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.
INTRODUCTION

Birch pollen allergy ranks among the most prevalent pollen allergies in Northern and Central Europe. Estimated 100 million individuals worldwide suffer from this IgE-mediated disease leading to clinical manifestations such as hay fever causing a major health and economic burden. Bet v 1 is the sole major birch pollen allergen. It sensitizes more than 90% of individuals allergic to birch pollen and mainly contributes to pollen-associated food allergy. Representing such a clinically important allergen, Bet v 1 is one of the best-characterized allergens and was in the focus of various strategies to treat and ameliorate birch pollen allergy. 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. While the contribution of blocking IgG antibodies for the clinical success of allergen-specific immunotherapy (AIT) has been proven, 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. 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.

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. They combine several advantageous qualities like unique biochemical characteristics and low immunogenicity. 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.

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 $1 \times 10^7$ 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).

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 VHs 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 VH 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).

FIGURE 1 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%
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.

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).

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 \( k_d = 2 \times 10^{-4}/s \) which equals a complex half-life of ~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).

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).

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 \( \beta \)-hexosaminidase release was more pronounced for patients 3, 4, 9, and 11 ranging between 30-fold (ie, patient 11) to threefold (ie., 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 observations that aside from 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.

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, 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. 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. 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.

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. Since Bet v 1 contains multiple IgE epitopes, 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. 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%). 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. Since those monoclonal antibodies were effective in proof-of-principle clinical studies, 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, the first allergen-specific nanobodies have been described very recently. Nanobodies specific for the major peanut allergen Ara h 3 and for the newly identified macadamia nut allergen Mac j 1 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

### 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

| Patient | Nb32 % Inhibition to Bet v 1 | Buffer % Inhibition to Bet v 1 | Nb32 % Inhibition to Aln g 1 | Buffer % Inhibition to Aln g 1 | Nb32 % Inhibition to Cor a 1 | Buffer % Inhibition to Cor a 1 |
|---------|-----------------------------|-------------------------------|-----------------------------|-------------------------------|-----------------------------|-------------------------------|
| Patient 1 | 0.662 (68.9) | 0.699 (47.9) | 0.743 (22.4) | 0.757 (29.4) | 0.757 (29.4) | 0.757 (29.4) |
| Patient 2 | 0.492 (62.1) | 0.162 (47.1) | 0.301 (29.4) | 0.426 (29.4) | 0.426 (29.4) | 0.426 (29.4) |
| Patient 3 | 0.852 (62.0) | 0.390 (18.9) | 0.705 (57.8) | 0.0 (0.0) | 0.0 (0.0) | 0.0 (0.0) |
| Patient 4 | 0.948 (45.7) | 0.464 (39.4) | 1.194 (30.9) | 1.309 (8.8) | 1.309 (8.8) | 1.309 (8.8) |
| Patient 5 | 0.899 (64.9) | 0.365 (60.7) | 0.972 (10.9) | 1.093 (11.1) | 1.093 (11.1) | 1.093 (11.1) |
| Patient 6 | 1.050 (70.5) | 0.753 (65.0) | 0.959 (17.6) | 45.7 (45.7) | 45.7 (45.7) | 45.7 (45.7) |
| Patient 7 | 0.693 (74.3) | 0.453 (39.3) | 0.443 (40.8) | 0.0 (0.0) | 0.0 (0.0) | 0.0 (0.0) |
| Patient 8 | 0.521 (73.2) | 0.114 (71.0) | 1.108 (47.6) | 1.476 (24.9) | 1.476 (24.9) | 1.476 (24.9) |
| Patient 9 | 1.087 (60.1) | 0.400 (61.9) | 1.405 (16.4) | 1.644 (16.4) | 1.644 (16.4) | 1.644 (16.4) |
| Patient 10 | 0.319 (51.5) | 0.255 (46.7) | 0.371 (42.0) | 0.422 (11.7) | 0.422 (11.7) | 0.422 (11.7) |
| Patient 11 | 0.630 (42.5) | 0.117 (61.4) | 0.412 (42.2) | 2.3 (2.3) | 2.3 (2.3) | 2.3 (2.3) |
| Patient 12 | 0.589 (41.5) | 0.185 (26.8) | 0.411 (48.0) | 14.4 (14.4) | 14.4 (14.4) | 14.4 (14.4) |
| Patient 13 | 0.867 (40.6) | 0.123 (67.8) | 0.772 (81.6) | 5.4 (5.4) | 5.4 (5.4) | 5.4 (5.4) |
| Patient 14 | 1.963 (47.7) | 0.449 (65.0) | 2.211 (25.2) | 12.6 (12.6) | 12.6 (12.6) | 12.6 (12.6) |
| Patient 15 | 0.711 (45.0) | 0.309 (28.0) | 0.322 (22.3) | 0.0 (0.0) | 0.0 (0.0) | 0.0 (0.0) |
| Patient 16 | 0.275 (49.2) | 0.084 (28.0) | 0.292 (28.0) | 0.0 (0.0) | 0.0 (0.0) | 0.0 (0.0) |
| Patient 17 | 0.394 (51.5) | 0.089 (4.3) | 0.198 (17.0) | 0.0 (0.0) | 0.0 (0.0) | 0.0 (0.0) |
| Patient 18 | 1.637 (57.5) | 0.668 (65.6) | 0.818 (107.3) | 23.8 (23.8) | 23.8 (23.8) | 23.8 (23.8) |
| Patient 19 | 0.696 (74.0) | 0.456 (61.2) | 0.879 (121.2) | 27.5 (27.5) | 27.5 (27.5) | 27.5 (27.5) |
| Patient 20 | 0.819 (53.7) | 0.758 (32.1) | 0.325 (34.9) | 7.1 (7.1) | 7.1 (7.1) | 7.1 (7.1) |
| Patient 21 | 0.451 (60.2) | 0.158 (47.6) | 0.692 (78.2) | 11.6 (11.6) | 11.6 (11.6) | 11.6 (11.6) |
| Patient 22 | 0.825 (57.3) | 0.329 (21.8) | 0.276 (17.9) | 0.0 (0.0) | 0.0 (0.0) | 0.0 (0.0) |
| Nonallergic | 0.083 (0.047) | 0.069 (0.056) | 0.062 (0.055) | 0.0 (0.0) | 0.0 (0.0) | 0.0 (0.0) |

**Mean**

| Patient 1 | 56.9 | 43.5 | 12.4 |

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.
FIGURE 5  (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 FcεRI 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 (y-axes) is displayed on the y-axes in relation to total β-hexosaminidase amount of lysed cells. Values are shown as means of triplicates ± 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 ± SDs.
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.

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SUPPORTING INFORMATION
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