

Allergy / Early View

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Isolation of nanobodies with potential to reduce patients' IgE binding to Bet v 1

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Abstract

Background

Recent studies showed that a single injection of human monoclonal allergen-specific IgG antibodies significantly reduced allergic symptoms in birch pollen-allergic patients. Since the production of full monoclonal antibodies in sufficient amounts is laborious and expensive, we sought to investigate if smaller recombinant allergen-specific antibody fragments, that is, nanobodies, have similar protective potential. For this purpose, nanobodies specific for Bet v 1, the major birch pollen allergen, were generated to evaluate their efficacy to inhibit IgE-mediated responses.

Methods

A cDNA-VHH library was constructed from a camel immunized with Bet v 1 and screened for Bet v 1 binders encoding sequences by phage display. Selected nanobodies were expressed, purified, and analyzed in regards of epitope-specificity and affinity to Bet v 1. Furthermore, cross-reactivity to Bet v 1-homologues from alder, hazel and apple, and their usefulness to inhibit IgE binding and allergen-induced basophil activation were investigated.

Results

We isolated three nanobodies that recognize Bet v 1 with high affinity and cross-react

with Aln g 1 (alder) and Cor a 1 (hazel). Their epitopes were mapped to the alpha-helix at the C-terminus of Bet v 1. All nanobodies inhibited allergic patients' polyclonal IgE binding to Bet v 1, Aln g 1, and Cor a 1 and partially suppressed Bet v 1-induced basophil activation.

Conclusion

We identified high-affinity Bet v 1-specific nanobodies that recognize an important IgE epitope and reduce allergen-induced basophil activation revealing the first proof that allergen-specific nanobodies are useful tools for future treatment of pollen allergy.

Abbreviations

ABTS

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

AIT

Allergen-specific immunotherapy

BAT

Basophil activation test

BSA

Bovine serum albumin

CDR

Complementarity-determining region

HCAb

Heavy Chain-only Antibody

HSA

Human serum albumin

ILZ

Isoleucine zipper

KLH

Keyhole limpet hemocyanin

PBMC

Peripheral blood mononuclear cell

PBS

Phosphate-buffered saline

PBST

Phosphate-buffered saline +0.05% Tween 20

RBL

Rat basophilic leukemia

SPR

Surface plasmon resonance

VHH

variable domain of heavy chain of Heavy chain-only antibodies

1 INTRODUCTION

Birch pollen allergy ranks among the most prevalent pollen allergies in Northern and Central Europe.¹ Estimated 100 million individuals worldwide suffer from this IgE-mediated disease leading to clinical manifestations such as hayfever causing a major health and economic burden.^{2,3} Bet v 1 is the sole major birch pollen allergen. It sensitizes more than 90% of individuals allergic to birch pollen^{1,4} and mainly contributes to pollen-associated food allergy.⁵ Representing such a clinically important allergen, Bet v 1 is one of the best-characterized allergens⁶ and was in the focus of various strategies to treat and ameliorate birch pollen allergy.⁷⁻¹⁰ Several attempts have developed hypoallergenic Bet v 1 derivatives for vaccination of birch pollen-allergic patients with the aim to induce protective IgG antibodies competing with IgE antibodies for allergen binding and thereby causing a variety of effects on distinct immune cells.¹¹⁻¹⁵ While the contribution of blocking IgG antibodies for the clinical success of allergen-specific immunotherapy (AIT) has been proven,¹⁵⁻¹⁷ current forms of vaccination still bear limitations including long-term administration of (modified) allergens and variable efficacy. Very recently, studies have demonstrated that passively administered allergen-specific monoclonal antibodies comparably reduce allergic responses as those induced in the course of AIT if they are directed to or overlap with IgE epitopes.¹⁸⁻²¹ These trials showed for the first time that passive immunization with allergen-specific IgG antibodies able to inhibit patients' IgE binding to major allergens is a rapid, effective, and well-tolerated treatment to reduce allergic inflammation.¹⁸⁻²¹

Since the generation and identification of blocking monoclonal human or humanized antibodies are laborious and expensive, recombinant antibody fragments including nanobodies turned out to be attractive molecules extensively studied for diverse applications in the recent past. Thus, nanobodies deriving from naturally occurring heavy chain-only antibodies of camelids have been proven to be useful tools in imaging technology, diagnosis, prevention and therapy of several diseases as cancer, viral infection, and blood disorders.²²⁻²⁴ They combine several advantageous qualities like unique biochemical characteristics and low immunogenicity.²⁵ Owing to their particular paratopes, that is, finger-like extensions that facilitate the recognition of a broad panel of various epitopes on antigens, and their reported antigen affinities comparable with affinities of monoclonal antibodies they recently aroused our interest to evaluate their potential to compete with IgE antibodies for allergen binding.^{22, 26, 27}

Within this study, we pursued the question if nanobodies raised against Bet v 1 may block IgE binding similar to protective IgG antibodies and thereby prevent activation of effector cells. For the first time, Bet v 1-specific nanobodies were isolated and their affinity, inhibitory capacity, and cross-protection were investigated.

2 METHODS

Methods are described in the Data [S1](#).

3 RESULTS

3.1 Construction of a phage-displayed cDNA-VHH-library from a camel immunized with rBet v 1 and Bet v 1-derived peptides and isolation of Bet v 1-specific nanobodies

A camel received five subcutaneous immunizations with Bet v 1 and Bet v 1-derived peptides according to a defined scheme (Figure 1A, Table S1). PBMCs to isolate RNA for the construction of the cDNA-VHH library were obtained 6 days after the fifth vaccination. At this time point, the camel had developed IgG antibodies specific for Bet v 1 (Figure 1B). The cloning of cDNA-sequences encoding the entire set of VHHs of specific heavy chain-only antibodies led to a final library size of 1×10^7 independent clones. A PCR analysis of randomly picked clones from the original library revealed that about 85% of picked clones comprise inserts of the expected size displaying high divergence (data not shown).

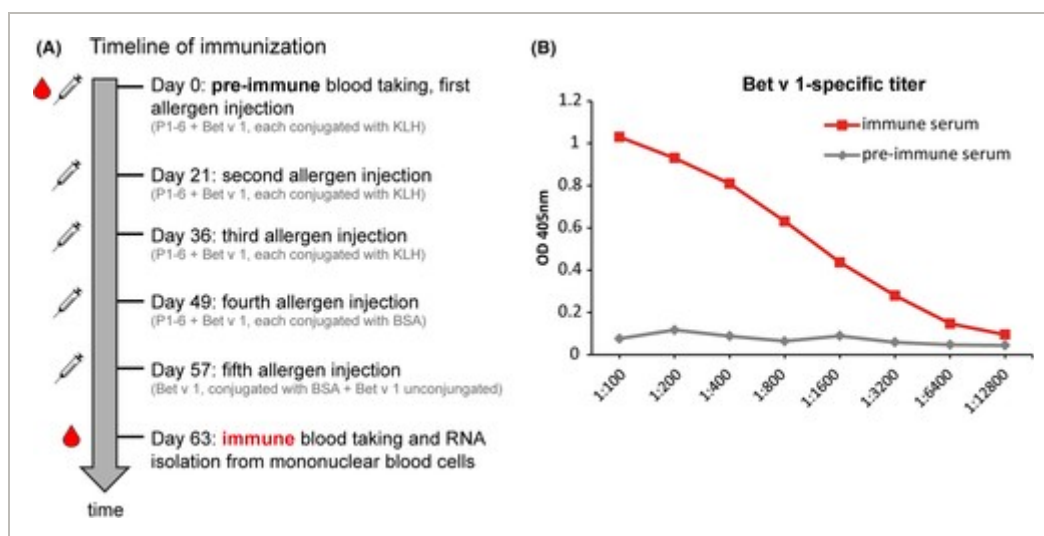


FIGURE 1

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Camel immunization for the generation of anti-Bet v 1 antibodies. (A) The time course of the camel's immunization with Bet v 1 ± a mixture of peptide 1-6 (P1-6) is shown. Taking the pre-immune and immune blood for sera preparations are indicated. The time point of the isolation of mononuclear blood cells for the construction of the cDNA-VHH-library is marked. (B) Development of Bet v 1-specific IgG responses in the serum of the immunized camel after five immunizations with Bet v 1 and Bet v 1-derived peptides (Day 63). OD values (y-axis) correspond to the amount of bound IgG antibodies and are shown as mean of duplicates with a variation of less than 5%

Three rounds of panning were conducted against Bet v 1 resulting in a strong enrichment of phages displaying Bet v 1-specific nanobodies. Forty-eight clones were picked after the third round of panning and their nanobody-coding sequences were PCR amplified and investigated using fingerprint-like restriction analysis. This analysis allowed us to identify three major groups (variants) of VHH sequences in a ratio of 25 clones to 17 clones to 2 clones.

