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A hypoallergenic variant of the major birch pollen allergen shows distinct characteristics in antigen processing and T-cell activation

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Abstract

Background: BM4 is a novel genetically engineered variant of the major birch pollen allergen Bet v 1 that lacks the typical Bet v 1-like fold and displays negligible IgE-binding but strong T cell-activating capacity. The aim of this study was to elucidate possible differences between BM4 and Bet v 1 in internalization, antigen processing, and presentation.

Methods: Proliferative responses to BM4 and Bet v 1 of peripheral blood mononuclear cells and Bet v 1-specific T-cell clones were compared. Fluorescently labeled BM4 and Bet v 1 were used to study surface binding, endocytosis, and intracellular degradation by monocyte-derived DC (mdDC). Both proteins were digested by endolysosomal extracts of mdDC. BM4- and Bet v 1-pulsed mdDC were employed to assess the kinetics of activation of Bet v 1-specific T-cell clones and the polarization of naïve T cells.

Results: BM4 displayed a significantly stronger T cell-activating capacity than Bet v 1. Furthermore, BM4 showed increased surface binding and internalization as well as faster endolysosomal degradation compared with Bet v 1. BM4-pulsed mdDC induced enhanced proliferative responses at earlier time-points in Bet v 1-specific T-cell clones and promoted less IL-5 production in T cells than Bet v 1-pulsed mdDC.

Conclusion: The loss of the Bet v 1-fold changes the protein's interaction with the human immune system at the level of antigen-presenting cells resulting in altered T-cell responses. By combining low IgE-binding with strong and modulating T cell-activating capacity, BM4 represents a highly interesting candidate for specific immunotherapy of birch pollen allergy.

Worldwide, around 100 million people suffer from birch pollen allergy, 93% of whom are sensitized to the major allergen Bet v 1 (1). Due to cross-reactivity, Bet v 1 frequently induces IgE-mediated allergic symptoms to pollen from other

trees of the *Fagales* family and to certain foods (1–3). Among at least 14 naturally occurring isoforms of Bet v 1, Bet v 1.0101 is the most abundant one (4, 5). Recombinant Bet v 1.0101 is routinely produced for allergy diagnosis and has been well characterized regarding physicochemical properties (6), 3D structure (7), IgE epitopes (8–10), and T-cell reactivity (11).

The only curative measure currently available for IgE-mediated allergy is allergen-specific immunotherapy (SIT), which modulates the allergic immune response at the levels of antigen-presenting cells (APC), T cells, and B cells (12).

Abbreviations

Aa, amino acid; APC, antigen-presenting cells; cpm, counts per minute; DC, dendritic cells; mDC, myeloid dendritic cells; mdDC, monocyte-derived DC; PBMC, peripheral blood mononuclear cells; SD, standard deviation; SI, stimulation index; SIT, allergen-specific immunotherapy; TCC, T-cell clones; UC, Ultra Culture.

Recombinant Bet v 1.0101 has been shown to be as effective as birch pollen extract in SIT, but various local IgE-mediated side effects have been reported (13). To reduce such undesired side effects, the use of hypoallergenic variants of allergens, which display reduced IgE-binding capacity and retained T cell-activating ability (14), has been proposed. BM4 is a novel hypoallergenic variant of Bet v 1.0101, which due to the exchange of 8 amino acid residues (aa) in the region Bet v 1.0101₁₀₉₋₁₁₆ is unable to attain the typical Bet v 1-like fold (15–17). Consequently, BM4 shows only residual IgE-binding and mediator release capacity (17). In mouse immunization experiments, BM4 induced a mixed T_H1/T_H2 immune response while Bet v 1.0101 induced a clear T_H2 response (17). These observations suggested an influence of the fold on the inherent allergic properties of the major birch pollen allergen.

We evaluated whether the loss of the Bet v 1-fold leads to differences in uptake, processing and presentation by human APC, and in subsequent T-cell responses. We utilized fluorescently labeled Bet v 1.0101 and BM4 to study internalization by different APC and to follow the proteins through the endocytic pathway in mdDC. The effects of presenting protein to either effector or naïve T cells were investigated by employing peripheral blood mononuclear cells (PBMC), Bet v 1-specific T-cell clones (TCC), and naïve T lymphocytes from birch pollen-allergic patients.

Materials and methods

Patients and proteins

Birch pollen-allergic patients showed typical case histories, positive skin prick tests to birch pollen extract and birch pollen-specific IgE of >3.5 kU_A/l (ImmunoCAP; Thermo Fisher Scientific, Uppsala, Sweden). The study was approved by the local ethics committee. Patients gave informed consent.

Recombinant Bet v 1.0101 (hereinafter referred to as Bet v 1) and Bet v 2 were purchased from Biomay (Vienna, Austria). Recombinant BM4 was expressed and purified as published previously (17). Proteins were quantified by UV spectrometry. Endotoxin levels in Bet v 1 and BM4 were below 1 EU/mg (LAL assay; Lonza, Basel, Switzerland). Proteins were conjugated to pHrodo-succinimidyl ester, FITC-succinimidyl ester, and Alexa Fluor 488®-succinimidyl ester (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Cell preparation

Monocytes were isolated from PBMC by immunomagnetic cell sorting (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany) resulting in >95% CD14⁺ cells. mdDC and microsome thereof were generated as described previously (18–20). Cryopreserved Bet v 1-specific TCC [generated as described (21)] were thawed, expanded, and re-stimulated with Bet v 1 or BM4 in duplicate in the presence of irradiated autologous (60 Gy) PBMC. Naïve CD4⁺ T cells were isolated from PBMC using the Naïve CD4⁺ T Cell Isolation Kit II (Miltenyi Biotec). Isolated populations were >97%

CD4⁺CD45RA⁺ and stored in liquid nitrogen until autologous mdDC had been generated.

