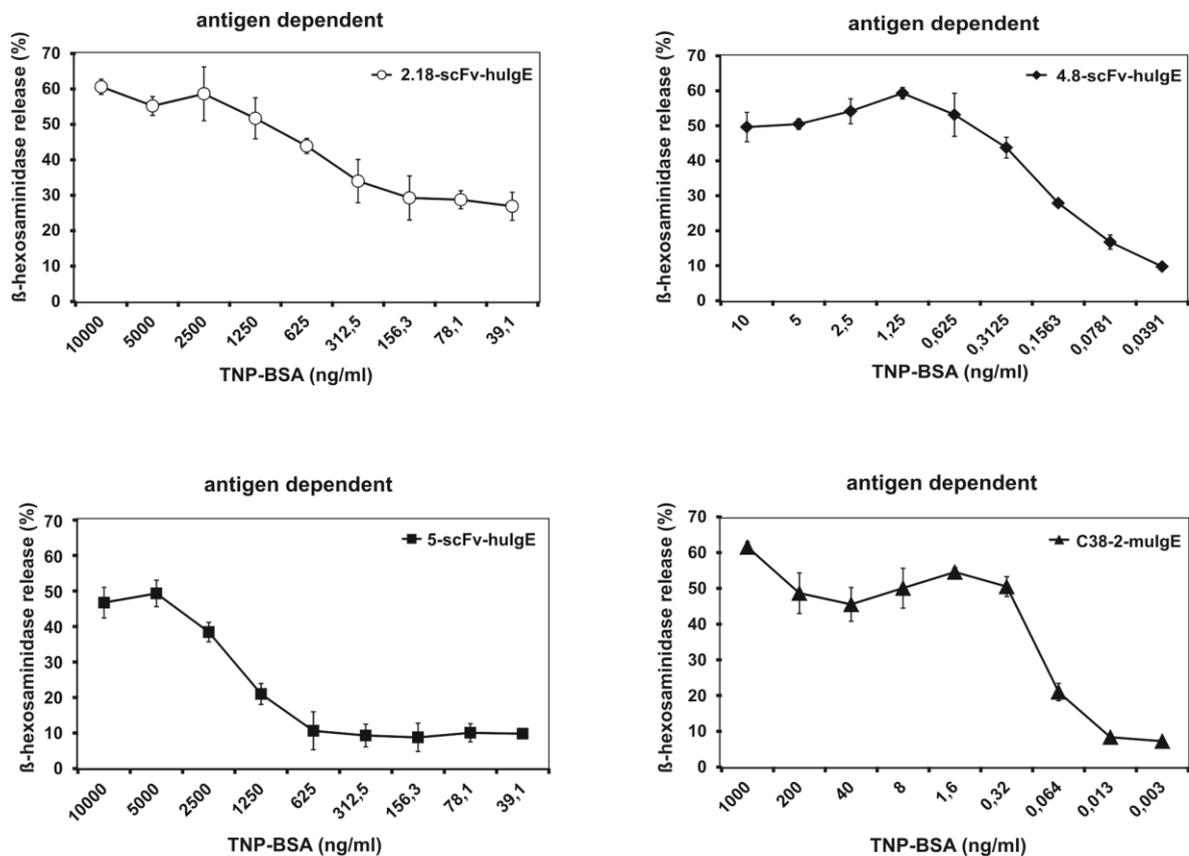
Fig. 3.38: Mediator release of humanized RBL-SX38 cells by antigen dependent crosslinking. RBL-SX38 cells were sensitized with 1:20 supernatant dilutions of the scFv-IgE constructs 2.18, 4.8, and 5 as well as the murine C38-2-IgE with a starting concentration of 1 µg/ml. Degranulation was induced by addition of different concentrations of TNP-BSA and was monitored by β-hexosaminidase activity released into culture supernatants. Data are mean +/- SD of triplicate measurements.

When applied to IgE-sensitized cells, activation was induced by the oligomeric TNP-BSA, confirming direct antigen induced cellular activation. Effector cell activation in a concentration-dependent manner was also observed.

All antibodies showed a mediator release, but obviously different antigen concentrations were needed for different antibody-affinities in order to mediate cell activation.

4 Discussion

In the last decades a significant progress in the field of allergy regarding characterizations of allergic diseases has been made and diverse factors influencing allergy have been described, such as genetic and environmental factors, participating cell populations or cytokines. However, the factors and properties determining the allergenicity of allergenic substances like proteins or haptenic structures such as CCDs are still not characterized completely. Obviously the mode and degree of exposure of a substance play essential roles in the induction of allergic responses, but also structural characteristics are important for the capacity of a molecule to modulate the immune response towards allergic reactions [219]. One deciding feature for the differentiation of immunogen or allergen substances is the recognition by IgE antibodies which are produced during sensitization. Therefore the characterizations of IgE epitopes as well as the evaluation of their interactions with their corresponding antibodies offer possibilities for detecting specific structural features of allergens. So far the knowledge of common structural characteristics of IgE epitopes is very limited. Available monoclonal antibodies enable specific molecular interaction analyses but one main limitation for approaches such as X-ray crystallography or STD-NMR is the very low concentration of natural monoclonal IgE antibodies in the human system, especially in the context of allergic reactions, and thus IgE epitope identification remains a major challenge.

The murine hybridoma technology is a well-established method for the generation of monoclonal antibodies and also an affinity increase by iterative immunizations of mice can lead to highly specific antibodies. Using recombinant technologies these antibodies can be employed for humanization procedures resulting in humanized or chimeric allergen specific isotypes [220]. Another technology for the generation of antigen specific antibody constructs is the phage display technology, where different libraries like human, murine or avian and additionally synthetic repertoires are used for the selection of antibody fragments [128, 139], enabling the generation of designed antibody constructs and isotypes with targeted specificities [221, 222] opening up new possibilities of investigations and interaction analyses.

In this work the characterization of allergenic epitopes and analyses of interactions with corresponding antibodies on different molecular levels are described. In the context of allergic reactions the hymenoptera venom allergy is a suitable model system for the investigation of type I hypersensitivity reactions. Additionally several hymenoptera venom components are CCD-carrier and CCDs are probably the most widely occurring IgE epitopes [223], however, in contrast to their diagnostic relevance the clinical relevance still remains unsettled.

Using different prokaryotic and eukaryotic cells as well as established systems of constructed vectors for the antibody expression in eukaryotic systems [138] it was possible to generate recombinant allergens and allergen specific monoclonal antibodies for antibody/epitope interaction analyses, epitope characterizations and investigations regarding antibody affinities and their influence of following cellular mechanisms.

For the investigation of CCD depending IgE reactivities the recombinant honeybee venom major allergen phospholipase A2 was produced for the first time in insect cells as eukaryotic expression system, providing posttranslational modifications such as glycosylation. Detailed evaluation of different protein variants resulted in variable and defined CCD phenotypes serving as useful tools in the assessment of patients IgE immunoreactivities and in effector cell activation analyses. As second hymenoptera venom derived molecule the carbohydrate-rich protein Api m 10 was produced for the first time in insect cells. This component is highly glycosylated and also exhibits a strong IgE-sensitizing potential beyond its CCD-reactivity, emphasizing the clinical relevance of this allergen.