Two to three representatives from each identified group of clones as well as all unique

variants (4 variants out of 48 were left ungrouped) were further used to produce the corresponding soluble nanobody (in the format of VHH with C-terminal linker, HA-tag, and His-tag) in bacterial periplasmic extracts. These extracts were analyzed for their reactivity to Bet v 1 and for control purposes to Casein/BSA by ELISA. All tested clones were confirmed to recognize Bet v 1 but not Casein/BSA. After verified binding to Bet v 1, detailed sequence analysis of the binders revealed typical VHH sequences having hallmark amino acid substitutions (from hydrophobic to more hydrophilic amino acids: V37F, G44E/K, L45R, W47G) in the region corresponding to the framework 2 of VH sequence of a classical antibody, which represents the site of the interface between VH and VL (Figure S1). Furthermore, all three VHH variants show sequences with two cysteine residues characteristically for VHHs reflecting possible bond formation between complementarity-determining region (CDR)1 and CDR3 to stabilize the nanobody paratope conformation (Figure S1). For further analysis, we selected one representative clone from each VHH variant and designated them Nb23, Nb24, and Nb32. These sequence data have been submitted to the GenBank database (www.ncbi.nlm.nih.gov/genbank) under accession numbers MZ708605, MZ708606, and MZ708607. The detailed comparison of their sequence revealed that they share highly homologous frameworks but differ slightly in their CDR hypervariable regions resulting in one amino acid residue substitution in each CDR (Figure S1).

3.2 Expression and purification of Bet v 1-specific nanobodies

Nanobodies (Nb23, Nb24, and Nb32) were expressed as C-terminally HA- and His-tagged proteins in *E. coli* XL1-Blue and purified via affinity chromatography. Theoretical isoelectric points of 7.00 (Nb23), 8.62 (Nb24), and 8.62 (Nb32) and molecular masses of 19.37 kDa, 18.93 kDa, and 19.30 kDa, respectively, were determined for each nanobody (13–15 kDa) including linker, HA-tag, and His-tag (~5 kDa). The analysis by SDS-PAGE and Coomassie staining as well as Western blot both under reducing (Figure 2A) and non-reducing conditions (Figure 2B) revealed purified nanobodies of a size of approximately 20 kDa corresponding to the estimated molecular masses due to the C-terminally added tags.

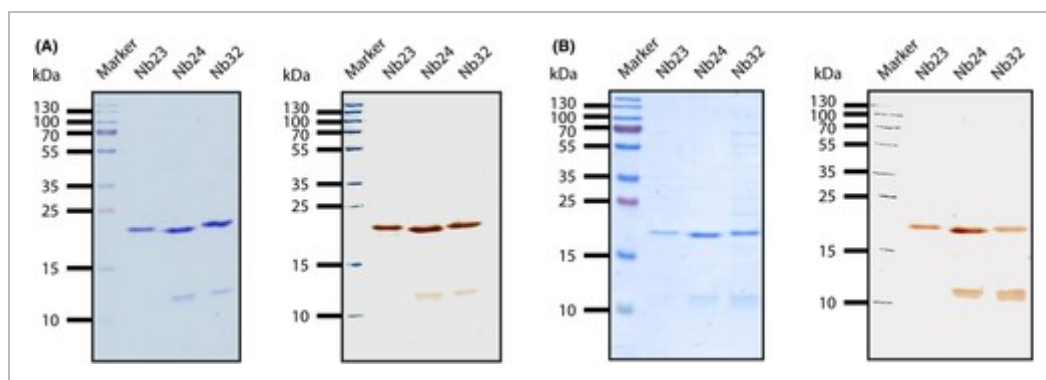


FIGURE 2

Coomassie brilliant blue-stained SDS-PAGE of Bet v 1-specific nanobodies and nitrocellulose-blotted Bet v 1-specific nanobodies detected with HRP-labeled mouse anti-HA-tag antibody under (A) reducing and (B) non-reducing conditions. Lane 1: protein molecular mass marker; lane 2: purified Nb23, 0.5 μ g; lane 3: purified Nb24, 1 μ g; lane 4: purified Nb32, 1 μ g. Molecular masses (in kDa) are indicated on the left margin

3.3 Cross-reactivity and epitope mapping of Bet v 1-specific nanobodies

To investigate the reactivity of Bet v 1-specific Nb23, Nb24, and Nb32 to the Bet v 1-homologous allergens Aln g 1 (alder), Cor a 1 (hazel), and Mal d 1 (apple), ELISA experiments were performed (Figure 3A). All three nanobodies recognized Aln g 1 and Cor a 1 with similar intensity but did not bind to Mal d 1. After increasing the avidity of Bet v 1-specific nanobodies by formatting nanobody trimers (as previously described²⁸), Mal d 1 was recognized but with lower intensity compared to Aln g 1 and Cor a 1 (data not shown). Incubation of allergens with detection antibodies only gave no signal (Figure 3A: buffer). Additionally, we tested the binding capability of Nb23, Nb24, and Nb32 to allergens in birch, alder, and hazel pollen extracts (Figure 3B). All three nanobodies reacted with natural (n)Bet v 1 and nAln g 1 and to a lower degree to nCor a 1 presumably caused by a mixture of several nCor a 1 isoforms available in hazel pollen extracts.^{29, 30} These results were confirmed by the detection of bound nanobodies to blotted extracts (Figure S2).

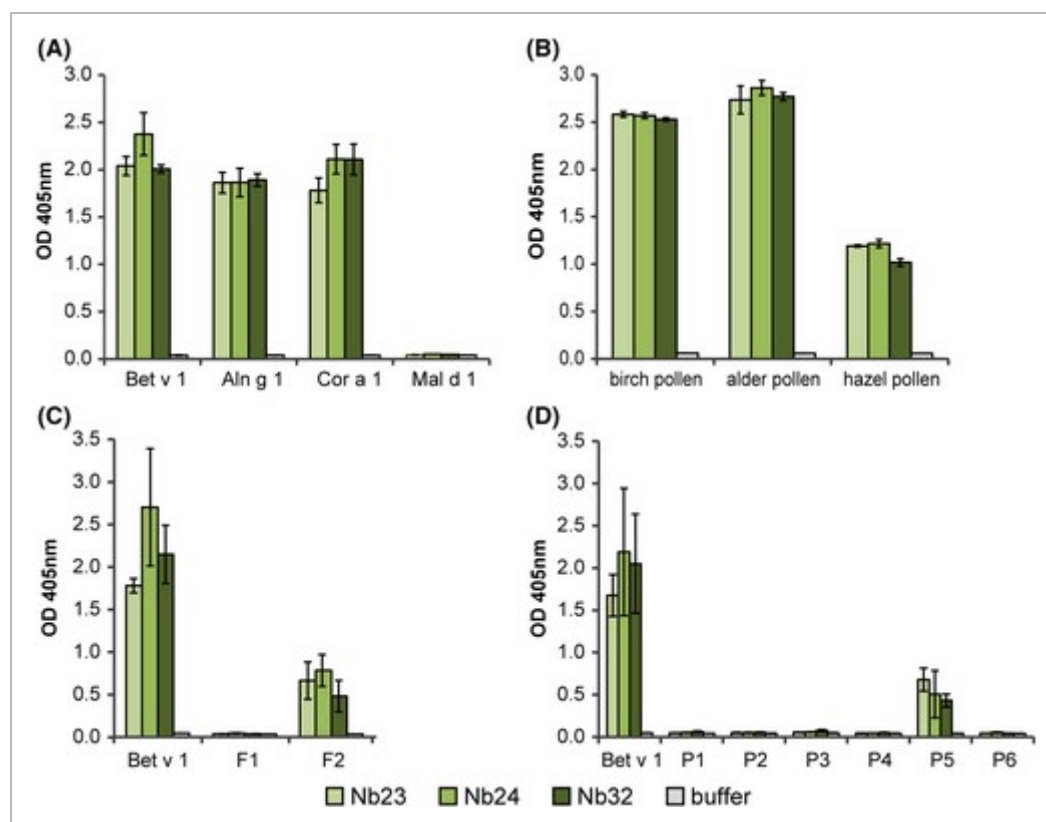


FIGURE 3

(A) Reactivity of Nb23, Nb24, and Nb32 to plate-bound Bet v 1 and related allergens from alder (Aln g 1), hazel (Cor a 1), and apple (Mal d 1). (B) Reactivity of Nb23, Nb24, and Nb32 to natural Bet v 1, Aln g 1, and Cor a 1 from birch, alder, and hazel pollen extracts, respectively. (C) Reactivity of Nb23, Nb24, and Nb32 to two Bet v 1 fragments (F1 and F2) and (D) to six Bet v 1-derived peptides (P1-6). OD values (y-axes) correspond to the amount of bound nanobodies and are shown as mean of triplicates $\pm$ SDs

Next, we intended to map the binding sites of all three nanobodies on Bet v 1 by using ELISA-plated recombinant Bet v 1 fragments (F1 and F2) (Figure 3C) and, for a more detailed analysis, six smaller synthetic Bet v 1-derived peptides (P1-6, Table S1), containing each 25 to 32 amino acids covering almost the whole allergen sequence (Figure 3D). It revealed that Nb23, Nb24, and Nb32 bound specifically to the C-terminal fragment 2 (F2; aa 75–160) (Figure 3C) and to the C-terminal peptide 5 (P5; aa 130–160) (Figure 3D).