Flow cytometry

The following monoclonal antibodies were used: CD1a-APC, CD3-PerCP, CD4-PE, CD8-APC, CD14-PerCP, CD19-APC, CD56-PE, CD80-PE, CD83-PE, CD86-PE, HLA-DR-FITC (BD Biosciences, San Jose, CA, USA), BDCA-2-PE, CD1c-APC, and CD141-APC (Miltenyi Biotec). Isotype controls were used to detect nonspecific binding.

Flow cytometry was performed on a FACS Canto (BD Biosciences) and analyzed using FACS Diva (BD Biosciences) and FlowJo software (TreeStar, Inc., Ashland, OR, USA).

Proliferation assays

Freshly isolated PBMC (2×10^5) were cultured in triplicate with Bet v 1 or BM4 (3–25 µg/ml) for 6 days. During the last 16 h of culture [³H]thymidine (0.5 µCi/well) was added. Proliferation of TCC was analyzed after 48 h of incubation of T cells ($2-5 \times 10^4$) and irradiated autologous PBMC (1×10^5) or mdDC (3.5×10^4) pulsed with protein (10 µg/ml) for up to 72 h (21). Stimulation indices (SI) were calculated as the ratio of counts per minute (cpm) of stimulated cultures to cpm of unstimulated cultures.

Surface binding, endocytosis, and degradation of labeled proteins

To assess protein uptake, Alexa 488-labeled proteins (400 ng/ml) were preincubated at a ratio of 1 : 5 with autologous serum or medium for 1 h at 37°C. Freshly isolated PBMC (2×10^6) were added, incubated for 1 h at 4°C, transferred to 37°C, incubated for another 20 h, and labeled with cell type-specific surface markers.

To assess surface binding, mdDC (1×10^6) were incubated with Alexa 488-labeled proteins (400 ng/ml) for 3 h at 4°C and fixed in 2% paraformaldehyde.

To determine endocytosis, mdDC (2×10^5) were incubated with pHrodo-labeled proteins (3 µg/ml) for 1 h at 4°C, further incubated at 37°C in fresh medium, and analyzed at the indicated time-points.

To assess intracellular degradation, mdDC (1×10^6) were incubated with FITC-labeled proteins (15 µg/ml) and incubated for 1 h at 37°C, washed, and further incubated at 37°C for the indicated time periods. The percentage of FITC-positive cells was measured using flow cytometry. The percentage of degradation of antigen is represented as the ratio of FITC⁺ cells at the indicated time to FITC⁺ cells at 0 h.

Polarization experiments

mdDC (1×10^4) were pulsed with protein for 24 h, washed, irradiated, and incubated with thawed naïve T cells (1×10^5) for 6 days (22). Culture supernatants were collected and analyzed for IL-5 and IFN-γ using the Luminex System 100 (detection limit 30 pg/ml) (Luminex, Austin, TX, USA).

Maturation of mdDC

mdDC were incubated with protein (17.5 µg/ml) or LPS (0.5 µg/ml) for 48 h. One part of these cells were analyzed for upregulation of maturation markers (CD83, CD80, CD86 and CD40) using flow cytometry. The other part of these cells was irradiated, and 2.5 , 5 , and 10×10^3 cells were co-cultured with allogeneic PBMC (1×10^5) for 6 days to determine mixed leukocyte reactions. LPS was used as a positive control because it has been shown to stimulate the production of cytokines and the expression of co-stimulatory molecules and HLA-DR on DC (23).

Statistical analysis

Data were analyzed using IBM SPSS 16.0 (SPSS, Chicago, IL, USA). Data from different patients were not normally distributed (as assessed by the Shapiro–Wilk Test) and are presented as median and interquartile ranges. Statistical differences were determined by the Wilcoxon signed ranks test and considered statistically significant for $P < 0.05$. Data from duplicate experiments are shown as means with standard deviation (SD).

Results

BM4 elicits stronger T-cell responses than Bet v 1

PBMC from 13 birch pollen-allergic patients were stimulated with titrated amounts of Bet v 1 or BM4. At all concentrations used, the response to BM4 was significantly stronger than to equal amounts of Bet v 1 (25 µg/ml, $P = 0.007$; 12.5 µg/ml, $P = 0.001$; 6.3 µg/ml, $P = 0.006$; 3.1 µg/ml, $P = 0.003$; Fig. 1). Furthermore, seven Bet v 1-specific TCC from five different birch pollen-allergic donors recognizing relevant T cell-activating regions of Bet v 1, that is, Bet v 1₄₋₁₈, Bet v 1₆₁₋₇₅, Bet v 1₈₂₋₉₆, Bet v 1₁₁₂₋₁₂₃, and Bet v 1₁₄₂₋₁₅₃ (11), were stimulated with either variant. TCC specific for Bet v 1₁₁₂₋₁₂₃ (TCC5, TCC6) were nonreactive to BM4 because this T cell-activating region contains the mutated sequence of aa 109-116 (TIKSISNKYHTK). All other clones proliferated substantially better in response to BM4 than to Bet v 1 even at very low protein concentrations (Table 1).

