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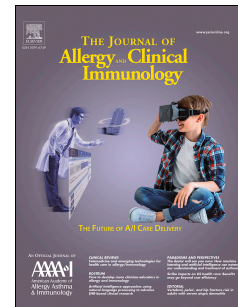
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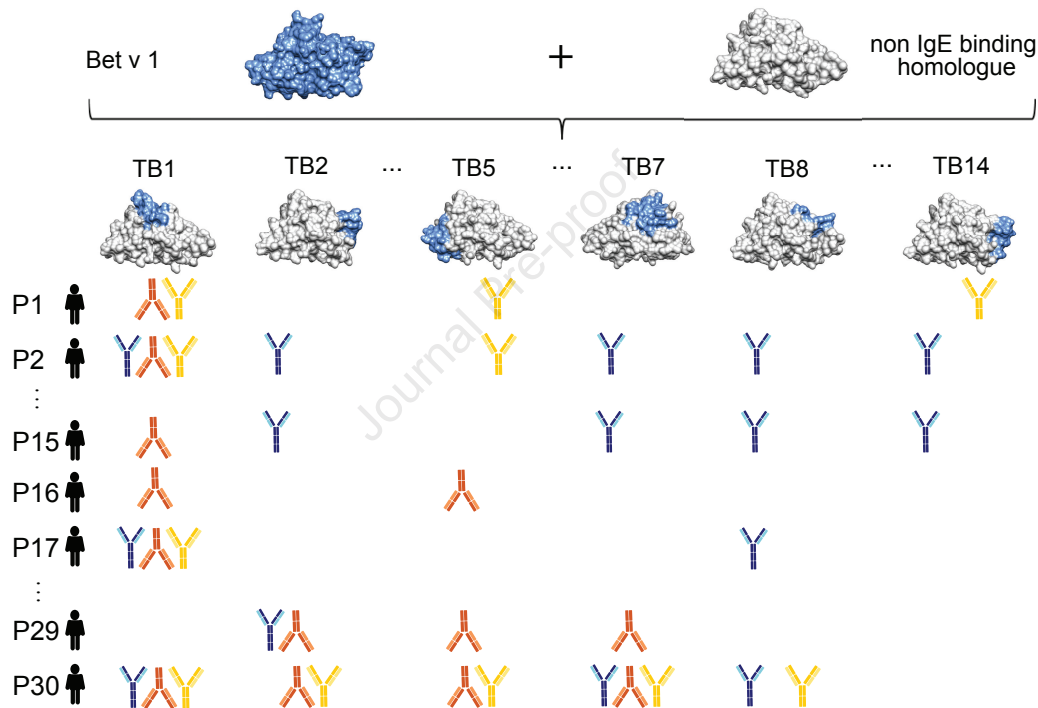
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ABSTRACT

Background: Birch pollen is an important elicitor of respiratory allergy. The major allergen, Bet v 1, binds IgE exclusively via conformational epitopes.

Objective: To identify Bet v 1-specific epitope repertoires of IgE and IgG from birch pollen-allergic and non-allergic subjects.

Methods: Chimeric proteins were created by grafting individual epitope-sized, contiguous surface patches of Bet v 1 onto a non-allergenic structural homologue and expressed in *Escherichia coli*. Binding of IgE, IgG1 and IgG4 from sera of 30 birch pollen-allergic and 11 non-allergic subjects to Bet v 1, 13 chimeric proteins and four bacterial Bet v 1 homologues were measured by ELISA. The proportion of epitope-specific in total Bet v 1-specific IgE and the cross-reactivity of Bet v 1-specific IgE with bacterial homologues were determined by competitive ELISA.

Results: Thirteen soluble, correctly folded chimeric proteins were produced. IgE from 27/30 birch pollen-allergic patients bound to 1-12 chimeric proteins (median 4.0) with patient-specific patterns. Three chimeras binding IgE from the majority of sera were identified, whose grafted patches overlapped with previously published epitopes. Patterns of IgG1 and IgG4 binding to the chimeric proteins did not correspond to the binding patterns of IgE. Sera of 19/30 birch pollen-allergic patients contained low amounts of IgE to bacterial homologues. Bacterial proteins were able to partially inhibit IgE binding to Bet v 1.

Conclusion: Epitopes recognized by Bet v 1-specific antibodies from birch pollen-allergic patients are specific to each patient and differ between IgE, IgG1 and IgG4.

56 Key messages:

- 57 • The repertoire of conformational epitopes recognized by Bet v 1-specific IgE is highly
58 variable.
- 59 • The epitope recognition profiles of Bet v 1-specific IgG1 and IgG4 differ from the
60 patterns observed for IgE.
- 61 • Despite their low sequence similarity to Bet v 1, bacterial structural homologues of
62 Bet v 1 are recognized by IgG1 and low amounts of IgE and IgG4 from some birch
63 pollen-allergic patients.

64 Capsule summary:

65 Mapping of conformational epitopes of the major birch pollen allergen, Bet v 1, using
66 chimeric proteins showed that epitope recognition profiles were specific to each patient and
67 differed between IgE, IgG1 and IgG4.

68 Key words:

69 birch pollen allergy; Bet v 1; conformational epitopes; epitope mapping

70 Abbreviations:

71	AP	alkaline phosphatase
72	BSA	bovine serum albumin
73	HRP	horseradish peroxidase
74	mAb	monoclonal antibody
75	NP	4-hydroxy-3-nitrophenyl acetyl
76	sIg	specific immunoglobulin
77	TBST	Tris-buffered saline, 0.05% Tween 20

INTRODUCTION

In the temperate zones of the northern hemisphere, birch (*Betula spp.*) and related trees from the order Fagales are among the most important elicitors of pollen allergy.^{1, 2} Most birch pollen-allergic patients are sensitized to the major allergen Bet v 1, with sensitization rates of over 90% in northern and central Europe.³ Bet v 1 belongs to a ubiquitous, conserved family of plant proteins. As a consequence of IgE cross-reactivity between Bet v 1 and homologous allergens in other plants, Bet v 1-sensitized patients frequently experience allergic symptoms to pollen from related trees, such as hazel, alder and oak, and also suffer from pollen-associated food allergy to certain fruits, vegetables and nuts.² However, not all Bet v 1-sensitized individuals develop plant food allergy, and there is no association between IgE-reactivity to certain Bet v 1 homologues and allergy to the respective foods.⁴

Knowledge about epitopes of allergens for IgE and other immunoglobulins is important for basic and clinical research, such as elucidating mechanism in forming the IgE repertoire, delineating the molecular basis of IgE cross-reactivity, increasing the accuracy of diagnostic tests by using epitope-based diagnostics, and design of hypoallergens for allergen immunotherapy.⁵ The interaction of Bet v 1 with human IgE depends on its native conformation. This was shown by the lack of IgE binding and allergenic activity of Bet v 1-derived synthetic peptides, recombinant fragments and unfolded mutants.⁶⁻⁸ Identification of conformational epitopes was possible only with time-consuming or indirect methods, such as computationally mapping phage display-derived IgE-binding peptide mimics to the Bet v 1 surface,⁹ epitope mapping of murine monoclonal antibodies (mAbs) that inhibited serum IgE binding to Bet v 1,^{10, 11} or of human mAbs isolated from IgE-expressing B cells.^{12, 13} The epitope of the murine IgE-inhibiting mAb BV16 was identified by co-crystallization of its Fab-fragment with Bet v 1 and confirmed by site-directed mutagenesis.^{10, 14} The center of this epitope is located on the so-called p-loop, a surface-exposed glycine rich stretch highly conserved among Bet v 1-related allergens.

A direct method of identifying conformational epitopes is measuring IgE binding to chimeric proteins generated by grafting contiguous surface patches of Bet v 1 onto a structurally related protein with decreased or absent IgE binding. This method was used to confirm the importance of the p-loop epitope^{15, 16} and to identify the epitope of a human IgE mAb selected by phage display.¹² We characterized the Bet v 1-specific IgE (sIgE) epitope repertoire from a large cohort by grafting four non-overlapping surface-exposed patches of Bet v 1, each comprising about 25% of its molecular surface, to Api g 1, a Bet v 1 homologue from celeriac with low IgE binding capacity.¹⁷ We showed that the sIgE epitope repertoire of birch pollen-allergic patients is highly individual and IgE epitopes are distributed across most parts of the Bet v 1 surface.