3.4 Nanobodies bind to Bet v 1 with high affinity and have a slow dissociation rate

Interaction between Bet v 1-specific nanobodies and Bet v 1 was assessed using surface plasmon resonance (SPR). For all nanobodies, a comparable binding behavior could be observed with almost identical complex stabilities (dissociation rates $k_d = 2 \cdot 10^{-4}$ /s which equals a complex half-life of $\sim$ 1 h). When nanobodies were applied in lower concentrations (<30 nM), recorded (black), and calculated (red) curves were superimposable using the 1:1 binding model indicating a monomeric binding between nanobodies and Bet v 1 (Figure 4). If administered in higher concentrations (>30 nM) neither the 1:1 binding model nor the bivalent analyte model perfectly described recorded curves, which pointed to a mixture of nanobody monomers and dimers in solution above a certain threshold (Figure S3).

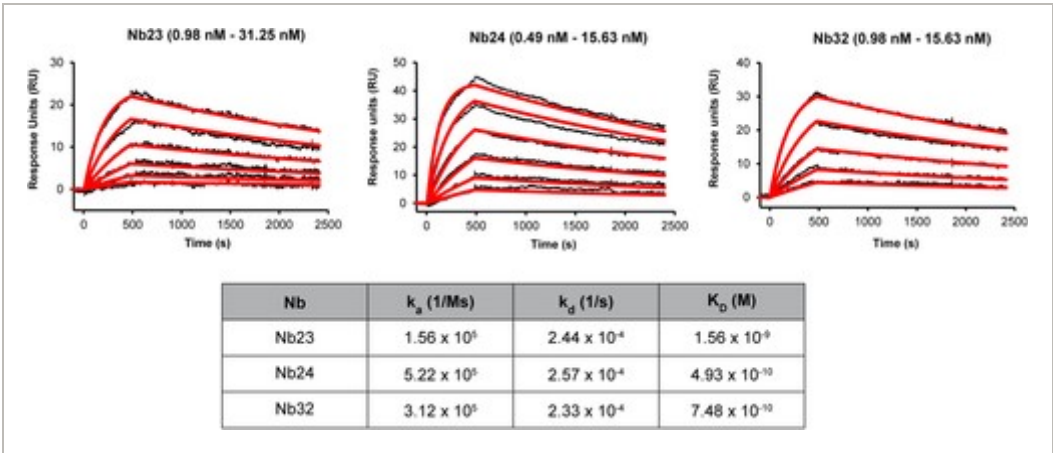


FIGURE 4

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Sensor chip-based study of the interaction between Bet v 1 and Bet v 1-specific nanobodies Nb23, Nb24, and Nb32. Bet v 1 was immobilized on the chip and nanobodies were injected in twofold increasing concentrations. Recorded (black lines) and calculated (red lines) curves, which represent fittings of the data to a 1:1 binding model, were superimposed.

Signal intensities (RU) are displayed (y-axes) versus time (in seconds; x-axes). Association rate constants (k_a), dissociation rate constants (k_d) and dissociation constants (K_D) are indicated

3.5 Bet v 1-specific nanobodies compete with polyclonal IgE for binding to Bet v 1, Aln g 1, and Cor a 1

To check whether the isolated nanobodies can inhibit polyclonal IgE from binding to Bet v 1, plate-bound allergen was incubated with a 10-fold excess of purified nanobodies and then exposed to birch allergic patients' sera containing specific IgE. Figure S4A shows a similar blocking activity of all nanobodies for three representative patients (Patient 1, 2, and 3). Combining all three nanobodies (ie. each still 10-fold excess) did not enhance the inhibition of IgE binding to Bet v 1. Since all three nanobodies yielded comparable IgE inhibition, Nb32 was selected as representative nanobody for further experiments. To determine the critical nanobody titer for competition with polyclonal IgE binding to Bet v 1, increasing concentrations of Nb32 were applied. By employing sera of patients 1, 3, and 5 a nanobody concentration of at least 5 µg/mL was found to achieve the maximum inhibitory effect (Figure S4B). Testing the cross-protectivity of Nb32, the effect of 10 µg/mL Nb32 (to ensure 10-fold molecular excess) on IgE binding to allergens Bet v 1, Aln g 1, and Cor a 1 was investigated with sera from 22 birch pollen-allergic patients (Table 1). Nb32 caused a mean inhibition of IgE binding to Bet v 1 of 56.9% ranging from 40.6 to 74.3% (Table 1, column 4). The IgE binding to Bet v 1-related allergens was reduced by mean values of 43.2% for Aln g 1 (ranging from 0–71.0%) and 12.4% for Cor a 1 (ranging from 0 to 45.7%) (Table 1, column 7 and 10).

TABLE 1. Inhibition of birch allergic patients' IgE binding to Bet v 1, Aln g 1, and Cor a 1 which had been pre-incubated with Nb32 or buffer

	Nb32	buffer	% Inhibition to Bet v 1	Nb32	buffer	% Inhibition to Aln g 1	Nb32	buffer	% Inhibition to Cor a 1
Patient 1	0.662	2.129	68.9	0.699	1.342	47.9	0.743	0.957	22.4
Patient 2	0.492	1.300	62.1	0.162	0.307	47.1	0.301	0.426	29.4
Patient 3	0.852	2.240	62.0	0.390	0.481	18.9	0.705	0.578	0.0
Patient 4	0.948	1.748	45.7	0.464	0.766	39.4	1.194	1.309	8.8
Patient 5	0.899	2.562	64.9	0.365	0.928	60.7	0.972	1.093	11.1
Patient 6	1.050	3.555	70.5	0.753	2.150	65.0	0.959	1.767	45.7
Patient 7	0.693	2.702	74.3	0.453	0.747	39.3	0.443	0.408	0.0

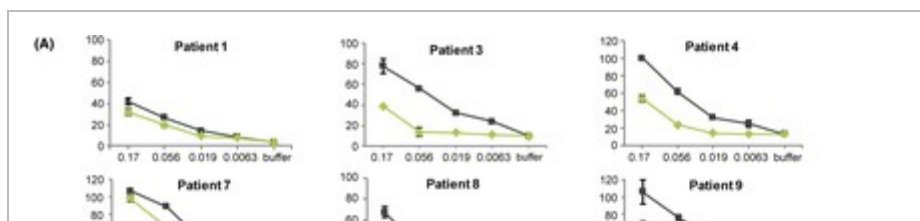
	Nb32	buffer	% Inhibition to Bet v 1	Nb32	buffer	% Inhibition to Aln g 1	Nb32	buffer	% Inhibition to Cor a 1
Patient 8	0.521	1.942	73.2	0.114	0.395	71.0	1.108	1.476	24.9
Patient 9	1.087	2.723	60.1	0.400	1.050	61.9	1.405	1.644	14.5
Patient 10	0.319	0.658	51.5	0.255	0.478	46.7	0.371	0.420	11.7
Patient 11	0.630	1.096	42.5	0.117	0.304	61.4	0.412	0.422	2.3

Notes

ELISA plate-bound allergens were pre-incubated with Nb32 or buffer and subsequently exposed to patients' sera. Displayed OD values correspond to allergen-bound IgE antibodies and are shown as mean of triplicates. Percentage of reduction of IgE-reactivity to Bet v 1, Aln g 1, and Cor a 1 pre-incubated with Nb32 in comparison to buffer are indicated in columns 4, 7, and 10, respectively.

3.6 Bet v 1-specific nanobodies reduce Bet v 1-induced basophil activation

To further analyze the capacity of the Bet v 1-specific nanobodies to block IgE-mediated activation of effector cells, basophil activation assays using rat basophilic leukemia (RBL) cells and Nb32 were performed. For this purpose, RBL cells were loaded with IgE from 12 birch pollen-allergic patients and the mediator release induced by Bet v 1 compared to Bet v 1 complexed with Nb32 was measured. Figure 5A shows that Nb32 reduced basophil degranulation of all tested patients but to a varying degree. While the reduction of β -hexosaminidase release was more pronounced for patients 3, 4, 9, and 11 ranging between 30-fold (ie, patient 11) to threefold (i.e., patient 3), Nb32 had limited protective effect for all other patients. These results revealed that Nb32 is a potential competitor for polyclonal IgE binding for certain but not all birch pollen-allergic individuals. This was also evidenced by testing human basophils from four Bet v 1-allergic patients incubated with Bet v 1 alone or complexed with Nb32. As shown in Figure 5B, Nb32 slightly reduced the percentage of activated basophil in all tested samples. These results further confirmed earlier observations³¹ that besides the prominent IgE epitope on the C-terminus of Bet v 1, additional IgE epitopes on Bet v 1 need to be blocked to achieve efficient prevention of mediator release.