Uptake of Bet v 1 and BM4 by different types of APC

The stronger T-cell reactivity to BM4 might result from enhanced endocytosis and/or processing of this protein. To evaluate possible differences in the uptake of Bet v 1 and BM4 by different types of APC, we analyzed their internalization by PBMC from birch pollen-allergic patients. Uptake of BM4 was enhanced compared with Bet v 1 in monocytes (CD14⁺) and in the two types of myeloid DC (mDC1: CD1c⁺CD14⁺CD19⁺; mDC2: CD14⁺CD141⁺), respectively (Fig. 2A). The number of plasmacytoid DC (CD14⁺CD303⁺) in PBMC was generally too low to assess protein uptake (data not shown). B cells (CD19⁺) did not endocytose protein under the conditions used, neither did NK cells

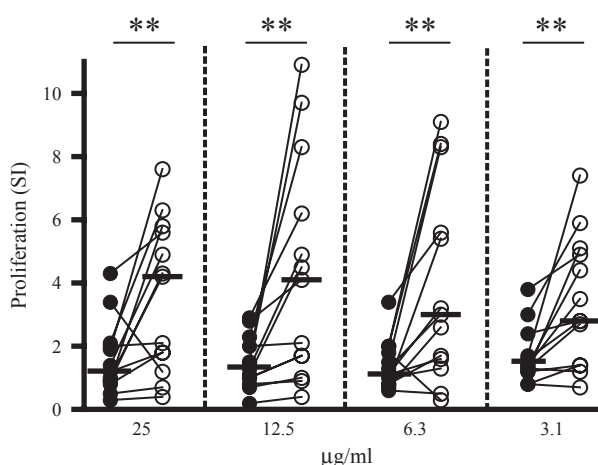


Figure 1 Proliferation of PBMC in response to Bet v 1 and BM4. PBMC of 13 birch pollen-allergic donors were stimulated with different concentrations of Bet v 1 (filled circles) or BM4 (open circles) for 6 days, and proliferation was measured using thymidine incorporation. SI values are shown, which represent the ratio between the cpm of stimulated cultures and the cpm of unstimulated cultures. Black lines indicate median values. ** $P < 0.01$ (Wilcoxon signed ranks test). Background levels of PBMC left in medium without stimulant ranged from 2.500 to 35.000 cpm.

(CD3⁺CD56⁺) nor T cells (CD3⁺CD4⁺ and CD3⁺CD8⁺, respectively) (data not shown). Preincubation of the proteins with autologous serum enhanced only the internalization of Bet v 1 by monocytes (Fig. 2A).

Uptake and intracellular degradation of Bet v 1 and BM4 by mdDC

Since PBMC contain very limited numbers of DC, we used immature mdDC from birch pollen-allergic individuals to study surface binding, endocytosis, and processing of Bet v 1 and BM4 in more detail. Surface binding was assessed by incubation of mdDC with Alexa 488-labeled protein at 4°C to abrogate endocytosis. Figure 2B shows that BM4 was significantly more efficiently bound to the surface of mdDC than Bet v 1 at 400 ng/ml of protein ($P = 0.028$), and a trend toward more binding was seen at the lower concentrations used.

To track endocytosis, we used Bet v 1 and BM4 conjugated to pHrodo, a pH-dependent dye that is nonfluorescent at neutral pH and turns bright red in the acidic environment of endosomes. At all time-points investigated (4, 6, 24 h), the percentage of BM4-positive cells was significantly higher than of Bet v 1-positive cells (Fig. 2C; $P = 0.043$ each).

Intracellular protein degradation was analyzed over a time period of 24 h after pulsing mdDC with FITC-conjugated proteins. In contrast to Bet v 1-pulsed cells, a substantial number of cells were negative for BM4 already after 1, 2, and 4 h (Fig. 2D). After 6 h of incubation, BM4 was fully degraded while about 60% of cells were still positive for Bet v 1. Furthermore, BM4 and Bet v 1 were subjected to

Table 1 Proliferation of TCC in response to Bet v 1 and BM4

TCC	Specificity		Bet v 1 [$\mu\text{g/ml}$]				BM4 [$\mu\text{g/ml}$]			
	aa	aa sequence	5	2.5	1.3	0.6	5	2.5	1.3	0.6
TCC 1	4–18	NYETTETSVIPAARL	29*	18	13	n.d.	65	64	64	n.d.
TCC 2	61–75	GFPFKYVKDRVDEVD	11	n.d.	n.d.	n.d.	23	n.d.	n.d.	n.d.
TCC 3	82–96	YNYSVIEGGPIGDTL	84	66	48	n.d.	269	166	139	n.d.
TCC 4	82–96	YNYSVIEGGPIGDTL	n.d.	27	16	7	n.d.	206	156	107
TCC 5	112–123	SILKISNKYHTK	52	n.d.	n.d.	n.d.	1	n.d.	n.d.	n.d.
TCC 6	112–123	SILKISNKYHTK	177	n.d.	n.d.	n.d.	1	n.d.	n.d.	n.d.
TCC 7	142–153	TLLRAVESYLLA	57	28	15	9	209	221	215	186

aa, amino acid residues; n.d., not determined.

