

# IgE and allergen-specific immunotherapy-induced IgG<sub>4</sub> recognize similar epitopes of Bet v 1, the major allergen of birch pollen

N. Groh<sup>1</sup>, C. S. von Loetzen<sup>2</sup>, B. Subbarayal<sup>3</sup>, C. Möbs<sup>4</sup>, L. Vogel<sup>1</sup>, A. Hoffmann<sup>1</sup>, K. Fötisch<sup>1</sup>, A. Koutsouridou<sup>1</sup>, S. Randow<sup>1</sup>, E. Völker<sup>1</sup>, A. Seutter von Loetzen<sup>1</sup>, P. Rösch<sup>2</sup>, S. Vieths<sup>1</sup>, W. Pfützner<sup>4</sup>, B. Bohle<sup>3</sup> and D. Schiller<sup>1</sup>

<sup>1</sup>Division of Allergology, Paul-Ehrlich-Institut, Langen, Germany, <sup>2</sup>Department of Biopolymers, University of Bayreuth, Bayreuth, Germany, <sup>3</sup>Department of Pathophysiology and Allergy Research and Christian Doppler Laboratory for Immunomodulation, Medical University of Vienna, Vienna, Austria and

<sup>4</sup>Department of Dermatology and Allergology, Philipps University Marburg, Marburg, Germany

## Clinical & Experimental Allergy

### Summary

**Background** Allergen-specific immunotherapy (AIT) with birch pollen generates Bet v 1-specific immunoglobulin (Ig)G<sub>4</sub> which blocks IgE-mediated hypersensitivity mechanisms. Whether IgG<sub>4</sub> specific for Bet v 1a competes with IgE for identical epitopes or whether novel epitope specificities of IgG<sub>4</sub> antibodies are developed is under debate.

**Objective** We sought to analyze the epitope specificities of IgE and IgG<sub>4</sub> antibodies from sera of patients who received AIT.

**Methods** 15 sera of patients (13/15 received AIT) with Bet v 1a-specific IgE and IgG<sub>4</sub> were analyzed. The structural arrangements of recombinant (r)Bet v 1a and rBet v 1a<sub>11x</sub>, modified in five potential epitopes, were analyzed by circular dichroism and nuclear magnetic resonance spectroscopy. IgE binding to Bet v 1 was assessed by ELISA and mediator release assays. Competitive binding of monoclonal antibodies specific for Bet v 1a and serum IgE/IgG<sub>4</sub> to rBet v 1a and serum antibody binding to a non-allergenic Bet v 1-type model protein presenting an individual epitope for IgE was analyzed in ELISA and western blot.

**Results** rBet v 1a<sub>11x</sub> had a Bet v 1a – similar secondary and tertiary structure. Monomeric dispersion of rBet v 1a<sub>11x</sub> was concentration and buffer-dependent. Up to 1500-fold increase in the EC<sub>50</sub> for IgE-mediated mediator release induced by rBet v 1a<sub>11x</sub> was determined. The reduction of IgE and IgG<sub>4</sub> binding to rBet v 1a<sub>11x</sub> was comparable in 67% (10/15) of sera. Bet v 1a-specific monoclonal antibodies inhibited binding of serum IgE and IgG<sub>4</sub> to 66.1% and 64.9%, respectively. Serum IgE and IgG<sub>4</sub> bound specifically to an individual epitope presented by our model protein in 33% (5/15) of sera.

**Conclusion and Clinical Relevance** Patients receiving AIT develop Bet v 1a-specific IgG<sub>4</sub> which competes with IgE for partly identical or largely overlapping epitopes. The similarities of epitopes for IgE and IgG<sub>4</sub> might stimulate the development of epitope-specific diagnostics and therapeutics.

**Keywords** Bet v 1, epitope, IgE, IgG<sub>4</sub>, immunotherapy

Submitted 26 July 2016; revised 14 September 2016; accepted 26 September 2016

### Correspondence:

Dirk Schiller, Paul-Ehrlich-Institut,  
Division of Allergology, Paul-Ehrlich-  
Str. 51-59, 63225 Langen, Germany.  
E-mail: Dirk.Schiller@pei.de

Cite this as: N. Groh, C. S. von  
Loetzen, B. Subbarayal, C. Möbs,  
L. Vogel, A. Hoffmann, K. Fötisch,  
A. Koutsouridou, S. Randow, E. Völker,  
A. Reuter, P. Rösch, S. Vieths,  
W. Pfützner, B. Bohle and D. Schiller.  
*Clinical & Experimental Allergy*, 2017  
(47) 693–703.

## Introduction

Allergen-specific immunotherapy (AIT) is currently the only causative treatment of respiratory allergy to pollen and house dust mites [1]. The induction of allergen-specific Immunoglobulin G (in particular IgG<sub>4</sub>) upon AIT has been associated with successful allergy treatment in some cases [2, 3]. IgG<sub>4</sub> competes with IgE for allergen binding, which leads to an inhibition of IgE-mediated hypersensitivity mechanisms and thus alleviates allergic

symptoms [4–8]. Whether the allergen's epitope repertoire for IgG<sub>4</sub> and IgE is identical, different, or similar is currently under debate. It has been reported that upon AIT the IgG<sub>4</sub> repertoire expands in a polyclonal fashion with novel epitope specificities [9]. In contrast, a mainly similar and largely overlapping epitope repertoire for both IgE and IgG<sub>4</sub> has been suggested [10, 11]. To understand the allergen's antibody binding sites on the molecular level, the conformation of Bet v 1 has been determined and several recombinant (r)Bet v 1

variants with multiple amino acid substitutions have been generated to analyze potential B and T cell epitopes [12–19]. Furthermore, rBet v 1 variants were tested as potential therapeutics in birch pollen AIT [20–25]. However, still only very little information on the location and clinical relevance of IgE and IgG<sub>4</sub> binding sites of Bet v 1 is available.

The only structurally solved epitope of a PR10 allergen was analyzed as complex structure of rBet v 1 and BV16, a mouse monoclonal antibody specific for Bet v 1 [26]. BV16 competes with serum IgE for Bet v 1 binding suggesting overlapping epitopes for both antibody classes [26, 27]. Similarly, recent studies based on phage-displayed antibody binding peptides suggested that the majority of AIT-induced IgG<sub>4</sub> and Bet v 1-specific IgE recognized highly overlapping epitopes indicating a competition of both antibodies for similar/identical epitopes [10, 11].

These observations prompted us to analyze whether the epitope specificities of AIT-induced IgG<sub>4</sub> antibodies are novel or similar to those of IgE using structurally well-characterized variants of proteins harbouring the Bet v 1 type fold. For this purpose, we used sera of birch pollen allergic subjects who received AIT and analyzed serum antibody binding to rBet v 1a as well as to a rBet v 1a variant harbouring eleven substitutions (rBet v 1<sub>11x</sub>) of amino acids located in five putative distinct epitopes for IgE or IgG<sub>4</sub> [10, 11, 28]. We evaluated the Bet v 1-type structure of the variant by circular dichroism (CD) and nuclear magnetic resonance (NMR). IgE and IgG<sub>4</sub> binding of rBet v 1 and rBet v 1<sub>11x</sub> was analyzed in ELISA, cellular mediator release assays, and in the presence of Bet v 1-specific murine monoclonal antibodies. Epitope specificities of serum IgE and IgG<sub>4</sub> were tested with a variant of norcochlorine synthase (NCS), a previously published non-allergenic Bet v 1-type model protein harbouring an individual antibody binding site [19].

## Material and methods

### Study population

Birch pollen allergic patients suffering from moderate-to-severe rhinoconjunctivitis and/or mild-to-moderate allergic asthma to early flowering tree pollen who received their first AIT were included as serum donors, as described [10, 11]. Blood samples (sera of patients 1–13) were collected before and after 36 months of AIT (Table 1). Specific sensitization was documented by quantitative determination of rBet v 1a-specific IgE and IgG<sub>4</sub> using ImmunoCAP™ (Thermo Fisher Scientific, Uppsala, Sweden). Patients were recruited from the Department of Dermatology and Allergology, Philipps University, Marburg (Patients 1–13); Serum of patient