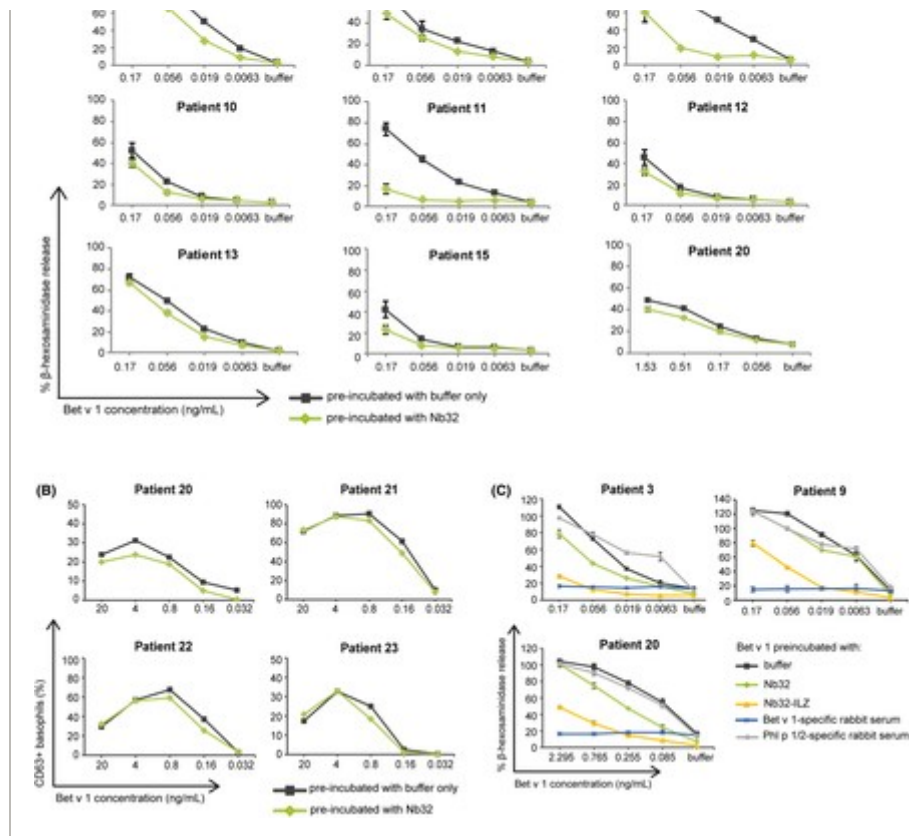


FIGURE 5

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(A) Rat basophilic leukemia (RBL) cell assay to determine the potential of Nb32 to suppress Bet v 1-induced IgE-mediated degranulation of basophils. RBL cells transfected with human Fc ϵ RI were loaded with sera from 12 birch pollen-allergic patients. Decreasing concentrations of Bet v 1 (x-axes) were pre-incubated with Nb32 (green lines) or buffer (black lines) and then added to the IgE-loaded cells. The percentage of β -hexosaminidase release induced by Bet v 1 is displayed on the y-axes in relation to total β -hexosaminidase amount of lysed cells. Values are shown as means of triplicates $\pm$ SDs. (B) Basophil activation test (BAT) to determine the potential to reduce activation of human basophils. Decreasing concentrations of Bet v 1 (x-axes) were pre-incubated with 10 times excess of Nb32 (green line) or buffer (black line) before being added to blood samples of four birch pollen-allergic patients (Patient 20–23). CD63+ basophils are displayed on the y-axes as percentage of the total basophil count. (C) RBL cell assay with sera from three patients and Bet v 1 pre-incubated with buffer (black lines), Nb32 (green lines), Nb32 trimer (Nb32-ILZ, yellow lines), Bet v 1-specific rabbit serum (blue lines), and Phl p 1/2-specific rabbit serum gray lines). Percentage of β -hexosaminidase release induced by Bet v 1 is displayed on the y-axes in relation to total β -hexosaminidase amount of lysed cells. Values are shown as means of triplicates $\pm$ SDs

To address this issue, we tested the efficiency of multivalent nanobody constructs (i.e., Nb32 trimer) and of polyclonal Bet v 1-specific rabbit immune serum on the IgE-mediated degranulation of RBL cells and compared the blocking effects with the capacity of Nb32 to reduce β -hexosaminidase release. It revealed that binding to the same epitope on multiple Bet v 1 molecules, as exemplified by the multivalent Nb32 trimer, had a superior blocking effect compared to the monovalent Nb32 (Figure 5C). Bivalent antibodies targeting various

different epitopes on one Bet v 1 molecule, as contained in the polyclonal Bet v 1-specific rabbit serum, caused the strongest reduction of β -hexosaminidase while bivalent antibodies specific for unrelated allergens (i.e., Phl p 1 and Phl p 2 from grass pollen) had no effect on the mediator release (Figure 5C).

4 DISCUSSION

Our study is the first to report the immunological characterization of Bet v 1-specific nanobodies. Three nanobodies (Nb23, Nb24, and Nb32) were isolated from an immune library derived from a camel that was immunized with recombinant Bet v 1 and synthetic Bet v 1-derived peptides. The immunization induced high affinity nanobodies that not only recognize Bet v 1 but also homologous pollen allergens from alder and hazel. Since Bet v 1 exhibits a broad cross-reactivity with pollen allergens resulting in the prolongation of the allergic symptoms beyond the birch pollen season,^{1, 32, 33} the ability of an antibody/nanobody to bind those cross-reactive allergens is crucial for developing a protective treatment to reduce birch pollen-allergic patients' symptoms.³⁴ On the other hand, the nanobodies showed limited binding to Mal d 1 from apple which may be explained by the lower sequence identity between Mal d 1 and Bet v 1 in the mapped epitope compared to Aln g 1 and Cor a 1, respectively.³⁵ However, it has already been demonstrated that patients vaccinated with recombinant Bet v 1 also fail to develop Mal d 1 cross-blocking IgG antibodies.¹⁷

The nanobodies' binding site is located in the C-terminal alpha-helix of Bet v 1, which has been identified as one important IgE epitope.³¹ Since Bet v 1 contains multiple IgE epitopes,³⁶ one single antibody or nanobody cannot suffice to completely block IgE binding and IgE-mediated basophil activation. However, formatted nanobody trimers capable to bind three Bet v 1 molecules simultaneously exhibited pronounced reduction of mediator release. In fact, several studies have shown that two or more antibodies are needed to fully inhibit allergen-induced basophil activation.^{18, 20, 37, 38} Nevertheless, an observed mean inhibition of 57% of IgE binding exhibited by Nb32 is in line with monoclonal Bet v 1-specific antibodies or antibody fragments using similar *in vitro* assays (ranging from 20% to 77%),^{20, 35, 36, 39-42} indicating that our Bet v 1-specific nanobodies comprise great potential to represent one component of a future mixture of protective nanobodies for treatment of birch pollen allergy.

SPR-detected high affinity of $10^{-9}/10^{-10}$ M of all three nanobodies resulted from measured fast association and slow dissociation rates. We cannot completely exclude that the slightly varying association rates of our nanobodies derived from different ratios of active and non-active amounts in applied nanobody fractions due to purification or storage but the difference could also be caused by the distinct usage of amino acids in the three CDRs, most prominently in the CDR3. Dissociation rates that are determined independently from the concentration of injected reagents, were proven for all three nanobodies to be in the range of 10^{-4} /s demonstrating equal dissociation rates reported for allergen-specific monoclonal

antibodies.^{18, 20} Since those monoclonal antibodies were effective in proof-of-principle clinical studies,^{19, 21} our generated Bet v 1-specific nanobodies represent promising tools as well for prospective clinical use.

Allergy is a comparatively new field for application of nanobodies. Apart from an anti-IgE nanobody as a novel class of disruptive IgE inhibitor,⁴³ the first allergen-specific nanobodies have been described very recently.^{44, 45} Nanobodies specific for the major peanut allergen Ara h 3⁴⁴ and for the newly identified macadamia nut allergen Mac j 1⁴⁵ have been developed with the aim to detect allergen concentration in food sources. Notably, both studies yielded nanobodies with affinity in the range of $10^{-7}/10^{-8}$ M representing medium affinity binding which points to the remaining difficulty of isolating allergen-specific nanobodies with high affinity similar to allergen-specific monoclonal antibodies.

To the best of our knowledge, we are the first to generate allergen-specific nanobodies with high affinity and slow dissociation rates that compete with IgE antibodies for allergen binding. Our isolated nanobodies recognize an important IgE binding site, an area also bound by REGN5713, one antibody component of the Bet v 1-specific antibody cocktail recently proven to be efficient to block acute allergic symptoms.²⁰

This study provides evidence for the applicability of nanobodies for passive immunotherapy of allergy, similar to monoclonal antibodies as has been shown only recently.¹⁸⁻²¹ Since nanobodies *per se* have a very short *in vivo* half-life, several strategies to extend the *in vivo* half-life of nanobody-based constructs have been developed.⁴⁶ As most appropriate formats, we consider to engineer bi-specific nanobodies with specificities against Bet v 1 and albumin due to its high abundance and long circulation in the blood. Furthermore, we intend to elaborate on multivalent Bet v 1-specific nanobody constructs to increase molecular masses.^{27, 28, 47} Both approaches will foster the biological activity and hence reduce the frequency of administration of nanobodies for treatment over the duration of a 3 months tree pollen season. In this context, it was recently demonstrated that large quantities of Bet v 1-specific monoclonal antibodies (i.e., 900 mg/patient) were needed to sufficiently inhibit allergen-induced mast cell degranulation during the entire birch pollen season.^{20, 21} Once reformatted, Bet v 1-specific nanobodies may represent an attractive cost-effective alternative to monoclonal antibodies worth to be tested for their clinical efficacy. Furthermore, passive administration of pre-selected formatted Bet v 1-specific blocking nanobodies may also be considered as more convenient treatment approach compared to anti-histamines which have to be applied every day and still bear some side effects.