*Stimulation indices (SI) are shown. Background values of medium control ranged from 99 to 988 cpm.

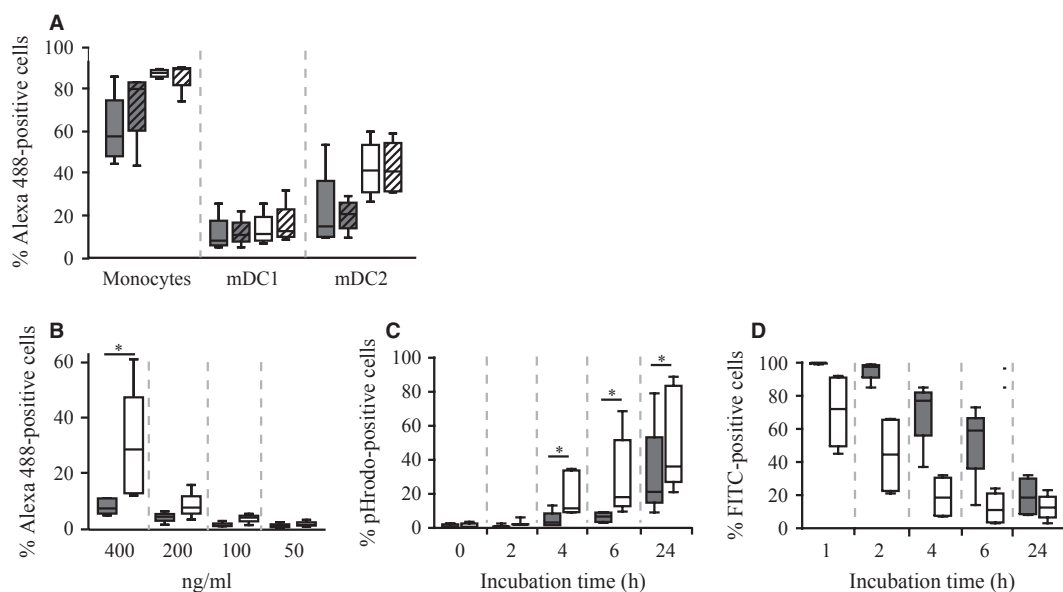


Figure 2 Bet v 1 and BM4 in the endocytic pathway. (A) PBMC from birch pollen-allergic donors were incubated with Bet v 1-Alexa488 (gray) or BM4-Alexa488 (white) preincubated with (hatched) or without (plain) autologous serum for 1 h. The percentage of Alexa 488-positive cell populations was determined by flow cytometry ($n = 4$). Surface binding (B), endocytosis (C), and intracellular degradation (D) by mdDC incubated with equal concentrations of Bet v 1 (gray) or BM4 (white) conjugated to different

fluorophores. (B) Alexa 488-positive cells after 3 h at 4°C ($n = 6$ for 400 ng/ml, $n = 3$ for the other concentrations). (C) pHrodo-positive cells after 1 h at 4°C and shift to 37°C for the indicated periods of time ($n = 6$). (D) Cells were incubated with FITC-labeled proteins for 3 h at 37°C, positive cells at time-point 0 were set to 100%, and the relative percentage of positive cells at the subsequent time-points is shown ($n = 4$). * $P < 0.05$ (Wilcoxon signed ranks test).

degradation assays employing endo/lysosomal proteases isolated from mdDC of birch pollen-allergic donors (19). Degradation of BM4 proceeded rapidly during the first hour of incubation (Fig. S1). In contrast, Bet v 1 was degraded more slowly and in a more continuous fashion.

Presentation of BM4 and Bet v 1 to Bet v 1-specific effector T cells

To investigate whether faster uptake and processing of BM4 by mdDC resulted in an earlier peptide presentation to T cells, we performed time course experiments employing different Bet v 1-specific TCC. Proliferation was stronger in the

presence of mdDC pulsed with BM4 for 6, 11, and 24 h as compared to mdDC pulsed with Bet v 1 (Fig. 3). Comparable proliferation of TCC was observed when mdDC had been pulsed with either protein for 48 h. After 72 h of pulsing, the clones responded stronger to Bet v 1-pulsed mdDC. No response to mdDC pulsed with Bet v 2 was observed (Fig. 3).