Here, we grafted epitope-sized Bet v 1-derived surface patches onto a bacterial homologue with high structural but low sequence similarity to Bet v 1, which was expected to lack IgE binding. Thereby we aimed to characterize conformational epitopes of Bet v 1 with an increased resolution. In addition, we compared the epitope binding repertoires of sIgE with those of other immunoglobulins from birch pollen-allergic and non-allergic donors. Prompted by an unexpected binding of IgE from some patients to the bacterial scaffold protein, we also investigated binding of antibodies from birch-allergic patients to other structurally similar proteins from bacterial species that are part of the normal human microbiota.

122

METHODS

Patients

Serum or plasma samples from 30 Bet v 1-sensitized, birch pollen-allergic patients were analyzed (Table E1 in the Online Repository). Patients were selected based on typical symptoms (seasonal allergic rhinoconjunctivitis or asthma in spring), positive skin-prick tests with birch pollen extract and sIgE to birch pollen and Bet v 1 (ImmunoCAP, Thermo-Fisher). Twenty-four patients (B1-B24) had been recruited for a previously published study,¹⁸ six patients were recruited at the Outpatient Clinic Reumannplatz, Vienna, Austria. Except for B26 and B27, none of the patients had received allergen immunotherapy. In addition, sera from eleven non-atopic donors with reported lack of allergic symptoms to common allergen sources were included as negative controls. All patients provided written informed consent to the use of their serum samples and clinical data. The study was approved by the Ethics Committee of the Medical University of Vienna (Registration Number: 2158/2018).

Design of Bet v 1-TTHA0849 chimeras

Based on a structural alignment, surface-exposed residues of the bacterial Bet v 1-related protein TTHA0849 from *Thermus thermophilus*¹⁹ were replaced by corresponding ones of Bet v 1 while preserving the hydrophobic core. Thereby, we created 13 chimeric proteins carrying epitope-sized, partially overlapping Bet v 1-derived surface patches (Fig. 1, Fig. E1 in the Online Repository). A detailed protocol can be found in the Online Repository.

Protein expression, purification and characterization

The chimeric proteins, Bet v 1 and its bacterial structural homologues, TTHA0849, SMU440 from *Streptococcus mutans*, PA1206 from *Pseudomonas aeruginosa* and SA2116 from *Staphylococcus aureus* (Fig. E2 and Table E2 in the Online Repository), were expressed in *Escherichia coli*. All proteins were purified by chromatography and characterized by SDS-

PAGE, mass spectrometry, circular dichroism spectroscopy and dynamic light scattering. The full protocol is available in the Online Repository.

Quantitative immunoglobulin ELISA

sIgE, sIgG1 and sIgG4 from patients' or non-atopic donors' sera were measured using a quantitative ELISA. Microtiter plates were coated with antigens and probed with sera diluted 1:10-1:300 or dilution buffer as a control. Binding of specific antibodies was detected by isotype-specific enzyme-labeled secondary antibodies followed by incubation with chromogenic or fluorogenic substrates. Quantification was achieved by coating plates with Bet v 1 or 4-hydroxy-3-nitrophenyl acetyl (NP)-conjugated bovine serum albumin and detection with serial twofold dilutions (0.3-10 ng/ml) of isotype-matched recombinant human mAbs to Bet v 1 or the NP hapten. A detailed protocol and the results of quality control experiments are available in the Online Repository.

All samples were measured in duplicates. For antibody binding to chimeric proteins, the absorbance or fluorescence values of TTHA0849 were subtracted in order to exclude the contribution of antibodies bound by the scaffold. Resulting values surpassing three times the standard deviation of the buffer controls were considered positive. Concentrations of specific antibodies were calculated by comparing the background-corrected signals with the standard curve and multiplying the values with the serum dilution.

For competitive ELISA, sera, diluted 1:100-1:300, were incubated for 2 h with 20 µg/ml or serial fivefold dilutions of the competitor proteins or dilution buffer as a control and subsequently added to the antigen-coated plate overnight at 4°C. Detection by secondary antibodies was performed as described above. Percent inhibition was calculated, after background subtraction, as follows: %inhibition = $100 * (1 - [\text{fluorescence of the inhibited sample}] / [\text{fluorescence of the reference sample}])$. For inhibitions with Bet v 1 and the bacterial

171 proteins, the non-inhibited serum was used as a reference, while for inhibitions with chimeric
172 proteins, the samples inhibited with the scaffold, TTHA0848, were used.

173 **Statistical analyses**

174 Two-way ANOVA was used to evaluate differences in the numbers of recognized chimeras
175 between isotypes and between the allergic and non-allergic group for each isotype.

176 Correspondence between chimera binding patterns of different isotypes compared to the
177 extent of correspondence expected by chance was quantified using Cohen's Kappa. P-values
178 < 0.05 were considered significant. All statistical analyses were conducted using Prism 5
179 (GraphPad Software) or SPSS Statistics 24 (IBM).

RESULTS

Expression of Bet v 1-TTHA0849 chimeras yielded soluble, correctly folded proteins

To characterize the repertoire of conformational epitopes of Bet v 1, we created 14 chimeric proteins (TB1-TB14), each displaying a distinct Bet v 1-derived contiguous patch on the surface of the bacterial Bet v 1-homologue TTHA0849 (Fig. 1A). Stabilizing mutations were introduced into TB3, TB7, TB11 and TB12 to improve expression yields (Fig. 1B). One chimeric protein (TB12) still remained insoluble. Nevertheless, the grafted patches of the successfully produced 13 chimeras covered the complete solvent-exposed surface of Bet v 1 with overlaps between neighboring patches (Fig. 1B, Fig. E1 in the Online Repository). All chimeric proteins adopted secondary structures similar to the scaffold TTHA0849 and Bet v 1 (Fig. E3 in the Online Repository) and were monomeric in solution with proportions of oligomeric or aggregated molecules < 5%, as determined by dynamic light scattering (data not shown).

To test whether the chimeric proteins were suitable for mapping conformational epitopes, we performed an ELISA with the anti-Bet v 1-mAb BV16, whose known epitope is nearly identical with the Bet v 1-derived patch transferred to TB1. As expected, BV16 bound to Bet v 1 and TB1, but not to the other chimeras or the scaffold protein, TTHA0849 (Fig. E4A in the Online Repository).

sIgE from most Bet v 1-sensitized patients binds to multiple conformational epitopes

We analyzed binding of Bet v 1-sIgE, sIgG1 and sIgG4 from 30 Bet v 1-sensitized birch-pollen-allergic patients (B1-B30; Table E1 in the Online Repository) and 11 non-allergic donors (N1-N11) to the chimeric proteins in a quantitative ELISA (Fig. 2).

Sera from 27 of 30 (90%) birch-allergic patients contained IgE binding to 1-12 chimeric proteins (median of all sera: 4.0), and 24 (80%) bound to at least 2 chimeras (Fig. 2A; Table

E3 in the Online Repository). Chimera binding profiles were distinct for each patient. The non-allergic donor group (N1-N11) showed no IgE reactivity for Bet v 1 or any of the chimeric proteins.

Three chimeric proteins displaying partially overlapping patches were bound by IgE from the majority of sera (Fig. 1C): TB2, TB7 and TB8 were recognized by 17 (57%), 16 (53%) and 16 sera, respectively. The p-loop epitope, represented by TB1 (Fig. 1A),¹⁰ bound IgE from 12/30 (40%) sera. One chimeric protein (TB9) was not recognized by any of the sera.

