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Bet v 1 from birch pollen is a hypoallergen with vitamin D3 in the pocket

To the Editor,

Based on our previous molecular studies with ligands for lipocalin and lipocalin-like proteins, we predicted that the active vitamin D metabolite vitamin D3 (VD3) could bind into the intramolecular pocket of the major birch pollen allergen Bet v 1 (Bet v 1.0101),[1,2] transforming it from *apo* (empty) to *holo*-Bet v 1. As VD3 *per se* is able to modulate innate and adaptive immune responses,[3] we hypothesized that the binding of this immunomodulatory molecule would modify the allergenicity of Bet v 1.

In a first step, we analysed VD3 binding to Bet v 1 by *in silico* docking analysis, *in vitro* ANS competition assay and IZ-VDRE cell reporter assay (Figure 1). *In silico* calculations based on the crystal structure of the Bet v 1-naringenin complex (PBD entry 4A87) (Figure 1A) rendered an affinity energy of −9.9 kcal/mol, corresponding to a dissociation constant of 0.055 µmol/L. The calculated affinity of VD3 to Bet v 1, being thus 6-times stronger than of vitamin A metabolite retinoic acid,[1] could be supported by *in vitro* assays. First, in an ANS competition assay, VD3 dose-dependently displaced ANS from Bet v 1 (Figure 1B). For the second assay, we used the human IZ-VDRE cell line stably transfected with vitamin D response elements VDRE-I from the human *CYP24A1* promoter and expressing the gene for luciferase under the control of VDRE.[4] The

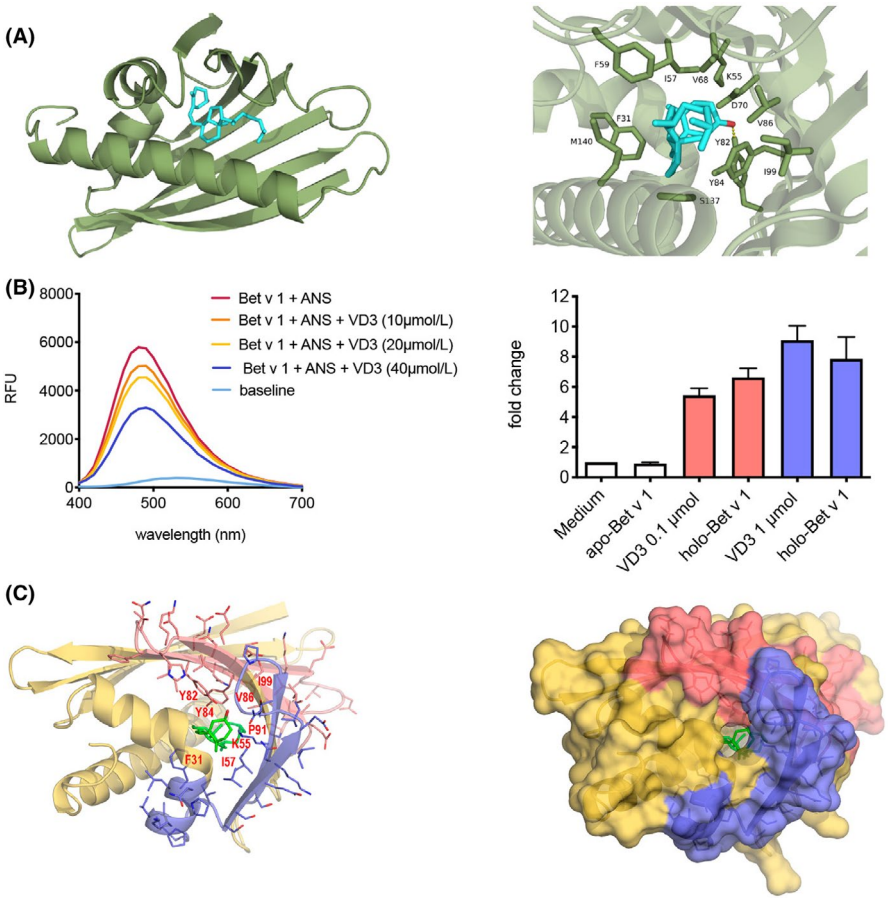

FIGURE 1 VD3 is a ligand to Bet v 1. (A) *In silico* docking analysis: crystal structure (left) and close-up view of the cavity (right) of Bet v 1 with ligand VD3 (sticks in turquoise); (B) ANS competition assay (left, RFU: relative fluorescence units) and IZ-VDRE reporter cell assay (right) with luciferase activity shown as fold change to medium control as means +/− SD from three independent experiments: (VD3 concentrations for stimulation and preincubation: salmon bars 0.1 µmol, violet bars 1 µmol); (C) Geometry of Bet v 1-VD3 complex and localization of B-cell epitopes: left diagram shows side chains of amino acids in both B-cell epitopes as sticks with carbons in slate blue (epitope 1: residues 29–58) and salmon (epitope 2: residues 73–103). Ligand VD3 is shown as stick with carbons in green. Red labels indicate all epitope residues that are within 4 Å of the ligand (epitope 1: F31, K55, I57; epitope 2: Y82, Y84, V86, P91, I99). Right diagram displays partially transparent renderings of surface views of the geometries shown in the left diagram