Polarization of naïve T cells in response to BM4

To finally test whether increased uptake, processing, and earlier presentation of BM4 affected the differentiation of naïve human T cells, we co-cultured $\text{CD4}^+ \text{CD45RA}^+$ T cells

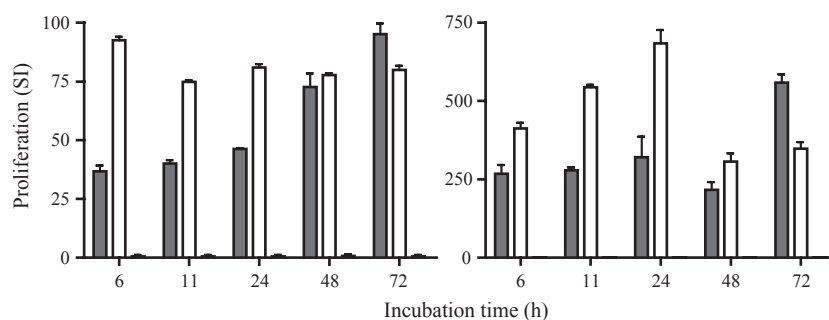


Figure 3 Activation kinetics of Bet v 1-specific TCC. mdDC were incubated with Bet v 1 (gray), BM4 (white), or Bet v 2 (black) for the indicated periods of time, washed, irradiated, and used to stim-

ulate autologous Bet v 1-specific TCC. After 48 h, T-cell proliferation was assessed. Two independent experiments of three are shown. Graphs represent mean plus SD of duplicates.

isolated from 7 birch pollen-allergic donors with mdDC that had been pulsed with Bet v 1 or BM4 for 24 h. After 6 days, culture supernatants were assessed for levels of IL-5, a T_H2 -associated cytokine, and IFN- γ , a T_H1 -associated cytokine. T cells primed by Bet v 1-pulsed mdDC produced significantly more IL-5 ($P = 0.028$) than those stimulated with BM4-pulsed mdDC, while the levels of IFN- γ were identical (330 and 400 ng/ml, respectively, Fig. 4). IL-4 and IL-10 were not detectable under the culture conditions used.

BM4 does not induce functional maturation of mdDC

To exclude the possibility that the more pronounced T-cell reactivity to BM4 was due to functional maturation of mdDC, immature mdDC from 6 birch pollen-allergic patients were left untreated or incubated with Bet v 1 or BM4. The positive control LPS induced significant expression

of CD80 ($P = 0.028$), CD83 ($P = 0.018$), CD86 ($P = 0.043$), and CD40 ($P = 0.028$; Fig. 5A). None of these molecules were upregulated after incubation with Bet v 1 or BM4. Accordingly, neither BM4- nor Bet v 1-treated mdDC-induced proliferation of allogeneic T cells, whereas LPS-matured mdDC evoked strong proliferative responses (Fig. 5B).

Discussion

Genetically engineered variants of naturally occurring allergens with reduced IgE-binding capacity and retained T cell-activating capacity have been developed with the aim of increasing the doses that can be administered in SIT while at the same time reducing therapy-associated side effects (24). Different approaches to generate hypoallergenic variants of the major birch pollen allergen have been introduced previously (25–28). Two hypoallergenic halves of the molecule and a Bet v 1 trimer have already been tested in clinical studies and diminished the pathological IgE response combined with a trend toward an improvement in well-being and reduced reactivity to specific allergen provocation (29, 30). BM4 is a genetically engineered variant of the most abundant isoform of Bet v 1 with an almost identical aa sequence but lacking the Bet v 1-fold, displaying dramatically reduced IgE-binding and retained T cell-activating capacity (17). Mice immunized with BM4 developed high levels of Bet v 1-reactive antibodies, demonstrating the protein's immunogenicity (17). Beyond that, immunization with BM4 induced IFN- γ -producing T cells whereas immunizations with Bet v 1 or Bet v 1 A 1-6, a hypoallergenic variant with a Bet v 1-fold (26), did not. These results suggest that reshaping the Bet v 1-fold does not only reduce IgE-binding but also the capacity to promote strong Th2-like responses (17).

In the present study, the cellular response of human cells to BM4 was investigated in great detail. We observed that PBMC and Bet v 1-specific TCC from birch pollen-allergic patients proliferated significantly stronger upon stimulation with BM4 than with Bet v 1 (Fig. 1, Table 1). This divergent T-cell stimulatory capacity might originate at the level of APC, either from differences in uptake, processing, or

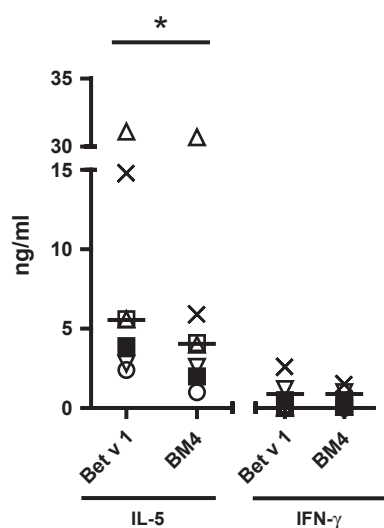


Figure 4 Polarization of naïve T cells. Naïve $CD4^+$ T cells from 7 birch pollen-allergic donors were stimulated for 6 days with autologous mdDC pulsed with Bet v 1 or BM4 for 24 h. Cytokine levels in the supernatants were determined. Black lines represent median values. * $P < 0.05$ (Wilcoxon signed ranks test).