The inhalative major allergen Phl p 5 was investigated on a proteinic level and the localization and evaluation of an authentic IgE epitope was possible using Phl p 5-specific antibody fragments in different assay systems as well as for molecular modeling and IgE epitope mapping procedures. The nonglycosylated small allergen and component of honeybee venom Api m 6 was investigated regarding its immunochemical and structural characteristics. It could be shown that also a protein with only a small, nearly haptenic molecular size like Api m 6 can exhibit an appreciable IgE-sensitizing potential.

For a closer look on antibody/antigen interaction on an atom level the clinical relevant CCD structure α-Gal was chosen and several α-Gal specific antibody formats were generated. By using STD NMR analysis it was possible to get further insights into the interaction of a monoclonal specific IgE antibody as well as polyclonal antibodies with the glycan structure α-Gal.

Against the background of IgE epitope structures as well as CCDs and the long-lasting and controversial discussions regarding their clinical relevance, a model system with several monoclonal IgE antibody constructs and TNP as target structure was established, considering glycans as small haptenic structures and to design a system with defined and constant conditions for detailed investigations of antibody interactions with their haptenic epitopes. In the context of allergy the focus was concentrated on the IgE isotype and IgE effector mechanisms and therefore different scFv-based human monoclonal TNP-specific IgE antibodies were generated and their immunoreactivities, binding affinities and characteristics in basophil degranulation assays were investigated.

4.1 Recombinant hymenoptera venom components and CCDs

One particular CCD-reactivity associated allergy is the allergy to hymenoptera venoms. This IgE-mediated hypersensitivity reaction of the type I can cause life-threatening and sometimes fatal anaphylactic reactions in humans. The prevalence of sensitization to hymenoptera venoms is estimated between 9.3 % and 28.7 % in adults [224] and in children of 3.7 % [225]. The prevalence of large local reactions in the general population ranges from 2.4 % to 26.4 %, up to 38 % in beekeepers [224], systemic reactions due to hymenoptera stings were reported ranging between 0.5 % to 3.3 % in the U. S. [226] and between 0.3 % and 7.5 % in Europe as reported in European epidemiological studies [224].

Sensitization is indicated by a positive skin test and/or the detection of specific IgE antibodies in patients with no previous case history. So far therapies as well as diagnostic approaches are based on the raw and pure venoms which are not sufficiently characterized. Thereby allergen specific IgE reactivities interfere with reactivities against crossreactive carbohydrate determinants [65, 185] present in venoms and inhalant allergens. This results in false positive tests in diagnostic *in vitro* methods and interferes with evaluations of epidemiological studies [111].

A detailed knowledge of allergological relevance of single venom components, their natural functions and the influence of their modifications like CCDs on the reactivity as well as the characterization of allergen epitope interactions with their corresponding antibodies is crucial for a successful understanding of molecular mechanisms in allergy.

4.1.1 Insect cell-derived recombinant Api m 1 with defined CCD phenotypes

The secretory phospholipase A2 (Api m 1, PLA2) is the most important major allergen in honeybee venom representing about 12 % of the dry weight of crude venom [227, 228]. More than 95 % of patients with *systemic allergic reactions* (SAR) to honeybees show Api m 1 specific serum IgE antibodies if looked for within a year after SAR [175]. The 16 kDa enzyme Api m 1 is a typical type III secreted PLA2 [181] with five conserved disulfide bonds. It hydrolyses fatty acids of membrane phospholipids by specific recognition of sn-2 positions of phospholipids [229] leading to damage of structural membranes. The protein contains only one carbohydrate epitope which is attached to the Asparagine on position 13 [81]. This single glycan contains an α-1,3-fucose residue which is responsible for CCD-reactivity of native PLA2 leading to false positive results in diagnostic tests with no or only low clinical relevance and therefore represents a challenge for diagnostic approaches [154].

The availability of single recombinant protein components is a prerequisite for the design of protein variants with defined characteristics such as glycosylation which are essential for proper folding and activity of a protein as well as its CCD reactivity.

In order to evaluate allergens depending on their glycosylation patterns different insect cell lines that were found to be suitable hosts for the production of hymenoptera enzymes [123, 230] and deliver variable CCD phenotypes [168] were used. The cell line HighFive, which was originally isolated from *Trichoplusia ni*, exhibits a distinct level of α-1,3-core-fucosylation and thereby the addition of CCDs compared to the cell line Sf9, which was isolated from *Spodoptera frugiperda*, showing no detectable α-1,3-core-fucosylation. Notably the glycosylation of the proteins is not further influenced, excepting the one single linked $\alpha-$1,3-core-fucose, providing an optimal system for the investigation of glycoproteins and the dissection of cross-reactivities. Additionally recombinant allergens such as PLA2 with only one defined glycosylation represent essential tools in order to address allergenic and diagnostic cross-reactivity.

The recombinant prokaryotic expression of Api m 1 resulted in an unfolded protein showing no biologic activity by intracutaneous skin testing and only extensive refolding procedures resulted in an allergenic and catalytic activity of the recombinant protein similar to the native one [227]. To improve the production of recombinant soluble PLA2 from honeybee, an eukaryotic expression and thereby glycosylation dependent native folding of the protein was striven, using cell lines with variant capacities of α-1,3-fucosylation. Due to its enzyme activity and expected cytotoxicity in cellular assays the PLA2 was inactivated by substituting the catalytically essential histidine residue [178, 179] 34 with glutamine (H34Q) by site-directed mutagenesis. To investigate the impact of glycosylation of the recombinant protein on IgE reactivities from sera obtained from hymenoptera venom allergic patients, an aglycosylated PLA2 version was produced by generating an allergen version without glycosylation site, substituting the relevant asparagine 13 with glutamine (H34Q N13Q).

The baculovirus-mediated expression in Sf9 and HighFive insect cells resulted in different soluble and secreted inactive recombinant protein variants, PLA2 H34Q from Sf9, PLA2 H34Q from HighFive and PLA2 H34Q N13Q from HighFive, exhibiting a varying degree of CCDs. The recombinant protein bands showed apparent molecular weights between 18 and 22 kDa, what is in good agreement with the described native molecular weight forms between 16 to 20 kDa [231] keeping the added tags in mind. Beside of investigations verifying the presence of N-linked glycans using a mannose specific lectin, a detailed evaluation of the Api m 1 variants regarding their CCD phenotypes was performed. Therefore an anti-HRP rabbit serum detecting specifically the plant-derived CCDs α-1,3-core-fucose and β-1,2-xylose, the causative structures for CCD-based cross-reactivities,

was applied. The immunoblot analyses revealed a distinct α-1,3-fucosylation of recombinant Api m 1 H34Q produced in HighFive cells as well as of native Api m 1 that was employed for comparison. The recombinant Api m 1 H34Q produced in Sf9 on the other hand exhibited no CCD-based reactivity. Keeping the single glycosylation site of Api m 1 in mind and using the different cell lines with or with no ability of detectable fucosylation, it was possible to detect the glycosylation influencing one single linked α-1,3 core-fucose structures on the recombinant and native molecules.