Table 1. Human sera used in this study

| Patient | IgE [kU <sub>A</sub> /L] |       |              |       | IgG <sub>4</sub> [mg <sub>A</sub> /L] |       |
|---------|--------------------------|-------|--------------|-------|---------------------------------------|-------|
|         | Bet v 1                  |       | Birch pollen |       | Birch pollen                          |       |
|         | m0                       | m36   | m0           | m36   | m0                                    | m36   |
| 1       | 2.66                     | 2.60  | 2.69         | 3.27  | 0.07                                  | 3.71  |
| 2       | 23.65                    | 29.40 | 23.60        | 29.10 | 0.01                                  | 9.12  |
| 3       | 6.28                     | 4.45  | 5.74         | 4.66  | 0.71                                  | 3.60  |
| 4       | 0.80                     | 0.40  | 0.97         | 0.44  | 0.02                                  | 1.61  |
| 5       | 60.70                    | 36.90 | 81.70        | 54.10 | 0.87                                  | 1.45  |
| 6       | 21.20                    | 43.50 | 16.80        | 47.60 | 0.26                                  | 28.20 |
| 7       | 42.90                    | 32.80 | 41.30        | 31.20 | 0.53                                  | 18.70 |
| 8       | 16.10                    | n.d.  | 16.80        | n.d.  | 0.14                                  | n.d.  |
| 9       | 16.35                    | 13.40 | 13.60        | 15.40 | 0.04                                  | 1.03  |
| 10      | 5.45                     | 3.69  | 5.33         | 4.45  | 0.47                                  | 7.41  |
| 11      | 5.08                     | 5.78* | 5.51         | 7.00* | 0.11                                  | 2.88* |
| 12      | 3.99                     | 3.49  | 3.89         | 3.31  | 0.13                                  | 2.87  |
| 13      | 11.50                    | n.d.  | 10.20        | n.d.  | 0.29                                  | n.d.  |
| 14      | >100                     |       | n.d.         |       | n.d.                                  |       |
| 15      | >100                     |       | >100         |       | n.d.                                  |       |

Allergen-specific levels of IgE and IgG<sub>4</sub> were determined by ImmunoCAP. Patients 1–13 received allergen-specific immunotherapy (AIT) with birch pollen extract. Patients 14–15 are allergic to birch pollen; however, reception of AIT is not known. m0, m36: months after start of AIT. \*: value from month 18 after start of AIT. n.d. – not determined.

14, a subject allergic to birch pollen, was purchased from DLab Diagnose GmbH (Hamburg, Germany). Patient 15 was donor of the serum collection of the Paul-Ehrlich-Institut. Ethical approval by the Ethics Committee of the Medical Faculty of the Philipps University Marburg (Az. 24/13) included consent form and consent procedure. Serum from a non-allergic subject was used as negative control for the specific IgE measurements. Patients 14 and 15 did not receive AIT (to our knowledge) but still had Bet v 1a-specific serum IgG<sub>4</sub> (only patient 14). Serum of patient 15 did not have IgG<sub>4</sub> against Bet v 1a but was included as additional control for IgE binding assays. Serum of patient 15 was published elsewhere [19]. If not stated otherwise, sera from blood drawings taken 36 months after start of AIT were analyzed. The sera and their Bet v 1a-specific IgE and IgG<sub>4</sub> levels are summarized in Table 1.

### Determination of allergen-specific IgE and IgG<sub>4</sub>

Specific IgE and IgG<sub>4</sub> levels to rBet v 1 and birch pollen were determined by ImmunoCAP-FEIA using a Phadia 250 instrument (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's instructions.

### Generation and purification of recombinant Bet v 1a (Bet v 1.0101) and Δ29NCS variants

rΔ29NCS and Δ29NCS<sub>5x</sub> were cloned and purified as described [19]. The open reading frame of Bet v 1.0101

(designated 'Bet v 1a' in the manuscript) optimized for codon usage of *Escherichia coli* was purchased from GeneArt (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) and cloned into bacterial expression vector pET15b (Novagen, Merck, Darmstadt, Germany) using the Clontech InFusion Kit (Mountain View, CA, USA). The variant rBet v 1a<sub>11x</sub> (rBet v 1a<sub>E6A/T9A/N28K/F30V/K55A/K65A/K115A/K119A/D125N/K134A/E138A</sub>) was generated with the QuickChange Multi Lighting mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA) using the Bet v 1a (Bet v 1.0101) ORF in pET15b\_Bet v 1a as DNA template and mutagenic primers Bet v 1<sub>E6A</sub> (*ggc gtg ttt aac tat gca acc gaa acc acc ag*), Bet v 1<sub>T9A</sub> (*gcc tat gca acc gaa gcc acc agt gtt att ccg*), Bet v 1<sub>N28K/F30V</sub> (*att ctg gat ggc gat aaa ctg gtt ccg aaa gtt gca ccg*), Bet v 1<sub>K55A</sub> (*ccg ggt acg atc aaa gca atc agc ttt ccg gaa g*), Bet v 1<sub>K65A</sub> (*ggt ttc ccg ttc gca tat gtt aaa gat cgt*), Bet v 1<sub>K115A/K119A</sub> (*ggt ggt agc att ctg gca att agc aac gca tat cac acc aaa ggc*), Bet v 1<sub>D125N</sub> (*tat cac acc aaa ggc aac cat gaa gtt aaa gca*), Bet v 1<sub>K134A/E138A</sub> (*gca gaa cag gtt gca gcc agc aaa gca atg ggt gaa acc ctg*) according to the manufacturer's instructions. The genes were expressed in *E. coli* strain BL21(DE3) cells, growing at 37°C in 1 liter of LB medium, containing 50 µg/mL carbenicillin to an OD<sub>600</sub> of 0.7. A final concentration of 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to induce protein expression. The cells were harvested 4 h after IPTG induction by centrifugation (10 000 g, 15 min, 4°C), and the cell pellets were stored at -20°C after washing with ice-cold water.

rBet v 1a variants were purified from inclusion bodies. The inclusion bodies were resuspended in 10 mL buffer (50 mM Tris/HCl pH 8.5, 5 mM EDTA, 1 Complete Mini EDTA free Protease Inhibitor Cocktail Tablet, 200 U/mL lysozyme, 200 U/mL benzonase) and incubated for 20 min rotating at room temperature. The suspension was sonicated on ice using pulses (15 s) and pauses (15 s) for a total of 2 min (amplitude 80%). The suspension was sedimented (12 000 g, 4°C, 20 min), and the supernatant (SF1) was stored at 4°C. The pellet was resuspended with 10 mL 50 mM Tris/HCl pH 8.5, 5 mM EDTA, 1 Complete Mini EDTA free Protease Inhibitor Cocktail Tablet, 1% sodium deoxycholate, and sonicated and sedimented as above. The supernatant (SF2) was stored at 4°C. The pellet was resuspended with 10 mL buffer (50 mM Tris/HCl pH 8.5, 1 Complete Mini EDTA free Protease Inhibitor Cocktail Tablet, 1% sodium deoxycholate) and incubated overnight at 37°C, followed by sonication, centrifugation, and storage at 4°C as above (SF3). The pellet was resuspended in 10 mL 50 mM Tris/HCl pH 8.5, incubated for 20 min (20°C), centrifuged, and stored at 4°C (SF4). Isolated inclusion bodies (IB) were resuspended with 100 mM Tris/HCl, pH 12.5 + 2 M urea. All SFs and IBs were checked via SDS-PAGE. Samples containing rBet v 1a

(SF1-4) were pooled and dialyzed in lysis buffer containing 0.5% sodium deoxycholate.

Proteins were bound to Ni-NTA Superflow (Qiagen, Hilden, Germany), washed with 50 mM Tris/HCl, pH 8.5. Buffer was exchanged to 10 mM potassium phosphate (KPi), pH 7.4 during chromatography, and proteins were eluted with 10 mM KPi, pH 7.4, 0.5 M imidazole. Fractions containing the rBet v 1 variants were pooled, concentrated, aliquoted, frozen in liquid nitrogen, and stored at -80°C. Protein concentrations were determined with bicinchoninic acid using bovine serum albumin as standard (BCA™ protein assay kit; Thermo Scientific, Rockford, IL, USA).

#### Circular dichroism spectroscopy

Far UV CD spectra of the Bet v 1 variants were acquired at 293 K using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Gross-Umstadt, Germany) at a bandwidth of 1 nm and a sensitivity of 100 mdeg in a 0.2-cm cell. All proteins were analyzed at a concentration of 10 µM in 10 mM KPi, pH 7.4. Each measurement comprised the average of 10 repeated scans between 255 and 185 nm.

#### Nuclear magnetic resonance spectroscopy

NMR samples were prepared in 10 mM KPi at pH 7.4, and 10% D<sub>2</sub>O containing 15 µM of rBet v 1a-6xHis or rBet v 1a<sub>11x</sub>-6xHis. Standard 1D <sup>1</sup>H spectra with WATERGATE solvent suppression [29] were recorded on a Bruker Avance 600 MHz spectrometer at 298 K. NMR data were processed and visualized with the Bruker spectrometer software TOP SPIN (Bruker, Billerica, MA, USA).

#### Dynamic light scattering

Dynamic light scattering analysis was used to determine hydrodynamic radii (R<sub>H</sub>) in nm using a Zetasizer Nano ZS (Malvern, Herrenberg, Germany). 10 µM of recombinant Bet v 1 and Bet v 1<sub>11x</sub> in 10 mM KPi, pH 7.4 or TBST 0.05% were analyzed at 25°C. Three individual measurements (3 × ≥ 10 runs per measurement) per protein were carried out.

