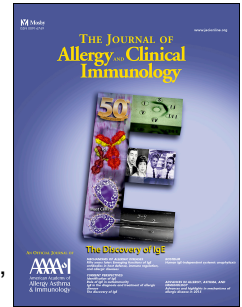


Accepted Manuscript

Tackling Bet v 1- and associated food allergies with a single hybrid protein

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PII: S0091-6749(16)31441-5

DOI: [10.1016/j.jaci.2016.09.055](https://doi.org/10.1016/j.jaci.2016.09.055)

Reference: YMAI 12491

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 14 March 2016

Revised Date: 19 August 2016

Accepted Date: 19 September 2016

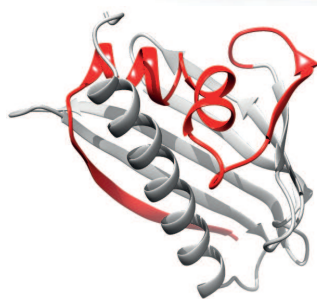
Please cite this article as: Hofer H, Asam C, Hauser M, Nagl B, Laimer J, Himly M, Briza P, Ebner C, Lang R, Hawranek T, Bohle B, Lackner P, Ferreira F, Wallner M, Tackling Bet v 1- and associated food allergies with a single hybrid protein, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2016.09.055.

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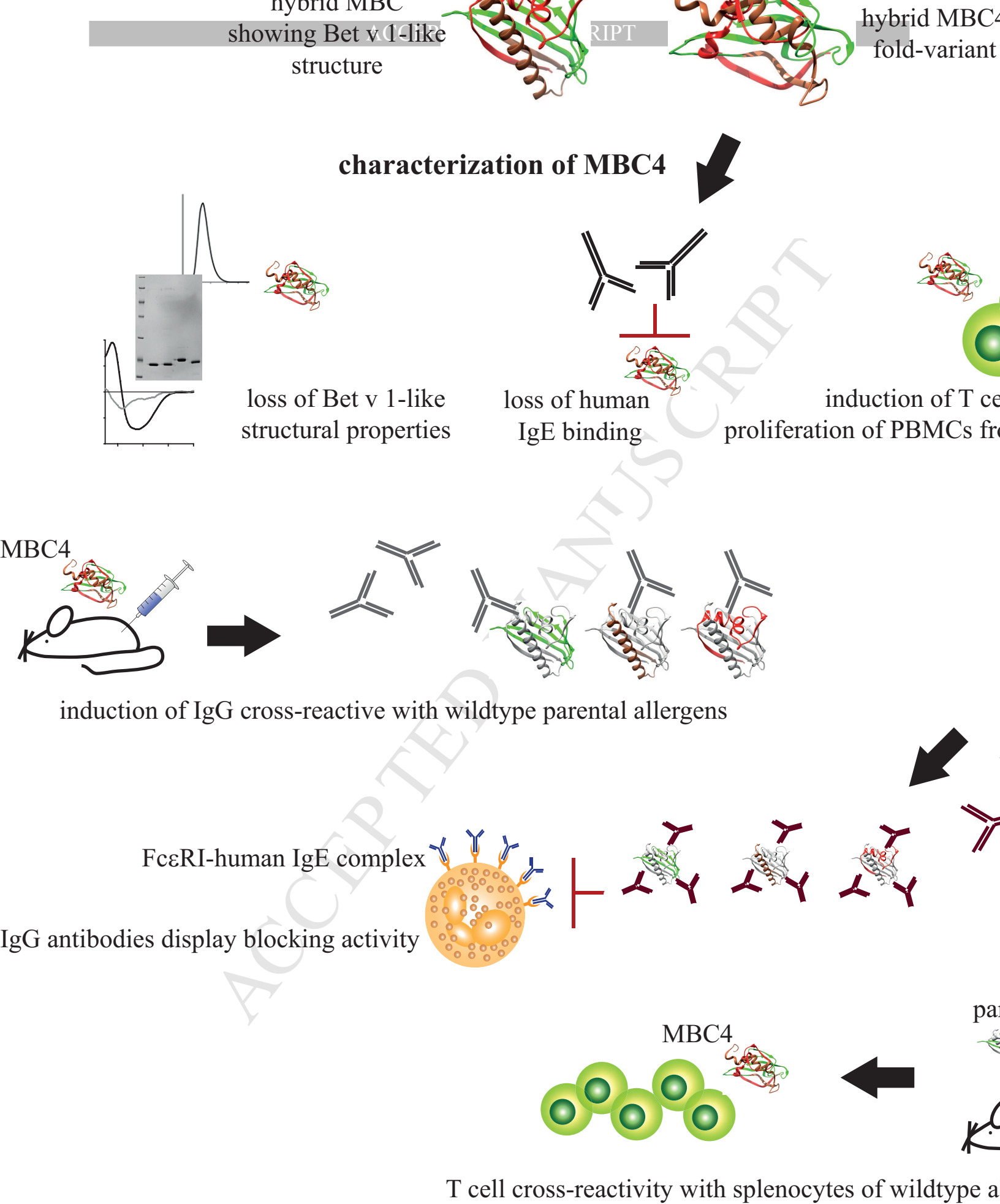


birch
Bet v 1

hyb
fo



apple
Mal d 1



MBC4 is a candidate for simultaneous treatment of Bet v 1- and concomitant

Tackling Bet v 1- and associated food allergies with a single hybrid protein

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Supported by the the Austrian Science Fund (FWF) grant L688, the Austrian National Bank (ÖNB) grant 12533, and the priority program “Allergy-Cancer-BioNano Research Centre” of the University of Salzburg

Disclosure of potential conflict of interest: F. Ferreira is a board member for HAL Allergy (NL), Indoor Bio-technologies, and AllergenOnline and has been supported by Austrian Science Funds (FWF). B. Bohle has been supported by Austrian Science Funds and the Christian Doppler Laboratory for Immunomodulation. M. Wallner has been supported by the University of Salzburg and the Austrian National Bank (ÖNB). The rest of the authors declare that they have no relevant conflicts of interest.

Key words

Birch pollen allergy, Bet v 1, birch pollen-associated food allergy, allergy vaccine candidate, molecular allergology

Clinical implications (29 words, max 30)

Targeted epitope recombination in combination with a knowledge-based structural modification allowed the production of a single molecule as allergy vaccine candidate to tackle birch pollen and associated food allergies

Capsule summary (31 words, max 35)

Therapy of birch pollen and associated food allergies is still precarious. A hybrid protein designed through epitope recombination shall allow the simultaneous treatment of Bet v 1- and concomitant food allergies.

Abbreviations

AIT: allergen-specific immunotherapy

ANS: 1-anilino-8-naphthalene sulfonate

APC: antigen-presenting cell

BSA: bovine serum albumin

CD: circular dichroism

Cpm: counts per minute

DC: dendritic cell

DLS: dynamic light scattering

DTT: dithiothreitol

ELISA: enzyme linked immunosorbent assay

ELISpot: enzyme linked immuno spot

EU: endotoxin unit

FTIR: Fourier transform infrared

GMP: good manufacturing practice

HEK: human embryonic kidney

IL: interleukin

IFN: interferon

mAb: monoclonal antibody

MBC: name of folded hybrid molecule, deduced from the consecutive order of epitopes from the parental allergens Mal d 1, Bet v 1, and Cor a 1.04, assembled to design the protein

MBC4: name of unfolded hybrid molecule, deduced from the hybrid MBC, where a distinct mutation was introduced into the sequence using the previously published protein BM4 as template ¹

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

PFS: pollen-food syndrome

RBL: rat basophil leukemia

SCIT: sub-cutaneous immunotherapy

SDS-PAGE: sodium dodecyl sulfate-poly acrylamide gel electrophoresis

SEM: standard error mean

SI: stimulation index

SLIT: sub-lingual immunotherapy

SSF: statistical scoring function

TBS: Tris buffered saline

Th: T helper

TLR: toll-like receptor

WT: wildtype

Abstract (250 words / 250 max)

Background: Allergy vaccines should be easily applicable, safe and efficacious. For Bet v 1-mediated birch pollen and associated food allergies a single wildtype allergen does not provide a complete solution.

Objective: Thus, we aimed to combine immunologically relevant epitopes of Bet v 1 and the two clinically most important related food allergens from apple and hazelnut to a single hybrid protein, termed MBC4.

Methods: After identification of T cell epitope-containing parts on each of the three parental allergens, the hybrid molecule was designed to cover relevant epitopes and evaluated *in silico*. Thereby, a mutation was introduced into the hybrid sequence, which should alter the secondary structure without compromising immunogenic properties of the molecule.

Results: MBC4 as well as the parental allergens were purified to homogeneity. Analyses of secondary structure elements revealed substantial changes rendering the hybrid *de facto* non-reactive with patients' serum IgE. Nevertheless, the protein was monomeric in solution. MBC4 was able to activate T cell lines from birch pollen allergic donors and from mice immunized with the parental allergens. Moreover, upon immunization of mice and rabbits MBC4 induced cross-reactive IgG antibodies, which were able to block the binding of human serum IgE.

Conclusion: Directed epitope rearrangements combined with a knowledge-based structural modification resulted in a protein unable to bind IgE from allergic patients. Still, properties to activate specific T cells or to induce blocking antibodies were conserved. This suggests that MBC4 is a suitable vaccine candidate for the simultaneous treatment of Bet v 1- and associated food allergies.

Introduction

Birch pollen allergy is dominated by a single disease-eliciting allergen, namely Bet v 1, showing reactivity rates beyond 90% among birch pollen allergic patients. Moreover, it has been reported that over 70% of birch pollen allergic individuals react to at least one Bet v 1-associated, allergenic food source including pomaceous and stone fruits, vegetables, nuts, and legumes. With 80% reactivity among food allergic subjects, apple was most frequently recognized followed by hazelnut, which triggered allergic symptoms in 59% of patients suffering from pollen-food-syndrome (PFS)².