To confirm that IgE binding to chimeric proteins was mediated by Bet v 1-sIgE, we performed an inhibition ELISA using 8 sera with varying binding patterns in the direct ELISA (Fig. 3A). Twelve of 13 chimeras were able to inhibit IgE binding to Bet v 1. TB8, which bound IgE from 16/30 sera in the direct ELISA (including 2/8 sera used in the inhibition assay), failed to inhibit IgE binding to Bet v 1. However, IgE binding to TB8 was inhibited by Bet v 1 by 80% (data not shown). The chimeras with the highest inhibitory capacities were TB1 and TB2, which inhibited IgE binding to Bet v 1 with all sera by 8-30% (mean: 19%), and 18-56% (mean: 33%), respectively. The other chimeric proteins caused partial IgE inhibition < 25% with 2-6 sera. In a concentration-dependent assay with TB1, TB2 and a mixture of these chimeras (Fig. 3B), we observed partial inhibition of IgE binding to Bet v 1 only at very high concentrations of chimeric proteins, pointing to a low affinity of the chimeras for Bet v 1-specific IgE. Due to the low amount and affinity of sIgE to the chimeric proteins, we were not able to perform the assay in the reverse format, i.e. inhibition of IgE binding to the chimeric proteins by Bet v 1.

The epitope binding patterns of Bet v 1-sIgG1 and sIgG4 are distinct from that of IgE

Sera from all tested birch pollen-allergic patients contained Bet v 1-sIgG1 (range: 15-6852 ng/ml; median: 244 ng/ml) and sIgG4 (range: 2-789 ng/ml; median: 71 ng/ml), albeit with distinct epitope specificities (Fig. 2B, C).

IgG1 from 29/30 sera recognized 1-7 chimeric proteins (median 2.0; Figs. 2B, 4A). TB1, bound by 28 sera, overlapped with the only immunodominant epitope. TB14 was not bound by IgG1 from any of the sera. Four of eleven sera from non-allergic donors had sIgG1 to Bet v 1 and TB1.

sIgG4 to Bet v 1 and chimeric proteins was detected only in sera from birch pollen-allergic, but not from non-allergic donors (Fig. 2C). Sera from 27/30 patients bound to 1-11 chimeras (median: 4.0; Fig. 4A). The protein most frequently recognized (19/30) was TB5. Three additional chimeras (TB1, TB3 and TB6) were recognized by 16 sera. The protein with the lowest IgG4-reactivity was TB10, bound by a single serum.

A comparison of the antibody binding frequencies of the chimeric proteins (Fig. 4B) showed that the chimeras with the highest frequencies of antibody binding were specific to each isotype: TB2, TB7 and TB8 for IgE, TB1 for IgG1 and TB1, TB3, TB5 and TB6 for IgG4.. Similar differences were observed when comparing the chimeras with the lowest antibody binding frequencies: TB9 did not bind IgE, TB14 lacked IgG1 binding, whereas TB10 bound IgG4 only from a single serum. An overall comparison of chimera binding patterns of all pairs of isotypes (Fig. 4C, Table E4 in the Online Repository) showed that, apart from similar binding patterns of IgG1 and IgG4 in some patients, the extent of correspondence was not significantly different from a random correspondence given the observed chimera binding frequencies (as shown by kappa values close to zero).

Bacterial structural Bet v 1 homologues are recognized by antibodies from birch pollen-allergic and non-allergic individuals

The bacterial scaffold protein TTHA0849, used as a negative control in the ELISA, bound antibodies from some sera despite its low sequence similarity to Bet v 1 (Table E2 in the Online Repository). This prompted us to test three additional structural Bet v 1 homologues

from bacterial species that are, unlike *Thermus thermophilus*, part of the normal human microbiota (Table E2 in the Online Repository).

About half of the birch-allergic patients had IgE to three of the four tested proteins (TTHA0849, SA2116 and SMU440), while PA1206 bound IgE from only 2 sera (Fig. 5A, Fig. E5 in the Online Repository). In total, 19 of 30 sera had sIgE to at least one bacterial protein. Nevertheless, the amounts of IgE to the bacterial proteins (medians of positive sera between 4.7 ng/ml for SMU440 and 9.0 ng/ml for TTHA0849) were considerably lower than the concentration of Bet v 1-sIgE (median 246 ng/ml). Moreover, 2/11 sera from non-allergic donors had IgE to one bacterial protein each.

We observed patient-specific patterns of IgG1 and IgG4 binding to the bacterial proteins in sera of allergic and non-allergic donors (Fig. E5 in the Online Repository). Like for IgE, the amounts of sIgG4 to bacterial proteins were considerably lower than the concentration of Bet v 1-sIgG4 (Fig. 5A), while the amounts of sIgG1 to bacterial proteins were similar to that of Bet v 1-sIgG1.

To examine cross-reactivity of Bet v 1-sIgE with its bacterial homologues, we performed an IgE inhibition ELISA with 7 sera of birch pollen-allergic patients (Fig. 5B). All bacterial proteins were able to partially inhibit IgE binding to Bet v 1 (median extents of inhibition between 8% for TTHA0849 and 18% for SA2116). Due to the low amounts of sIgE to the bacterial proteins, we were not able to perform the assay in the reverse format, i.e. inhibition of IgE binding to the bacterial proteins by Bet v 1.

DISCUSSION

In this work, we characterized the epitope binding repertoires of Bet v 1-specific antibodies in sera of birch pollen-allergic and non-allergic donors. To this end, we used structure-based grafting of surface areas containing potential conformational epitopes of Bet v 1 to a non-allergenic structural homologue. Thereby, we created a panel of chimeric proteins displaying overlapping patches covering the complete surface of Bet v 1 (Figs. 1, Fig. E1 in the Online Repository). We showed that the Bet v 1-sIgE repertoires of birch pollen-allergic patients are polyclonal and highly individual (Figs. 2 and 3). Moreover, epitopes of IgG1 and IgG4 differ from IgE binding sites (Figs. 2 and 4).

IgE specific to respiratory allergens mainly binds to conformational epitopes. This has been shown, among others, for Bet v 1⁶⁻⁸ and the major grass pollen allergen Phl p 5.²⁰ These epitopes cannot be mapped using efficient peptide mapping techniques, such as microarrays or bead-based multiplex assays. Instead, more sophisticated and indirect methods have to be employed.²¹

Structure-based grafting of potential epitopes of allergens to non-allergenic homologues or *vice versa* allows for direct localization of areas overlapping with conformational epitopes. This method was first used for epitope mapping of Ber e 1 from Brazil nut²² and later applied to the olive pollen allergens Ole e 1 and Ole e 15^{23, 24} as well as to selected epitopes of Bet v 1.^{12, 15, 16} In a previous study, we grafted four large non-overlapping Bet v 1-derived surface patches to its low-allergenic homologue Api g 1 from celeriac.¹⁷ In the present work, we further developed this approach by reducing the size of the grafted patches to being slightly greater than the typical epitope size,²⁵ generating an increased number of chimeric proteins with overlapping grafted areas, and using a bacterial Bet v 1-homologue with low sequence similarity and, consequently, almost absent IgE binding as the acceptor protein (Fig. 1, Fig. E1 in the Online Repository). By selectively transferring surface-exposed residues, we

generated folded, soluble proteins and successfully purified 13 of 14 designed chimeras (Fig. 1, Figs. E1 and E3 in the Online Repository).

We identified three partially overlapping surface areas (TB2, TB7 and TB8; Fig. 1C) that bound IgE from more than 50% of the patients and another six areas that bound IgE from more than one-third of the sera (Fig. 2A). Hence, most parts of the surface of Bet v 1 are highly allergenic. Our data corroborate the results of previous epitope mapping studies, each of them identifying IgE binding sites on a different part of the surface.⁹⁻¹³ Likewise, epitope mapping of Gly m 4, the Bet v 1 homologue from soybean,²⁶ Phl p 1²⁷ and Phl p 5²⁰ from grass pollen and the mite allergen Der f 21²⁸ showed polyclonal IgE binding to large parts of the allergen surface. Accordingly, clonality of sIgE has been identified as an important determinant of the extent of mast cell activation²⁹.

The most frequently recognized areas identified here overlap with previously identified important IgE epitopes (Fig. E5 in the Online Repository). TB1 bound IgE from 40% of birch pollen-allergic patients (Fig. 2A) and accounted for up to 30% of Bet v 1-sIgE (Fig. 3). Its surface patch is almost identical with the contact surface of the mAb BV16, which inhibited 40% of IgE binding to Bet v 1 (Fig. E6A in the Online Repository).¹⁰ Gieras and coworkers identified three Bet v 1 peptides as minimal epitopes of a set of strongly IgE-inhibiting murine mAbs.¹¹ One peptide (49-58) overlaps with the p-loop epitope also recognized by BV16 and represented by TB1, whereas the peptide 73-88 overlaps with the patch of TB7 (Fig. E6B), and the peptide 88-103 is part of the patches of TB2, TB8 and TB13 (Fig. E6C). These four chimeras bound IgE from about half of the sera each (Fig. 2A).