Furthermore, the immuno- and crossreactivities of the recombinant and native Api m 1 variants were characterized, assessing specific IgE reactivities of insect venom allergic patients sera by ELISA with the protein variants and a CCD marker. The sIgE reactivities of 5 selected individual patient sera exhibited distinct recognition patterns and it was possible to distinguish between sIgE reactivity due to proteinic epitope recognition or due to α-1,3-fucose based crossreactivity.

In a final step the ability of native Api m 1 and recombinant Api m 1 H34Q to mediate degranulation of RBL cells was tested, demonstrating that the inactivated protein was not able to induce IgE independent effector cell activation, compared to the native enzymatically active protein.

In these specific IgE based assays the recombinant produced molecules showed advantageous properties regarding cross-reactivity. The use of recombinant molecules with defined CCD phenotypes might provide further insights and can have intriguing consequences for the understanding of hymenoptera venom IgE reactivities [168] and therefore represents a novel strategy with major implications for diagnostic and therapeutic approaches.

4.1.2 Api m 10 as high glycosylated allergen and with allergenicity independent of CCDs

Another honeybee venom allergen is the protein Api m 10, also known as Icarapin or due to its high glycosylation level as carbohydrate-rich protein. Two independent groups identified peptides of this protein in 2005 with a molecular weight of about 22 kDa [140, 186]. First, the protein was prokaryotically expressed in *E. coli* resulting in an instable molecule without glycosylation but with IgE reactivity [143].

In order to evaluate its relative abundance in honeybee venom as well as the importance of Api m 10 as allergen in the context of allergy and VIT the cDNA of splicing variant 2 was amplified from venom gland cDNA and the protein was eukaryotically expressed in different insect cells.

Again, HighFive cells as well as Sf9 cells were used in order to obtain different protein variants with varying glycosylation patterns with and without CCDs. An aglycosylated form

of Api m 10 for comparisons was also obtained by prokaryotic expression in *E. coli*. All variants were assessed for their IgE reactivity and basophil activation tests were performed comparing eukaryotic and prokaryotic protein variants. By generation of a monoclonal human IgE antibody using phage display technology and subsequent conversion into a scFv-IgE construct a specific tool for detection and quantification of the protein in *Apis mellifera* venom as well as therapeutic venom preparations was received.

For the aglycosylated Api m 10 variant the protein was prokaryotically produced without fusion partner using the strategy of chitin binding domain (CBD) fusion followed by autocatalytic intein-mediated cleavage [187]. This resulted in pure and soluble target protein released from the intein-CBD tag that showed an apparent molecular weight of about 35 kDa, suggesting a modified migration behavior because of the low pI of the protein. Glycosylated protein variants using the different insect cell lines resulted in recombinant Api m 10 with an apparent molecular mass of about 50-55 kDa. This difference of molecular weight obviously stem from extensive posttranslational modifications, such as glycosylation.

The evaluation of the immunoreactivities of the three recombinant variants by immunoblot verified the expected CCD-based crossreactivity for Api m 10 produced in α-1,3-fucosylation providing HighFive cells, compared to the glycosylated Sf9 variant as well as aglycosylated *E. coli*-derived protein that showed no CCD reactivity.

The screening of patient sera for IgE reactivity with the different variants revealed that about 50 % of honeybee venom allergic patients showed reactivity with recombinant Api m 10 beyond CCD reactivity. This observation was confirmed by basophil activation tests giving rise to the conclusion that Api m 10 is an important sensitizing component of honeybee venom. Furthermore beekeeper sera showed in over 50 % of cases a pronounced reactivity of IgG4 antibodies, which is in accordance of the previous results since IgG4 induction is accompanied with allergens which are good inducers of IgE as well.

Using the monoclonal anti-Api m 10 IgE antibody for specific detection of the native protein it was shown that Api m 10 is present in honeybee venom with a concentration of about 0.8 %, but surprisingly it was vastly underrepresented in three therapeutic honeybee venom preparations. The aspect of low abundant allergens in therapeutic extracts and their influence on the efficacy of a VIT has to be analyzed more in detail.

The obtained results demonstrate on the one hand that the characteristics of allergens are strongly influenced by their glycosylation and that the state of glycosylation can be defined by the host [168]. On the other hand obviously Api m 10 plays an important role as sIgE-sensitizing component in honeybee venom beyond CCD-crossreactivity.

4.2   IgE epitopes in the context of allergy

For the development of allergic diseases such as allergic asthma or venom allergies the synthesis of specific IgE is required, but many individuals exhibit allergen-specific IgE without developing any symptoms [232]. The connection of positive IgE, sensitization and the relation to clinical symptoms is still a topic of discussion. The IgE repertoire of an allergic and also non-allergic person is a complex and very individual mixture of distinct IgE properties regarding the total and allergen-specific IgE concentrations, affinities or the IgE clonality [220]. Effector cells like basophils or mast cells contain high affinity IgE receptors on their cell surfaces and after receptor bound IgE cross-linking by allergens the cells release their potent mediators, leading to type I allergic reactions and symptoms. The extent to which individual properties of the IgE antibodies contribute to the effector cell degranulation is still not clear. The difficulties in isolating individual monoclonal IgE antibodies from patient's sera are one major barrier for detailed characterization procedures or antibody/epitope interaction analyses.

A variety of approaches have investigated the structural basis of IgE B cell epitopes on allergens [233, 234] but in the majority of cases polyclonal IgE were applied resulting only in indirect information.

Detailed analyses of IgE epitopes and their interaction with corresponding monoclonal antibodies as well as the understanding of the role of IgE affinities and of specific allergens and its ability to elicit allergen-mediated basophil activation is important for the understanding of allergic mechanisms.

4.2.1   Epitope analyses of monoclonal antibodies specific for Phl p 5a

Almost all cereal and agricultural crops belong to the family of poaceae, which provide the main resource of grass pollen allergens. Poaceae are also distributed in grassland containing a high capacity distributing their pollen, and worldwide about 50 % of allergic people suffering from type I allergy show reactions to grasspollen proteins [196]. Typical clinical symptoms elicited by these potent allergens are rhinitis, conjunctivitis and asthma [196]. Phleum p 5 (Phl p 5) is one major pollen allergen of timothy grass (*Phleum pratense*) and it was shown that the recombinant protein bound IgE of about 80 % when tested with grass pollen-allergic patients [197]. The allergen occurs in two isoforms, Phl p 5a and Phl p 5b, which show a high amino acid sequence homology with 70-75 % and exhibit very similar molecular weights and biochemical characteristics [194, 198]. In 2002 the crystal structure of a C-terminal fragment of Phl p 5b was clarified showing a bundle of four α-helices arranged around a hydrophobic core [211]. This folding was also predicted for the N-terminal fragment due to the homologous and alanine-rich repeats forming the

α−helices. A very similar protein folding was observed for the allergen Phl p 6 and this structure was used in this work as matrix for N-terminal Phl p 5 fragment modeling [235].