For the sustainable treatment of birch pollen allergy it has been shown that extract-based therapeutics can be successfully substituted by purified recombinant Bet v 1 applied either via the sub-cutaneous (SCIT) or via the sub-lingual (SLIT) route^{3,4}. However, successful birch pollen allergen-specific immunotherapy (AIT) does not necessarily correlate with the amelioration of concomitant food allergies. In a study by Kinaciyan *et al.* SLIT performed with birch pollen extract induced protective Bet v 1-specific IgG4 antibodies in test subjects, which were not cross-reactive with Mal d 1 from apple. Moreover, the treatment reduced exclusively the responsiveness of Bet v 1-specific T cells, the T cell response to Mal d 1 remained practically unaltered⁵.

In a follow-up, the presence of specific blocking IgG4 antibodies as well as the ratio IgG4/IgE was linked to food tolerance. Such blocking antibodies can be either innately present in food tolerant patients or they can be induced via AIT^{2,6}. The majority of AIT-induced IgG4 is thought to recognize IgE epitopes on the allergens; however also allergen-specific unique IgG4 specificities, which do not overlap with IgE epitope, will eventually develop during therapy⁶.

In light of the above, it is necessary to tackle Bet v 1-associated food allergies at the B cell as well as at the T cell level and AIT based on wildtype (WT) Bet v 1 seems insufficient for this task. The structure of Bet v 1, which is immunologically distinct from the structures of the related food allergens from apple and hazelnut, may therefore be the cause of this observation⁷.

Thus, we aimed to design a hybrid molecule by combining immunologically relevant epitopes of Mal d 1 from apple and Cor a 1.04 from hazelnut with the major birch pollen allergen. Recently, we successfully developed a strategy to produce a vaccine candidate for patients, who are multi-sensitized to Bet v 1-like Fagales pollen allergens, by generating a hybrid molecule. Sequence regions of the five most important allergens from birch, hazel, alder, oak and hornbeam were combined to a single protein, which showed reduced patients' IgE binding but preserved immunogenicity⁸. In analogy to this study, we identified T cell epitopes on Mal d 1, Bet v 1, and Cor a 1.04 and combined T cell-reactive stretches to a hybrid protein (MBC) showing the same overall length as the parental allergens. To reduce IgE binding of the protein, we generated a fold-variant of our hybrid (MBC4) by introducing a mutation previously identified as important for Bet v 1 to adopt its native fold^{1,9}. This particular mutation was shown to conserve sufficient potential IgG-reactive secondary structure

elements on the surface of Bet v 1 to efficiently induce blocking antibodies while preexisting IgE epitopes would be eliminated. In addition, the structural modification should not affect the immunogenic properties of the fold-variant.

Material and Methods

Patients and sera

Patients with birch pollen allergy and concomitant pollen-food syndrome (PFS) were selected on case history, positive skin prick testing and/or *in vitro* IgE detection (CAP system, Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden) (**Tab. S1**). Inclusion criteria were a CAP class >3 to birch, as well as >1 to apple and hazelnut. Experiments with patients' sera were approved by the ethics committee of the University of Vienna (EK028/2006) and Salzburg (415-E/1398/4-2011). Informed written consent was obtained from all subjects included in the study.

Design, *in silico* evaluation, and cloning of MBC and MBC4

Hybrid proteins were designed and evaluated *in silico* as described in the article's online Supporting Information. Thereafter, the hybrid allergen MBC was cloned by PCR-recombination of three overlapping fragments from the genes *mal d 1.0108* (AF126402), *bet v 1.0102* (X77266) and *cor a 1.0401* (AF136945), respectively. The gene was thereafter cloned into a pET28b vector (Novagen, Merck Millipore, Billerica, MA, USA). Using MBC as template, a mutation was introduced in the Bet v 1.0102 part of the molecule to generate the MBC4. Moreover, in MBC4 the C-terminal cysteine of Cor a 1.0401 was mutated to a serine to abrogate dimerization⁷. A detailed description is provided in the article's online Supporting Information.

Expression and purification of recombinant proteins

Parental allergens, Bet v 1.0101, Mal d 1.0108, and Cor a 1.0401, respectively, were produced as non-fusion proteins as described and are referred to as Bet v 1, Mal d 1, and Cor a 1.04 in the following^{1,7}. Recombinant MBC was expressed in *E. coli* and produced from the soluble fraction whereas MBC4 was produced from inclusion *E. coli* bodies and refolded after purification. Both methods are described in the article's online Supporting Information. Recombinant Bet v 1.0201 used as reference material was produced as described¹⁰. Endotoxin levels of recombinant proteins were determined by HEK-Blue™ mTLR4 (InvivoGen, San Diego, CA, USA) cell assays according to manufactures instructions.

Physicochemical analyses of recombinant proteins

Recombinant proteins were analyzed as previously described in terms of quantity, primary and secondary structure, aggregation behavior as well as ligand-binding by amino acid analysis, mass spectrometry, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopy, dynamic light scattering (DLS) and 1-anilino-8-naphthalene sulfonate (ANS)-binding assays, respectively^{1,7}.

In vitro assessment of endo-/lysosomal proteolysis

The proteolytic stability of all proteins was determined by degradome assays as previously described¹¹. Briefly, the microsomal fraction of the JAWS II cells (ATCC® number CRL-11904™) was obtained by ultracentrifugation. Incubation of 5 µg protein and 7.5 µg of microsomes in 100 mM citrate buffer pH 4.8 and 2 mM dithiothreitol (DTT) was performed for 0, 0.5, 1, 3, 6, 12, 24 and 48 h at 37°C. The reaction was stopped at 95°C for 5 min. Samples were analyzed by SDS-PAGE and MS/MS.

Human T cell studies

Bet v 1-specific T cell lines (TCL) were expanded from PBMC of birch pollen-allergic patients as previously described¹². TCLs were stimulated with recombinant proteins or with a panel of synthetic 12-mer peptides covering the sequence of Bet v 1.0101 in the presence of irradiated (60 Gray) autologous PBMC as antigen-presenting cells (APC). Stimulation indices were calculated as ratio between counts per minute (cpm) obtained in cultures with T cells plus APCs and antigenic stimulus and cpm obtained in T cell cultures containing only APCs.

Antibody binding analyses

Binding of allergic patients' serum IgE to parental allergens as well as MBC4 was compared by ELISA and mediator release assays using RBL-2H3 cells transfected with the alpha chain of the FcεRI receptor and passively sensitized with patients' IgE as described in the article's online Supporting Information. Antigen-specific murine IgG1 and IgG2a levels were determined by ELISA, and murine IgE by mediator release assays, respectively. Both methods are described in the article's online Supporting Information.

Mouse immunization model

Eight week old female BALB/c mice purchased from Janvier (Saint Berthevin, France) were housed under specific pathogen-free conditions. Mice were immunized with 5 µg of protein adsorbed to Alu-Gel S (Serva, Heidelberg, Germany) given in two injections of 50 µL sub-cutaneously into the back of the animal, and boosted on day 7, 14, and 21. Sera were collected on day -3 and 28, respectively. Per group 5 animals were tested. At day 28 animals were sacrificed and lymphocytes were harvested from spleen as previously described¹³. Briefly, spleens were homogenized and after lysis of erythrocytes, cells were counted and used for cytokine analysis. ELISpot assays were performed according to manufactures instructions (Merck Millipore). Splenocytes (2x 10⁵ cells/well) were re-stimulated with either 40 µg/mL of parental allergen or MBC4 protein, respectively, and secreted cytokines were analyzed using matched pair mAbs for IL-4, IL-5 and IFN-γ detection (eBioscience, San Diego, CA, USA). Splenocyte supernatants were analyzed using the ProcartaPlex® multiplex system

according to the manufacturer's instructions (eBioscience), measurements were performed with a Magpix® system (Merck Millipore). All mouse experiments were conducted according to the national guidelines approved by the Austrian Federal Ministry of Science, Research and Economy (BMW-F-66.012/0010-II/3b/2013).

Rabbit immunization model

Two New Zealand White rabbits (NZW) were immunized with 200 µg of MBC4 adsorbed to Alu-Gel S (Serva) in a volume of 500 µl, booster immunizations were given on day 14, 28, and 42, final bleeding was performed on day 69 (Charles River, Chatillon sur Chalaronne, France). Rabbit total IgG was purified from immune sera via Protein G sepharose (GE Healthcare). Inhibition mediator release assays were performed using sera of patients with Bet v 1-mediated birch pollen allergy and concomitant OAS to apple and hazelnut. 0.1 ng of Bet v 1 or 1 ng of Cor a 1.04 and Mal d 1, respectively, were pre-incubated with 15 µg of total rabbit IgG for 1h in Tyrode's buffer at room temperature and thereafter used to trigger mediator release of IgE-loaded RBL cells. A serum of a ragweed pollen allergic patient was used as control; here mediator release was triggered with 1 ng Amb a 1 after pre-incubation with rabbit IgG.

Results

In silico design and cloning of hybrid molecules

The hybrid molecule MBC was designed by combining T cell epitope containing stretches of Mal d 1, Bet v 1, and Cor a 1.04, respectively (**Fig S1**). In general, high levels of T cell cross-reactivity between Bet v 1 and homologous food allergens have been reported¹⁴⁻¹⁷. To assemble the hybrid allergen we employed the isoform Bet v 1.0102, formerly Bet v 1d which shows limited IgE-reactivity but high immunogenicity¹⁶. Within Bet v 1, T cell epitopes are scattered throughout the entire sequence¹², whereas the central region of Bet v 1 was shown to be important for the structure of the allergen¹. T cell epitope mapping of Mal d 1 (isoform 0108) revealed a clustering of epitopes at the N-terminus and within the central region of the allergen¹⁸. Moreover, hazelnut Cor a 1.04 harbors a unique immuno-dominant T cell epitope (residues 142-153), which shows limited cross-reactivity with pollen allergens, but cross-reacts with the carrot homologue of Bet v 1, Dau c 1¹⁵.