#### Binding of serum IgE, serum IgG<sub>4</sub>, and mouse monoclonal antibodies to variants of rBet v 1a and Δ29NCS

Nunc Maxisorp plates (Fisher Scientific, Schwerte, Germany) were coated overnight at room temperature with 50 ng/100 µL (rBet v 1a and rBet v 1a<sub>11x</sub>) or 300 ng/100 µL (Δ29NCS and Δ29NCS<sub>5x</sub>), in phosphate-buffered saline (PBS). After blocking with PBS containing

2% BSA, sera (dilution 1 : 10) or murine monoclonal antibodies (dilution 1 : 10) were added for 1 h at room temperature in PBS containing 0.05% Tween 20 and 0.1% BSA. Allergen-specific human IgE/IgG<sub>4</sub> and murine IgG were detected with horseradish peroxidase (HRP)-conjugated mouse anti-human IgE and mouse anti-human IgG<sub>4</sub> antibody (Clone B3102E8 and Clone HP6025 respectively, SouthernBiotech via Biozol, Eching, Germany) diluted 1 : 1000 and HRP-conjugated goat anti-mouse IgG antibody (A3673, SigmaAldrich, Taufkirchen, Germany) diluted 1 : 3000 in PBS containing 0.05% Tween 20 and 0.1% BSA. As substrate 3,3',5,5'-tetramethylbenzidine (Roth, Karlsruhe, Germany) was used for the HRP and the absorbances at 450 and 630 nm were measured after stopping the reaction with 25% H<sub>2</sub>SO<sub>4</sub>.

#### *IgE and IgG<sub>4</sub> inhibition ELISA*

For IgE/IgG<sub>4</sub>-ELISA inhibition experiments, Nunc Maxiisorp plates (Fisher Scientific, Schwerte, Germany) were coated overnight at room temperature with 300 ng Δ29NCS<sub>5x</sub>/100 μL of phosphate-buffered saline (PBS), pH 7.4. After blocking with PBS containing 2% BSA, plates were incubated with human sera (patient 6 : 1 : 25 for IgE; 1 : 100/1 : 150 for IgG<sub>4</sub>; patient 11 : 1 : 25 for IgE; 1 : 100 for IgG<sub>4</sub>; patient 14 : 1 : 10 for IgE; 1 : 100/1 : 200 for IgG<sub>4</sub>) with increasing concentrations (3 fg to 30 μg/well) of recombinant inhibitor (rBet v 1a) for 3 h at room temperature in PBS containing 0.05% Tween 20 and 0.1% BSA. Allergen-specific IgE and IgG<sub>4</sub> was detected with HRP-conjugated mouse anti-human IgE/IgG<sub>4</sub> antibody (IgE: Clone B3102E8, IgG<sub>4</sub>: Clone HP6025, SouthernBiotech via Biozol) diluted for IgE 1 : 1000 and for IgG<sub>4</sub> 1 : 2000 with PBS containing 0.05% Tween 20 and 0.1% BSA. The substrate for HRP was 3,3',5,5'-tetramethylbenzidine (Roth), and the reaction was stopped with 25% H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at 450 nm.

#### *SDS-PAGE and immunoblot analysis with purified recombinant Δ29NCS*

SDS-PAGE was performed with 15% separating gels and 5% stacking gels using a discontinuous buffer system [30]. For immunoblot analysis, equimolar amounts of rΔ29NCS/Δ29NCS<sub>5x</sub> were transferred onto 0.2 μm nitrocellulose membranes by semi-dry blotting at 0.8 mA/cm<sup>2</sup> for 1 h [31]. After blocking with Tris-buffered saline (TBS, pH 7.4) containing 0.3% (v/v) Tween 20, blots were incubated overnight at room temperature with 1 : 55/1 : 110 dilution of human sera with TBS containing 0.05% Tween 20 (TBST 0.05%) and 0.1% BSA (TBST 0.05%–0.1% BSA). After

incubation for 1.5 h with HRP-labelled mouse anti-human IgE/IgG<sub>4</sub> antibody (IgE: Clone B3102E8, IgG<sub>4</sub>: Clone HP6025, SouthernBiotech via Biozol), diluted 1 : 70 000 for IgE and 1 : 200 000 for IgG<sub>4</sub> with TBST 0.05–0.1% BSA, the IgE/IgG<sub>4</sub>-binding proteins were visualized by chemiluminescence (LumiGLO Reserve diluted 1 : 3; KPL via Medac, Wedel, Germany) using a luminescence imager (Fusion Fx; Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany).

#### *β-hexosaminidase release from humanized rat basophil leukaemia (RBL) cells*

The mediator release assay was performed as described [32]. Briefly, RBL cells expressing the α-chain of the high affinity receptor FcεRI for human IgE were sensitized overnight with serum of birch pollen allergic subjects (diluted 1 : 40). After washing, cells were stimulated with serial dilutions of rBet v 1a and rBet v 1a<sub>11x</sub>, respectively. Degranulation was quantified by photometric measurement of β-hexosaminidase activity in the culture supernatants. The percentage of β-hexosaminidase activity relative to cells lysed with Triton X-100 (Sigma-Aldrich, Steinheim, Germany) was calculated and corrected for spontaneous release (sensitized cells without allergen). Allergen concentrations for half-maximal release (EC<sub>50</sub>) were calculated using a four-parameter sigmoid curve fit.

#### *Mass spectrometry analysis of recombinant Bet v 1 preparations*

The identity of rBet v 1a and rBet v 1a<sub>11x</sub> (rBet v 1a<sub>E6A/T9A/N28K/F30V/K55A/K65A/K115A/K119A/D125N/K134A/E138A</sub>) was confirmed by liquid chromatography mass spectrometry (LC-MS<sup>E</sup>) after SDS-PAGE separation as described elsewhere [33] with slight modifications. Peptides were eluted with 25 mM NH<sub>4</sub>HCO<sub>3</sub>; 10% acetonitrile (ACN) and the digestion were stopped by adding 5% formic acid. The peptides were analyzed using a nano-ultra performance LC system coupled to a nano-ESI-MS (nano Acquity UPLC nanoESI Synapt-MS; Waters, Milford, CT, USA) with a 5 μm symmetry 180 μm × 20 mm c18 pre-column and a 1.7 μm BEH 130 100 μm × 100 mm c18 separation column. A 30-min gradient (3–40% ACN at 500 nl/min) after 3 min of trapping (99% water at 5 μL/min) was applied to separate peptides. MS was operated in V mode, acquiring MSE data and applying standard parameters. Data analysis was performed with ProteinLynx Global Server version 2.4 (Waters), searching an in-house database consisting of the Uniprot database (as of May 2011, restricted to reviewed entries of eukaryotic organisms) and the amino acid sequences of rBet v 1a and rBet v 1a<sub>11x</sub>. Protein hits were accepted at a false-positive rate of <4%. The MS data are summarized in the

supporting information (Table S1 and Fig. S1). The peptides harbouring the individual substitutions were individually double checked and detected with mass errors of 1.4–3.2 ppm, respectively.

#### Mouse monoclonal antibodies against Bet v 1a and Bet v 1-homologous allergens

Allergen-specific murine monoclonal antibodies were essentially produced as described [34]. Briefly, monoclonal antibodies were obtained after immunization of Balb/c mice with birch pollen extract and subsequent selection for Bet v 1a-specific binding. Antibodies were either purified via Protein G -affinity chromatography (HiTrap Protein G HP; GE Healthcare Life Sciences, GE Healthcare Europe GmbH, Freiburg, Germany) or applied as cell culture supernatants.

#### Miscellaneous

Three-dimensional protein models were visually analyzed and illustrated using PyMol [35].

#### Results

##### Generation of rBet v 1a<sub>11x</sub>, a recombinant 11-fold substitutional variant of Bet v 1a

Recently, we identified 12-mer peptides binding IgE and IgG<sub>4</sub> specific for Bet v 1a which allowed us to bioinformatically predict epitope profiles of the molecular surface of rBet v 1a (PDB: 1BV1) for both IgE and IgG<sub>4</sub> antibodies from sera of subjects allergic to birch pollen, who received AIT [10, 11]. Based on this information, we substituted eleven amino acids within five