For generation of different antibody formats a Phl p 5-specific antibody fragment selected from an immune library, was used as template for gene synthesis [161] and converted into different allergen-specific immunoglobulins of IgG, IgE and also IgA isotypes, representing the first full set of allergy related antibody isotypes. Notably the IgE is the first fully human Phl p 5 specific monoclonal IgE antibody and the identification of its epitope defines the first authentic IgE epitope on Phl p 5. Furthermore a murine hybridoma line, which was also specific for Phl p 5a, was established, recloned and produced as human chimeric IgE.

First the antibodies were purified and their immunoreactivities with the antigen were assessed. Notably, the isoform Phl p 5b was neither bound by the human isotypes nor the chimeric IgE antibody in ELISA, suggesting that the epitope is exclusively present in Phl p 5a. In detailed immunoblot analyses determining the bound epitope region the cIgE showed a reduced reactivity with Phl p 5b, suggesting a rather weak binding to the C-terminal part of the isoform. The functional binding of the antibodies to the high-affinity receptors FcεRI and FcγRI could be confirmed, thereby verifying their general applicability in allergy diagnostic and immunological backgrounds.

In the context of allergy there are still controversial discussions about antibody affinities of different isotypes specific for the same allergen and their corresponding physiological relevance [236]. Both monoclonal IgE antibodies exhibited apparently medium to high affinities as shown by the fact that both constructs were able to mediate basophil degranulation in the presence of Phl p 5a. Christensen *et al.* reported in their study that lower affinities of individual allergen-specific IgE significantly decrease basophil sensitivity [220], as also discussed in detail in chapter 4.2.4. In contrast to this findings it could be observed that in cellular assays by using particle-based versus soluble Phl p 5b microspheres and the monoclonal chimeric IgE antibody exhibiting rather reduced affinity due to the murine origin, the mediator release was reduced.

In order to determine the specific binding site of the monoclonal human IgE with Phl p 5a and b, N-terminal fragments and chimeras were designed forming different parts of the antigen. By these immunoblot analyses in combination with molecular modeling of the N-terminus of the allergen, an IgE epitope mapping was performed. The epitope of the authentic human IgE could be assigned to a looped stretch exclusively present in Phl p 5a. The strongly exposed loop region from the protein represents an unique feature of Phl p 5a and could already be observed in NMR analyses [211]. This finding contradicts the concept of IgE architecture declaring that IgE epitopes are defined by large, planar surface areas comprising several structural elements [57, 58] and that IgE epitopes might

be different from IgG epitopes [57]. Based on crystal structures of allergens in complex with patient derived and murine Fab fragments IgG epitopes are characterized by small exposed and continuous regions.

Using characterized recombinant monoclonal IgE antibodies, different methodologies and approaches in combination it was possible to dissect the structural basis of an authentic human IgE B cell epitope of Phl p 5a for the first time.

4.2.2 Recombinant and structural evaluation of the low molecular weight hymenoptera allergen Api m 6

Api m 6 is an honeybee venom allergen and with a molecular weight of only about 8 kDa it is an interesting molecule that despite of its small size is able to cause IgE sensitization in allergic persons. Api m 6 was identified some years ago by separation of honeybee venom [142] and it shows only a proportion of about 0.8-2 % [142, 145] to the dry venom. So far relatively little is known about such lower abundance components in hymenoptera venoms, as well as their allergenicity, their sensitizing potential, clinical relevance and finally their function. Four different Api m 6 isoforms have been described named as Api m 6.01 to 6.04 and having a primary structure with a common core of 67 amino acids and only varying sequences at the amino and carboxy terminus of at most six residues [142]. The protein contains 10 cysteine residues forming 5 stabilizing disulfide bonds and the protein exhibits no putative glycosylation site, excluding any CCD reactivity. The natural function of Api m 6 is still not proven, but the protein exhibits a trypsin inhibitor like (TIL) cysteine-rich domain indicating its function as protease inhibitor.

For the first time the protein was recombinantly expressed in *E. coli* and in Sf9 insect cells and the specific IgE reactivity of the prokaryotically expressed allergen was evaluated in ELISA. The screening of honeybee venom allergic patients sera with Api m 6 variants demonstrated a specific IgE reactivity of about 26 % what is in accordance of the results of Kettner *et al.*, who demonstrated specific IgE recognition of about 40 % of honeybee venom-sensitized patients in immunoblotting as well as T cell proliferation [142]. These results suggest a relevant role of Api m 6 as sensitizing venom component. Furthermore the amino acid sequence of the mature Api m 6 was aligned with two related serine protease inhibitors. With a sequence homology of about 30 %, basically due to the presence of the 10 typical cysteine residues, Api m 6 can be sorted within the serine protease inhibitors in the class of canonical inhibitors.

In order to get more insights in structure function relationship of Api m 6 a three-dimensional model of the mature allergen was created using the known NMR structure of the *Apis mellifera* chymotrypsin/cathepsin G inhibitor-1 (AMCI-1) as template. Additionally the structure of a third serine protease inhibitor, the chymotrypsin/elastase inhibitor

(C/E-1) from *Ascaris suum* which was solved by crystallization, was used for structural comparison. The molecular modeling of Api m 6 and the both protease inhibitors revealed a typical common fold of canonical protease inhibitors, dominated by an exposed binding loop including the protease binding site and thereby supporting the putative function of Api m 6. With the modeling it was also possible to predict the electrostatic potential of the protein surfaces as well as their epitopes. The illustration of the electrostatic potential ranging from basic to acidic surface properties revealed a strong basic character of the Api m 6 surface and compared to the related proteins a highly variant surface charge. Anyhow the predicted epitope distribution appeared to be similar to the other protease inhibitors.

The honeybee venom component and putative protease inhibitor Api m 6 was recombinantly produced for the first time and evaluated using immunochemical and structural methodologies. The function of Api m 6 as putative serine protease inhibitor could be underlined by the design of a structural model and in comparison with two other related protease inhibitors. It could be shown that also a protein with only a low molecular weight such as Api m 6 and with no putative glycosylation site, shows a noticeable IgE sensitizing effect. In further studies the potential of such small molecules to induce pronounced effector cell activation and thus clinical symptoms should be addressed.