Taken this into consideration, the arrangement of the protein stretches were Mal d 1, Bet v 1, and Cor a 1.04. For the cross-over events structurally conserved regions were selected. Therefore, primers were designed spanning those regions and coding at the 5'-end for the N-terminal and at the 3'-end for the C-terminal fusion partner. Using these primer pairs, regions of the three parental allergens with overlapping ends were amplified and recombined to full length genes by PCR.

We applied statistical scoring functions (SSFs), which are versatile tools in structural bioinformatics, to derive a qualitative ranking of the fit of the different allergen sequences to the canonical Bet v 1 fold. This allowed a knowledge-based evaluation and prediction of the structural behavior of hybrid molecules. One hundred models were generated per allergen sequence. Randomly sampled pairs of models were superimposed, resulting in a root mean square deviation of 0.3 Å (standard deviation 0.2 Å, 10000 samples) indicating that the models closely resemble the Bet v 1 fold. Statistical scoring functions were applied to determine, how well different sequences would fit to a given structure. The higher the score, the less the given fold represents the native folding behavior of Bet v 1-like proteins. We found that the sequence of the hybrid molecule MBC was still compatible with the canonical Bet v 1 fold. Thus a variant of MBC, MBC4, was designed by incorporating amino acids, previously identified as detrimental for the Bet v 1 structure, into the backbone of MBC¹. This resulted in a protein, which had clearly the lowest compatibility with the canonical Bet v 1 fold (**Fig. S2A**). In order to reduce the impact of the local structure on the score, we removed the pair interactions between residues close in sequence (sequence distance < 10 residues). The differences in the scores between MBC4 and the other allergens remained, indicating the occurrence of larger structural rearrangements (**Fig. S2B**).

Recombinant production of the hybrid proteins

First, the hybrid protein MBC was produced in *E. coli* (**Fig S3, A**), purified to homogeneity and subjected to screening by CD spectroscopy (**Fig S3, B**). Both CD spectra from Bet v 1 and MBC, showed identical curve shape, whereas differences in amplitudes accounted for different ratios of secondary structure elements. As predicted, CD data confirmed that MBC still contained a high percentage of native secondary structure elements¹. Comparison of patients' IgE binding of MBC with the low IgE-binding isoform Bet v 1.0102 by ELISA revealed an almost identical IgE response (**Fig S3, C**). The residual IgE-binding was considered a risk factor for a potential therapeutic application. Our *in silico* data indicated that by introducing a mutation into the backbone of MBC (**Fig. S1**) the resulting molecule MBC4 (**Fig 1**) should not be able to adopt the typical Bet v 1 structure¹. Previously, Bet v 1 has been altered using the same strategy. The resulting protein, termed BM4, did not react with patients' serum IgE; however in a mouse model, the molecule was able to induce IgG antibodies cross-reactive with Bet v 1¹. The hybrid MBC4, as well as parental WT allergens, were produced in *E. coli* and purified to homogeneity (**Fig. 1B**). Protein identity was confirmed by mass spectrometry (**Fig S4A**). Endotoxin levels were found below 3 EU/mg recombinant protein (**Tab S4B**).

MBC4 is monomeric in solution showing unique secondary structure elements

In CD measurements, MBC4 showed the typical curve shape of proteins with high amounts of unordered structural elements whereas the curves of the parental WT allergens were indicative for the typical Bet v 1-like fold (**Fig 1C**). This was confirmed by FTIR measurements which indicated an alpha helix content of 12 % for MBC4 compared to 33 % for Bet v 1, 23 % for Mal d 1, and 18 % for Cor a 1.04 (**Fig 1D**). The beta sheet structures of MBC4 accounted for 16 % compared to 40 % in Bet v 1, 40 % in Mal d 1, and 32 % in Cor a 1.04. The substrates deoxycholate as well as ANS were previously reported to bind to the hydrophobic cavity typical for members of the Bet v 1 allergen family¹⁹. Thus, ANS displacement assays were performed to analyze whether this structural property was conserved in MBC4. In our experimental setup we could not measure ligand binding to MBC4 indicative for an inaccessible cavity, which is very likely a result of the structural changes induced into the molecule (**Fig S5**). The aggregation state of the four proteins was determined by DLS measurements (**Fig S4C**). Bet v 1 was found monomeric with a hydrodynamic radius of approx. 1.8 nm, Mal d 1 as well as Cor a 1.04, represented as a mixture of monomers and cysteine-induced multimers⁷, whereas 99% of MBC4 was found in a monomeric state in solution showing a hydrodynamic radius of 3.2 nm indicating the partially unfolded nature of the fold-variant.

Binding of patients' IgE to MBC4 is drastically reduced

IgE-binding to MBC4 was compared to parental allergens in ELISA using sera of Bet v 1-allergic patients with PFS to apple and hazelnut (**Fig 2A**). The mean IgE-binding to MBC4 (0.48 arbitrary units) was significantly lower than IgE-binding to Bet v 1 (1.86), Mal d 1 (1.64), or Cor a 1.04 (2.02). Mediator release assays with RBL cells confirmed the data whereas in mean 0.055 ng/mL Bet v 1 were necessary to trigger half maximal mediator release of cells passively sensitized with patients' serum IgE. 1.16 ng/mL Cor a 1.04, 123.4 ng/mL Mal d 1, and 65.82 ng/mL MBC4 were required to obtain the same levels of basophil activation indicating an 1196-fold reduction in allergenicity of MBC4 compared to the highly reactive allergen Bet v 1 (**Fig 2B**).

MBC4 effectively activates T cell lines from allergic donors

Bet v 1-specific T cell lines (TCL) were stimulated with either Bet v 1, Mal d 1, Cor a 1.04, or MBC4 (**Fig 2C** and **Tab S2**). As expected, Bet v 1 was a significantly stronger T cell stimulus than Mal d 1 and Cor a 1.04. Though MBC4 was less potent in activating T cells as Bet v 1, the T cell stimulating capacity was approx. 3-fold elevated compared to the two food allergens. Since the TCLs were generated upon stimulation of PBMCs from birch pollen allergic donors with Bet v 1, this might induce a T cell bias, which could explain the higher T cell activating property of MBC4 compared to both food allergens. However, epitope mapping of Bet v 1-specific TCLs with overlapping 12-mer peptides revealed T cell epitopes spreading the entire sequence including the parts replaced by Mal d 1 and Cor a 1.04, respectively (**Fig 3**). To compare antigen-processing of MBC4 with Bet v 1, either protein was digested with a cocktail of proteases from DCs and the degradation pattern were compared at different time points. Two representative time points are shown in **Figure 3**, a high resolution image showing five different time points for MBC4 is provided in the article's online Supporting Information (**Fig. S6**). Detailed analyses of degradation pattern of Bet v 1, Mal d 1, and Cor a 1.04 have recently been published ^{7, 11}. Degradations of Bet v 1-like molecules with endo-/lysosomal proteases usually result in the formation of peptide clusters over a period of more than 24h. This was also observed here. Initially, MBC4 degraded faster than Bet v 1 resulting in more peptides available for MHCII loading after 1h; however, after 12h we could not find any difference in the peptide pattern of both proteins. Moreover, we found essentially the same degradation pattern for both molecules suggesting that the endo-/lysosomal degradation process is comparable. The peptide clusters found for both proteins overlapped very well with peptides that were identified in epitope mapping experiments with T cell lines of birch pollen allergic donors. The data indicates that MBC4 will be processed similar as its parental allergens resulting in the effective activation of allergen specific T cell.

MBC4 is able to induce cross-reactive IgG antibodies in a mouse model

BALB/c mice were immunized either with parental allergens or with MBC4 (**Fig 4A**). After 3 booster injections IgG1 as well as 2a titers were determined by ELISA (**Fig 4B**). Therefore, parental WT allergens were coated and sera of mice, immunized with the parental allergen, were compared to sera of MBC4-immunized animals. Moreover, anti-MBC4 antibody titers were determined. For IgG1, we found that MBC4 immunization induced the highest titers against itself and against Mal d 1, followed by Bet v 1, and Cor a 1.04. There was no significant difference between titers induced by immunizations with wildtype allergens or by MBC4 immunization when tested with the respective immobilized wildtype proteins. For IgG2a, the picture was essentially similar; titers however, were generally lower and MBC4 immunization led to higher antibody titers against Bet v 1 and Cor a 1.04, than Mal d 1.

Murine immunization-induced IgE antibodies show significantly lower cross-reactivity

Despite the highly cross-reactive IgG response, murine immunization-induced IgE antibodies showed a statistically significant lower cross-reactivity (**Figure 5A**). In mediator release assays, we found that stimulation of cells, passively sensitized with murine Bet v 1 immune sera, with Bet v 1 itself led to an mean maximal activation of 23.5% whereas stimulation with MBC4 activated 4.8% of the cells. Similar results were found for Mal d 1 and Cor a 1 (58.9% vs. 0.9% and 15.8% vs. 2.9% mediator release), respectively. Though MBC4 induced high levels of IgE against itself, the cross-reactivity with Bet v 1, Cor a 1, and Mal d 1 was significantly reduced (68.3% vs. 8.6%, 3.2%, and 3.5% activation).