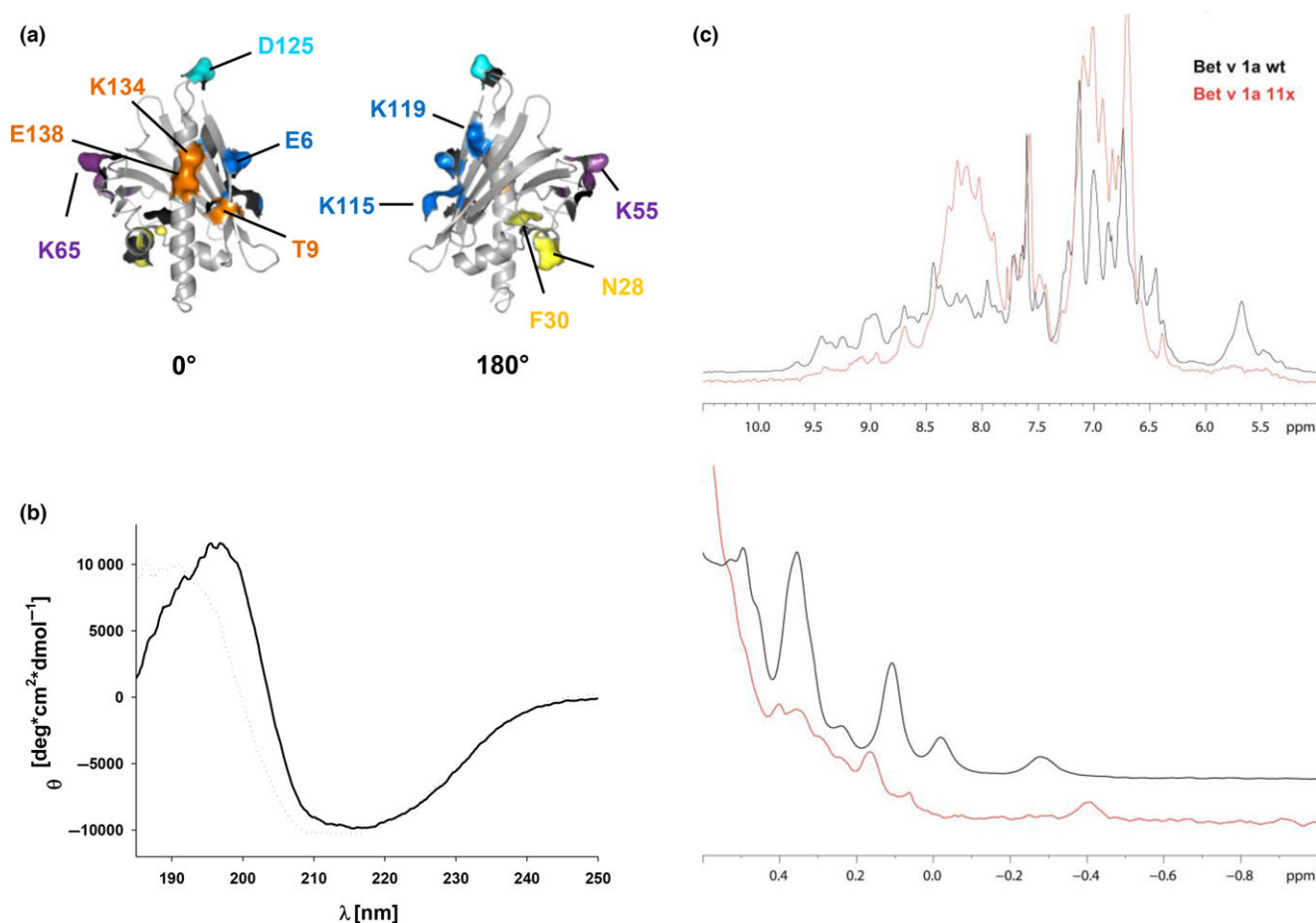


Fig. 1. Structural integrity of rBet v 1a<sub>11x</sub>. (a) Secondary structure topology of Bet v 1a (PDB: 1BV1). The following amino acids were substituted in rBet v 1a to generate rBet v 1a<sub>11x</sub>: E6A, T9A, N28K, F30V, K55A, K65A, K115A, K119A, D125N, K134A, and E138A. Amino acids potentially representative of similar individual antibody binding sites are shown in the same colour. (b) Circular dichroism of rBet v 1a (solid line) and rBet v 1a<sub>11x</sub> (dotted line). (c) Overlay of the 1D-<sup>1</sup>H-NMR spectra of 15  $\mu$ M rBet v 1a (black) or rBet v 1a<sub>11x</sub> (red) in 10 mM potassium phosphate, pH 7.4 at 298 K. A close-up of the amide proton region (upper panel) and of the methyl proton region (lower panel) is shown. The spectrum of rBet v 1a<sub>11x</sub> is scaled up by a factor of 2–3 compared to rBet v 1a.

predicted distinct epitopes of Bet v 1a to study their impact on binding of both serum IgE and IgG<sub>4</sub> and purified the allergen variant rBet v 1a<sub>11x</sub> (Fig. 1a and Fig. S1). Secondary structure of rBet v 1a<sub>11x</sub> was analyzed by CD spectroscopy and revealed a Bet v 1-type spectrum with high content of  $\beta$ -strands and  $\alpha$ -helices (Fig. 1b). However, the minimum ellipticity of the variant is broadened (rBet v 1a<sub>11x</sub>: 216–209 nm, rBet v 1a: 216 nm) while the maximum ellipticity and the zero transition are slightly shifted by approximately 4 nm to lower wavelengths compared to rBet v 1a.

Next, we recorded 1D <sup>1</sup>H-NMR spectra to further analyze the structural arrangement of rBet v 1a<sub>11x</sub> in comparison with rBet v 1a (Fig. 1c). Both proteins showed similar spectra with a high degree of resonance dispersion resulting from stable secondary and tertiary structure [36–38]. Compared to rBet v 1a, the relative intensities of rBet v 1a<sub>11x</sub> signals typical for structured conformations (8.5–11 ppm and <1 ppm) are slightly lower [38]. We assumed that rBet v 1a<sub>11x</sub> either partially unfolds or tends to oligomerize as the signal-to-noise ratio for the variant is lower than for rBet v 1a under identical experimental conditions. We observed a buffer and storage-dependent slight oligomerization tendency of rBet v 1a<sub>11x</sub>. However, immediate preparation of the protein without intermediate freezing or the presence of low concentrations of detergents yielded

monomeric protein with hydrodynamic radii of  $2.0 \pm 0.2$  nm (rBet v 1a) and  $2.8 \pm 1.6$  nm (rBet v 1a<sub>11x</sub>). We concluded that the substitutions of eleven amino acids might slightly interfere with the local secondary structure but not significantly with the tertiary PR-10 type structure of rBet v 1a<sub>11x</sub>.

#### *rBet v 1a<sub>11x</sub> only marginally binds serum IgE of subjects allergic to birch pollen*

To address whether the eleven amino acid substitution in rBet v 1a<sub>11x</sub> affects the allergenic potential of the protein, we measured its capacity to induce IgE-mediated mediator releases of humanized RBL cells individually sensitized with serum IgE of six selected patients with a range of specific IgE for rBet v 1a between 4.45 (patient 3) and >100 kU<sub>A</sub>/L (patients 14 and 15) (Fig. 2). Whereas rBet v 1a induced mediator releases of 26%, 57%, 43%, 47%, 87%, and 65%, and its variant rBet v 1a<sub>11x</sub> elicited releases of 21%, 54%, 35%, 33%, 90%, and 56% for sera of patients 3, 5, 6, 7, 14, and 15, respectively. However, the effective allergen concentrations needed for half-maximal releases (EC<sub>50</sub>) were 139 to 1585 times higher when rBet v 1a<sub>11x</sub> instead of rBet v 1a was offered. Thus, functional interaction of receptor-bound IgE with rBet v 1a<sub>11x</sub> on the surface of basophils was not fully diminished but strongly reduced

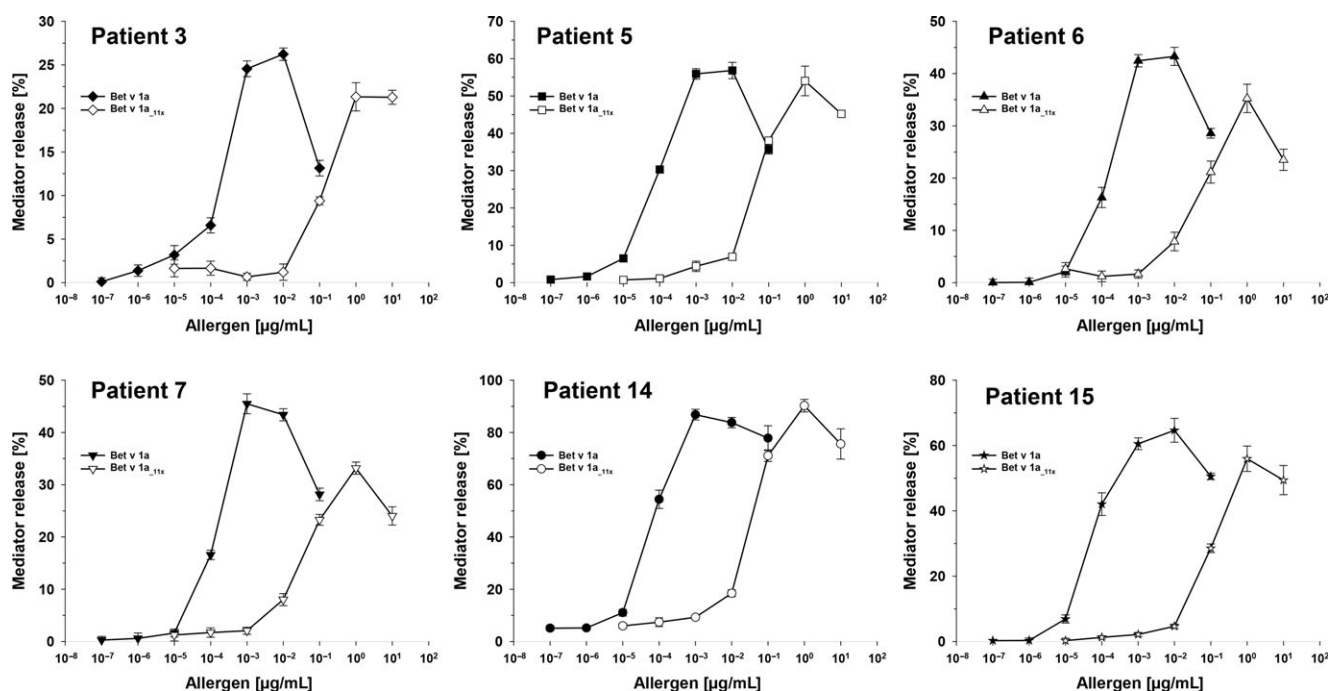


Fig. 2. Mediator release of humanized rat basophils induced by rBet v 1a and rBet v 1a<sub>11x</sub>.  $\beta$ -hexosaminidase release of humanized rat basophil leukaemia cells individually sensitized with sera of subjects allergic to birch pollen. Cross-linking of membrane-bound human IgE by IgE-Bet v 1 complex formation and subsequent dose-dependent mediator release was determined in the presence of either rBet v 1a (filled symbols) or rBet v 1a<sub>11x</sub> (open symbols).