The amounts of IgE binding to the chimeric proteins were considerably lower than Bet v 1-specific IgE (Table E3 in the Online Repository). Moreover, inhibition assays revealed a very low affinity of patients' IgE for the chimeric proteins, as very high concentrations of chimeric proteins were required to partially inhibit IgE binding to Bet v 1 (Fig. 3). Both effects might

be caused by antibodies that bind only to the edge of the patch with low affinity. An alternative explanation could be subtle conformational differences between the grafted patches and the corresponding regions on the surface of Bet v 1. The low affinity of chimera-specific IgE might also explain the incomplete inhibition of IgE binding to immobilized chimeras by the chimeric protein itself and by Bet v 1 (data not shown). Moreover, the high serum concentrations required for this assay due to the low chimera-specific signal might have caused interference by Bet v 1-specific IgG and IgA. In summary, the method of designing chimeric proteins may still be optimized regarding patch size and criteria for including surface-exposed residues.

Sera of all Bet v 1-sensitized, birch pollen-allergic patients contained Bet v 1-sIgG1 and sIgG4 (Fig. 2B, C), albeit with different epitope specificities than sIgE. We did not find a correlation between epitope binding patterns of different antibody classes (Fig. 4C). The origin of IgE-expressing B cell has been a matter of debate. Studies that sequenced heavy chain transcripts from B cells of allergic patients either found no clonal relationship between IgE and other isotypes³⁰ or concluded that IgE originated mainly from indirect class switch from IgG1 and to a lesser extent from other switched B cells.³¹ The non-relatedness of IgE and IgG repertoires in our sera supports the notion that development of IgE-producing B cells occurs independently of IgG-producing ones by direct class switch from IgM. However, high throughput single-cell sequencing of Bet v 1-specific B cells will be required to corroborate this hypothesis.

We also tested binding of serum antibodies to bacterial structural homologues of Bet v 1, including three proteins from bacteria that are part of the normal human microbiota but can also be pathogenic.³²⁻³⁴ Few data, other than their structures, on the Bet v 1 homologues in those species are available.³⁵ Despite their low sequence identities with Bet v 1, we detected binding of IgE and IgG4 mainly from birch pollen-allergic patients as well as IgG1 from

allergic and non-allergic donors to all four tested bacterial proteins (Fig. 5A, Fig. E5 in the Online Repository). While the concentrations of IgE to the bacterial proteins were 10-100 times lower than Bet v 1-sIgE concentrations, the amount of sIgG1 to the bacterial proteins and Bet v 1 were similar. These data point to a role of bacterial Bet v 1 homologues in the development of birch pollen allergy. One may speculate that in atopic patients, B cells specific to Bet v 1-related proteins from common bacteria might undergo additional affinity maturation and class switch triggered by Bet v 1 and pollen-derived adjuvants, leading to IgE with high affinity for Bet v 1, but only minimal residual binding to their original bacterial antigens. A similar mechanism was recently shown in patients with pemphigus vulgaris, whose autoantibodies had originally been directed to walnut proteins.³⁶ However, supporting this mechanism will require extensive examination of cross-reactivity between Bet v 1 and bacterial proteins for different isotypes as well as single-cell sequencing of B cells specific to Bet v 1 and bacterial proteins in order to establish their clonal relationship.

In summary, we showed that epitope grafting is an approach suitable for delineating the repertoire of conformational epitopes bound by allergen-specific IgE and other isotypes. Chimeric proteins presenting individual epitopes of Bet v 1 will be useful tools for gaining insights into the mechanism of allergen immunotherapy by high-resolution epitope monitoring of sIgE and sIgG during birch pollen immunotherapy³⁷. Moreover, analyzing epitope repertoires of birch pollen-allergic patients with varying extents of pollen-associated plant food allergy may shed light on factors that cause the variable clinical consequences of sensitization to Bet v 1.

369 REFERENCES

- 370
371 1. D'Amato G, Cecchi L, Bonini S, Nunes C, Annesi-Maesano I, Behrendt H, et al.
372 Allergenic pollen and pollen allergy in Europe. *Allergy* 2007; 62:976-90.
- 373 2. Biedermann T, Winther L, Till SJ, Panzner P, Knulst A, Valovirta E. Birch pollen
374 allergy in Europe. *Allergy* 2019; 74:1237-48.
- 375 3. Movérare R, Westritschnig K, Svensson M, Hayek B, Bende M, Pauli G, et al.
376 Different IgE reactivity profiles in birch pollen-sensitive patients from six European
377 populations revealed by recombinant allergens: an imprint of local sensitization. *Int*
378 *Arch Allergy Immunol* 2002; 128:325-35.
- 379 4. Tolkki L, Alanko K, Petman L, Skydtsgaard MB, Milvang PG, Seppälä U, et al.
380 Clinical characterization and IgE profiling of birch (*Betula verrucosa*)--allergic
381 individuals suffering from allergic reactions to raw fruits and vegetables. *J Allergy*
382 *Clin Immunol Pract* 2013; 1:623-31.e1.
- 383 5. Aalberse RC, Crameri R. IgE-binding epitopes: a reappraisal. *Allergy* 2011; 66:1261-
384 74.
- 385 6. Focke M, Linhart B, Hartl A, Wiedermann U, Sperr WR, Valent P, et al. Non-
386 anaphylactic surface-exposed peptides of the major birch pollen allergen, Bet v 1, for
387 preventive vaccination. *Clin Exp Allergy* 2004; 34:1525-33.
- 388 7. Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, et al.
389 Division of the Major Birch Pollen Allergen, Bet v 1, into Two Non-Anaphylactic
390 Fragments. *International Archives of Allergy and Immunology* 1997; 113:246-8.
- 391 8. Wallner M, Hauser M, Himly M, Zaborsky N, Mutschlechner S, Harrer A, et al.
392 Reshaping the Bet v 1 fold modulates T(H) polarization. *J Allergy Clin Immunol*
393 2011; 127:1571-8 e9.

- 394 9. Mittag D, Batori V, Neudecker P, Wiche R, Friis EP, Ballmer-Weber BK, et al. A
395 novel approach for investigation of specific and cross-reactive IgE epitopes on Bet v 1
396 and homologous food allergens in individual patients. *Mol Immunol* 2006; 43:268-78.
- 397 10. Mirza O, Henriksen A, Ipsen H, Larsen JN, Wissenbach M, Spangfort MD, et al.
398 Dominant epitopes and allergic cross-reactivity: complex formation between a Fab
399 fragment of a monoclonal murine IgG antibody and the major allergen from birch
400 pollen Bet v 1. *J Immunol* 2000; 165:331-8.
- 401 11. Gieras A, Cejka P, Blatt K, Focke-Tejkl M, Linhart B, Flicker S, et al. Mapping of
402 conformational IgE epitopes with peptide-specific monoclonal antibodies reveals
403 simultaneous binding of different IgE antibodies to a surface patch on the major birch
404 pollen allergen, Bet v 1. *J Immunol* 2011; 186:5333-44.
- 405 12. Hecker J, Diethers A, Schulz D, Sabri A, Plum M, Michel Y, et al. An IgE epitope of
406 Bet v 1 and fagales PR10 proteins as defined by a human monoclonal IgE. *Allergy*
407 2012; 67:1530-7.
- 408 13. Levin M, Davies AM, Liljekvist M, Carlsson F, Gould HJ, Sutton BJ, et al. Human
409 IgE against the major allergen Bet v 1--defining an epitope with limited cross-
410 reactivity between different PR-10 family proteins. *Clin Exp Allergy* 2014; 44:288-99.
- 411 14. Spangfort MD, Mirza O, Ipsen H, Van Neerven RJ, Gajhede M, Larsen JN.
412 Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen,
413 characterized by X-ray crystallography and site-directed mutagenesis. *J Immunol*
414 2003; 171:3084-90.
- 415 15. Holm J, Ferreras M, Ipsen H, Wurtzen PA, Gajhede M, Larsen JN, et al. Epitope
416 grafting, re-creating a conformational Bet v 1 antibody epitope on the surface of the
417 homologous apple allergen Mal d 1. *J Biol Chem* 2011; 286:17569-78.