It has been repeatedly shown that nanobodies intrinsically comprise a low immunogenicity due to shared similarities with the VH domains of human IgG3.^{48, 49} This high homology facilitates the humanization process and renders humanized camel antibody less immunogenic than humanized mouse antibody, for example, Omalizumab.⁵⁰ It was further demonstrated recently that even non-humanized nanobodies have a low capacity to activate

dendritic cells and consequently a low potential to induce T-cell proliferation.⁴⁹ Humanization of our bi-specific and multivalent nanobody constructs will be the next step prior to their usage in *in vivo* studies based on the knowledge of safety studies of nanobody-based drugs already used in clinical trials like Caplacizumab, the first therapeutic nanobody approved by the FDA and EMA.⁵¹

Their robustness, single domain organization, ease of formatting and production, and low immunogenicity combined with illustrated high affinity to a crucial IgE epitope of Bet v 1 make our Bet v 1-specific nanobodies attractive tools for development of novel nanobody-based drugs. In summary, our results reveal for the first time Bet v 1-specific nanobodies being suitable first candidates for a nanobody cocktail to ameliorate symptoms of birch pollen-allergic patients.

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CONFLICT OF INTEREST

The authors have no financial conflicts of interest. The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

SF and ST designed the experiments, IZ, TI, MS, CW, OG, EK, MR, and ST performed experiments, IZ, MS, AD, BB, SF, and ST analyzed data. MF supported CW to generate peptides. MF provided peptides. IZ and SF wrote the manuscript. All authors critically revised the article and finally approved the version to be published.

Supporting Information



Filename	Description
all15191-sup-0001-FigS1.tif TIFF image, 24.9 MB	Fig S1
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all15191-sup-0003-FigS3.tif TIFF image, 24.9 MB	Fig S3
all15191-sup-0004-FigS4.tif TIFF image, 24.9 MB	Fig S4
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Nb23 MAQVQLQVQLVESGGSVYRGGSLRLSCAASGYSYSSYGMANFRQAPGKERESLAFLDWDGSLDYADSVKG (71)
 Nb24 ---MALQVQLVESGGGVQSGGSLRLSCAASGYTYSYGMANFRQAPGKKEQVAIEDIDGSTDYADSVKG (68)
 Nb32 MAQVQI QVQLVTEGGGSVYSGGSLRLSCAASGYTYSYGMANFRQAPGKKEEVAFLDWDGSLDYADSVKG (71)

 CDR1 CDR2
 Nb23 RFTISQDAAKNTLYLQNNSLAPKDTANYYCAAYGGSCARGGSGSYWGQGTQVTVSSLEPKLPQPKPQPKP (142)
 Nb24 RFATISQDAKNTLYLQNNSLKPEDTANYYCAAYGGSCARGGSGSYWGQGTQVTVSSLEPKLPQPKPQPKP (139)
 Nb32 RFATISQDAKNTLYLQNNSLKPEDTANYYCAAYGGSCARGGSGSYWGQGTQVTVSSLEPKLPQPKPQPKP (142)

 CDR3
 Nb23 QPQPKPQPKPFRPSGRYPYDWFDDYASGRGSHHHHHH (178)
 Nb24 QPQPKPQPKPFRPSGRYPYDWFDDYASGRGSHHHHHH (175)
 Nb32 QPQPKPQPKPFRPSGRYPYDWFDDYASGRGSHHHHHH (178)

 HA-tag 6x His-tag

Figure S1_Zettl et al.

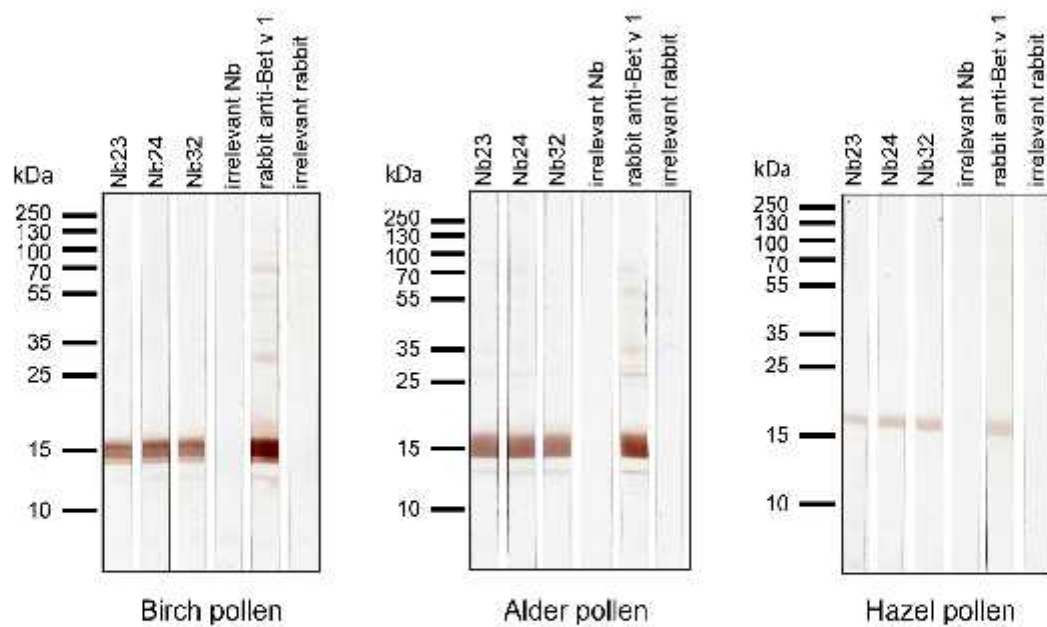


Figure S2_Zettl et al.

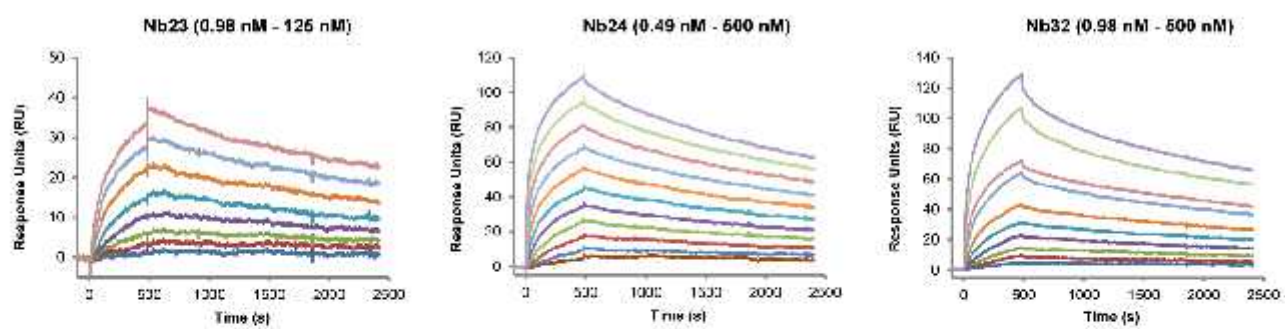


Figure S3_Zettl et al.

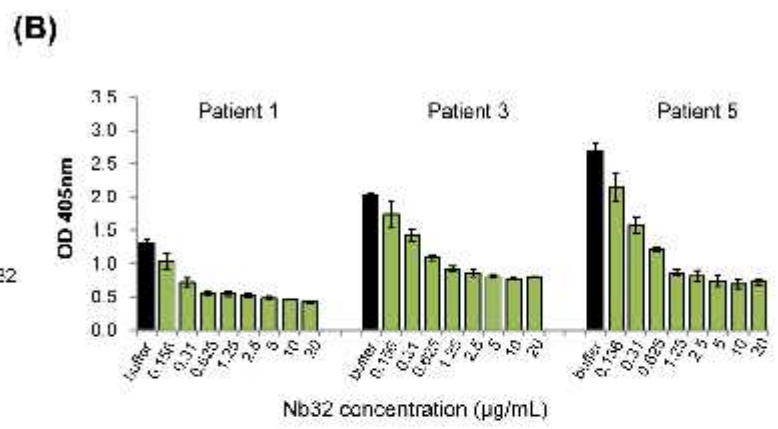
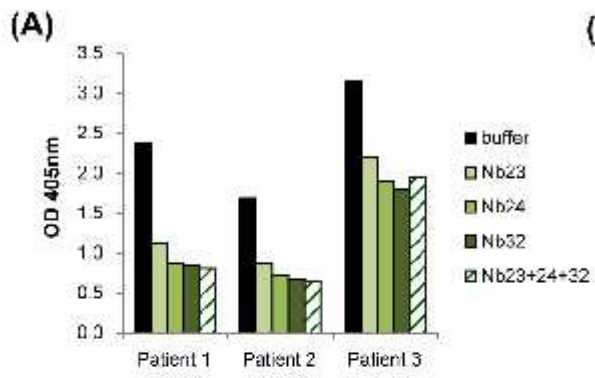


Figure S4_Zettl et al.