4.2.3 Close-up of an α-Gal specific monoclonal IgE

One of the most abundant cell surface carbohydrate epitopes is the α-Gal epitope produced by the glycosylation enzyme α1,3galactosyltransferase (α1,3GT), whose gene is highly active in nonprimate mammals, prosimians and New World monkeys, but not in Old World primates such as humans, apes and Old World monkeys [90, 91]. Because of the complete inactivation of the α1,3GT gene, probably during the adaptation of the immune system acting against α-Gal expressing pathogens [91, 92] and the subsequent elimination of the α-Gal epitope [93, 94], Old World primates including humans produce very large amounts of natural antibodies against this glycan structure. The anti-Gal antibody which is in particular of the isotype IgG2 constitutes about 1 % of circulating immunoglobulins in the serum [92, 102] and it prevents xenotransplantation of organs and tissues from nonprimate mammals, such as pigs, into humans through their binding to α-Gal on the xenografts leading to hyperacute rejections due to the immunological barrier [237]. Another impressive influence of anti-Gal antibodies was observed when patients showed anaphylactic reactions after administrations of the therapeutic antibody cetuximab [89]. This chimeric mouse-human IgG1 monoclonal antibody is approved for the use in colorectal cancer and squamous-cell carcinoma of the head and neck and due to its

murine origin it exhibits the α-Gal epitope on the Fab portion of its heavy chain. Interestingly it was shown that in most cetuximab-treated subjects, IgE antibodies against cetuximab or rather α-Gal specific IgE were already present in serum before therapy [89], raising the question of sensitization. In this context α-Gal specific IgE were associated with meat induced delayed symptoms of anaphylaxis [106] as well as a possible stimulation of IgE production by tick bites [107].

In order to dissect the interaction between α-Gal and its specific antibodies and to get a closer look on the detailed epitope, an α-Gal specific chimeric antibody was generated and applied in different assay systems. Therefore the murine hybridoma IgM clone M86, which was established galactosyltransferase knock-out mice, was chosen for conversion into the human/mouse chimeric IgG and IgE isotypes, resembling the human system better than any other animal model [162]. The purified antibodies were assessed by immunoblots and their immunoreactivities with antigens as well as with Fc receptor molecules were verified, confirming their functional binding and applicability in immunological approaches. The apparent affinities of the recombinant IgE with different defined α−Gal carriers were determined by SPR analyses, resulting in dissociation constants at equilibrium in the middle nanomolar range. α−Gal specific antibodies in humans are reported to have affinities in the low micromolar range of about 10^{-5} to 10^{-6} M [162, 238] which is a little bit inferior to the results of the M86 derived antibody. Anyhow, this rather medium affinity of M86 can be expected due to its murine origin as IgM that has not undergone affinity maturation processes. The medium affinity is also in accordance to known KD values of carbohydrate-specific binders [163, 238]. Nevertheless, the real affinity is only difficult to assess because of influencing valence effects of both the multivalent α-Gal proteins and the bivalent antibodies.

Interestingly the recombinant IgE was not able to induce mediator release in basophil degranulation assay in the presence of different multivalent α−Gal carrying conjugates, compared to the murine reference antibody C38-2 IgE that exhibits also a medium affinity but showed a clear mediation of basophil degranulation by binding a haptenic target structure. It was observed that limited affinities of allergen specific IgE can affect effector cell degranulation and thereby significantly decrease basophil sensitivity [220]. Furthermore the availability and the architecture of the α-Gal epitope on the carrier surface might affect the capability of cellular activation. The inability of cellular activation of the M86 derived IgE might reflect the delayed type of anaphylaxis observed for α-Gal based allergy [106], although underlying mechanisms remain speculative.

Having a closer look on antibody/carbohydrate interaction by human IgE and IgG on a molecular level, the antibodies were used for epitope characterization with α-Gal by STD-

NMR. This method allows a detailed analysis of binding properties of low-molecular ligands to proteins. It is also possible to characterize not only monoclonal but also polyclonal antibodies such as present in human serum. Due to spin diffusion throughout the ligand, low STD effects of less than 50 % should be considered as irrelevant without further evidence and thereby these effects were not depicted. In contrast protons with medium to strong STD effects represent carbohydrate positions important for interaction. For the interaction footprint the glycan structure Galα1,3GalαOMe was used and the epitope characterization revealed a participation of both galactose residues in interaction of the recombinant IgE as well as the affinity-purified serum. Also the contributions of the carbohydrate residues were similar. The terminal galactose exhibited more pronounced signals suggesting a stronger implication in the carbohydrate/antibody interaction. These findings are in good accordance with the relevance of the 1,3-linkage, which is crucial for recognition, and with a for glycan specific binding molecules reported end-on binding mode. Furthermore the second galactose is also essential for the interaction. The terminal OMe group only shows an insignificant STD effect, reflecting an inferior role of the third glycan residue. Other studies done with chrystallographic and also STD NMR analyses and with galactose binding lectins also reported a predominant binding of the terminal non-reducing galactose residue [239-241]. Compared to the STD epitope of the 120-kDa lectin *Ricinus communis* agglutinin I (RCA(120)) that was analyzed binding the glycan methyl beta-D-galactoside, the epitope mapping showed that the H2, H3, and H4 protons are saturated to the highest degree, what is a nearly identical distribution of STD effects [242].

The generated antibodies specific for the clinical relevant CCD structure α-Gal enabled antibody/carbohydrate interaction analyses on a molecular level and for the first time a STD NMR-based epitope mapping of a human carbohydrate IgE B cell epitope was generated. Thereby combining NMR-based and cellular methodologies a detailed dissection of the recognition and binding mode is possible.

4.2.4   Dissecting IgE reactivities with their haptenic epitopes using TNP as model system

Haptens are small nonpeptidic epitopes with a molecular weight of only 5 kDa or below. Their origin can be quite variable, ranging from antibiotics or hormones to pesticides, but also glycans can be considered as small haptenic structures that are not able to elicit an immune response on their own, but when they are attached to an immunogenic carrier. TNT or rather its surrogate TNP was used as model system because of its haptenic nature providing a minimal epitope. TNT shows a molecular weight of 227 g/mol, is still one of the most important military explosives and is also used as safety explosive in both

military and industrial field. TNT is an environmental toxin and has very high toxic effects on different organisms, since aromatic nitro compounds are enzymatically reduced to amines or their primary metabolites. Skin contact with high TNT doses can cause skin irritation and chronic expose can lead to anemia, abnormal liver and kidney functions as well as cyanosis and formation of methemoglobin. The wide application of TNT in past and presence in combination with its dangerous to health is one of the reasons for the development of many immunoassays for the detection of TNT [243]. Keeping this original intended use in mind different TNT/TNP-specific antibody fragments were selected as detection tools. Here the fragments were used for the generation of monoclonal antibody constructs with specificity for one defined haptenic epitope and in the context of allergy and antibody/antigen interactions the focus was concentrated on the IgE isotype and IgE effector mechanisms.

Three different TNP-specific human monoclonal antibody constructs were successfully generated and the analyses of their immunoreactivities and specificities were verified. The interaction affinities of the three antibodies were determined by SPR measurements, resulting in dissociation constants in equilibrium in the nanomolar range. Notably all three constructs exhibited something different apparent KD values that were 8 nM for 4.8-scFv-IgE, 90 nM for 2.18-scFv-IgE and 794 nM for 5-scFv-IgE. As reference the murine IgE C38-2 was investigated showing a KD of 43 nM. The origin of this antibody was a hybridoma cell line in where the antibodies undergo no affinity maturation processes so that a medium affinity can be assumed.

In the following experiments the influence of different antibody affinities on degranulation levels in cellular *in vitro* mediator release assays was directly investigated. First as a reference, cross-linking was achieved by the addition of polyclonal anti-human IgE serum. Additionally the medium affinity of the murine C38-2-IgE was used as activation control.