16. Berkner H, Seutter von Loetzen C, Hartl M, Randow S, Gubesch M, Vogel L, et al. Enlarging the toolbox for allergen epitope definition with an allergen-type model protein. *PLoS One* 2014; 9:e111691.
17. Gepp B, Lengger N, Bublin M, Hemmer W, Breiteneder H, Radauer C. Chimeras of Bet v 1 and Api g 1 reveal heterogeneous IgE responses in patients with birch pollen allergy. *Journal of Allergy and Clinical Immunology* 2014; 134:188-94.
18. Geroldinger-Simic M, Kinaciyan T, Nagl B, Baumgartner-Durchschlag U, Huber H, Ebner C, et al. Oral exposure to Mal d 1 affects the immune response in patients with birch pollen allergy. *J Allergy Clin Immunol* 2013; 131:94-102.
19. Nakabayashi M, Shibata N, Komori H, Ueda Y, Iino H, Ebihara A, et al. Structure of a conserved hypothetical protein, TTHA0849 from *Thermus thermophilus* HB8, at 2.4 Å resolution: a putative member of the StAR-related lipid-transfer (START) domain superfamily. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 2005; 61:1027-31.
20. Levin M, Rothhus S, Wendel S, Najafi N, Källström E, Focke-Tejkl M, et al. Multiple independent IgE epitopes on the highly allergenic grass pollen allergen Phl p 5. *Clin Exp Allergy* 2014; 44:1409-19.
21. Breiteneder H. Mapping of conformational IgE epitopes of food allergens. *Allergy* 2018; 73:2107-9.
22. Alcocer MJ, Murtagh GJ, Wilson PB, Proglas P, Lin J, Archer DB. The major human structural IgE epitope of the Brazil nut allergen Ber e 1: a chimaeric and protein microarray approach. *J Mol Biol* 2004; 343:759-69.
23. Marazuela EG, Hajek R, Villalba M, Barber D, Breiteneder H, Rodríguez R, et al. A non-allergenic Ole e 1-like protein from birch pollen as a tool to design hypoallergenic vaccine candidates. *Mol Immunol* 2012; 50:83-90.

24. San Segundo-Acosta P, Oeo-Santos C, Navas A, Jurado A, Villalba M, Barderas R. Ole e 15 and its human counterpart -PPIA- chimeras reveal an heterogeneous IgE response in olive pollen allergic patients. *Sci Rep* 2019; 9:15027.
25. Pomés A, Mueller GA, Chruszcz M. Structural Aspects of the Allergen-Antibody Interaction. *Front Immunol* 2020; 11:2067.
26. Husslik F, Nurnberg J, Seutter von Loetzen C, Mews T, Ballmer-Weber BK, Kleine-Tebbe J, et al. The conformational IgE epitope profile of soya bean allergen Gly m 4. *Clin Exp Allergy* 2016; 46:1484-97.
27. Madritsch C, Gadermaier E, Roder UW, Lupinek C, Valenta R, Flicker S. High-density IgE recognition of the major grass pollen allergen Phl p 1 revealed with single-chain IgE antibody fragments obtained by combinatorial cloning. *J Immunol* 2015; 194:2069-78.
28. Pang SL, Ho KL, Waterman J, Rambo RP, Teh AH, Mathavan I, et al. Crystal structure and epitope analysis of house dust mite allergen Der f 21. *Sci Rep* 2019; 9:4933.
29. Christensen LH, Holm J, Lund G, Riise E, Lund K. Several distinct properties of the IgE repertoire determine effector cell degranulation in response to allergen challenge. *J Allergy Clin Immunol* 2008; 122:298-304.
30. Levin M, Levander F, Palmason R, Greiff L, Ohlin M. Antibody-encoding repertoires of bone marrow and peripheral blood-a focus on IgE. *J Allergy Clin Immunol* 2017; 139:1026-30.
31. Looney TJ, Lee JY, Roskin KM, Hoh RA, King J, Glanville J, et al. Human B-cell isotype switching origins of IgE. *J Allergy Clin Immunol* 2016; 137:579-86 e7.
32. Ogonowska P, Gilaberte Y, Barańska-Rybak W, Nakonieczna J. Colonization With *Staphylococcus aureus* in Atopic Dermatitis Patients: Attempts to Reveal the Unknown. *Front Microbiol* 2020; 11:567090.

33. Bedoya-Correa CM, Rincón Rodríguez RJ, Parada-Sanchez MT. Genomic and phenotypic diversity of *Streptococcus mutans*. *J Oral Biosci* 2019; 61:22-31.
34. Maurice NM, Bedi B, Sadikot RT. *Pseudomonas aeruginosa* Biofilms: Host Response and Clinical Implications in Lung Infections. *Am J Respir Cell Mol Biol* 2018; 58:428-39.
35. Nan J, Brostromer E, Liu XY, Kristensen O, Su XD. Bioinformatics and structural characterization of a hypothetical protein from *Streptococcus mutans*: implication of antibiotic resistance. *PLoS One* 2009; 4:e7245.
36. Lin L, Moran TP, Peng B, Yang J, Culton DA, Che H, et al. Walnut antigens can trigger autoantibody development in patients with pemphigus vulgaris through a "hit-and-run" mechanism. *J Allergy Clin Immunol* 2019; 144:720-8 e4.
37. Gepp B, Lengger N, Mobs C, Pfutzner W, Radauer C, Bohle B, et al. Monitoring the epitope recognition profiles of IgE, IgG1, and IgG4 during birch pollen immunotherapy. *J Allergy Clin Immunol* 2016; 137:1600-3 e1.

484

485 **FIGURE LEGENDS**

486 **Fig. 1. Chimera design.** **A** Design of chimeric proteins showing TB1 as an example. **A**
 487 surface-exposed residue of Bet v 1 (here: E45) is selected as the center of the patch (1), the
 488 patch is created by adding adjacent surface-exposed residues up to a surface area of 1000 Å²
 489 (2), and the chimeric protein is created by replacing structurally equivalent residues on the
 490 surface of the scaffold protein, TTHA0849, by those from Bet v 1 (3). **B** Structure-based
 491 sequence alignment of Bet v 1, the scaffold TTHA0849 and the chimeric proteins. Bet v 1-
 492 derived residues introduced into the chimeric proteins are shaded, solubility enhancing
 493 mutations outside the grafted patch are shown in bold, secondary structure elements of Bet v 1
 494 are shown below the alignment (s: strand, h: helix). **C** Models of the chimeric proteins TB2,
 495 TB7 and TB8 with the grafted patches in dark grey.