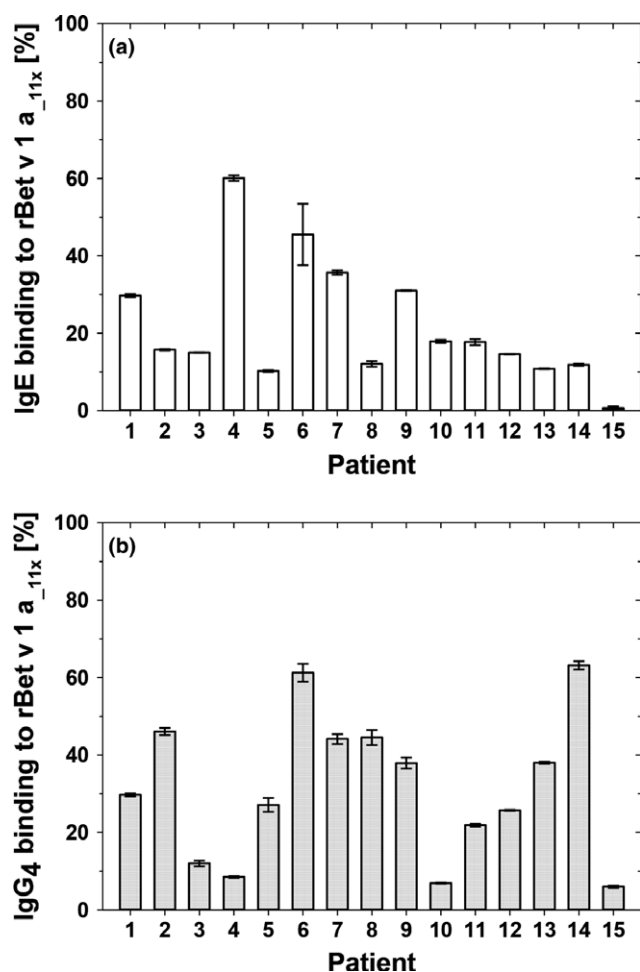


Fig. 3. Binding of serum IgE or IgG<sub>4</sub> to rBet v 1a and rBet v 1a<sub>11x</sub>. Binding of IgE (a) or IgG<sub>4</sub> (b) from sera of subjects allergic to birch pollen to equimolar amounts of surface-coated rBet v 1a and rBet v 1a<sub>11x</sub> in ELISA. The figures represent the percentage of IgE or IgG<sub>4</sub> binding to rBet v 1a<sub>11x</sub> in relation to IgE or IgG<sub>4</sub> binding to rBet v 1a (set to 100%) for each patient.

compared to rBet v 1a. Next, we analyzed *in vitro* IgE binding of purified rBet v 1a<sub>11x</sub> in ELISA (Fig. 3a). All of the individual sera had specific IgE to rBet v 1a; however, binding of serum IgE to rBet v 1a<sub>11x</sub> was reduced to 0.7% (patient 15) up to 60.1% (patient 4) as compared to IgE binding to rBet v 1a, indicating that the amino acid substitutions in rBet v 1a<sub>11x</sub> lowered the binding capacity of rBet v 1a for IgE in a serum-specific fashion, as already observed in the *in vitro* cellular assay above.

#### *rBet v 1a<sub>11x</sub> exhibits reduced binding of serum IgG<sub>4</sub> of birch pollen allergic subjects*

Allergen-specific immunotherapy induces the development of specific IgG<sub>4</sub> antibodies competing with IgE for allergen binding, thereby blocking IgE-mediated

hypersensitivity mechanisms. Whether allergen-specific IgE and IgG<sub>4</sub> recognize identical epitopes is currently under debate. Since 87% (13/15 patients) of our study population had received AIT, we wondered whether rBet v 1a<sub>11x</sub> would compromise binding of IgG<sub>4</sub> in a similar manner as it did for IgE. Therefore, we tested IgG<sub>4</sub> binding of rBet v 1a<sub>11x</sub> in ELISA in a similar fashion as we did for IgE above (Fig. 3b). IgG<sub>4</sub> binding to rBet v 1a<sub>11x</sub> was reduced to 6.9% up to 63.2%, which was largely comparable to the binding capacity of the Bet v 1a variant for IgE in particular for patients 1, 3, 5–7, 9–12, and 15. In contrast, patients 2, 8, and 13–14 had a more reduced IgE and patient 4 a more reduced IgG<sub>4</sub> binding to rBet v 1a<sub>11x</sub>. These results suggested that amino acids of rBet v 1a which are critical for the interaction with both IgE and IgG<sub>4</sub> are lacking in rBet v 1a<sub>11x</sub>.

#### *Bet v 1-specific monoclonal antibodies inhibit binding of both serum IgE and IgG<sub>4</sub> to Bet v 1*

To test our hypothesis that IgE and IgG<sub>4</sub> address similar epitopes of Bet v 1a, we tested a panel of Bet v 1a-specific murine monoclonal antibodies that did not bind to rBet v 1a<sub>11x</sub> and thus potentially had similar epitope specificities as serum IgE/IgG<sub>4</sub> did in the previous section (Fig. 4, cf. Fig. 3). We incubated patients' sera with rBet v 1a in the presence of these monoclonal antibodies and observed inhibition of IgE and IgG<sub>4</sub> binding to rBet v 1a by 2.2–66.1% (IgE) and 3.4–64.9% (IgG<sub>4</sub>), respectively (Table 2). Interestingly, we also found inhibition of IgE binding to rBet v 1a only, pointing to a more diverse IgG<sub>4</sub> antibody repertoire in the sera of these patients. Since we observed competitive binding of IgE, IgG<sub>4</sub>, and monoclonal antibodies to rBet v 1a, we concluded that Bet v 1 epitopes recognized by these antibodies are at least partially overlapping.

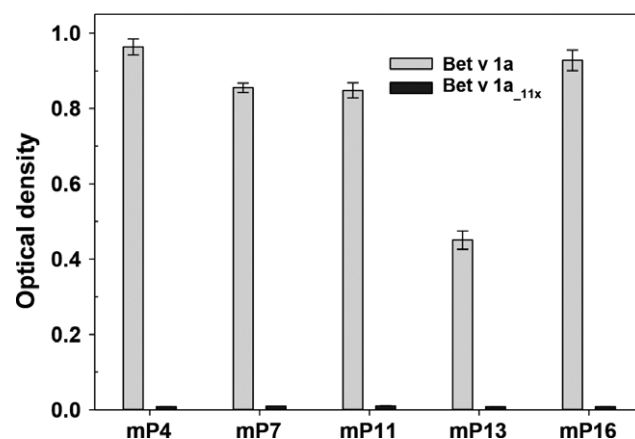


Fig. 4. Binding of murine monoclonal IgG to rBet v 1a and rBet v 1a<sub>11x</sub>. Binding of murine monoclonal IgG to equimolar amounts of either surface-coated rBet v 1a or rBet v 1a<sub>11x</sub> in ELISA. Absorbance was measured at 450/630 nm.