Table S1. Details of synthetic peptides used for immunization

	Position aa	Sequence	Number of aa
Peptide 1	1-24	MGVFNYETETTSVIPAARLFKAFIC	25
Peptide 2	30-59	LFPKVAPQAISSVENIEGNGGPGTIKKISFC	31
Peptide 3	50-79	CGPGTIKKISFPEGFPFKYVKDRVDEVDHTN	31
Peptide 4	110-139	DGGSILKISNKYHTKGDHEVKAEQVKASKEC	31
Peptide 5	130-160	CKAEQVKASKEMGETLLRAVESYLLAHSDAYN	32
Peptide 6	75-104	CVDHTNFKYNYSVIEGGPIGDTLEKISNEIK	31

Position, sequence and length of Bet v 1-derived synthetic peptides

Data S1

Isolation of nanobodies with potential to reduce patients' IgE binding to Bet v 1

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METHODS

Allergens, pollen extracts, antibodies, patients' sera

Recombinant allergens Bet v 1 (birch), Aln g 1 (alder), Cor a 1 (hazel) and Mal d 1 (apple) were obtained from Biomay AG (Vienna, Austria). Bet v 1 fragments 1 and 2 were kindly provided^{E1}. Bet v 1-derived peptides 1-6 were freshly synthesized for the camel's immunization as previously described (Table S1)^{E2}. Birch, alder and hazel pollen were purchased from Allergon (Ängelholm, Sweden). Pollen (3 g) were suspended in 20 mL 10 mM potassium phosphate buffer (pH 7.0) containing 200µL protease inhibitor cocktail (EDTA-free, Stock: 1 tablet in 10 mL H₂O; Sigma-Aldrich, St. Louis, MO, USA), stirred for two hours at room temperature and centrifuged for one hour (20,000 rpm, 4°C). The supernatant was dialyzed against ddH₂O for 48 hours at 4°C and lyophilized. The dry extract was dissolved in ddH₂O and the total protein content was determined by MicroBCA (Thermo Fisher Scientific, Waltham, MA, USA). Mouse anti-HA-tag antibody labeled with HRP was purchased from Sigma-Aldrich. Donkey anti-His₆-tag antibody labeled with HRP was purchased from Invitrogen (Carlsbad, CA, USA). Mouse anti-human-IgE labeled with AKP was purchased from BD Pharmingen (San Diego, CA, USA). Sera were obtained from patients suffering from birch pollen allergy according to case history, skin prick testing and IgE serology after informed consent was obtained. Serum and blood samples were analyzed in an anonymized manner with permission from the Ethics committee of the Medical University of Vienna (EK641/2014).

Camel immunization with the major birch pollen allergen, Bet v 1 and Bet v 1-derived peptides

All animal work was undertaken in strict accordance with recommendations of the National Standard of the Russian Federation GOST R 53434–2009. The camel immunization was carried out at the Scientific-Experimental Base "Chernogolovka" of the Severtsov Institute of Problems of Ecology and Evolution at the Russian Academy of Sciences (Chernogolovka, Russia) using a camel kept at the Center for Collective use "Live Collection of Wild Mammals". The work was approved on 11.02.18 (registration number 17) by the Commission on Bioethics formed on May 3, 2017 in the Severtsov Institute of Problems of Ecology and Evolution.

A young 1.5-year-old male camel (*Camelus bactrianus*) was immunized for the first time in its life. Before starting the immunization process, we verified that no Bet v 1-specific antibodies were available in the camel's pre-immune serum. The immunization was carried out by 5-stage subcutaneous injections (each time the injection was carried out at 4-5 sites relatively accessible for piercing and located at the upper part of the sides of the body or in the neck region). The second injection was carried out 3 weeks after the first, and the next 3 injections 8-14 days after the previous one. Complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA) for the first stage and incomplete Freund's adjuvant (Difco Laboratories) for the subsequent stages were used as adjuvants for the immunization. The adjuvants were mixed in equal proportions with the antigen solution right before immunization (typically, it was a mix of 4 and 4 mL).

To aim for a nanobody repertoire that besides being highly diverse and specific for a broad epitope spectrum also targets already identified important IgE epitopes of Bet v 1, we used both the full-length Bet v 1 protein and Bet v 1-derived peptides (25-32 aa long) corresponding to the surface-exposed areas of this protein (Table S1)^{E2}. Earlier

immunization of rabbits and mice with the selected peptides led to the successful generation of peptide-specific antibodies that inhibit patients' IgE binding to Bet v 1 by competing for these IgE epitopes^{E2,E3}.

For immunization, peptides and full-size protein conjugated to a carrier protein were used in the following order: conjugates with Keyhole Limpet Hemocyanin (KLH, My Bio Sourc Inc., San Diego, CA, USA) for the first three stages of immunization, and conjugates with Bovine Serum Albumin (BSA, Amresco, Solon, OH, USA) for the two final stages. For the first four steps, 500 µg of each peptide and the full-length protein conjugated with an equal weight of the carrier protein were taken for injection. For the last step, 200 µg of conjugates and 500 µg of full-length unconjugated Bet v 1 protein were taken. We used different carrier proteins in order to focus the spectrum of induced antibodies on Bet v 1 epitopes, and not on epitopes of the given carrier protein. Peptides were conjugated to the carrier protein in individual reactions using the glutaraldehyde cross-linking procedure as described^{E4}. Full-size recombinant Bet v 1 protein was conjugated to the carrier protein using disuccinimidyl suberate (DSS) crosslinker according to the protocol of the manufacturing company (Thermo Fisher Scientific).

The final blood sample (150 mL) was taken 6 days after the fifth injection to analyze the Bet v 1-specific IgG titer and to gain peripheral blood mononuclear cells (PBMCs) for the construction of a cDNA-VHH-library. To prevent clotting of the blood, an equal volume of phosphate buffered saline (PBS) containing heparin (100 U/mL) and EDTA (3 mM) was added to the blood.

Monitoring of the anti-Bet v 1 IgG titer available in the blood of the immunized camel was carried out using ELISA plates (MaxiSorp, Nunc, Roskilde, Denmark) immobilized with recombinant Bet v 1 (2µg/ml in 1x PBS). After several washing steps (once in 1x PBS, twice in 1x PBS with 0.05% Tween 20 (1x PBST), or 1x

PBST, and in 1x PBS once again), immune and pre-immune sera samples (diluted from 1:100 to 1:12800 in 1x PBS) were added for 2 hours incubation on a rotating platform at room temperature. After several washing steps, secondary anti-camel IgG polyclonal affinity-purified rabbit antibodies conjugated to HRP (previously obtained by Tillib *et al.*, unpublished) were added in concentration of 1 µg/ml. After 1 hour incubation and the final thorough washing step, the activity of the bound peroxidase was detected using ABTS (Sigma-Aldrich) as a chromogenic substrate. The optical density was measured at a wavelength of 405 nm using a microplate fluorometer. Results of the ELISA experiments are expressed as means of duplicate determinations with a deviation of less than 5%.

Construction of a cDNA-VHH library and selection of anti-Bet v 1 nanobody-coding sequences by phage display

The cloning of cDNA-sequences encoding the entire set of variable domains (VHHs) of specific heavy chain-only antibodies from PBMCs of immunized camel (RNA isolation, cDNA synthesis, two-stage PCR with specific primers, insert restriction and purification, and insertion into a phagemid vector) was performed as previously described^{E5–E9}. This procedure resulted in a new specific nucleotide sequence library of nanobody-coding sequences in the pHEN4 phagemid vector^{E10}. This library consisted of two sub-libraries that were different in the restriction sites used for insertion into the phagemid vector of sequences coding for antigen recognition domains, (i) NcoI + NotI and (ii) PstI + NotI. *E. coli* cells (strain TG1) were transformed by electroporation with recombinant plasmid DNA obtained by ligation of the two insert sub-libraries and two corresponding vector sequences. Standard cloning, bacterial and bacteriophage M13 procedures were performed following the

techniques described^{E11,E12}. The generated VHH-library satisfied the criteria of 1×10^7 clones and about 85% of inserts of the expected size.

The selection of nanobody-coding sequences by phage display, production of nanobodies in the bacterial periplasm, and the analysis of the nanobodies' ability to recognize a given antigen were performed using the previously described techniques^{E6-E8}. Briefly, recombinant Bet v 1 (10 μ g in 100 μ L of 1x PBS) was immobilized on ELISA plates (MaxiSorp) overnight at 4°C. Casein blocking buffer (Sigma-Aldrich) and 1% BSA (Sigma-Aldrich) in 1x PBS were used as alternating blocking agents. On average, approximately 10^{11} phage particles in 100 μ L from the original library were introduced into a well with the immobilized Bet v 1. After incubation and multiple washing steps (in 1x PBS and in 1x PBST), bound phages were eluted in 1.4% triethylamine and neutralized adding equal volume of 1 M Tris-HCl, pH 7.2. The eluted phages were used to infect growing bacterial *E. coli* K12/TG1 cells with a purpose to amplify the eluted phages for the next round of selection. M13KO7 bacteriophage (New England Biolabs, Ipswich, MA, USA) was used as a helper phage. The phage display selection procedure was performed with alternating blocking proteins for 3 cycles. After the final round of selection eluted phages were used to infect *E. coli* K12/WK6 cells and after 30 minutes of incubation infected cells were plated on LB agar (with 100 μ g/ml ampicillin and 1% glucose) in Petri dishes. Forty-eight clones of nanobody-coding sequences were analyzed in terms of DNA primary structures (fingerprint-like analysis as described^{E6}) or/and by custom DNA sequencing (Center of Collective Use «Genome», Engelhard Institute of Molecular Biology, Russian Academy of Science, Moscow, Russia). The binding of the corresponding nanobodies obtained from the bacterial periplasm to Bet v 1 was confirmed by ELISA. Three major groups (variants) of cloned and selected nanobody-coding sequences were identified representing 52% (including clone 24),

35% (including clone 32) and 4% (including clone 23) of all selected clones correspondingly. The selected cDNA sequences (clones 23, 24 and 32) encoding the three selected major nanobody groups/variants were subcloned (by conventional or PCR cloning) into the pHEN6 expression plasmid^{E13} together with the pelB leader sequence (for periplasmic production), camel upper hinge (the longest hinge variant) and two short tag sequences (HA-tag and (His)6-tag) at the C-terminus coding region to obtain the formatted nanobodies^{E7,8} that are easy to purify (using Ni-NTA-agarose) and to detect (with antibodies against HA-tag).