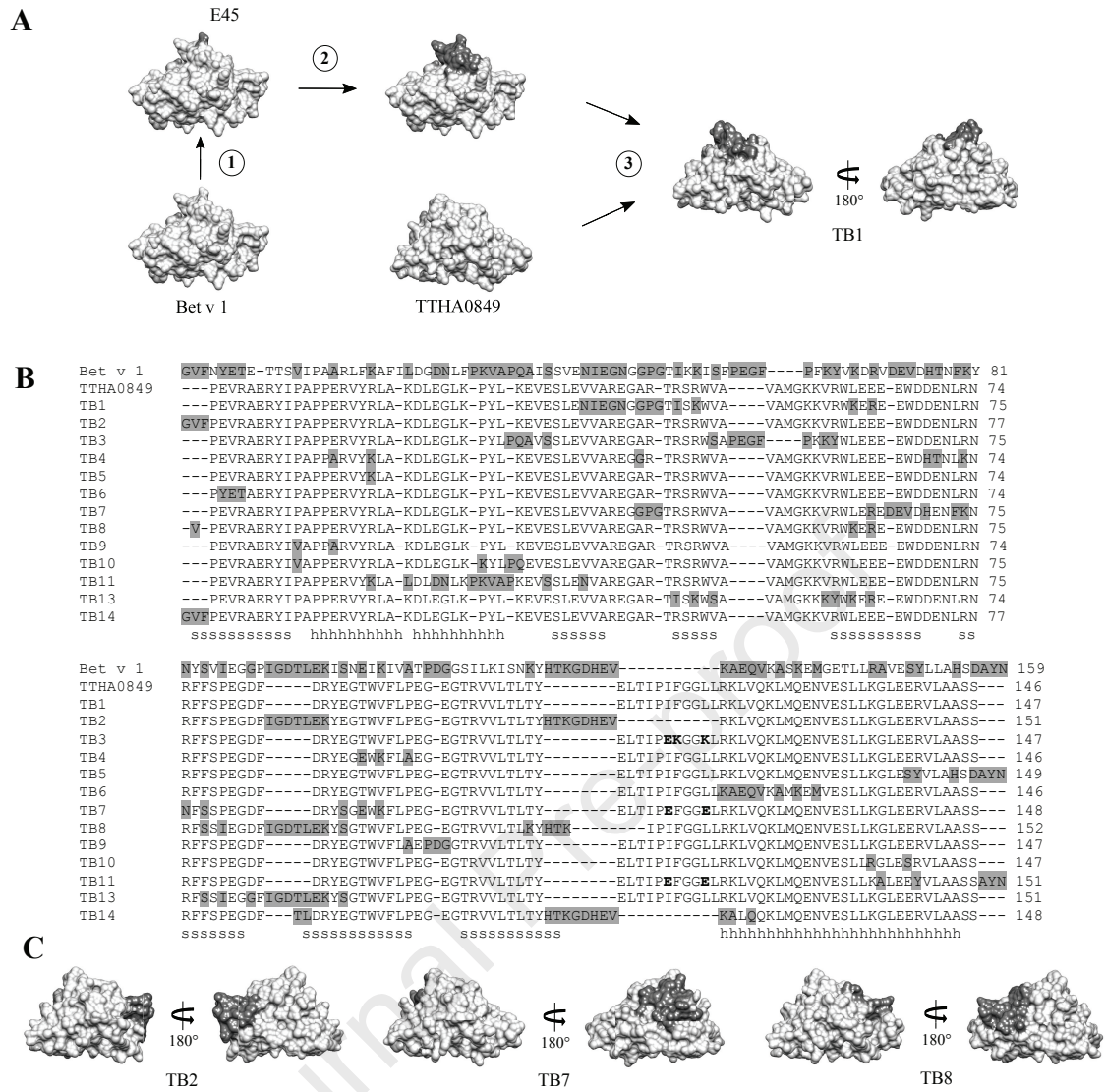
496 **Fig. 2. Binding patterns of Bet v 1-specific antibodies to chimeric proteins.** Sera from 30
 497 birch pollen-allergic patients (B1 to B30) and 11 non-allergic donors (N1 to N11) were
 498 assayed by a quantitative ELISA for sIgE (**A**), sIgG1 (**B**) and sIgG4 (**C**). Numbers of sera
 499 tested positive are indicated in the row beneath each heatmap, numbers of chimeric proteins
 500 recognized by each serum are summarized in the rightmost columns.

501 **Fig. 3. Inhibition of IgE-binding to Bet v 1 by chimeric proteins.** **A.** Sera from 9 birch
 502 pollen-allergic patients were preincubated with 20 µg/mL Bet v 1, chimeric proteins or
 503 TTHA0849 as a control before measuring unblocked Bet v 1-sIgE by ELISA. **B.** Titrated
 504 inhibition of IgE binding to Bet v 1 using selected chimeras as inhibitors. In both assays,
 505 inhibition values obtained with TTHA0849 were set to zero.

506 **Fig. 4. Comparison of isotype-specific chimera binding patterns.** **A.** Numbers of chimeric
 507 proteins bound by antibodies from birch pollen-allergic (B) and non-allergic (N) donors' sera.

Significant differences determined by 2-way ANOVA are indicated (* = $p < 0.05$; *** = $p < 0.001$). **B.** Antibody binding frequencies of each chimeric protein among birch pollen-allergic patients. **C.** Agreement of chimera binding patterns of each pair of isotypes calculated for each birch pollen-allergic patient's serum expressed by Cohen's kappa. A kappa value of 0 corresponds to the chance agreement as expected from the number of positive chimeras for each isotype.

Fig. 5. Antibody binding to bacterial Bet v 1 homologues. **A.** Concentrations of individual immunoglobulin isotypes in sera of 30 allergic patients for Bet v 1 and its bacterial homologues as determined by ELISA. Only positive values with their median indicated by a horizontal line and the number of reactive sera specified above the graph are shown. **B.** Inhibition of IgE binding to Bet v 1 by its bacterial homologues. Sera from 7 birch pollen-allergic patients were preincubated with Bet v 1 or bacterial proteins before measuring unbound Bet v 1-sIgE by ELISA. The graph shows inhibition of IgE binding relative to untreated sera. Horizontal lines indicate median values.