**Table 2.** Inhibition of Immunoglobulin E and G<sub>4</sub> binding to rBet v 1a by murine monoclonal antibodies. The reduction of IgE or IgG<sub>4</sub> binding to rBet v 1a is indicated as percentage of inhibition (100% = complete inhibition)

| Patient | mP4        |                  | mP7        |                  | mP11       |                  | mP13       |                  | mP16       |                  |
|---------|------------|------------------|------------|------------------|------------|------------------|------------|------------------|------------|------------------|
|         | IgE        | IgG <sub>4</sub> | IgE        | IgG <sub>4</sub> | IgE        | IgG <sub>4</sub> | IgE        | IgG <sub>4</sub> | IgE        | IgG <sub>4</sub> |
| P1      | 36.0 ± 0.3 | 59.6 ± 0         | 38.2 ± 1.0 | 62.8 ± 0.9       | 37.1 ± 0.5 | 64.9 ± 0.5       | 42.0 ± 0.5 | 64.9 ± 0.7       | 39.8 ± 0.1 | 64.4 ± 0.4       |
| P2      | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             |
| P3      | 33.5 ± 3.9 | 18.8 ± 0.2       | 32.5 ± 2.5 | 22.2 ± 0.2       | 32.0 ± 1.4 | 49.0 ± 0.1       | 34.0 ± 2.0 | 42.5 ± 0.1       | 29.2 ± 2.6 | 0.0              |
| P4      | 40.9 ± 2.9 | 2.4 ± 1.5        | 59.1 ± 1.0 | 0.0              | 66.1 ± 1.9 | 33.8 ± 1.7       | 59.9 ± 1.1 | 56.5 ± 0.3       | 54.8 ± 0.9 | 0.0              |
| P5      | 40.4 ± 2.0 | 29.1 ± 6.1       | 42.2 ± 2.0 | 43.5 ± 2.8       | 46.9 ± 0.5 | 14.6 ± 5.9       | 44.2 ± 3.8 | 15.4 ± 4.7       | 40.2 ± 0.6 | 5.0 ± 0.6        |
| P6      | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             |
| P7      | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             |
| P8      | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             | n.d.       | n.d.             |
| P9      | 28.6 ± 0.9 | 10.8 ± 0.9       | 24.9 ± 0.7 | 0.0              | 35.6 ± 0.9 | 5.6 ± 3.8        | 25.3 ± 1.7 | 0.0              | 0.0        | 0.0              |
| P10     | 26.5 ± 0.2 | 0.0              | 2.9 ± 1.5  | 5.5 ± 0.3        | 33 ± 1.3   | 0.0              | 37.9 ± 0.7 | 0.0              | 39.5 ± 0.2 | 8.3 ± 0.3        |
| P11     | 0.0        | 0.0              | 0.0        | 0.0              | 0.0        | 0.0              | 8.1 ± 1.3  | 0.0              | 1.1 ± 0.8  | 0.0              |
| P12     | 23.3 ± 0.0 | 0.0              | 19.8 ± 0.2 | 0.0              | 32.2 ± 0.4 | 0.0              | 28.0 ± 0.9 | 0.9              | 24.1 ± 0.2 | 3.8 ± 0.4        |
| P13     | 7.1 ± 0.3  | 3.4 ± 0.3        | 5.4 ± 0.1  | 0.0              | 13.5 ± 0.5 | 0.0              | 5.3 ± 0.6  | 0.0              | 0.2 ± 0.2  | 0.0              |
| P14     | 2.2 ± 2.8  | 6.2 ± 0.2        | 0.0        | 0.0              | 0.0        | 0.0              | 1.8 ± 4.2  | 0.0              | 2.7 ± 2.3  | 0.0              |
| P15     | 0.0        | 1.3 ± 0.8        | 10.8 ± 4.8 | 8.2 ± 2.2        | 11.3 ± 7.1 | 13.4 ± 3.5       | 22.2 ± 2.6 | 0.0              | 26.1 ± 4.5 | 17.0 ± 4.2       |

mP 4-16: Murine monoclonal antibodies specific for Bet v 1; n.d.: not detected due to limited sera availability; grey shading: reduction of IgE binding only.

### Epitope-specific competition of IgE and IgG<sub>4</sub>

Next, we analyzed antibody binding to an individual epitope and utilized our recently published Bet v 1-type model protein Δ29NCS<sub>5x</sub> that presents an individual conformational epitope for both Bet v1-specific serum IgE and murine monoclonal IgG [19]. When we tested the sera of our study population for IgE binding to Δ29NCS<sub>5x</sub> in western blot, we found that 40% (6 of 15 sera) of our study population (patients 5-8, 11, and 14) had IgE to the particular epitope displayed by the model protein (Fig. 5a). Likewise, these sera also had specific IgG<sub>4</sub> that bound to Δ29NCS<sub>5x</sub>. Remarkably, sera of patients 1, 2, and 9 only had IgG<sub>4</sub>, but no IgE, and serum of patient 15 only had IgE, but no detectable IgG<sub>4</sub> recognizing the individual epitope. To test whether the observed antibody binding to Δ29NCS<sub>5x</sub> is indeed Bet v 1-specific, we analyzed competitive IgE and IgG<sub>4</sub> binding to rBet v 1a in ELISAs (Fig. 5b,c). For this, we selected three sera (P6, P11, and P14), which most prominently showed binding of both IgE and IgG<sub>4</sub> to Δ29NCS<sub>5x</sub>. rBet v 1a competed in a dose-dependent fashion for both serum IgE and IgG<sub>4</sub> with maximal inhibition of 100%, 94%, and 84% for IgE and 99%, 92%, and 92% for IgG<sub>4</sub> for sera of patients 6, 11, and 14, respectively. Since neither IgE nor IgG<sub>4</sub> bound to Δ29NCS, the reference model protein lacking the epitope presented by Δ29NCS<sub>5x</sub>, we concluded that patients receiving AIT can develop allergen-specific IgE and IgG<sub>4</sub> antibodies with identical epitope specificities.

### Discussion

We analyzed the epitope specificities of Bet v 1a-specific IgE and IgG<sub>4</sub>. Binding of both serum IgE and IgG<sub>4</sub> to a rBet v 1a variant (rBet v 1a<sub>11x</sub>) which was modified in five putative epitopes was reduced in a largely comparable manner. Likewise, Bet v 1-specific murine monoclonal antibodies did not bind to rBet v 1a<sub>11x</sub> but competed with serum IgE and IgG<sub>4</sub> for binding to rBet v 1a. Finally, binding of IgE or IgG<sub>4</sub> to an individual epitope presented by a Bet v 1-type model protein Δ29NCS<sub>5x</sub> clearly demonstrated the presence of allergen-specific IgE and IgG<sub>4</sub> with identical epitope specificity in a subset of patients.

A native protein conformation of Bet v 1 is required for IgE-mediated hypersensitivity reactions. The variant rBet v 1a<sub>11x</sub> studied here was modified in five particular epitope areas to ensure a measurable effect on a compromised Bet v 1a-IgE and Bet v 1a-IgG<sub>4</sub> interaction. However, even though the goal of this study was not to determine patient-specific epitope profiles of Bet v 1a on the molecular level, the amino acids selected for substitution seemed to be similarly involved in binding of both IgE and IgG<sub>4</sub> antibodies.

Competitive binding of monoclonal IgG and IgE antibodies to Bet v 1 has been reported [14, 26, 38]. The epitope of Bet v 1a for the murine monoclonal antibody BV16 has been analyzed in a three-dimensional allergen-antibody complex. BV16 competes with serum IgE for Bet v 1 binding, and amino acid substitutions within the epitope for BV16 could reduce IgE-Bet v 1 complex formation suggesting overlapping binding sites