Immunization with MBC4 recruits both, cross-reactive Th1 and Th2 cells in mice

Splenocytes of immunized animals were stimulated by addition of protein antigens and cytokine-producing cells were detected in ELISpot assays (**Figure 5B**). Therefore, splenocytes of mice immunized with parental WT allergens were stimulated with the respective allergen or with MBC4 and numbers of INF- γ , IL-4, as well as IL-5 producing cells were determined. No significant differences between parental allergens and MBC4 were found. Further, we analyzed the supernatant of restimulated splenocytes and found essentially the same picture. Splenocytes of wildtype allergen immunized mice could be stimulated with MBC4, in most cases though to a lower level as with the immunogen. Restimulation with MBC4 could also induce IL-10 in splenocytes, which, with the exception of Mal d 1-immunized animals, did not significantly differ between MBC4 and the respective immunogen (**Figure 5C**).

MBC4 immunization is able to induce cross-reactive blocking IgG in rabbits

To test whether MBC4 could induce blocking IgG against parental allergens, two NZW rabbits were immunized with the antigen. Total rabbit IgG was purified by affinity chromatography and used to

block mediator release in RBL assays. Therefore, cells were passively sensitized with sera of Bet v 1-allergic patients with concomitant food allergies to apple and hazelnut. The addition of rabbit IgG to either parental allergen could significantly reduce mediator release (**Fig. 6**). MBC4-induced blocking antibodies were specific as they failed to inhibit mediator release of a ragweed allergic patient serum triggered by the addition of the major ragweed pollen allergen Amb a 1.

Discussion

Ideally, allergy treatment should be specific, efficacious, safe and easily applicable²⁰. Especially with widely cross-reactive allergens, simplicity becomes already a major challenge. In case of birch pollen allergy, the simultaneous treatment of concomitant pollen as well as food allergies seems essential; however, extract-based therapy – even though successful for pollen allergy - does not necessarily ameliorate food-related symptoms of Bet v 1-associated allergic reactions^{5, 21, 22}. Therefore, it is unlikely that a purified single allergen such as Bet v 1 will show better performance. Moreover, pollen-related food allergens seem to have unique immunologic properties distinct from Bet v 1⁷. Since AIT with a cocktail of different allergens might have disadvantages over a single molecule in terms of reproducibility and production costs, we aimed to produce a hybrid protein by recombining parts of Mal d 1, Bet v 1, and Cor a 1.04, which contained the most relevant T cell epitopes^{12, 15, 18}. Introducing point mutations further allowed us to modify the 3D structure of the hybrid abrogating the IgE-binding properties of the WT allergens but preserving their T cell reactivity – a crucial step to reduce IgE-binding and retain efficacy of a potential therapeutic agent.

The MBC4 hybrid protein displayed a significant change of secondary structure elements compared to the parental allergens. Nevertheless, MBC4 was monomeric in solution. Due to the fold variation the hydrophobic cavity, a characteristic feature of Bet v 1-like proteins, was blocked. The structural rearrangements translated into a strongly reduced IgE-binding capacity of MBC4 so that in mediator release assays using RBL cells loaded with patients' serum IgE the protein showed an 1196-fold reduced allergenic activity compared to Bet v 1.

To investigate its T cell activating properties, Bet v 1-specific TCLs containing T cells specific for a variety of epitopes spreading the entire sequence of the major birch pollen allergen were stimulated. MBC4 was slightly less active than Bet v 1; however, it still represented a more potent stimulus than either of the parental WT food allergens. In addition, endo-/lysosomal proteases were used to degrade MBC4 *in vitro*. When comparing the acquired peptide spectra with Bet v 1, essentially the same pattern were obtained. Moreover, in our murine model splenocytes of animals immunized with the WT allergens were isolated and stimulated either with the immunogen or with MBC4. In ELISpot assays, we did not find any differences in the stimulatory capacity of MBC4 compared to the parental allergens. When analyzing the splenocyte supernatants, we saw essentially the same pattern. Splenocytes of wildtype allergen immunized mice could be stimulated with MBC4 and we could also detect some IL-10 in the supernatants. All of this indicates, that the epitope recombination preserved, not to say expanded, the T cell epitope repertoire of MBC4. Therefore, it should allow the induction of specific T cell tolerance to the individual allergens in a therapeutic setup.

It is generally accepted that the induction of blocking antibodies is a hallmark of AIT²³. Therefore, proteins have to be immunogenic and, in case a modified allergen-derivative is used for vaccination,

able to induce antibodies cross-reactive with the WT allergen. In this case, MBC4-induced antibodies should be reactive with Bet v 1 and associated food allergens. We established a mouse model, immunized the animals with MBC4 and tested the antibody cross-reactivity to WT allergens. MBC4 was very immunogenic and induced high IgG1 as well as 2a antibody titers reactive to each of the parental WT allergens. Interestingly, the IgE antibodies induced by MBC4 immunization were not cross-reactive. We therefore speculate that the structural rearrangements within MBC4 prohibit the binding as well as the induction of cross-reactive IgE; however, within MBC4 sufficient secondary structure elements were preserved to stimulate a potent cross-reactive IgG response. This was also verified in a rabbit model where MBC4 immunization induced blocking antibodies against all three wildtype allergens, which could significantly inhibit allergen-induced degranulation of passively sensitized basophils.

Clinical studies using recombinant allergens, especially modified proteins, are still rare²⁴. Moreover, especially the treatment of food allergies remains problematic. Here, we present a modified allergen-derivative with drastically reduced IgE-binding properties but retained immunogenicity for the simultaneous treatment of birch pollen and associated food allergies. The concept can serve as archetype for modern AIT vaccine design and will prove itself especially valuable to approach problems of cross-reactivity frequently encountered in allergen families.

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Figure legends

Graphical abstract

Graphical abstract, photos were purchased from Fotolia, structural models were derived from the pdb entry 1bv1 (www.rcsb.org) and modified using UCSF Chimera 1.10.1 (www.cgl.ucsf.edu/chimera/).

Figure 1. Schematic representation of MBC4 sequence (A). Coomassie-stained 15% SDS-PAGE of purified proteins, 1 µg/lane was loaded (B). CD spectra of parental allergens compared to MBC4 were recorded at 20°C and are presented as mean residue molar ellipticity after baseline correction (C). FTIR spectra, recorded at 20°C, were used to calculate secondary structure elements (D).

Figure 2. IgE ELISA (A) and mediator release assays (B) using sera of birch pollen allergic donors with PFS to apple and hazelnut, Log(SI) values of TCLs from birch pollen allergic donors stimulated with 0.5µg antigen/well (C); *p*-values, calculated with ANOVA, of < 0.05 were considered significant. Data of mediator release assays was transformed prior to analysis [$y = \log(y)$].

Figure 3. Bars underneath protein sequences show peptide clusters derived from *in vitro* endo-/lysosomal proteolysis (black: frequently occurring peptides, grey: peptides of medium frequency, light grey: rare peptides). Peptide clusters above each sequence (boxed) represent TCL reactive peptides from birch pollen allergic donors (each color represents one donor).

Figure 4. Schematic representation of immunization schedule (A). Murine IgG1 as well as IgG2a antibody titers were determined as means ± SEM by ELISA (B). Statistical analysis was performed using ANOVA, *p*-values < 0.05 were considered significant.

Figure 5. RBL cells were passively sensitized with sera of immunized mice and mediator release was triggered by addition of antigen (A). Splenocytes of immunized animals were cultured in the presence of antigen and cytokine producing cells were identified in ELISpot assays (B). Splenocyte supernatants were analyzed with a multiplex approach (C). *P*-values < 0.05 were considered significant.

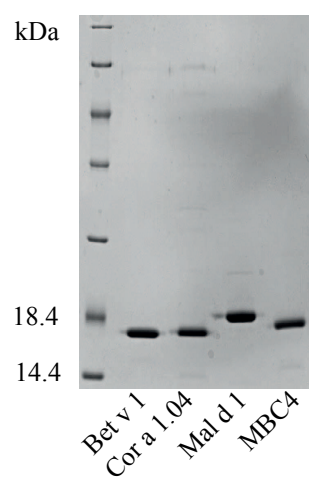
Figure 6. RBL cells were passively sensitized with sera of patients with Bet v 1-mediated birch pollen allergy and concomitant OAS (open symbols) or the serum of a ragweed allergic patient (filled symbol). Mediator release was triggered by addition of antigen in the presence or absence of IgG isolated from MBC4-immunized rabbits. *P*-values, calculated with ANOVA, < 0.05 were considered significant.