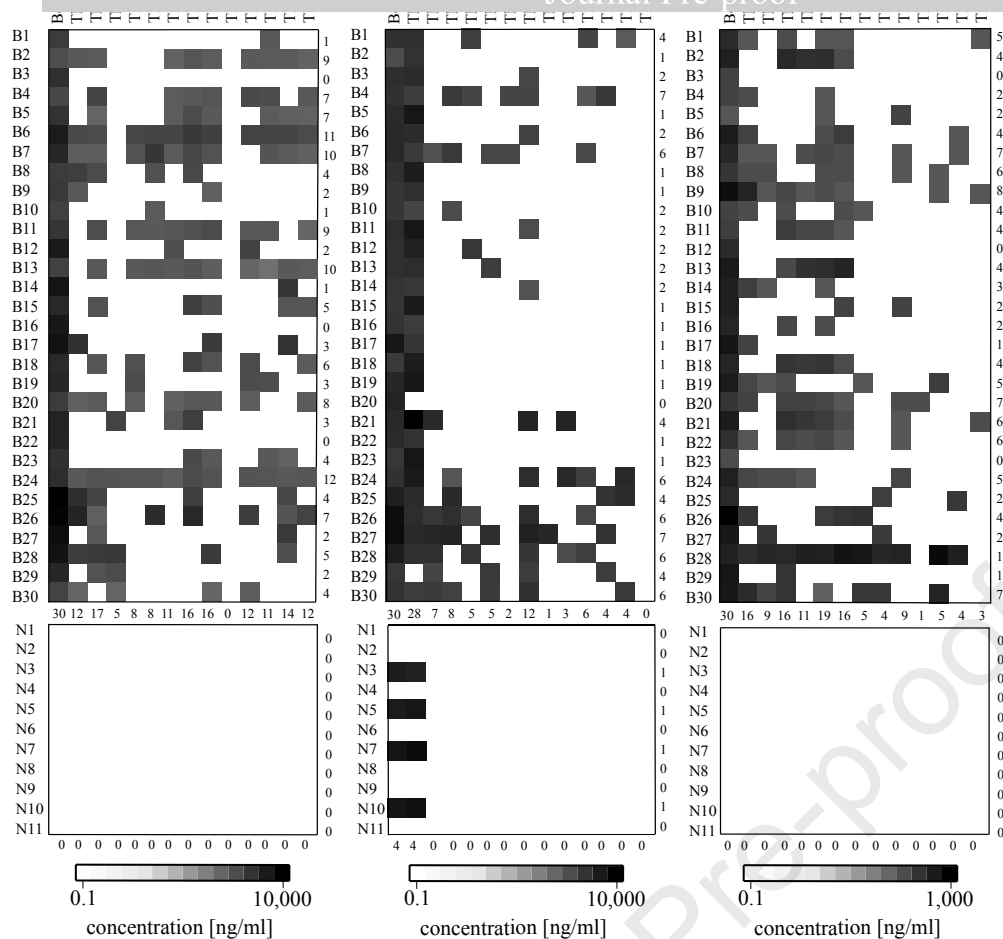


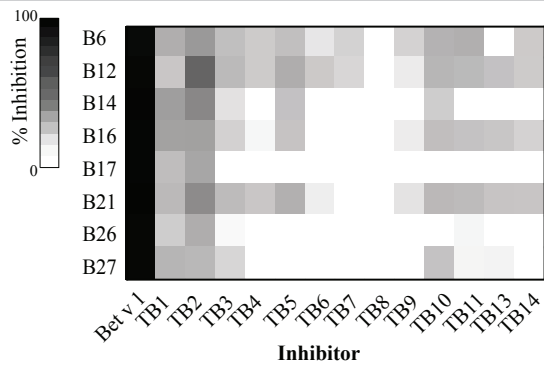
A IgE

B IgG1

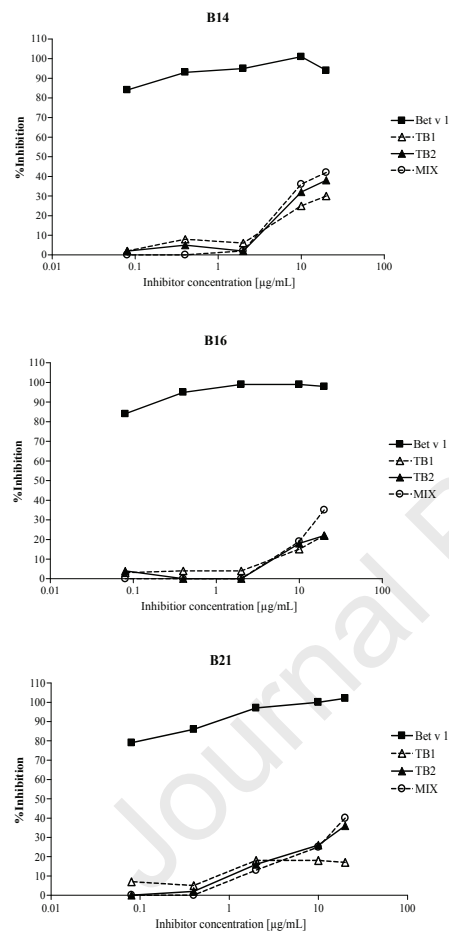
C IgG4

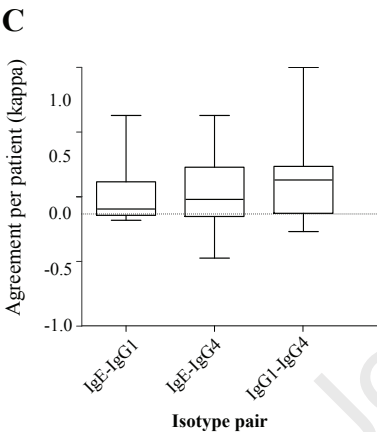
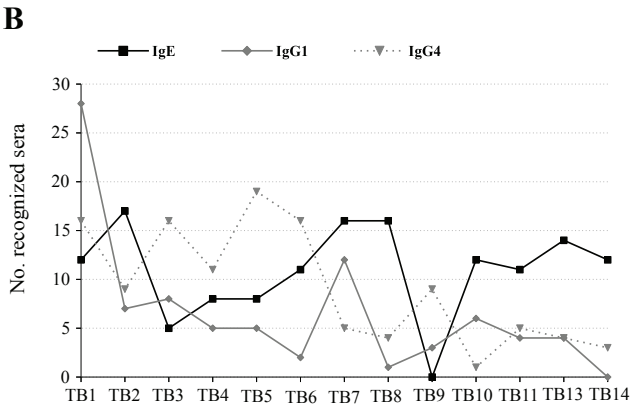
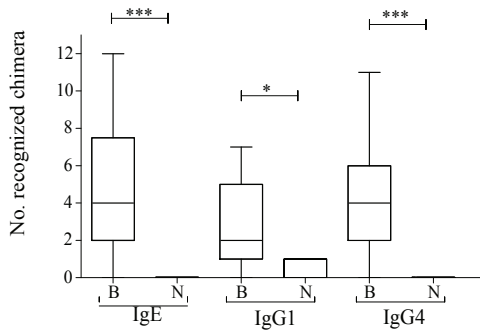
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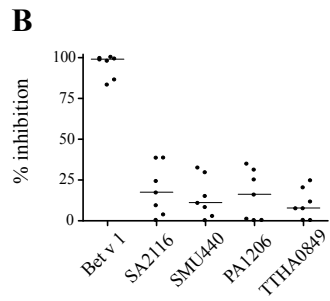
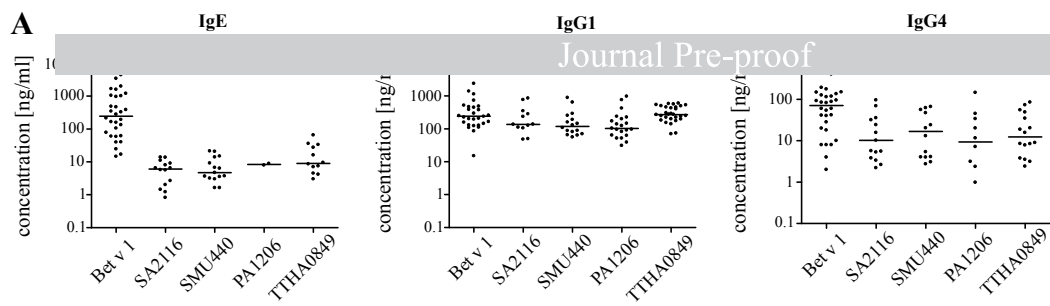