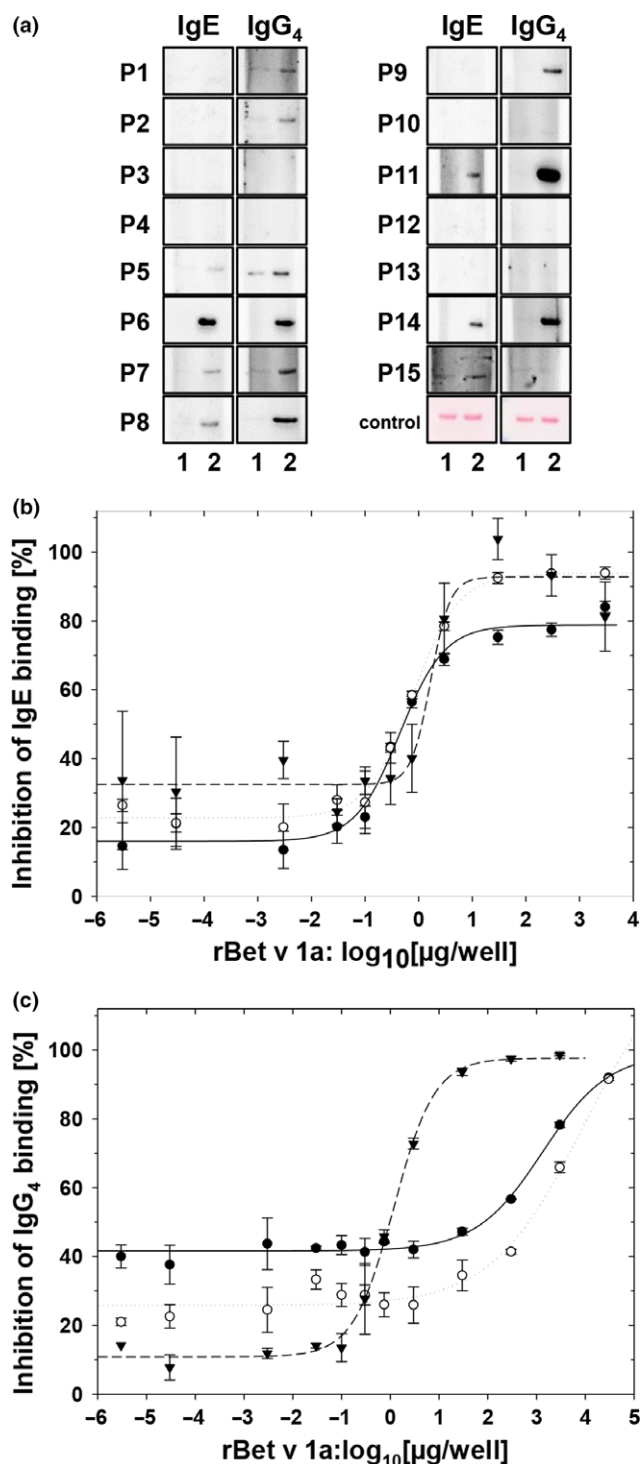


Fig. 5. Binding of serum IgE and IgG<sub>4</sub> to an individual Bet v 1-specific epitope. (a) Serum-specific IgE and IgG<sub>4</sub> binding to recombinant Bet v 1-type model proteins Δ29NCS (lane 1) and Δ29NCS\_5x (lane 2) with the latter presenting a Bet v 1-specific IgE-binding site as published previously [17]. Ponceau S stained protein bands of Δ29NCS and Δ29NCS\_5x are shown (control). Dose-dependent inhibitions of serum IgE (b) or IgG<sub>4</sub> (c) binding to surface-coated Δ29NCS\_5x by rBet v 1a. Sera of patient 6 (open circle), patient 11 (triangles), and patient 14 (closed circles) were used. Curves were fitted with a four-parameter, logistic sigmoidal curve fit.

mutual sterical hindrance and repulsion of the antibody moieties. Simultaneous binding of only up to three antibodies to Bet v 1 has been reported so far [39]. Our observation, that allergen binding of IgE and IgG<sub>4</sub> to a rBet v 1a variant is similarly reduced, indicates that the majority of epitopes for IgE and IgG<sub>4</sub> might be overlapping and thus are not distinct. This hypothesis is also supported by our observation that monoclonal antibodies reduced binding of IgE and IgG<sub>4</sub> to Bet v 1a to a similar extent. The strongest argument for our hypothesis, however, is the fact that allergen-specific IgE and IgG<sub>4</sub> recognized an identical individual epitope presented by our Bet v 1a-type model protein NCS showing that indeed a maximum of five amino acids of Bet v 1a is crucial for the binding of both IgE and IgG<sub>4</sub> antibodies. Thus, we provide evidence for our hypothesis that epitopes for IgE and IgG<sub>4</sub> are highly overlapping. This was suggested previously not only by bioinformatically mapping of IgE and IgG<sub>4</sub> binding peptides onto the molecular surface of Bet v 1, but also by IgE and IgG<sub>4</sub> binding data to Bet v 1 surface areas grafted onto recombinant Api g 1 [10, 11, 40].

With respect to the biosynthesis of antibodies, our observations are in line with the notion that successful AIT induces allergen-specific antibodies with similar paratopes, i.e. epitope specificities, but different Fc portions. As the epitope specificity of allergen-specific Ig is mostly genetically determined, antibody affinity maturation might allow for slight differences in the epitope diversity that may minimize the expansion of the epitope specificities. However, we also observed some distinct binding and patient-specific variations in the epitope specificities of the IgE and IgG<sub>4</sub> antibodies that we analyzed. Whether such variations in the epitope profiles of IgE and IgG<sub>4</sub> might prove useful to predict the courses of either allergy or efficacious AIT remains to be evaluated. Detailed knowledge on the epitope specificities of IgE and IgG antibodies is required to develop novel epitope-specific diagnostics and therapeutics. Since the present study population has significantly improved in their allergy symptoms, one may hypothesize that on the one hand only a limited number of IgG<sub>4</sub> epitope specificities is needed for a beneficial therapeutic effect. On the other hand, however,

that do not allow simultaneous binding of both murine IgG and serum IgE to Bet v 1a [27]. The epitope area in the BV16-Bet v 1 complex comprises 931 Å<sup>2</sup> and covers about 10% of the overall surface of Bet v 1 [26]. However, simultaneous binding of antibodies to adjacent epitopes is not just restricted by the actual epitope area covered by the antibody paratope but also by

whether or not allergen-specific IgG<sub>4</sub> represents the major factor determining a positive therapeutic effect is still under debate. Therefore, beyond the data presented here, studies on epitope specificities and diversities of allergen-specific IgE and AIT-induced IgG subclasses in early and later phases of immunotherapy in larger study populations would provide further valuable insights into the molecular mechanisms of AIT and increase the efficacy of current diagnosis and treatment of allergies.

In summary, the data presented here provides evidence that birch pollen allergic patients receiving AIT can develop Bet v 1a-specific IgG<sub>4</sub> with similar epitope

specificity as IgE. Our results also suggest that the Bet v 1a epitope repertoire for IgE and IgG<sub>4</sub> is not completely identical but may cover specific epitopes for either IgE or IgG<sub>4</sub> antibodies exclusively.

### Acknowledgement

We thank Ramona Heissmann and Luisa Schwaben for excellent technical assistance.

### Conflict of interest

The authors declare no conflict of interest.

### References

- Burks AW, Calderon MA, Casale T *et al.* Update on allergy immunotherapy: American Academy of Allergy, Asthma & Immunology/European Academy of Allergy and Clinical Immunology/PRACTALL consensus report. *J Allergy Clin Immunol* 2013; 131:1288–96.
- Wachholz PA, Durham SR. Mechanisms of immunotherapy: IgG revisited. *Curr Opin Allergy Clin Immunol* 2004; 4:313–8.
- Flicker S, Valenta R. Renaissance of the blocking antibody concept in type I allergy. *Int Arch Allergy Immunol* 2003; 132:13–24.
- van Neerven RJ, Wikborg T, Lund G *et al.* Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4 + T cells by inhibiting serum-IgE-facilitated allergen presentation. *J Immunol* 1999; 163:2944–52.
- Würtzen PA, Lund G, Lund K *et al.* A double-blind placebo-controlled birch allergy vaccination study II: correlation between inhibition of IgE binding, histamine release and facilitated allergen presentation. *Clin Exp Allergy* 2008; 38:1290–301.
- Lalek N, Kosnik M, Silar M *et al.* Immunoglobulin G-dependent changes in basophil allergen threshold sensitivity during birch pollen immunotherapy. *Clin Exp Allergy* 2010; 40:1186–93.
- Möbs C, Ipsen H, Mayer L *et al.* Birch pollen immunotherapy results in long-term loss of Bet v 1-specific TH2 responses, transient TR1 activation, and synthesis of IgE-blocking antibodies. *J Allergy Clin Immunol* 2012; 130:1108–16.
- Shamji MH, Ljørring C, Francis JN *et al.* Functional rather than immunoreactive levels of IgG<sub>4</sub> correlate closely with clinical response to grass pollen immunotherapy. *Allergy* 2012; 67:217–26.
- Vickery BP, Lin J, Kulis M *et al.* Peanut oral immunotherapy modifies IgE and IgG<sub>4</sub> responses to major peanut allergens. *J Allergy Clin Immunol* 2013; 131:128–34.
- Subbarayal B, Schiller D, Möbs C *et al.* Kinetics, cross-reactivity, and specificity of Bet v 1-specific IgG<sub>4</sub> antibodies induced by immunotherapy with birch pollen. *Allergy* 2013; 68:1377–86.
- Subbarayal B, Schiller D, Möbs C *et al.* The diversity of Bet v 1-specific IgG<sub>4</sub> antibodies remains mostly constant during the course of birch pollen immunotherapy. *J Allergy Clin Immunol* 2015; 136:1680–2.
- Gajhede M, Osmark P, Poulsen FM *et al.* X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat Struct Biol* 1996; 3:1040–5.
- Ferreira F, Ebner C, Kramer B *et al.* Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J* 1998; 12:231–42.
- Holm J, Gajhede M, Ferreras M *et al.* Allergy vaccine engineering: epitope modulation of recombinant Bet v 1 reduces IgE binding but retains protein folding pattern for induction of protective blocking-antibody responses. *J Immunol* 2004; 173:5258–67.
- Jahn-Schmid B, Radakovics A, Lüttkopf D *et al.* Bet v 1142–156 is the dominant T-cell epitope of the major birch pollen allergen and important for cross-reactivity with Bet v 1-related food allergens. *J Allergy Clin Immunol* 2005; 116:213–9.
- Klinglmayr E, Hauser M, Zimmermann F *et al.* Identification of B-cell epitopes of Bet v 1 involved in cross-reactivity with food allergens. *Allergy* 2009; 64:647–51.
- Hecker J, Diethers A, Schulz D *et al.* An IgE epitope of Bet v 1 and fagales PR10 proteins as defined by a human monoclonal IgE. *Allergy* 2012; 67:1530–7.
- Gepp B, Lengger N, Bublin M *et al.* Chimeras of Bet v 1 and Api g 1 reveal heterogeneous IgE responses in patients with birch pollen allergy. *J Allergy Clin Immunol* 2014; 134:188–94.
- Berkner H, Seutter von Loetzen C, Hartl M *et al.* Enlarging the toolbox for allergen epitope definition with an allergen-type model protein. *PLoS ONE* 2014; 9:e111691.
- Mahler V, Vrtala S, Kuss O *et al.* Vaccines for birch pollen allergy based on genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1. *Clin Exp Allergy* 2004; 34:115–22.
- Gafvelin G, Thunberg S, Kronqvist M *et al.* Cytokine and antibody responses in birch-pollen-allergic patients treated with genetically modified derivatives of the major birch pollen allergen Bet