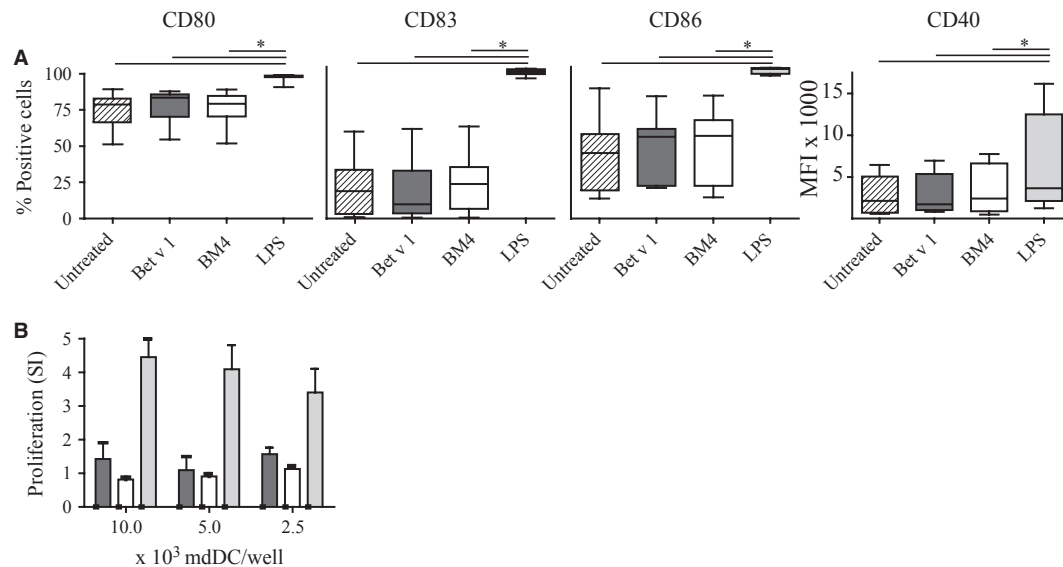


Figure 5 Functional maturation of mdDC. mdDC were left untreated (hatched) or incubated with Bet v 1 (gray), BM4 (white), or LPS (light gray) for 48 h and used for (A) surface marker expression analysis ($n = 6$) * $P < 0.05$ (Wilcoxon signed ranks test) or (B)

to stimulate allogeneic PBMC ($2.5, 5$ or 10×10^3 mdDC to 10^5 PBMC). SI values are calculated in relation to cells grown in the presence of unstimulated mdDC (mean values with SD of triplicates of one representative experiment of four are shown).

presentation of BM4. By being less compact than Bet v 1, BM4 represents a bulkier structure with a presumably higher potential to interact with the surface of APC. Indeed, we found that BM4 bound more efficiently to mdDC and was endocytosed more rapidly than Bet v 1 (Fig. 2). Moreover, BM4 was processed faster by mdDC than Bet v 1 (Fig. 2D). Employing microsomal proteases from human mdDC in degradation assays confirmed that BM4 was more rapidly degraded than Bet v 1 during the first hour of incubation (Fig. S1). We conclude that the less compact structure of BM4 is responsible for its differential behavior in the antigen-processing pathway, causing more efficient surface binding and uptake as well as improved access to endolysosomal proteases.

When comparing the kinetics of T-cell proliferation in response to BM4- and Bet v 1-pulsed mdDC, we found, in agreement with the faster endolysosomal degradation of BM4, that BM4-pulsed mdDC induced significantly stronger proliferative responses than Bet v 1-pulsed mdDC during the first 24 h of incubation (Fig. 3). This observation could not be referred to functional maturation of mdDC, as stimulation with BM4 induced neither an upregulation of co-stimulatory molecules nor a T-cell stimulatory potential in mixed leukocyte reactions (Fig. 5). We speculate that the more rapid uptake and processing of BM4 leads to the presentation of a larger numbers of peptide-loaded MHC class II complexes on the surface of APC at earlier time-points. The extent of T-cell receptor ligation by peptide MHC class II complexes is known to influence the differentiation fate of naïve T cells (31). High doses of antigen promote the induction of Th1-like responses whereas low doses support Th2-like responses. In accordance with this concept, naïve CD4⁺

T cells from birch pollen-allergic patients differentiated into cells producing significantly less IL-5 when primed with BM4-pulsed mdDC than with Bet v 1-pulsed mdDC (Fig. 3). Similarly, BM4 induced a less-pronounced Th2 response in mice than Bet v 1 (17). As expected, the differences in the polarization of naïve T cells from already Bet v 1-sensitized individuals were less clear-cut than those observed in naïve mice (17).

Our finding that the loss of the Bet v 1-fold changes the protein's interaction with the immune system at the level of APC and results in altered T-cell responses accords with the former work demonstrating that unfolded variants of bee venom phospholipase A₂ induce more pronounced Th1-like responses in bee venom-allergic patients *in vitro* than the correctly folded allergen (32). Together, these observations hint at the relevance of allergen conformation for the establishment of Th2-like responses.

Subcutaneous administration of recombinant Bet v 1.0101 has already been demonstrated to be effective for the treatment of birch pollinosis (13). As BM4 combines significantly reduced IgE-binding, strong T-cell reactivity with less Th2-promoting capacity and excellent production characteristics, namely stable unfolding, no tendency to aggregate, high expression yield, and low batch-to-batch variability (17), this hypoallergenic variant of Bet v 1.0101 represents a highly promising vaccine candidate for SIT of birch pollen allergy.

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Author contributions

C.K. and B.B. designed the experiments; C.K., M.W., S.D., S.M., and C.W. performed the experiments and analyzed the data; G.Z. helped with cytokine analyses; F.F. contributed to the experimental design and writing of the paper. C.K. and B.B. wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.