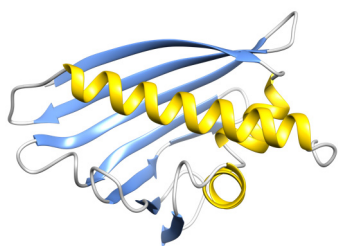


B

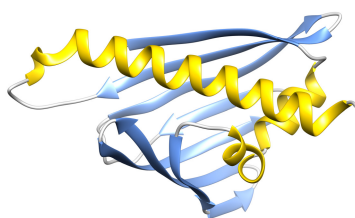




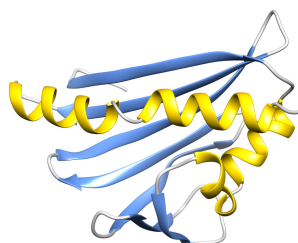




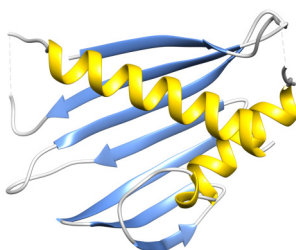
Bet v 1



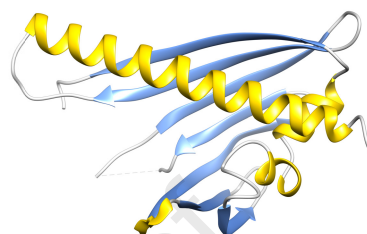
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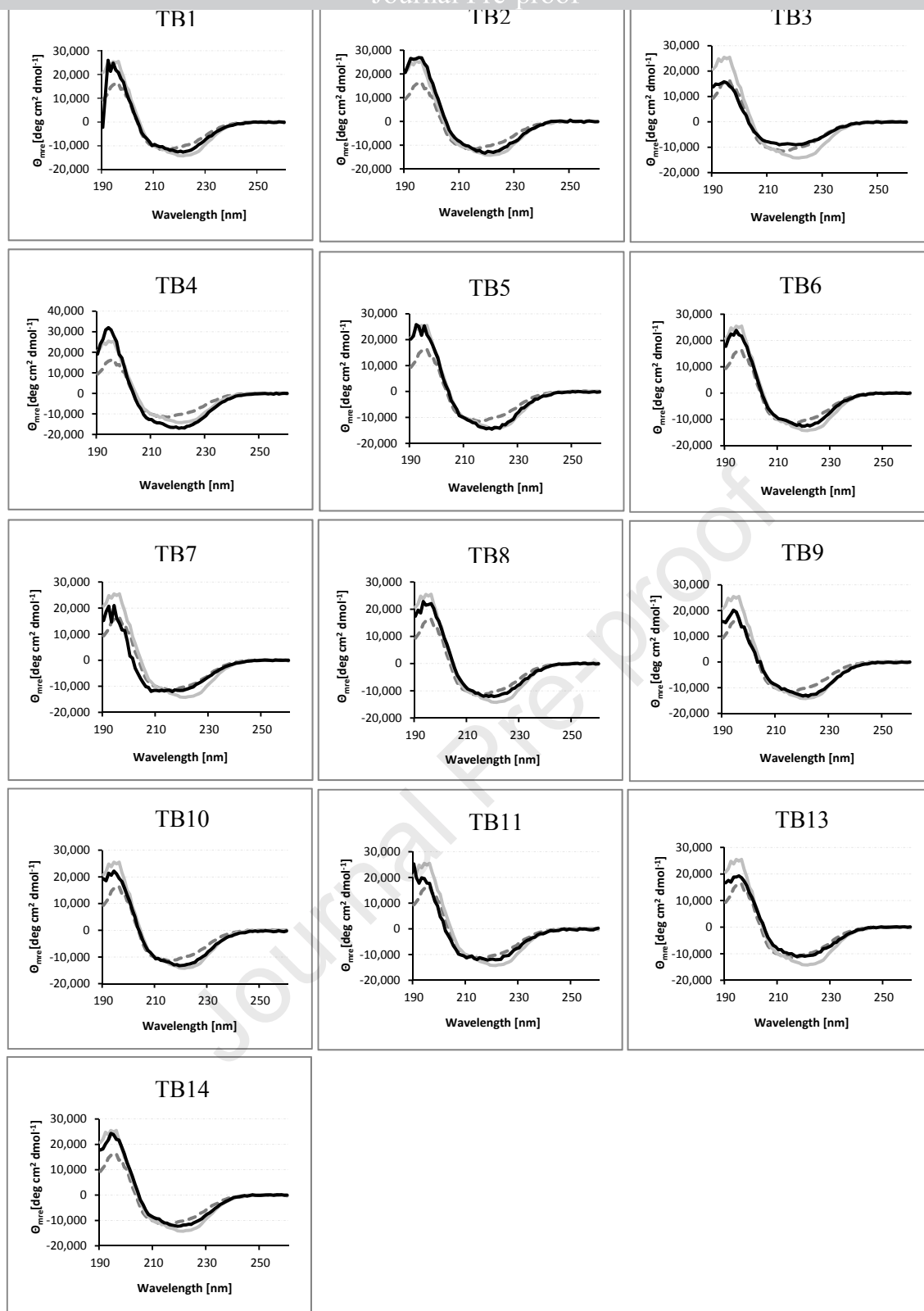
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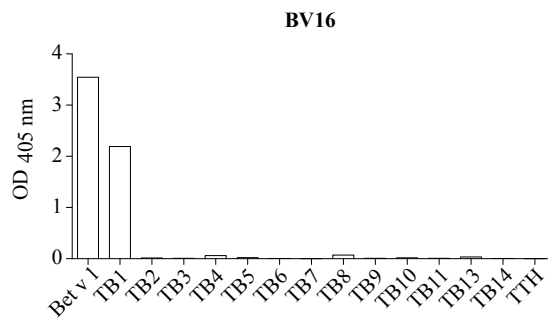
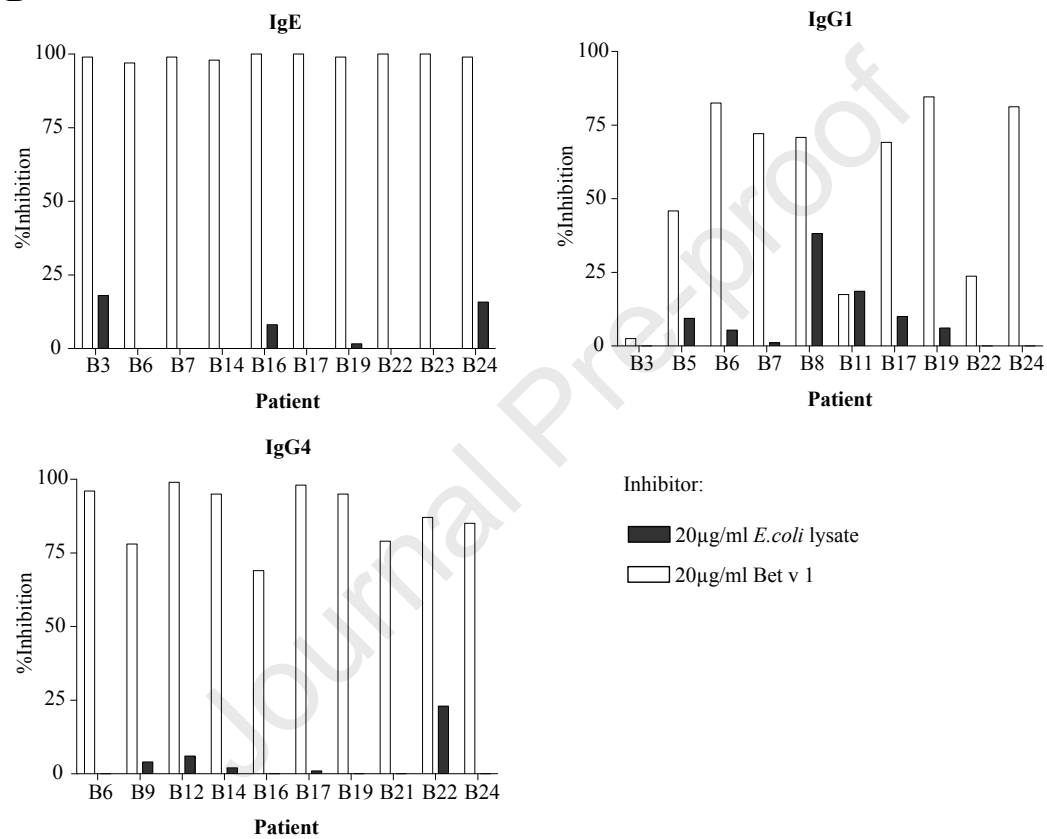


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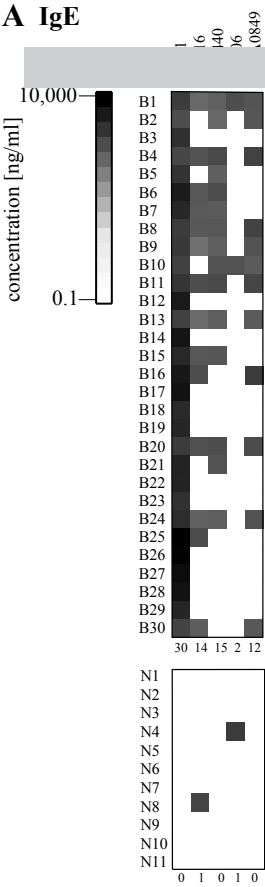


SA2116



A**B**

A IgE

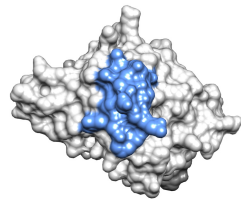


B IgG1

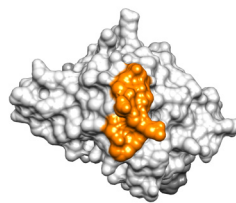


C IgG4

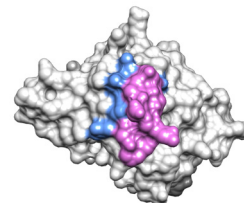


A

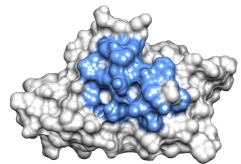
patch TB1



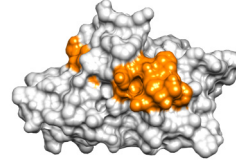
murine mAb BV16



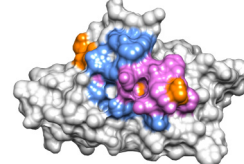
overlap

B

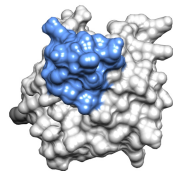
patch TB7



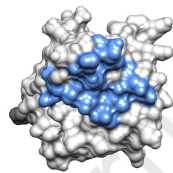
peptide 73-88



overlap

C

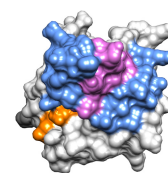
patch TB2



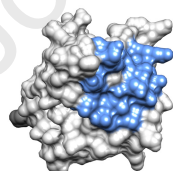
patch TB8



peptide 88-103



overlap



patch TB13