ACCEPTED MANUSCRIPT

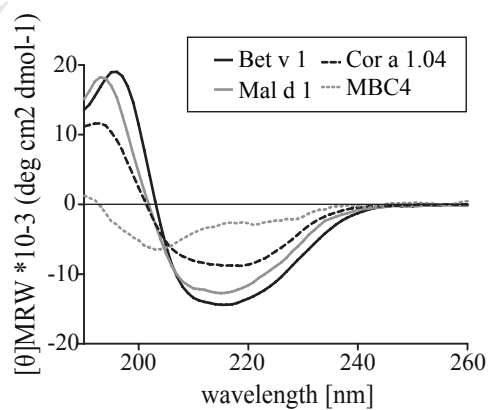
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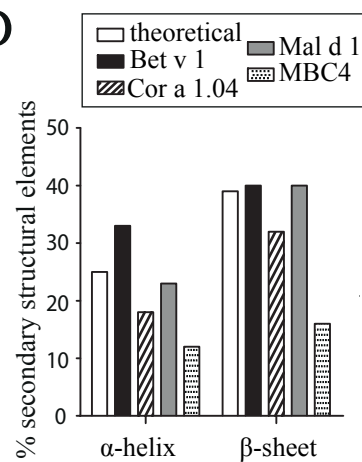
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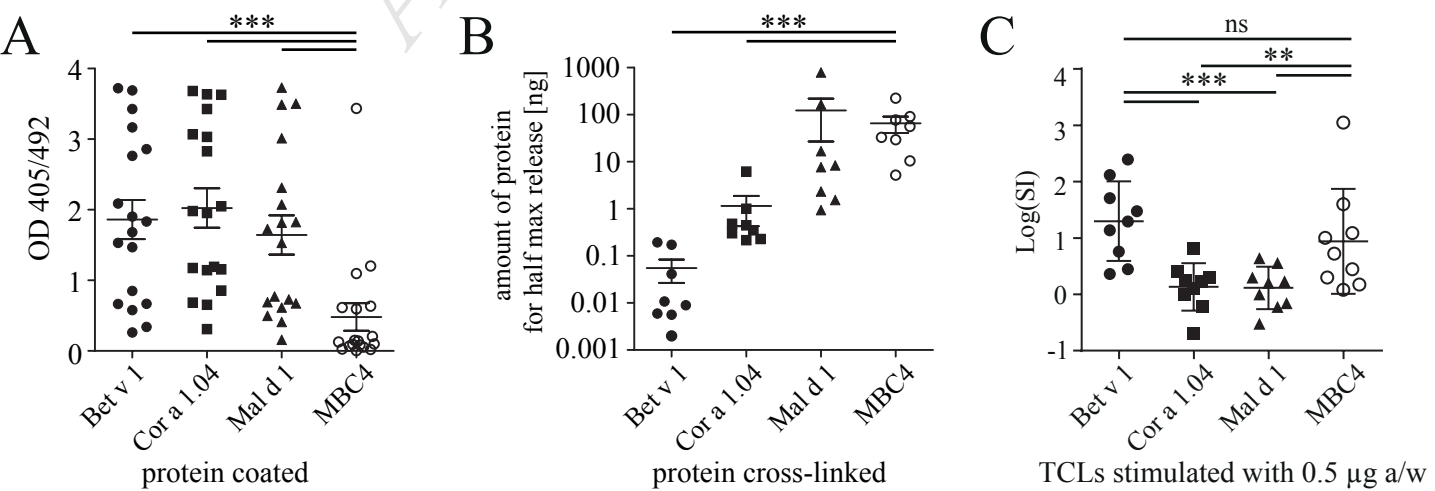


C



D



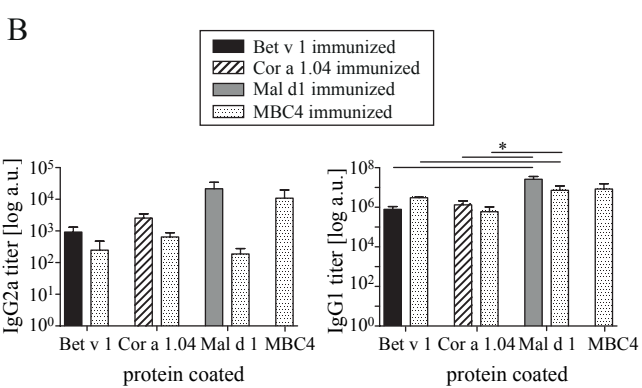
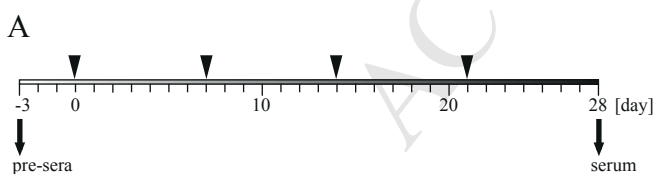


Bet v 1

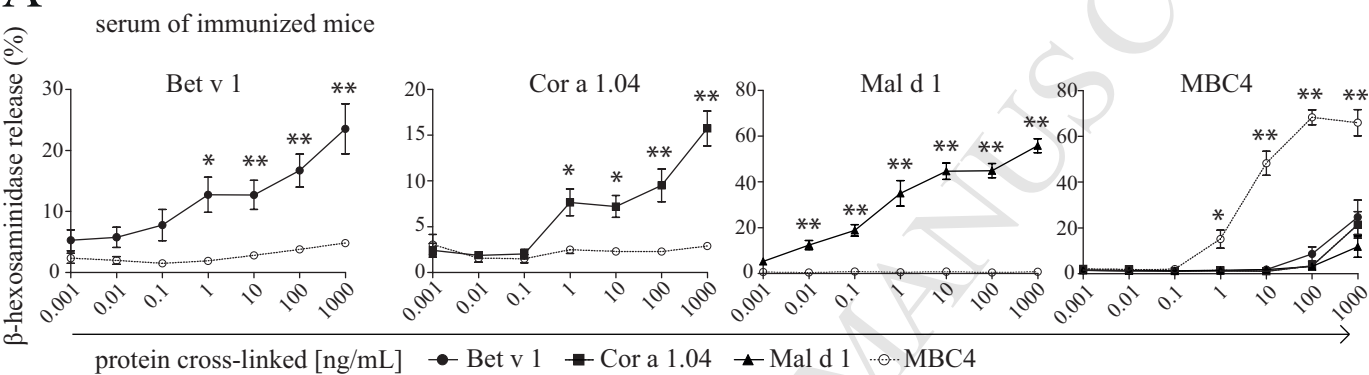


MBC4

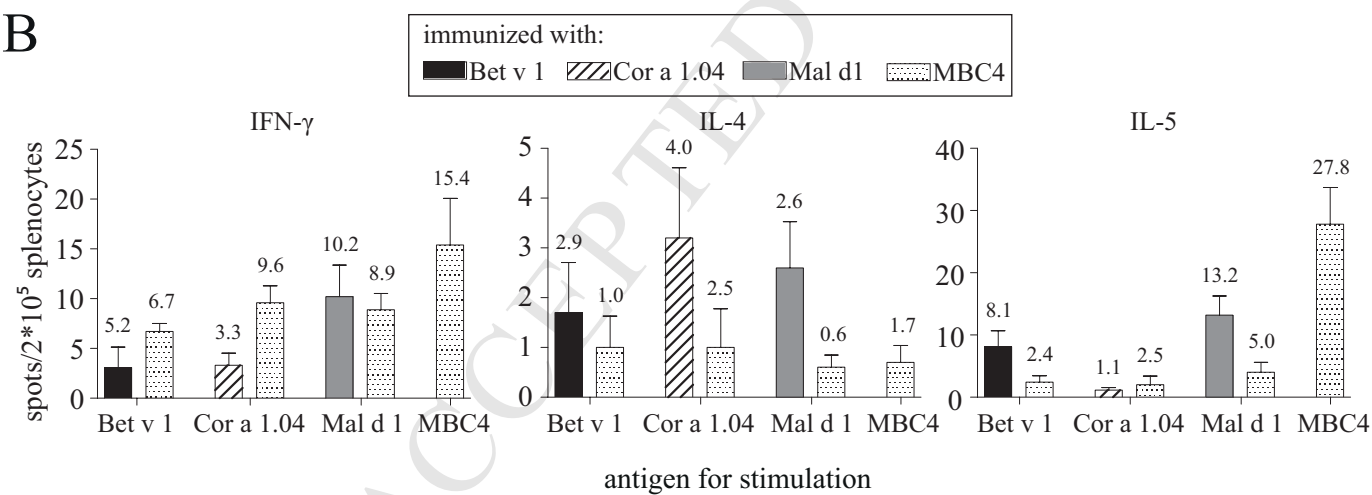




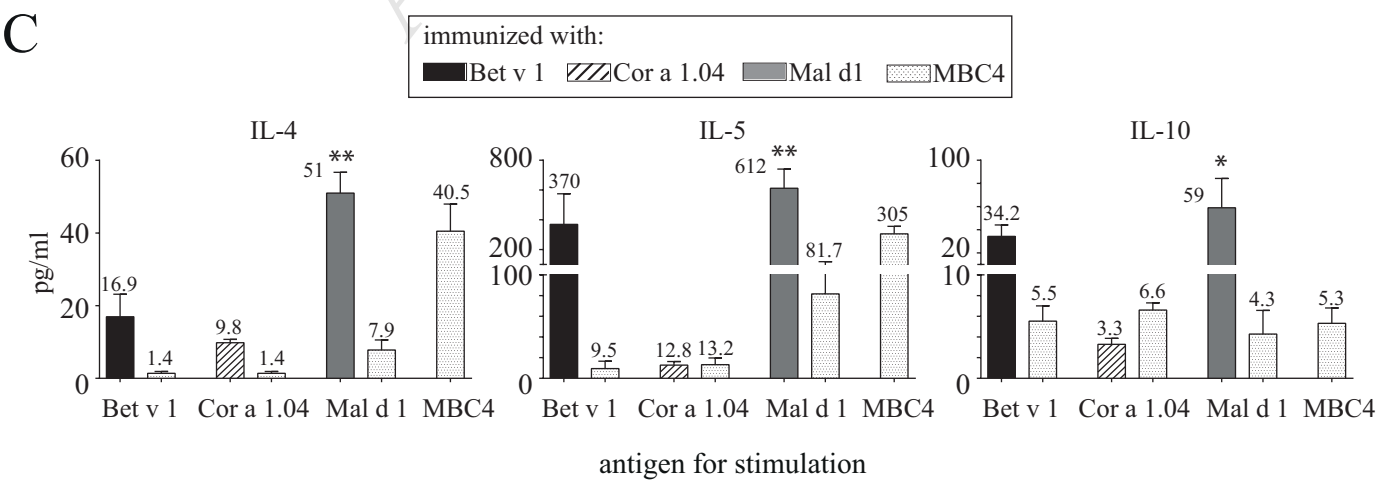
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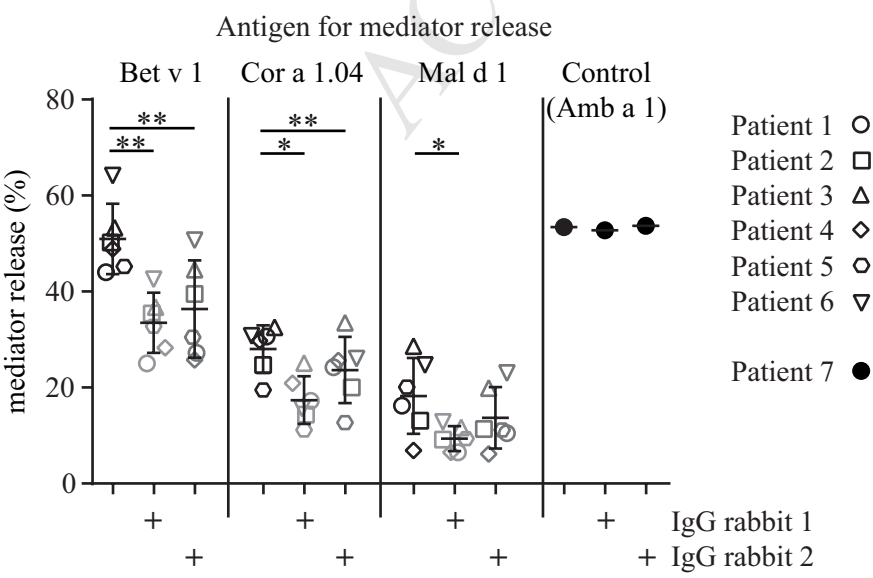