References

- Geroldinger-Simic M, Zelniker T, Aberer W, Ebner C, Egger C, Greiderer A et al. Birch pollen-related food allergy: clinical aspects and the role of allergen-specific IgE and IgG (4) antibodies. *J Allergy Clin Immunol* 2011;**127**:616–622. e611.
- Eriksson NE. Allergy to pollen from different deciduous trees in Sweden. An investigation with skin tests, provocation tests and the radioallergen sorbent test (RAST) in springtime hay fever patients. *Allergy* 1978;**33**:299–309.
- Eriksson NE, Formgren H, Svenonius E. Food hypersensitivity in patients with pollen allergy. *Allergy* 1982;**37**:437–443.
- Swoboda I, Jilek A, Ferreira F, Engel E, Hoffmann-Sommergruber K, Scheiner O et al. Isoforms of Bet v 1, the major birch pollen allergen, analyzed by liquid chromatography, mass spectrometry, and cDNA cloning. *J Biol Chem* 1995;**270**:2607–2613.
- Erlar A, Hawranek T, Kruckemeier L, Asam C, Egger M, Ferreira F et al. Proteomic profiling of birch (*Betula verrucosa*) pollen extracts from different origins. *Proteomics* 2011;**11**:1486–1498.
- Himly M, Nony E, Chabre H, Van Overtvelt L, Neubauer A, van Ree R et al. Standardization of allergen products: 1. Detailed characterization of GMP-produced recombinant Bet v 1.0101 as biological reference preparation. *Allergy* 2009;**64**:1038–1045.
- Gajhede M, Osmark P, Poulsen FM, Ipsen H, Larsen JN, Joost van Neerven RJ et al. X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat Struct Biol* 1996;**3**:1040–1045.
- Dall'Antonia F, Gieras A, Devanaboyina SC, Valenta R, Keller W. Prediction of IgE-binding epitopes by means of allergen surface comparison and correlation to cross-reactivity. *J Allergy Clin Immunol* 2011;**128**:872–879. e878.
- Gieras A, Cejka P, Blatt K, Focke-Tejkl M, Linhart B, Flicker S et al. Mapping of conformational IgE epitopes with peptide-specific monoclonal antibodies reveals simultaneous binding of different IgE antibodies to a surface patch on the major birch pollen allergen, Bet v 1. *J Immunol* 2011;**186**:5333–5344.
- Holm J, Ferreras M, Ipsen H, Wurtzen PA, Gajhede M, Larsen JN et al. Epitope grafting, re-creating a conformational Bet v 1 antibody epitope on the surface of the homologous apple allergen Mal d 1. *J Biol Chem* 2011;**286**:17569–17578.
- Jahn-Schmid B, Radakovic A, Luttkopf D, Scheurer S, Vieths S, Ebner C et al. Bet v 1 142–156 is the dominant T-cell epitope of the major birch pollen allergen and important for cross-reactivity with Bet v 1-related food allergens. *J Allergy Clin Immunol* 2005;**116**:213–219.
- Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* 2006;**6**:761–771.
- Pauli G, Larsen TH, Rak S, Horak F, Pastorello E, Valenta R et al. Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis. *J Allergy Clin Immunol* 2008;**122**:951–960.
- Valenta R, Linhart B, Swoboda I, Niederberger V. Recombinant allergens for allergen-specific immunotherapy: 10 years anniversary of immunotherapy with recombinant allergens. *Allergy* 2011;**66**:775–783.
- Vanek-Krebitz M, Hoffmann-Sommergruber K, Laimer da Camara Machado M, Susani M, Ebner C, Kraft D et al. Cloning and sequencing of Mal d 1, the major allergen from apple (*Malus domestica*), and its immunological relationship to Bet v 1, the major birch pollen allergen. *Biochem Biophys Res Commun* 1995;**214**:538–551.
- Radauer C, Lackner P, Breiteneder H. The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. *BMC Evol Biol* 2008;**8**:286.
- Wallner M, Hauser M, Himly M, Zaborsky N, Mutschlechner S, Harrer A et al. Reshaping the Bet v 1 fold modulates T(H) polarization. *J Allergy Clin Immunol* 2011;**127**:1571–1578. e1579.
- Mutschlechner S, Egger M, Briza P, Wallner M, Lackner P, Karle A et al. Naturally processed T cell-activating peptides of the major birch pollen allergen. *J Allergy Clin Immunol* 2010;**125**:711–718. e711–718, e712.
- Egger M, Jurets A, Wallner M, Briza P, Ruzek S, Hainzl S et al. Assessing protein immunogenicity with a dendritic cell line-derived endolysosomal degradome. *PLoS ONE* 2010;**6**:e17278.
- Delamarre L, Pack M, Chang H, Mellman I, Trombetta ES. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* 2005;**307**:1630–1634.
- Fritsch R, Bohle B, Vollmann U, Wiedermann U, Jahn-Schmid B, Krebitz M et al. Bet v 1, the major birch pollen allergen, and Mal d 1, the major apple allergen, cross-react at the level of allergen-specific T helper cells. *J Allergy Clin Immunol* 1998;**102**:679–686.
- Smole U, Wagner S, Balazs N, Radauer C, Bublin M, Allmaier G et al. Bet v 1 and its homologous food allergen Api g 1 stimulate dendritic cells from birch pollen-allergic individuals to induce different Th-cell polarization. *Allergy* 2010;**65**:1388–1396.
- Verhasselt V, Buelens C, Willems F, De Groote D, Haeflner-Cavaillon N, Goldman M. Bacterial lipopolysaccharide stimulates the production of cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells: evidence for a soluble CD14-dependent pathway. *J Immunol* 1997;**158**:2919–2925.
- Cromwell O, Hafner D, Nandy A. Recombinant allergens for specific immunotherapy. *J Allergy Clin Immunol* 2011;**127**:865–872.
- Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR et al. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. *J Clin Invest* 1997;**99**:1673–1681.
- Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ et al. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic vari-