The TNP-specific IgE formats bound to the FcεRI and induced mediator release in an antigen-independent manner using anti-IgE antibodies to an extent comparable with the murine reference. All TNP-specific antibodies showed a comparable maximal mediator release of about 60 %, but different antigen concentrations were needed at different antibody-affinities in order to mediate cellular activation. For the antibody with the highest affinity, 4.8-scFv-IgE, concentrations of less than 1 ng/ml antigen were sufficient for reaching maximal degranulation. For the clone 5-scFv-IgE with the lowest KD value the cellular activation required a 5000 fold higher antigen concentration to reach an equivalent degranulation level. The antibody 2.18-scFv-IgE exhibited an affinity in between and needed also about 2500 fold higher antigen concentrations than the 4.8-scFv-IgE, in order to reach the maximal mediator release, but the clone showed cellular activation also with lower TNP-BSA concentrations, such as the murine C38-2-IgE.

Christensen *et al.* described in a study with recombinant IgE specific for the major house dust mite allergen Der p 2 how individual properties of the IgE repertoire affect effector cell degranulation upon allergen challenge [220]. The panel of antibodies showed affinities in the pico- and nanomolar range, designated as high and low affinity antibodies, and in a mapping of their epitopes on Der p 2 seven individual binding patterns were identified. They performed basophil activation tests and demonstrated that the antibody composition, varying in antibody affinities, relative concentrations and clonality, is of major importance for basophil degranulation.

To go more into detail, they sensitized human basophils with different combinations of two recombinant IgE clones having different affinities for the protein, but still binding the same two epitope regions. They observed that basophil sensitivity increased with increasing affinity of individual IgE clones involved in the combinations. Interestingly basophils sensitized with two low-affinity IgE antibodies with KD values in the nanomolar range required a 500-1000-fold higher antigen concentration than basophils sensitized with high-affinity clones to reach equivalent degranulation levels. Furthermore a drastic effect was seen when a low affinity with a high affinity antibody was combined. As also observed with the TNP-specific antibodies all affinity combinations resulted in similar maximal basophil degranulation levels, but in their study they did not investigate the combination of two low-affinity antibodies and it was not tested whether this combination reached the same maximal response at higher antigen concentrations.

With regard to the influence of different affinities to the degranulation level and needed antigen concentrations, the observations of the TNP-specific antibodies are in good accordance with the results of Christensen *et al.*. However, they used protein specific antibodies binding different proteinic epitopes for their comprehensive characterizations in contrast to the experiments shown here, where a haptenic epitope with different but for the same target structure specific antibodies was analyzed.

In the context of CCDs, which show haptenic properties, there is still a controversial discussion of the clinical relevance of CCD specific IgE antibodies. For polyclonal patients' IgE with specificity for α1,3-fucose and β1,2-xylose high affinities of 10^{-10} M were detected [223], but the antibodies appear to be of only low clinical significance demonstrating that the low binding affinity of anti-CCD IgE antibodies cannot be the reason for their insignificance. Further the results which are shown here demonstrate the ability of cellular basophil activation using hapten specific antibodies with only low affinities, maintaining the open question of the underlying and crucial degranulation triggering factor.

The demonstrated recognition of haptenic structures such as carbohydrates by IgE is not in accordance with the hypothesis that IgE epitopes are preferably defined by large surface areas as shown in crystal structures so far [57, 58]. The influence of the involved

peptidic environment of glycan structure carrying molecules on the recognition mechanism still has to be evaluated. The structural and functional characterization of hapten-specific antibodies such as shown here can thereby serve as a suitable model system for detailed interaction analyses with defined experimental conditions and may contribute to the understanding of molecular interactions of antibodies or proteins with low molecular target structures.

4.3   Summary

Against the background of hypersensitivity reactions caused by hymenoptera venoms, the honeybee venom allergens Api m 1 and Api m 10 were investigated regarding IgE reactivities against CCDs or peptidic epitopes, and recombinant approaches for a detailed dissection could be established. The almost haptenic honeybee venom allergen Api m 6 characterized here exhibited in spite of its small molecular weight a pronounced IgE reactivity, raising again questions about IgE epitope distributions on allergens. For interaction analyses of an authentic IgE epitope of Phl p 5a, two recombinant IgE antibodies with different affinities were generated recognizing two different epitope regions on the allergen and showing mediation of basophil activation. In this regard the presented results are in good accordance with demonstrated binding studies of Christensen *et al.* [220]. Moreover, the authentic IgE epitope of Phl p 5a interestingly could be assigned to an exposed looped stretch, contradicting the concept of IgE architecture declaring that IgE epitopes are defined by planar, large surface areas comprising several structural elements [57, 58]. An α-Gal specific IgE with medium binding affinity was on the other hand not able to mediate cellular activation in degranulation assays, despite the fact that α-Gal as terminal glycan structure is also exposed. In addition, α-Gal represents a highly clinical relevant CCD structure in contrast to α1,3-fucose or β1,2-xylose, which obviously have no or only low clinical relevance even though polyclonal investigated human IgE specific for α1,3-fucose and β1,2-xylose showed high affinities of KD = 10^{-10} to 10^{-11} M [233]. The influence of the probably difficult accessibility of these core glycans remains speculative. Suitable model systems with known and defined experimental conditions for precise interaction studies, as shown here with the TNP-specific IgE antibodies, can help to understand the complex correlations of antibody/epitope interactions and connected cellular mechanisms. Due to the results presented here it can be postulated that an explicit epitope presentation in the periphery is not essential for IgE accessibility, but the question of specific characteristics or common features of IgE epitopes and their architecture still remains open.

The obtained molecular insights however can contribute to the understanding of complicate interactions of antibodies with their corresponding antigens and help elucidating the nature of IgE B cell epitopes.

4.4 Outlook

Currently available recombinant technologies open up a wide range of possibilities in order to obtain detailed molecular insights into complex interactions of antibodies with their antigens. In the context of their clinical relevance further analyses of haptenic structures such as CCDs are of significant interest. The investigation of e.g. α1,3-fucose and β1,2-xylose and corresponding specific recombinant human IgE antibodies and their ability to mediate degranulation in cellular assays as well as the ability of very small, nearly haptenic allergens like Api m 6, may contribute to understand molecular interactions and resulting cellular reactions. Moreover, comparisons of characteristics of monoclonal and polyclonal antibodies with different isotypes are of interest. Established model systems, as shown here with a set of monoclonal TNP-specific monoclonal IgEs with defined experimental conditions, can form the basis for further interaction analyses. Increasing the affinity of the α-Gal specific recombinant antibody by mutagenesis strategies could evidence if mediation of cellular degranulation is possible. Regarding the architecture of IgE epitopes further footprints of binding modalities using STD NMR analyses can help to assess the influence of accessibility of specific epitope regions or glycan structures. A main focus should also be the dissection of the interplay of multiple binding parameters clarifying if cumulative binding kinetics are essential.