B



C

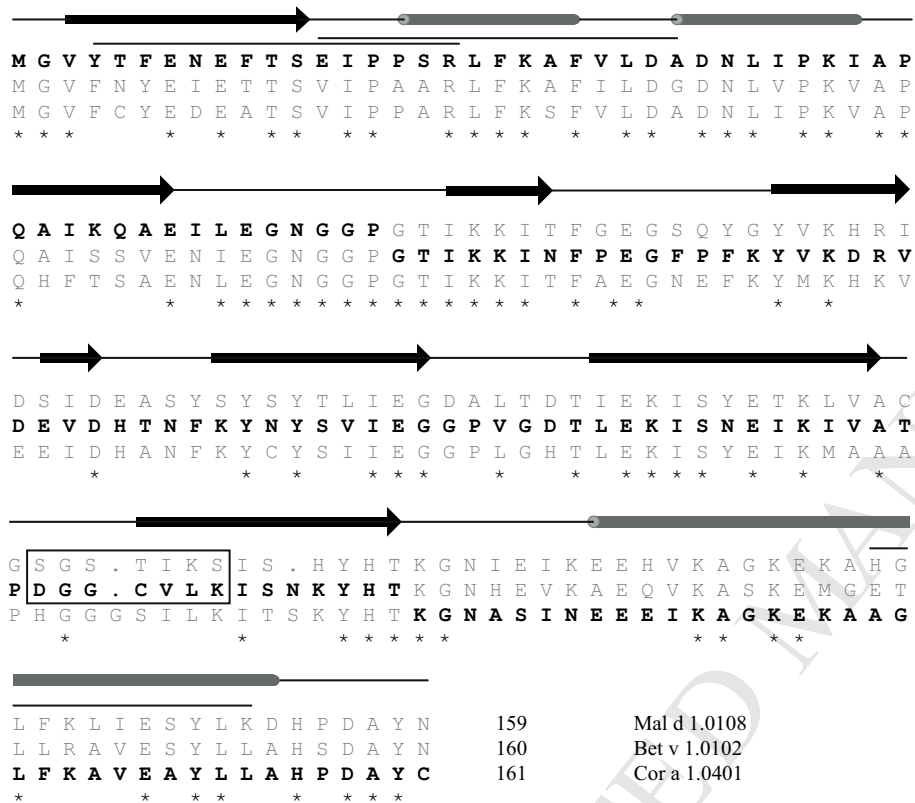




patient	code	total	birch pollen-		ACCEPTED MANUSCRIPT		SPT			diagnosis		pollen food					
		IgE	specific IgE	food specific IgE	class	B	Ap	Hn	AS	Po	Ap	Hn	Pn	Ca	Ki	Ro	
		(kU/L)	class	PRU	(kU/L)	class											
1	1	537	4	44.2	apple (2.49)	2	++	+++	+++		•	•	•				•
					hazelnut (1.0)	2											
					apple (7.41)												
2	5	721	4	39.5	hazelnut (10.6)	3	+	+	+	•	•	•	•		•	•	
3	6	8.38	3	42.6	apple (0.82)	2	+	+++	+	•	•	•	•		•	•	
4	8	1028	NA	NA	apple (4.64)	3											
					hazelnut (0.56)	1	+++			•	•	•	•		•		•
5	10	115	4	21.4	NA		+			•	•	•	•				•
6	20	16.4	3	8.24	apple (1.03)	2	+++	+++	+++		•	•	•				•
7	28	49.9	4	10.9	apple (1.26)		+++				•	•	•				•
					apple (11.5)												
8	33	1210	5	73.7	hazelnut (0.89)	3				•	•	•	•				•
						2	+++										
9	34	217	NA	NA	NA		+		+++		•	•	•				•
10	36	92.7	4	20	NA		+++	+	+++		•	•	•				
11	65	116	NA	NA	apple (6.41)	3											
					hazelnut (2.84)	2	+++	+++	+++	•	•	•	•			•	•
					apple (27.4)												
12	66	NA	6	>100	hazelnut (3.59)	4	+				•	•	•			•	•
						3											
					apple (30.1)												
13	67	594	6	>100	hazelnut (7.59)	4	+	+++	+++	•	•	•	•		•		•
						3											
14	95	491	6	>100	apple (8.66)	3	+++	+++	+++		•	•	•				•
					hazelnut (4.4)	3											
15	103	107	4	24.09	apple (6.16)	4	+++	++	+		•	•	•				
					hazelnut (1.91)	3											
16	108	426	NA	NA	apple (15.3)	3											
					hazelnut (4.15)	3	++	+++		•	•						
17	117	329	5	80.5	apple (24.2)	4											
					hazelnut (5.33)	3	+	++	+		•	•	•				•
18	131	29	4	135	apple (3.59)	3											
					hazelnut (0.78)	2	NA	NA	NA	•	•	•	•		•	•	
19	645843	51.1	5							•	•						
20	646859	51.6	5								•						
21	646494	73.6	5								•						
22	646951	>100	6							•	•	•				•	
23	646966	93.6	5								•	•					•
24	647859	59.3	5								•	•					

protein		patient 1	2	3	4	5	6	7	8	9	median
Bet v 1.0101	1 µg/w	32.4	4.4	2.5	54.4	20.0	2.5	9.1	133.9	362.4	20.0
	0.5 µg/w	29.8	5.7	2.8	50.9	19.7	2.3	13.7	130.9	246.2	19.7
	0.25 µg/w	24.3	3.4	2.7	51.5	17.1	2.5	8.6	129.1	99.4	17.1
	0.13 µg/w		2.7	2.6	55.4	16.4	2.8	9.5	135.1		9.5
Cor a 1.0401	1 µg/w	1.1	0.0	0.4	6.3	1.6	1.8	2.7	1.2	2.6	1.6
	0.5 µg/w	1.3	0.2	0.6	6.5	1.8	2.5	1.7	1.0	2.0	1.7
	0.25 µg/w	0.7	0.5	0.8	7.1	2.1	2.3	2.8	1.9	1.6	1.9
	0.13 µg/w		0.3	1.4	4.5	1.7	2.2	2.5	2.0		2.0
Mal d 1.0108	1 µg/w	5.6	0.2	0.5	5.3	0.3	0.8	1.3	3.2	1.2	1.2
	0.5 µg/w	4.4	0.3	0.7	3.6	0.6	1.0	1.6	2.0	1.7	1.6
	0.25 µg/w	4.4	0.2	1.2	1.7	1.0	1.0	1.4	2.3	3.4	1.4
	0.13 µg/w		0.1	0.7	1.5	1.1	0.9	1.5	2.1		1.1
MBC4	1 µg/w	10.7	1.7	1.3	35.3	4.5	2.2	10.7	2.8	935.2	4.5
	0.5 µg/w	12.2	1.5	2.0	39.7	5.3	2.8	10.1	1.2	1123.5	5.3
	0.25 µg/w	12.8	1.4	2.0	37.3	4.6	2.3	8.8	0.6	1180.3	4.6
	0.13 µg/w		2.4	1.9	37.5	6.6	2.9	10.8	0.4		2.9

A



B Amino acid sequence of MBC

MGVYTFENEFTSEIPPSRLFKAFVLDADNLI PKIAP
 QAIKQAEILEGNNGGPGTIKKKINFP EGF PFKYVKDRV
 DEV DHTNFKYNYSVIEGGPVGDTLEKISNEIKIVAT
 PDGGCVLKISN KYHTKGNASINEEEIKAGKEKAAGL
 FKAVEAYLLAHPDAYS

Mal d 1.0108 (residue M1-P51)

Bet v 1.0201 (residue G52-T123)

Cor a 1.0401 (residue K124-S160)

C Amino acid sequence of MBC4

MGVYTFENEFTSEIPPSRLFKAFVLDADNLI PKIAP
 QAIKQAEILEGNNGGPGTIKKKINFP EGF PFKYVKDRV
 DEV DHTNFKYNYSVIEGGPVGDTLEKISNEIKIVAT
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 F K A V E A Y L L A H P D A Y S

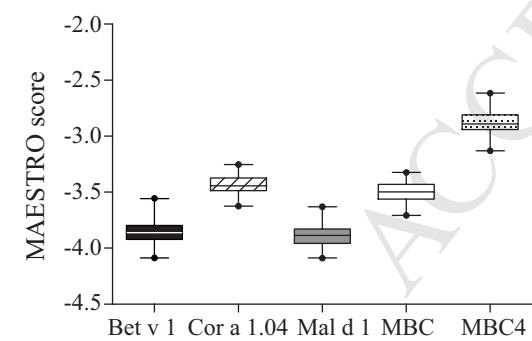
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Bet v 1.0201 (residue G52-T123)