Formatting of anti- Bet v 1 nanobodies (Nb-ILZ) to form trimers

Initially selected cDNA sequences encoding anti- Bet v 1 nanobodies (Nb23, Nb24 and Nb3) were formatted (by conventional or PCR cloning) with an addition to their C-terminal part of a trimerizing isoleucine zipper domain (ILZ) as described^{E7}. The ILZ sequence is responsible for the posttranslational trimerization of the formatted nanobody (Nb23-ILZ, Nb24-ILZ, Nb32-ILZ)^{E7}.

Determination of DNA sequences corresponding to nanobodies and their derivatives (Nb-ILZ)

DNAs were prepared (NucleoBond Xtra Maxi plus, Macherey-Nagel, Düren, Germany) and sequences were confirmed by custom DNA sequencing (Eurofins Genomics, Ebersberg, Germany). DNA sequences were translated (ExPASy, Swiss Institute of Bioinformatics, Geneva, Switzerland) and amino acid sequences were aligned with the multiple sequence alignment tool ClustalW (European Bioinformatics Institute, Cambridge, UK).

Expression and purification of nanobodies as monomers or trimers

Phagemid vector pHEN6 containing the DNA of nanobodies specific for Bet v 1 were transformed into *E. coli* XL1-Blue competent cells (Agilent Technologies, Santa Clara, CA, USA) and plated on LB-plates containing 100 µg/mL ampicillin and 1% glucose. Single colonies were picked and incubated in 4 mL 2x YT medium containing 100 µg/mL ampicillin and 1% glucose overnight at 37°C. Cultures were transferred into 200 mL fresh 2xYT-medium (supplemented with 80µg/mL ampicillin and 0.1% glucose) each, and after an OD600 of 0.5 – 0.7 was reached, the temperature was reduced to 30°C and expression was induced by adding 0.2 mM IPTG. After incubation at 30°C overnight, cultures were centrifuged (3,500 rpm, 4°C, 15 min) and nanobodies were extracted from the periplasm by incubation of the pellet with 4 mL TES buffer (50 mM Tris pH 8.0, 0.5 mM EDTA, 20% saccharose) containing 10 mM imidazole, 100 µg/mL PMSF and 5 mM β-mercaptoethanol, and subsequently with 6 mL 10 mM Tris buffer (pH 8.0) containing 1 mM MgCl₂ for 30 min on ice each. The suspension was centrifuged again (14,000 rpm, 4°C, 30 min) and the supernatant containing nanobodies was purified by affinity chromatography using HIS-Select® Nickel Affinity Gel (Sigma-Aldrich) according to protocol of Qiagen (The QIAexpressionist™, fifth edition, 2003). Purified nanobodies were dialyzed against DPBS (Gibco, Life Technologies, Carlsbad, CA, USA) containing 10 mM imidazole using 3K Amicon Ultra centrifugal filter tubes (Millipore, Billerica, MA, USA). Concentrations were determined by absorption at 280 nm (DeNovix DS-11 FX+ spectrophotometer, Wilmington, DE, USA) and Bradford-based colorimetric protein assay (Bio-Rad, Hercules, CA, USA). Extinction coefficients, theoretical isoelectric points and molecular masses for all nanobodies, Nb23, Nb24, Nb32 and Nb23-ILZ, Nb24-ILZ and Nb32-ILZ, were calculated using the Expasy tool ProtParam.

A successful purification was checked by SDS-PAGE (14%) under reducing and non-reducing conditions following Coomassie Brilliant Blue staining. Nanobodies were mixed with 4x sample buffer (62.5 mM Tris/HCl, 2.5% SDS, 8.5% glycerol, 0.025% bromophenolblue). To achieve reducing conditions 5% (v/v) beta-mercaptoethanol was added to the 4x sample buffer and samples were boiled at 95°C for 5 min before loading. Nanobodies were separated and blotted onto nitrocellulose membrane (0.2 µm, Amersham Protran, GE Healthcare). The membrane was blocked with buffer A (40 mM Na₂HPO₄, 0.6 mM NaH₂PO₄, 0.5% BSA, 0.5% Tween 20, 0.05% NaN₃) for 30 minutes and incubated with mouse anti-HA-tag (1:5000 in buffer A) or donkey anti His₆-tag antibodies (1:2000 in buffer A) coupled with HRP for two hours at room temperature. Bound antibodies were traced with HRP-substrate DAB (Diaminobenzidine, 0.6 mg/mL, Roth, Karlsruhe, Germany) in 50 mM Tris/HCl (pH 7.3).

Reactivity of nanobodies to Bet v 1 and to cross-reactive allergens Aln g 1, Cor a 1 and Mal d 1

4 µg/mL of recombinant Bet v 1, Aln g 1, Cor a 1 and Mal d 1 (Biomay) as well as 10µg/mL of birch, alder and hazel pollen extracts were coated onto ELISA plates (MaxiSorp) at 4°C overnight. Wells were washed three times with 1x PBST and blocked with 1x PBST containing 1% (w/v) BSA for three hours at room temperature. Plates were washed three times and purified Nb23, Nb24 and Nb32, diluted in 1x PBST containing 0.1% (w/v) BSA to a final concentration of 1 µg/mL, were incubated overnight at 4°C. Bound nanobodies were traced with HRP-labeled mouse anti-HA-tag antibody (1:4000 in PBST/0.1%BSA) and HRP substrate ABTS (1mg/ml, Sigma-Aldrich). ODs were measured at 405 nm and a reference wavelength of 495 nm with

a TECAN Infinite F50 microplate reader (Männedorf, Switzerland). Results are shown as means of triplicates and error bars indicate standard deviation (SD).

Reactivity of nanobodies to blotted pollen extracts

200 µL pollen extract (birch, alder and hazel) mixed with 4x sample buffer were loaded on 14% acrylamide gels and separated under reducing conditions. Separated proteins were blotted onto a nitrocellulose membrane which was subsequently cut into 0.5 cm wide stripes. Stripes were blocked with buffer A for 30 min and incubated with Nb23 (5µg/mL), Nb24 (5µg/mL), Nb32 (5µg/mL), unrelated Nb (negative control, 5µg/mL), Bet v 1-specific rabbit serum (1:5000) or unrelated rabbit serum (negative control, 1:5000) (diluted in buffer A) for two hours at room temperature. Stripes were washed again with buffer A and bound nanobodies and antibodies were detected by incubating with HRP-labeled mouse anti-HA-tag (1:5000 in buffer A) or donkey anti-rabbit (1:2000 in buffer A) antibodies, respectively, for two hours at room temperature. The stripes were washed once more and bound detection antibodies were visualized with HRP-substrate DAB (0.6 mg/mL, Roth, Karlsruhe, Germany) in 50 mM Tris/HCl (pH 7.3).

Identification of the binding sites of Bet v 1-specific nanobodies

For epitope mapping, ELISA plates were coated with 4 µg/mL Bet v 1, Bet v 1 fragment 1 (aa 1-74) and 2 (aa 75-160)^{E1} or 5µg/mL Bet v 1 peptides 1-6 encompassing the entire Bet v 1 sequence^{E2} and saturated as described. Purified nanobodies diluted 1:10 in PBS/0.1% BSA were incubated overnight at 4°C and

detected as detailed before. Results are shown as means of triplicates and error bars indicate standard deviation (SD).

Affinity studies of nanobodies by plasmon surface resonance

Affinity studies by plasmon surface resonance were performed on a Biacore 3000 (Cytiva, formerly GE Healthcare, Sweden) at 25°C. The surface of a sensor chip CM5 was activated by injecting a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) for 7 minutes at a flow rate of 5 µL/min. Bet v 1, diluted 10 µg/mL in 10 mM acetate buffer pH 4.5 according to pH scout, was immobilized. The surface was deactivated with 35 µL 1M ethanolamine at a flow rate of 5 µL/min. The reference flow cell was activated and deactivated as described above.

Multicycle kinetics were repeatedly conducted to determine kinetic rate constants for Nb23, Nb24 and Nb32. Nanobodies diluted in HBS-EP (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% vol/vol surfactant P20 (pH 7.4)) were injected in two-fold increasing concentrations (0.49 nM – 500 nM; 15.31nM as duplicate) over reference and Bet v 1 surface for 8 min with a flow rate of 30 µL/min. Dissociation was investigated for 30 min in buffer flow. Regeneration of the chip surface was established by injecting 10 mM glycine-HCl (pH 1.5) three times for 30 seconds at 30 µL/min followed by injecting HBS-EP for 60 min to guarantee a stable baseline for the next cycle. Dissociation constants (K_D) and rate constants (on-rate: k_a , off-rate: k_d) were calculated with BIAevaluation software 3.2 (GE Healthcare) using a 1:1 (Langmuir) binding model.