By making a direct connection between affinity and biological activity of a CCD specific antibody, the knowledge, diagnosis and treatment of allergies finally might be improved.

5 References

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Appendix

Curriculum Vitae

entfällt aus datenschutzrechtlichen Gründen

Publications as first author

Michel Y, McIntyre M, Ginglinger H, Ollert M, Cifuentes L, Blank S, Spillner E, "The putative serine protease inhibitor allergen Api m 6 from *A. mellifera* venom: recombinant and structural evaluation", *Journal of Investigational Allergy & Clinical Immunology*, *submitted,* Impact Factor: 1.5

Plum M, **Michel Y**, Wallach K, Raiber T, Blank S, Bantleon FI, Diethers A, Greunke K, Braren I, Hackl T, Meyer B, Spillner E, "Close-up of the Immunogenic α1,3-Galactose Epitope as Defined by a Monoclonal Chimeric Immunoglobulin E and Human Serum Using Saturation Transfer Difference (STD) NMR", *Journal of Biological Chemistry* **2011** Dec 16;286(50):43103-11, Impact Factor: 5.3

Blank S, **Michel Y**, Seismann H, Plum M, Greunke K, Grunwald T, Bredehorst R, Ollert M, Braren I, Spillner E, "Evaluation of Different Glycoforms of Honeybee Venom Major Allergen Phospholipase A2 (Api m 1) Produced in Insect Cells", *Protein & Peptide Letters* **2011** Apr 18:415-22, Impact Factor: 1.9

Other publications

Blank S, Seismann H, **Michel Y**, McIntyre M, Cifuentes L, Braren I, Grunwald T, Darsow U, Ring J, Bredehorst R, Ollert M, Spillner E, "Api m 10, a genuine *A. mellifera* venom allergen, is clinically relevant but underrepresented in therapeutic extracts", *Allergy* 2011, Oct;66(10):1322-9, Impact Factor: 6.3

Hecker J, Diethers A, Etzold S, Seismann H, **Michel Y**, Plum M, Bredehorst R, Blank S, Braren I, Spillner E, "Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a", *Molecular Immunology* **2011** May;48(9-10):1236-44, Impact Factor: 2.9

Abstracts

Blank S, **Michel Y**, Bantleon F, Seismann H, McIntyre M, Ollert M, Spillner E, "Evaluation of the IgE sensitizing potential of novel hymenoptera venom allergens", *Allergo Journal*, 2012

Plum M, **Michel Y**, Wallach K, Raiber T, Blank S, Bantleon F, Diethers A, Greunke K, Braren I, Hackl T, Meyer B, Spillner E, "Close-up of the alpha-1,3-Gal epitope as defined by a monoclonal chimeric IgE and human serum using saturation transfer difference (STD) NMR*", Allergo Journal*, 2012

Diethers A, Hecker J, **Michel Y**, Plum M, Schulz D, Sabri A, Mempel M, Ollert M, Blank S, Braren I, Spillner E, "A human monoclonal IgE derived from hybrid repertoire libraries defines an epitope genuine for Bet v 1 and fagales PR10 proteins", *Allergo Journal*, 2012

Michel Y, Plum M., Bantleon F, Blank S, Braren I, Spillner E, "Human monoclonal anti-Gal IgE for diagnostic and mechanistic analyses of alpha-Gal associated reactivities", *Allergo Journal,* 2011

Blank S, Bantleon F, Seismann H, **Michel Y**, Plum M, McIntyre M, Bredehorst R, Ollert M, Braren I, Spillner E, "Allergen composition of therapeutic and diagnostic venom preparations as assessed by monoclonal human IgE antibodies", *Allergy*, 2011, Impact Factor: 6.3

Hecker J, Diethers A, Etzold S, Seismann H, **Michel Y**, Plum M, Bredehorst R, Blank S, Braren I, Spillner E, "Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a", *Allergy* 2011, Impact Factor: 6.3

Diethers A, Hecker J, Etzold S, Seismann H, **Michel Y**, Plum M, Bredehorst R, Blank S, Braren I, Spillner E, "Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a", *Allergo Journal*, 2011

Oral Presentations

Michel Y, Plum M, Bantleon F, Blank S, Braren I, Spillner E, "Human monoclonal anti-Gal IgE for diagnostic and mechanistic analyses of alpha-Gal associated reactivities", DGAKI, Mainz, 2011

Conference Contributions

Blank S, **Michel Y**, Bantleon F, Seismann H, McIntyre M, Ollert M, Spillner E, „Evaluation of the IgE sensitizing potential of novel hymenoptera venom allergens", DGAKI, Mainz, 2012

Plum M, **Michel Y**, Wallach K, Raiber T, Blank S, Bantleon F, Diethers A, Greunke K, Braren I, Hackl T, Meyer B, Spillner E, "Close-up of the alpha-1,3-Gal epitope as defined by a monoclonal chimeric IgE and human serum using saturation transfer difference (STD) NMR", DGAKI, Mainz, 2012

Diethers A, Hecker J, **Michel Y**, Plum M, Schulz D, Sabri A, Mempel M, Ollert M, Blank S, Braren I, SpillnerE, "A human monoclonal IgE derived from hybrid repertoire libraries defines an epitope genuine for Bet v 1 and fagales PR10 proteins", DGAKI, Mainz, 2012

Blank S, Bantleon F, Seismann H, **Michel Y**, Plum M, McIntyre M, Bredehorst R, Ollert M, Braren I, Spillner E, "Allergen composition of therapeutic and diagnostic venom preparations as assessed by monoclonal human IgE antibodies", DGAKI, Mainz, 2011, EAACI, Istanbul, 2011

Diethers A, Hecker J, Etzold S, Seismann H, **Michel Y**, Plum M, Bredehorst R, Blank S, Braren I, Spillner E, "Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a", DGAKI, Mainz, 2011

Poster

Hecker J, Diethers A, Etzold S, Seismann H, **Michel Y**, Plum M, Bredehorst R, Blank S, Braren I, Spillner E, "Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a", EAACI, Istanbul, 2011

Blank S, Seismann H, Braren I, **Michel Y**, McIntyre M, Cifuentes L, Ring J, Grunwald T, Bredehorst R, Ollert M, Spillner E, "A recombinant approach to component-resolution in hymenoptera venom allergy", 4th International Symposium on Molecular Allergology, October 29 – 31, 2010, Munich, Germany

Blank S, Bantleon F, Seismann H, **Michel Y**, Braren I, Ollert M, Spillner E, "Allergen composition of therapeutic and diagnostic venom preparations as assessed by monoclonal human IgE antibodies", 4th International Symposium on Molecular Allergology, October 29 – 31, 2010, Munich, Germany

Gefahrstoffe und Sicherheitsdaten

Gefahrensymbole, Gefahrenhinweise (H-Sätze, Hazard Statements) und Sicherheitsratschläge (P-Sätze, Precautionary Statements) verwendeter Substanzen nach dem GHS-System:

Verbindung:	Gefahrensymbole:	H-Sätze:	P-Sätze:
ABTS	GHS07	315/319/335	261/305+351+338
Acrylamid	GHS06/08	350/340/361/301/372/332/312/319/317	201/280/301/310/305+351+338/308+313
Ammoniumpersulfat	GHS02/07/08	272/302/315/319/335/334/317	280/305+351+338/302+352/304+341/342+311
Ampicillin	GHS07/08	315/317/319/334/335	261/280/305+351+338/342+311
Chloroform	GHS07/08	302/315/351/373	302+352/314
Dimethylformamid	GHS02/07/08	360D/226/332/312/319	201/302+352/305+351+338/308+313
Dithiothreitol	GHS07	302/315/319	302+352/305+351+338
EDTA	GHS07	319	305/351/338
Eisessig	GHS02/05	226-314	280-301+330+331/307/310
Ethanol	GHS02	225	210
Ethidiumbromid	GHS06/08	341/330/302	281/302/352/305+351+338/304+340/309/310
Glutardialdehyd	GHS05/06/08/09	331/301/314/334/317/400	260/273/280/303+361+353 305+351+338/342+311
Imidazol	GHS05/07/08	302/314/361D	280/301+331/305+351+338/309/310
Kanamycinsulfat	GHS08	360D	281/201/305+351+338 308+313
Methanol	GHS02/06/08	225/331/311/301/370	210/233/280/302+352

Verbindung:	Gefahrensymbole:	H-Sätze:	P-Sätze:
Natronlauge	GHS05	314	280/301+330+331/310 305+351+338
Nickelsulfat	GHS08/07/09	350i/341/360D/372/ 332/302/315/334/317/ 410	201/280/273/308+313 342+311/302+352
Phenol	GHS05/06/08	341/331/311/301/373/ 314	280/302+352/301+330+ 331/309/310/305+351+338
2-Propanol	GHS02/07	225/319/336	210/233/305+351+338
Salzsäure	GHS05/07	314/335	260/301+330+331/361+ 353/305+351+338/405/501
SDS	GHS06/02	228/331/302/335/315 319	210/280/304+340/305+ 351+338/309+310
TEMED	GHS02/05/07	225/332/302/314	210/233/280/301+330+ 331/305+351+338/309+ 310
Triethylamin	GHS02/05/07	225/332/312/302/314	210/280/303+361+353/ 305+351+338/310/312
Tris	GHS07	315/319/335	216/305+351+338
Wasserstoffperoxid	GHS03/05/07	271/332/302/314	220/261/280/305+351+ 338/310
Zitronensäure	GHS05	318	305+351+338

Angaben zum individuellen Beitrag an den Publikationen, die im Appendix dieser Dissertation aufgeführt sind

Erstautorenschaft:

Michel Y, McIntyre M, Ginglinger H, Ollert M, Cifuentes L, Blank S, Spillner E, "The putative serine protease inhibitor allergen Api m 6 from *A. mellifera* venom: recombinant and structural evaluation", *Journal of Investigational Allergy & Clinical Immunology*, **submitted**, Impact Factor: 1.5

Eigener Anteil: ca. 55 % (Klonierung und Expression von Api m 6 in *E. coli*, Reinigung des rekombinanten Proteins mittels Affinitätschromatographie, Charakterisierung von rApi m 6 im ELISA und Immunoblot, Reaktivitätsanalyse von rApi m 6 mit Patientenseren im ELISA, Abgleich der Api m 6 Aminosäuresequenz mit verwandten Proteinen, Erstellung der Ergebnisabbildungen, Textverfassung)

Plum M, **Michel Y**, Wallach K, Raiber T, Blank S, Bantleon FI, Diethers A, Greunke K, Braren I, Hackl T, Meyer B, Spillner E, "Close-up of the Immunogenic α1,3-Galactose Epitope as Defined by a Monoclonal Chimeric Immunoglobulin E and Human Serum Using Saturation Transfer Difference (STD) NMR", *Journal of Biological Chemistry 2011* Dec 16;286(50):43103-11, Impact Factor: 5.3

Eigener Anteil: ca. 40 % (Expression des scFv in *E. coli*, Reinigung des in HEK-293-Zellen exprimierten monoklonalen scFv-IgE M86 mittels Affinitätschromatographie, immunologische und funktionelle Charakterisierung des Konstrukts im ELISA und Immunoblot, Charakterisierung der Effektorfunktion im Degranulations-assay, SPR-spektroskopische Messungen zur Bestimmung der Affinität des scFv-IgE mit verschiedenen Zielmolekülen, vorbereitende Präparation des scFv-IgE für STD NMR Messungen, Textbeiträge Methoden und Ergebnisse, Erstellung der entsprechenden Ergebnisabbildungen)

Blank S, **Michel Y**, Seismann H, Plum M, Greunke K, Grunwald T, Bredehorst R, Ollert M, Braren I, Spillner E, "Evaluation of Different Glycoforms of Honeybee Venom Major Allergen Phospholipase A2 (Api m 1) Produced in Insect Cells", *Protein & Peptide Letters* 2011 Apr 18:415-22, Impact Factor: 1.9

Eigener Anteil: ca. 40 % (Expression der *Apis mellifera* Phospholipase A2 in verschiedenen Zelllinien und Reinigungen der unterschiedlichen Proteinvarianten mittels Affinitätschromatographie, Charakterisierungen der Proteinvarianten im Immunoblot)

Weitere Publikationen:

Blank S, Seismann H, **Michel Y**, McIntyre M, Cifuentes L, Braren I, Grunwald T, Darsow U, Ring J, Bredehorst R, Ollert M, Spillner E, "Api m 10, a genuine *A. mellifera* venom allergen, is clinically relevant but underrepresented in therapeutic extracts", *Allergy* 2011, Oct;66(10):1322-9, Impact Factor: 6.3

Eigener Anteil: ca. 20 % (Klonierung und Expression von rekombinanten Api m 10 in *E. coli*, Reinigung mittels Affinitätschromatographie)

Hecker J, Diethers A, Etzold S, Seismann H, **Michel Y**, Plum M, Bredehorst R, Blank S, Braren I, Spillner E, "Generation and epitope analysis of human monoclonal antibody isotypes with specificity for the timothy grass major allergen Phl p 5a", *Molecular Immunology* 2011 May;48(9-10):1236-44, Impact Factor: 2.9

Eigener Anteil: ca. 15 % (Charakterisierungen diverser Phleum p 5 Fragmente im Immunoblot, Textbeitrag Material und Methoden)

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Eidesstattliche Versicherung

Hiermit versichere ich an Eides statt, die vorliegende Arbeit selbstständig und ohne fremde Hilfe sowie nur mit den angegebenen Hilfsmitteln und Quellen erstellt zu haben. Ergebnisse aus Bachelor- oder Schwerpunktarbeiten, die in diesem Arbeitskreis angefertigt wurden und die teilweise in diese Arbeit eingeflossen sind, wurden von mir initiiert und unter meiner Anleitung angefertigt.

Ebenfalls versichere ich, noch keinen weiteren Promotionsversuch an einer anderen Einrichtung unternommen zu haben.

Hamburg, den 28.03.2012

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Yvonne Michel