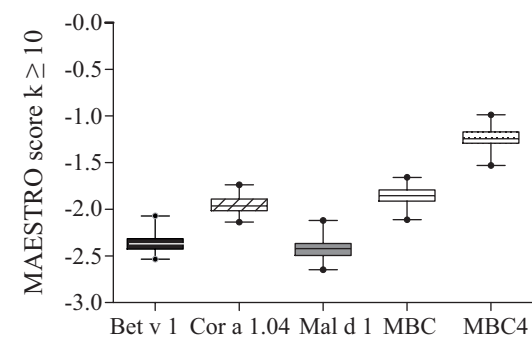
Cor a 1.0401 (residue K124-S160)

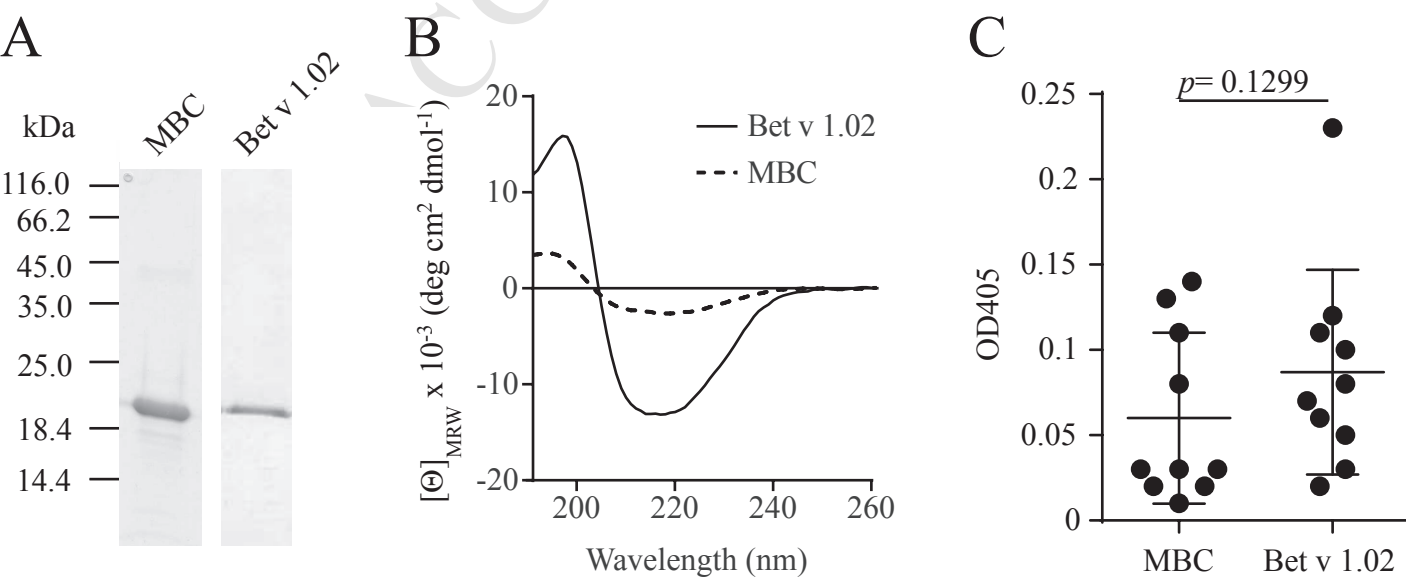
Mutation site (residue S110-S116)

A Stability calculation



B





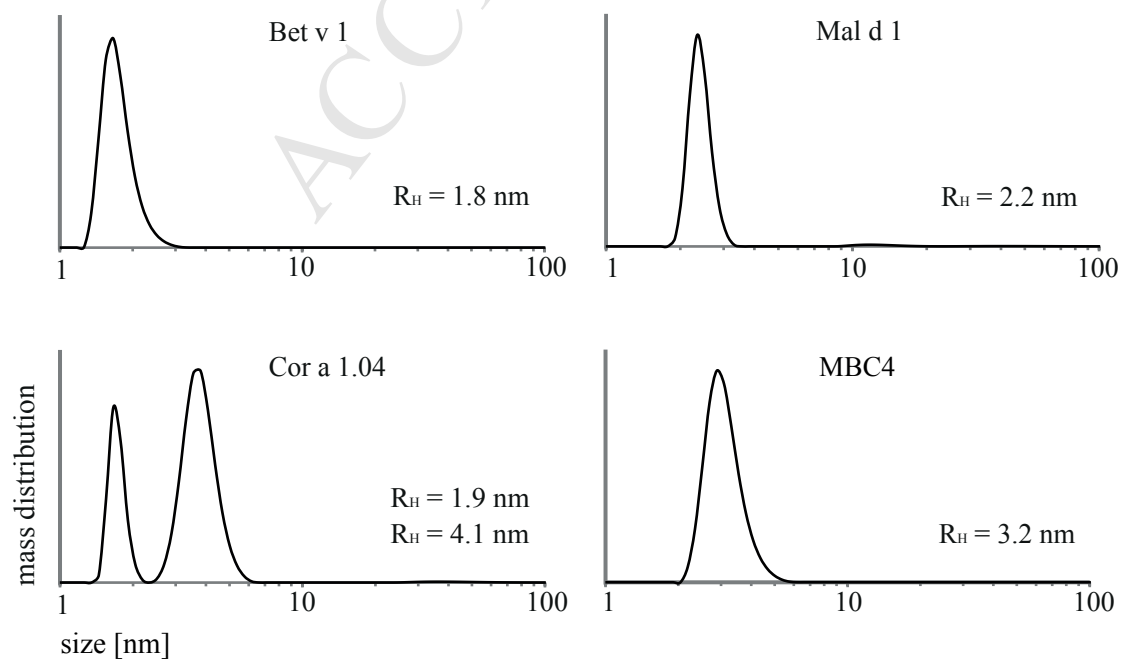
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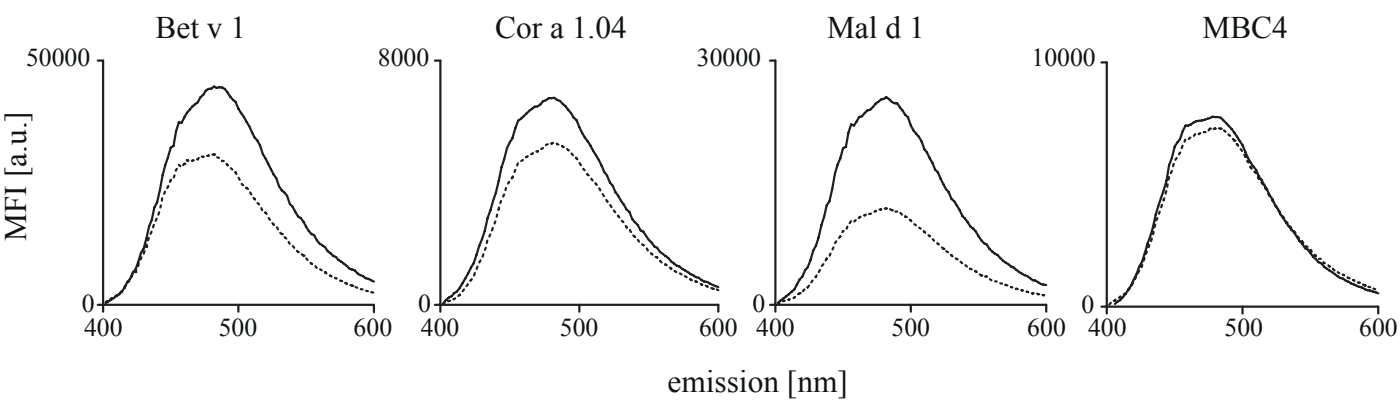
protein	theoretical mass [Da]	experimental mass [Da]	Δm [Da]
Bet v 1.0101	17439.6	17439.7	0.1
Cor a 1.0401	17621.0	17620.6	0.4
Mal d 1.0108	17548.7	17548.2	0.5
MBC4	17409.6	17409.7	0.1