Inhibition of allergic patients' IgE binding to ELISA plate bound Bet v 1, Aln g 1 and Cor a 1 by nanobodies

ELISA plates were coated with 1 µg/mL Bet v 1 overnight at 4°C. Wells were washed three times with 1x PBST and saturated with 2% (w/v) BSA in 1x PBST. In a pilot experiment, all three nanobodies applied individually and in combination (Nb23+24+32) were used at a predefined concentration of 10 µg/mL first to evaluate their specific potential to inhibit patients' IgE binding to Bet v 1. In a separate setting, Nb32 was applied in 2-fold serial decreasing dilutions ranging from 20 µg/mL to 0.15 µg/mL in 1x PBST containing 0.5% (w/v) BSA (PBST/0.5%BSA) to determine the optimal concentration of Nb32 for patients' IgE inhibition. Based on the results of these pilot experiments, for the following study Nb32 was used in a concentration of 10 µg/mL to ensure excess of nanobodies.

ELISA plates coated with 1 µg/mL Bet v 1, Aln g 1 and Cor a 1, washed and saturated as described above were incubated with Nb32 overnight at 4°C. After washing with 1x PBST sera from patients allergic to Bet v 1, diluted 1:10 in PBST/0.5%BSA, were added overnight at 4°C. Bound Bet v 1-specific IgE was traced with AKP-coupled mouse anti-human-IgE (BD Pharmingen) diluted 1:1000 in PBST/0.5%BSA and pNPP (1 mg/mL, Sigma-Aldrich). ODs were measured at 405 nm with a reference wavelength at 550 nm and are shown as means of triplicates. Percentage inhibition of patients' IgE binding after pre-incubation with Nb32 compared to buffer was calculated as follows: $100 - (\text{OD after pre-incubation with Nb32} \times 100 / \text{OD after pre-incubation with buffer})$.

Influence of Nb32 and Nb32 trimer on Bet v 1-induced basophil degranulation tested by rat basophilic leukemia cell-based mediator-release assay and human basophil activation test

Rat basophilic leukemia (RBL) cells (RS-ATL8^{E14}) transfected with the human FcεRI were cultivated in Minimal Essential Medium (Gibco) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (Gibco), 2 mM L-Glutamine (Gibco), 100 U/mL Penicillin-Streptomycin (Gibco), 0.2 mg/mL Geneticin (Life Technologies) and 0.2 mg/mL Hygromycin B (Life Technologies). 1.5x10⁵ cells/well were seeded in transparent, flat-bottomed, sterile 96-well cell culture plates (Costar, Corning Incorporated, Corning, New York, USA) and incubated with allergic patients' sera diluted 1:10 in medium or with medium alone overnight at 37°C. Cells were washed thrice with Tyrode's buffer (Tyrode's salt, Sigma-Aldrich; with added 24 nM NaHCO₃) containing 0.1% BSA and then exposed for one hour at 37°C to different concentrations of Bet v 1 or Bet v 1 preincubated with Nb32. In detail, Bet v 1 (three-fold serial dilutions) had been preincubated with 20 µg/mL Nb32 diluted in buffer B (Tyrode's buffer with 50% D2O and 0.1% BSA) or with buffer B only for two hours at room temperature in flat-bottomed, transparent 96-well microplates (Greiner, Kremsmünster, Austria) before being added to the cells. For control purposes, cells loaded with IgE were incubated with buffer B only and cells not loaded with IgE were incubated with the highest concentration of Bet v 1 with or without Nb32 (20 µg/mL) to determine the background release. After one hour, six wells with cells only were lysed using Triton-X100 with a final concentration of 1% to determine the total β-hexosaminidase content (100%). All cell supernatants were transferred to a fresh 96-well microplate (Greiner) and mixed 1:1 with 0.16 mM 4-Methylumbelliferyl N-acetyl-β-D-galactosaminide (4-MUC, Sigma-Aldrich) in 0.1 M citric acid buffer (pH 4.2). β-hexosaminidase amounts were measured one hour after adding 4-MUC by

fluorescence (excitation wavelength: 360nm; emission wavelength: 465nm) with a
TECAN infinite M200pro plate reader. To investigate the influence of multivalent
binding partners on basophil activation, we also tested a nanobody trimer (Nb32-ILZ)
and polyclonal Bet v 1-specific rabbit immune serum. Nb32-ILZ in equimolar
concentration to Nb32 and rabbit serum diluted 1:5 were pre-incubated with three-
fold serial dilutions of Bet v 1 as described before. For control purposes, rabbit sera
specific for Phl p 1 or Phl p 2 were used. To define background levels cells were
loaded with IgE and incubated with buffer only, and cells not loaded with IgE were
incubated with Bet v 1, Bet v 1 + Nb32, Bet v 1 + Nb32-ILZ or Bet v 1 + rabbit sera.
Release of β -hexosaminidase was determined as detailed above. Allergen-specific
releases are given as percentages of the total mediator content^{E15}. All results are
shown as means of triplicates and error bars indicate standard deviation (SD).

For basophil activation tests (BATs), Bet v 1 (five-fold serial dilutions, 20 ng/mL –
0.032 ng/mL) was incubated with or without 10-fold excess of Nb32 (200 ng/mL –
0.32 ng/mL) in a HEPES Calcium buffer (HCB, pH 7.4) supplemented with BSA
(1 mg/mL) and IL-3 (2 ng/mL) for two hours at room temperature prior to addition of
heparinized whole blood of birch pollen-allergic individuals. Stimulation with fMLP
(Sigma-Aldrich) and anti-IgE antibody (SeraCare, Milford, MA, USA) served as
positive control for basophil activation and HCB with IL-3 was used as a negative
control. After incubation for 15 min at 37°C the reaction was stopped by addition of
HEPES/EDTA buffer. The cells were then stained with anti-CCR3 (APC), anti-CD123
(PerCP) and anti-CD63 (PE) antibodies (BioLegend, San Diego, CA, USA). After
erythrocyte lysis, the cells were washed and acquired on a FACSCanto II using
FACSDiva Software Version 6.1.3 (BD Biosciences, San Jose, CA, USA). Basophils
were defined as CD123+/CCR3+ cells and activated basophils were distinguished by
CD63+ expression.

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TABLES

Table S1. Details of synthetic peptides used for immunization^{E2}

	Position aa	Sequence	Number of aa
Peptide 1	1-24	MGVFN YETETT SVIPAARLFKAFIC	25
Peptide 2	30-59	LFPKVAPQA ISSVENIEGNGGPGTIKKISFC	31
Peptide 3	50-79	CGPGTIKKISFPEGFPFKYVKDRVDEVDHTN	31
Peptide 4	110-139	DGGSILKISNKYHTKGDHEVKAEQVKASKEC	31
Peptide 5	130-160	CKAEQVKASKEMGETLLRAVESYLLAHSDAYN	32
Peptide 6	75-104	CVDHTNFKYNYSVIEGGPIGDTLEKISNEIK	31

Position, sequence and length of Bet v 1-derived synthetic peptides

FIGURES

Figure S1. Sequence alignment of Bet v 1-specific nanobodies Nb23, Nb24 and Nb32. Distinct amino acids are highlighted in grey, cysteines that form interloop disulfide bridges are colored in orange, hallmark VHH amino acid substitutions (V37F, G44E/K, L45R, W47G) are marked in blue, complementary determining regions (CDRs) are boxed in red and tag sequences (HA-tag, 6x His-tag) are highlighted in yellow.

Figure S2. Blotted pollen extracts (birch, left; alder, middle; hazel, right) incubated with Nb23 (lane 1), Nb24 (lane 2), Nb32 (lane 3) and Bet v 1-specific rabbit serum (lane 5). An ICAM1-specific nanobody and Phl p 5-specific rabbit serum (lanes 4 and 6, respectively) were used as negative controls. Molecular masses (in kDa) are indicated on the left margin.

Figure S3. SPR binding sensorgrams of the interaction between Bet v 1 and Bet v 1-specific nanobodies Nb23, Nb24 and Nb32. Recorded curves (colored) represent nanobodies (two-fold dilutions ranging from 0.49 nM to 500 nM) binding to and dissociating from immobilized Bet v 1. Signal intensities (RU) are displayed (y-axes) versus time (in seconds; x-axes).

Figure S4. Pilot experiments for inhibition ELISAs. **(A)** The capacity of Nb23, Nb24, Nb32 and of their combination (Nb23+24+32) to inhibit patients' IgE binding (patient 1-3) to ELISA-bound Bet v 1 was determined and compared to IgE binding without hindrance (buffer). OD values (y-axis) corresponding to the amount of bound IgE are displayed as means of duplicates. **(B)** Titration of Nb32 (x-axis: two-fold increasing concentrations starting from 0.156 µg/mL to 20 µg/mL) to determine the critical nanobody concentration for prevention of IgE binding exemplified for three patients (Patient 1, 3 and 5). Shown OD values correspond to bound IgE and are displayed as means of triplicates ± SDs.