- v 1. *Int Arch Allergy Immunol* 2005; 138:59–66.
- 22 Pree I, Reisinger J, Focke M *et al.* Analysis of epitope-specific immune responses induced by vaccination with structurally folded and unfolded recombinant Bet v 1 allergen derivatives in man. *J Immunol* 2007; 179:5309–16.
  - 23 Purohit A, Niederberger V, Kronqvist M *et al.* Clinical effects of immunotherapy with genetically modified recombinant birch pollen Bet v 1 derivatives. *Clin Exp Allergy* 2008; 38:1514–25.
  - 24 Meyer W, Narkus A, Salapatek AM *et al.* Double-blind, placebo-controlled, dose-ranging study of new recombinant hypoallergenic Bet v 1 in an environmental exposure chamber. *Allergy* 2013; 68:724–31.
  - 25 Pichler U, Asam C, Weiss R *et al.* The fold variant BM4 is beneficial in a therapeutic Bet v 1 mouse model. *Biomed Res Int* 2013; 2013:832404.
  - 26 Mirza O, Henriksen A, Ipsen H *et al.* Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. *J Immunol* 2000; 165:331–8.
  - 27 Spangfort MD, Mirza O, Ipsen H *et al.* Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. *J Immunol* 2003; 171:3084–90.
  - 28 Markovic-Housley Z, Degano M, Lamba D *et al.* Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J Mol Biol* 2003; 325:123–33.
  - 29 Piotto M, Saudek V, Sklenar V. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J Biomol NMR* 1992; 2:661–5.
  - 30 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
  - 31 Kyhse-Andersen J. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* 1984; 10:203–9.
  - 32 Vogel L, Luttkopf D, Hatahet L *et al.* Development of a functional *in vitro* assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* 2005; 60:1021–8.
  - 33 Albrecht M, Alessandri S, Conti A *et al.* High level expression, purification and physico- and immunochemical characterisation of recombinant Pen a 1: a major allergen of shrimp. *Mol Nutr Food Res* 2008; 52(Suppl. 2): S186–95.
  - 34 Wiche R, Gubesch M, König H *et al.* Molecular basis of pollen-related food allergy: identification of a second cross-reactive IgE epitope on Pru av 1, the major cherry (*Prunus avium*) allergen. *Biochem J* 2005; 385:319–27.
  - 35 DeLano WL. The PyMOL Molecular Graphics System. Version 1.8 Schrödinger, LLC.
  - 36 Williamson MP. Secondary-structure dependent chemical shifts in proteins. *Biopolymers* 1990; 29:1423–31.
  - 37 Wishart DS, Sykes BD, Richards FM. Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. *J Mol Biol* 1991; 222:311–33.
  - 38 Rehm T, Huber R, Holak TA. Application of NMR in structural proteomics: screening for proteins amenable to structural analysis. *Structure* 2002; 10:1613–8.
  - 39 Gieras A, Cejka P, Blatt K *et al.* Mapping of conformational IgE epitopes with peptide-specific monoclonal antibodies reveals simultaneous binding of different IgE antibodies to a surface patch on the major birch pollen allergen, Bet v 1. *J Immunol* 2011; 186:5333–44.
  - 40 Gepp B, Lengger N, Möbs C *et al.* *J Allergy Clin Immunol* 2016; 137:1600–1603.e1.

## Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Summary of mass spectrometric sequence confirmation.

Figure S1. Amino acid sequence of rBet v 1<sub>11x</sub> variant.

Figure S2. Binding of serum IgE and IgG<sub>4</sub> to rBet v 1<sub>a</sub> and rBet v 1a<sub>11x</sub>.

## Supporting information

### **IgE and allergen-specific immunotherapy-induced IgG<sub>4</sub> recognize similar epitopes of Bet v 1, the major allergen of birch pollen**

Nicole Groh<sup>1</sup>, Christian Seutter von Loetzen<sup>2</sup>, Brinda Subbarayal<sup>3</sup>, Christian Möbs<sup>4</sup>, Lothar Vogel<sup>1</sup>, Andreas Hoffmann<sup>1</sup>, Kay Fötisch<sup>1</sup>, Anna Koutsouridou<sup>1</sup>, Stefanie Randow<sup>1</sup>, Elke Völker<sup>1</sup>, Andreas Reuter<sup>1</sup>, Paul Rösch<sup>2</sup>, Stefan Vieths<sup>1</sup>, Wolfgang Pfützner<sup>4</sup>, Barbara Bohle<sup>3</sup>, Dirk Schiller<sup>1</sup>

<sup>1</sup> Division of Allergology, Paul-Ehrlich-Institut, Langen, Germany.

<sup>2</sup> Department of Biopolymers, University of Bayreuth, Bayreuth, Germany.

<sup>3</sup> Department of Pathophysiology and Allergy Research and Christian Doppler Laboratory for Immunomodulation, Medical University of Vienna, Vienna, Austria.

<sup>4</sup> Department of Dermatology and Allergology, Philipps University Marburg, Marburg, Germany.

Corresponding author:

Dirk Schiller

Paul-Ehrlich-Institut

Division of Allergology

Paul-Ehrlich-Str. 51-59

63225 Langen

Germany

Phone: +49 6103 775208

Fax: +49 6103 771258

Dirk.Schiller@pei.de

Running head: Similar Bet v 1 epitopes for IgE and IgG<sub>4</sub>

Keywords: IgE, IgG<sub>4</sub>, epitope, immunotherapy, Bet v 1

Table E1 Summary of mass spectrometric sequence confirmation

| <b>Description</b> | <b>S</b> | <b>SC</b> | <b>P</b> | <b>E</b> |
|--------------------|----------|-----------|----------|----------|
| Bet v 1 wt         | 9,306    | 75.0      | 21       | 5.0      |
| Bet v 1 11x        | 1,901    | 89.0      | 24       | 6.7      |

S: PLGS protein score; SC: sequence coverage in %; P: digest peptides including modified peptides & missed cleavage sites which were detected by MS; E: mass error in ppm

# Figure E1 Amino acid sequence of rBet v 1<sub>11x</sub> variant

Amino acids printed in bold were substituted in comparison to Bet v 1.0101 (Bet v 1a). Peptides detected with MS are underlined.

|              |                          |            |                     |                             |                     |
|--------------|--------------------------|------------|---------------------|-----------------------------|---------------------|
|              | 1                        | 11         | 21                  | 31                          | 41                  |
| Bet v 1.0101 | MGSSHHHHHH               | SSGLVPRGSH | MGVFNYETET          | TSVIPAAARLF                 | KAFILDGDNL          |
| Bet v 1 11x  | MGSSHHHHHH               | SSGLVPRGSH | MGVFNY <b>A</b> TEA | TSVIPAAARLF                 | KAFILDGD <b>K</b> L |
|              | 51                       | 61         | 71                  | 81                          | 91                  |
| Bet v 1.0101 | FPKVAPQAIS               | SVENIEGNNG | PGTIKKISFP          | EGFPFKYVKD                  | RVDEV DHTNF         |
| Bet v 1 11x  | <u>VPKVAPQAIS</u>        | SVENIEGNNG | PGTI <b>K</b> AISFP | EGFPF <b>A</b> YVKD         | RVDEV DHTNF         |
|              | 101                      | 111        | 121                 | 131                         | 141                 |
| Bet v1.0101  | <u>KYNYSVIEGG</u>        | PIGDTLEKIS | NEIKIVATPD          | GG <u>SILK</u> ISNK         | YHTKGDHEVK          |
| Bet v 1 11x  | <u>KYNYSVIEGG</u>        | PIGDTLEKIS | NEIKIVATPD          | GG <u>SIL</u> <b>A</b> ISNA | YHTK <b>G</b> NHEVK |
|              | 151                      | 161        | 171                 | 180                         |                     |
| Bet v1.0101  | AEQVKASKEM               | GETLLRAVES | YLLAHSDAYN          |                             |                     |
| Bet v 1 11x  | <u>AEQ<b>V</b>AASKAM</u> | GETLLRAVES | YLLAHSDAYN          |                             |                     |

**Figure E2 Binding of serum IgE and IgG<sub>4</sub> to rBet v 1<sub>a</sub> and rBet v 1a<sub>11x</sub>**

Serum-specific IgE and IgG<sub>4</sub> binding to rBet v 1<sub>a</sub> (lane 1) and rBet v 1a<sub>11x</sub> (lane 2). Ponceau S stained protein bands of rBet v 1<sub>a</sub> (lane 1) and rBet v 1a<sub>11x</sub> are shown (control).