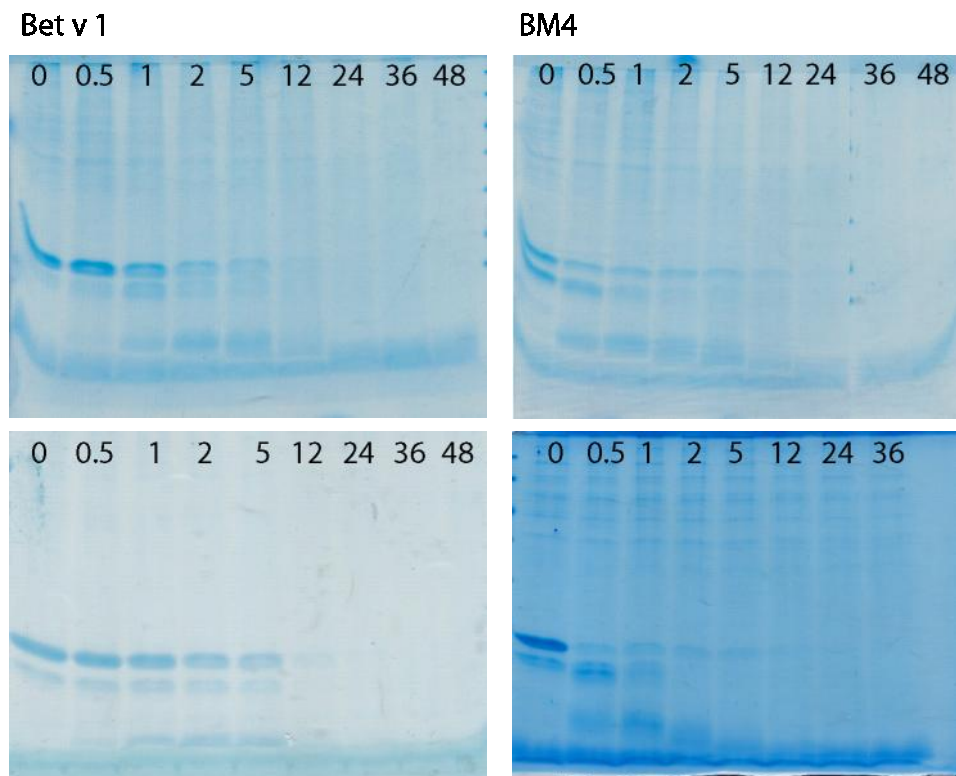
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Endolysosomal degradation of proteins.

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- ants for immunotherapy. *FASEB J* 1998;**12**:231–242.
27. Vrtala S, Fohr M, Campana R, Baumgartner C, Valent P, Valenta R. Genetic engineering of trimers of hypoallergenic fragments of the major birch pollen allergen, Bet v 1, for allergy vaccination. *Vaccine* 2011;**29**:2140–2148.
 28. Campana R, Vrtala S, Maderegger B, Jertschin P, Stegellner G, Swoboda I et al. Hypoallergenic derivatives of the major birch pollen allergen Bet v 1 obtained by rational sequence reassembly. *J Allergy Clin Immunol* 2010;**126**:1024–1031, 1031 e1021–1028.
 29. Niederberger V, Horak F, Vrtala S, Spitzauer S, Krauth MT, Valent P et al. Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc Natl Acad Sci USA* 2004;**101**(Suppl 2): 14677–14682.
 30. Purohit A, Niederberger V, Kronqvist M, Horak F, Gronneberg R, Suck R et al. Clinical effects of immunotherapy with genetically modified recombinant birch pollen Bet v 1 derivatives. *Clin Exp Allergy* 2008;**38**:1514–1525.
 31. Constant S, Pfeiffer C, Woodard A, Pasqualini T, Bottomly K. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4⁺ T cells. *J Exp Med* 1995;**182**:1591–1596.
 32. Akdis CA, Blesken T, Wymann D, Akdis M, Blaser K. Differential regulation of human T cell cytokine patterns and IgE and IgG4 responses by conformational antigen variants. *Eur J Immunol* 1998;**28**:914–925.



Supplementary figure 1: Endolysosomal degradation of proteins

Bet v 1 (left) and BM4 (right) were incubated with endo/lysosomal microsomes derived from mdDC of birch pollen allergic patients for the indicated periods of time. Lysates were loaded onto SDS-PAGE gels and stained by Coomassie Blue.