B

protein	EU/mg
Bet v 1.0101	1.12
Cor a 1.0401	2.50
Mal d 1.0108	1.21
MBC4	0.99

C

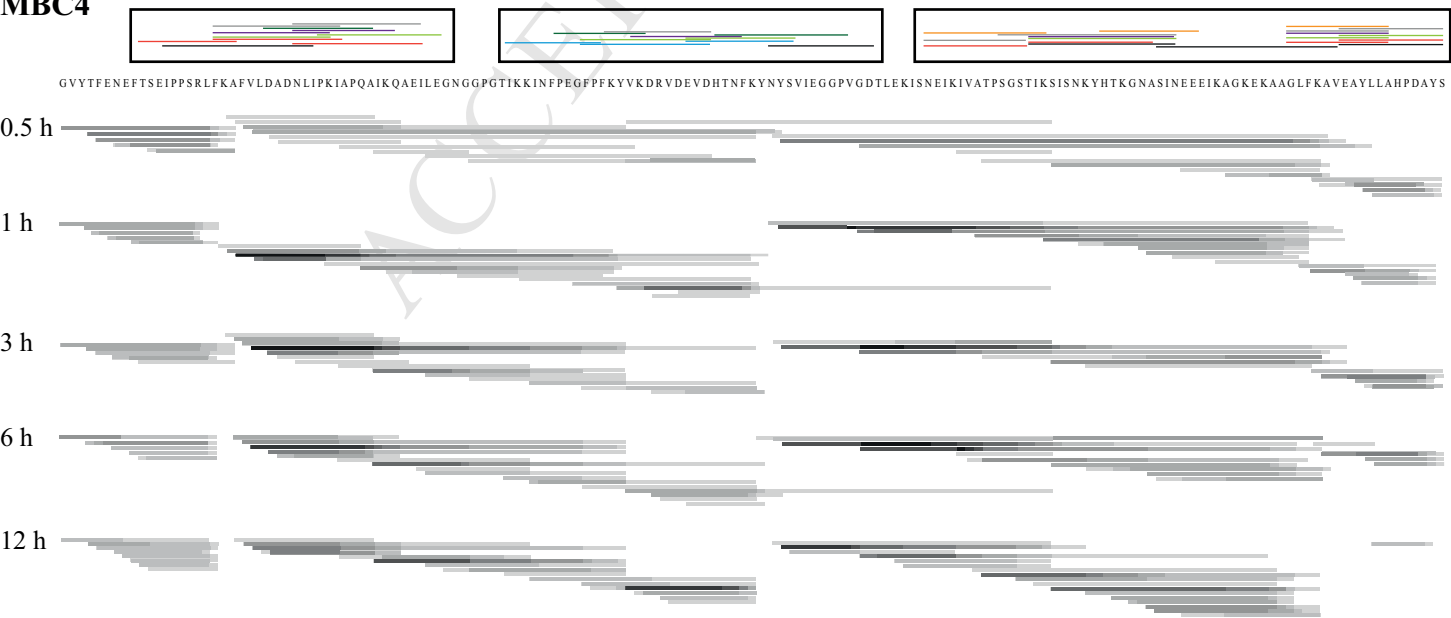




Bet v 1



MBC4



ONLINE SUPPORTING INFORMATION

Tackling Bet v 1- and associated food allergies with a single hybrid protein

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Bioinformatics analysis of sequence and fold variations

For the design of the hybrid molecules overlapping fragments of the parental allergens Bet v 1.0102, Cor a 1.0401, and Mal d 1.0108 were recombined. Structural models of Bet v 1.0102, Cor a 1.0401, Mal d 1.0108, MBC, and MBC4 were built using MODELLER¹. In order to obtain a common structural baseline for all models, the PDB entry 4a84 was selected. This PDB entry was identified by a BLAST search with all five sequences as query. PDB entry 4a84 was the hit with the best rank sum. Subsequently, for each target 100 models were generated by MODELLER in automodel mode. The models were then scored by two statistical scoring functions as provided by the MAESTRO software². The corresponding configuration files are available from the MAESTRO web site (<https://biwww.che.sbg.ac.at/maestro/>). The resulting scores reflect how well a certain sequence fits to a given fold. Structural similarities between the different models were determined with the MatchMaker tool as provided by UCSF Chimera³.

Cloning of MBC and MBC4

MBC was cloned by combining three overlapping fragments from *mal d 1.0108* (AF126402), *bet v 1.0102* (X77266) and *cor a 1.0401* (AF136945), respectively. Fragments were amplified by PCR from parental allergens using the primers MBC fwd (5'CATGCCATGGGTGTGTACACCTTCGAGAAC3') and MalBet rev (5'TGATCTTCTTGATGGTCCGGGGCCACCGTTTCCTTCAAGGATTCAGCTTGCTTG3'), MalBet fwd (5'CAAGCAAGCTGAAATCCTGAAGGAAACGGTGGCCCCGGAACCATCAAGAAGATCA3') and BetCor rev (5'CATTGATTGAAGCGTTGCCCTTGGTGTGGTACTTGTGTGC3'), and BetCor fwd (5'GCAACAAGTACCACACCAAGGGCAACGCTTCAATCAATG3') and MBC rev (5'CCGGAATTCTTAACAGTAGGCATCAGGGTGTG3'), restriction sites are underlined. Thereafter PCR products were separated on a 1.4% agarose gel, bands were excised and centrifuged at 14.000 g for 20 min at 4°C and subjected in a 1:1:1 ratio to 5 cycles of a primerless PCR. After addition of the primer pair MBC fwd and MBC rev, reassembled genes were amplified and cloned into a pET28b vector (Novagen, Merck Millipore, Billerica, MA, USA).

MBC4 was generated by PCR using MBC as template. The primer pairs MBC fwd and MBC4, BM4 F (5'GCAACCCCTagTGGAAgCaCCATCaaGAgtATCAGCAAC3'), as well as BM4 R (5'GTTGCTGATacTcttGATGGtgCtTCCActAGGGGTGTC 3') and MBC4 rev (5'CCGGAATTCTTACGAGTAGGCATCAGGGTGTG3') were used to amplify two fragments containing the desired mutations⁴. Both fragments were separated on a 1.4% agarose gel, bands were excised and centrifuged at 14.000 g for 20

min at 4°C and subjected in a 1:1 ratio to 5 cycles of a primerless PCR. After addition of the primer pair MBC fwd and MBC4 rev, reassembled genes were amplified and cloned into a pET28b vector (Novagen).

Recombinant production of MBC and MBC4

MBC was produced as recombinant protein in *E. coli* BL21 Star™ (DE3) (Thermo Fisher). Freshly transformed cells were grown in LB medium supplemented with 2 mM MgSO₄, 1% (v/v) glycerol, 0.2% (w/v) ammonium sulfate, 10 mM sodium phosphate pH 7.4, and 25 mg/L kanamycin, to an OD₆₀₀ of 0.8 at 37°C and thereafter induced with 0.5 mM IPTG for 5h at 37°C. After harvesting cells were resuspended in 20 mM NaP pH 8, 0.5 M urea, followed by three consecutive freeze/thaw cycles. Thereafter, 50 mM NaH₂PO₄ and 0.5 M NaCl were added on ice under constant stirring. The protein was subjected to a first purification step using a phenylsepharose column (GE Healthcare). MBC-containing fractions were dialyzed against 20 mM imidazole pH 7.4 and further purified with a DEAE column (GE Healthcare). Final polishing was performed using a Superdex 75 10/300 column (GE Healthcare). Purified proteins were stored at -20°C in 10 mM NaP pH 7.4.

Recombinant MBC4 was purified from bacterial inclusion bodies. Therefore, the MBC4 construct was transformed into competent *E. coli* BL21 Star™ (DE3) cells (Thermo Fisher). Positive transformants were cultured in LB medium supplemented with 2 mM MgSO₄, 1% (v/v) glycerol, 0.2% (w/v) ammonium sulfate, 10 mM sodium phosphate pH 7.4, and 25 mg/L kanamycin. Protein expression was induced at OD₆₀₀ of 0.7 by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and cultured for 5 h at 37°C. Bacterial cells were harvested after 6 h expression at 37°C by centrifugation at 4000 g for 20 min. and thereafter resuspended in 50 mM TrisBase, 1 mM EDTA and 0.1 % Triton X-100. Lysis was performed by three consecutive freeze/thaw cycles in liquid nitrogen and at 37°C, respectively. The pellet was washed with 50 mM TrisBase, 1 mM EDTA, 1 % Triton X-100, followed by a wash step in 5 mM sodium phosphate buffer pH 7.4 containing 25 % ethanol. Finally the pellet was resolved in 20 mM sodium phosphate buffer pH 7.4 and 6 M urea. The protein solution was filtered through a Millex-HPF HV filter unit (Merck Millipore) and loaded onto a Q FF anion exchange column (GE Healthcare). The flow through was collected, the urea concentration reduced to 4 M and after addition of 2.5 M of NaCl the protein solution was loaded on a Capto-Phenyl column (GE healthcare). Elution was performed with a 100 mL gradient to 20 mM sodium phosphate buffer pH 7.4, 6 M urea. Thereafter, the protein was renatured gradually by dialysis against 10 mM sodium phosphate buffer pH 7.4 and 4 M urea, 3 M urea, 1.5 M urea, and 0 M urea, respectively and thereafter stored at -20°C.

Antibody binding analyses

ELISA experiments were performed to measure human serum IgE binding to parental allergens as well as MBC4. Therefore, Maxisorp plates (Nunc, Thermo Fisher, Waltham, MA, USA) were coated with 100 ng/well protein antigen in 50 μ L PBS overnight at 4°C. After washing and blocking, plates were incubated with 1:10 diluted human serum samples in TBS pH 7.4, 0.05% (v/v) Tween, 0.5% (w/v) BSA) overnight at 4°C. Bound human IgE was detected using an AP-conjugated mouse anti human IgE antibody (BD Biosciences, Franklin Lakes, USA). Measurements were performed on a Tecan Sunrise (Tecan) plate reader at 405 nm using the substrate 4-nitrophenyl phosphate (Applichem GmbH, Darmstadt, Germany). Murine IgG1 as well as IgG2a were detected with AP-conjugated rat anti mouse IgG1 antibodies or AP-conjugated rat anti mouse IgG2a antibodies, respectively (both Southern Biotech, Birmingham, USA). After incubation for 1h at 37°C and 1h at 4°C, colorimetric detection with 10 mM 4-nitrophenyl phosphate (Applichem GmbH) was performed as described. Mediator release assays with human IgE antibodies were performed using RBL-2H3 cells transfected with the alpha chain of the human Fc ϵ RI. Cells were passively sensitized with patients' sera diluted in 1:20 in tissue culture medium over night at 37°C and 7% CO₂ ⁴. After several washing steps, antigen was added in serial dilutions in Tyrode's buffer (Sigma) supplemented with 1 g/L sodium bicarbonate, 0.1 % (w/v) BSA, and 50% (v/v) deuterium oxide (Sigma). Antigen-dependent β -hexosaminidase release into the supernatant was measured after 1 h incubation at 37°C by enzymatic cleavage of the fluorogenic substrate 4-methylumbelliferyl-N-acetyl- β -glucosaminide and expressed as % of total enzyme content of Triton X-100 treated cells. Alternatively, release assays were performed with RBL-2H3 cells, passively sensitized for 1h at 37°C and 7% CO₂ with a pool of murine sera 1:25 diluted in tissue culture medium following the protocol as described ⁵.

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