Abbreviations: ANS, 1-anilino-8-naphthalene sulfonate; BCA, bicinchoninic acid; BP, birch pollen; E_{aff}, affinity energy; FCS, foetal calf serum; IL, interleukin; K_D, binding constant; kDa, kilodalton; MFI, mean fluorescence intensity; OD, optical density; PBMCs, peripheral blood mononuclear cells; PDB, protein data bank; RA, retinoic acid; RFUs, relative fluorescence units; VD3, Vitamin D3; VDRE, vitamin D response element.

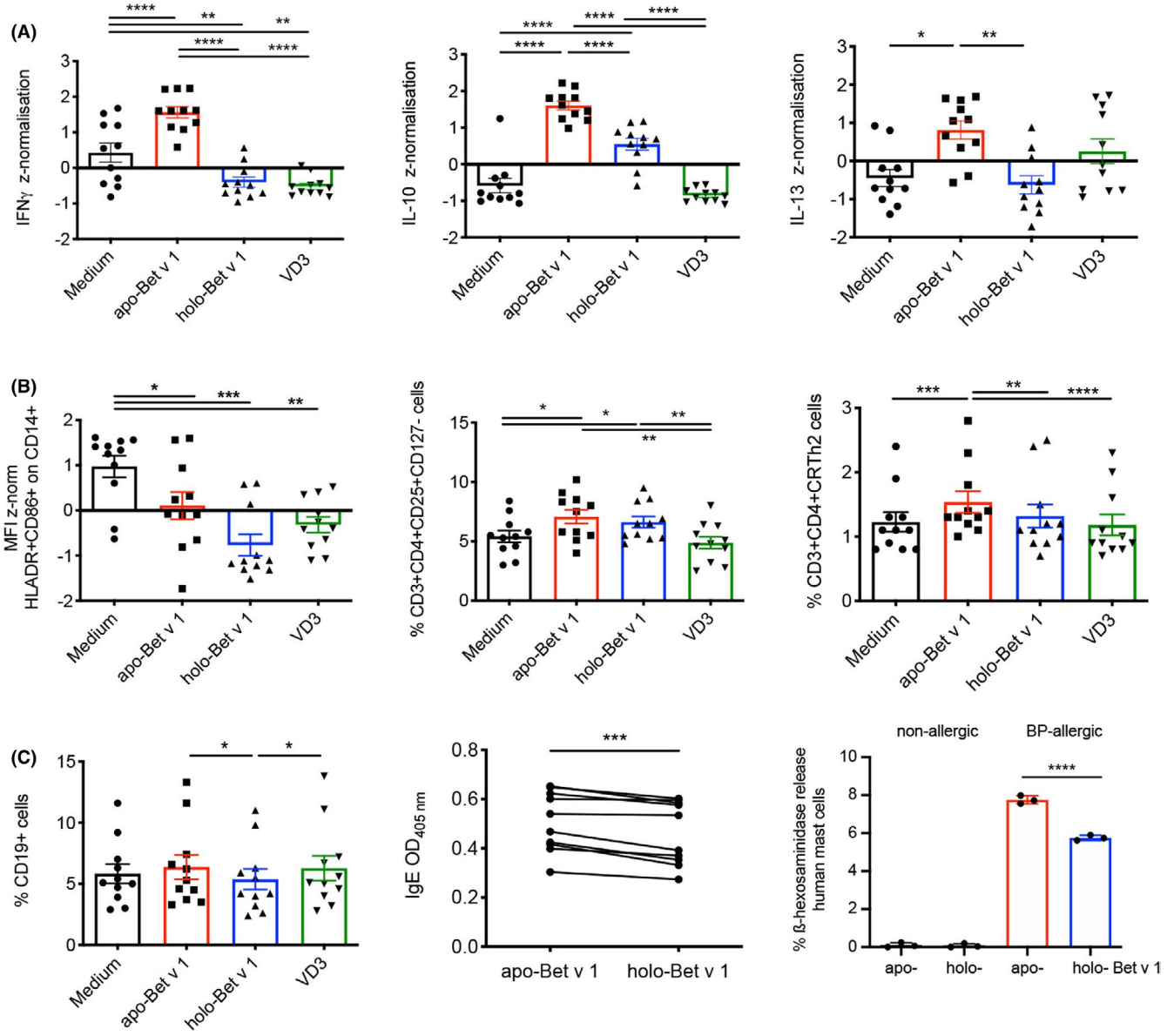

FIGURE 2 VD3 binding to Bet v 1 alleviates allergic immune response *in vitro*. (A) IFN-γ, IL-10 and IL-13 production in PBMCs from eleven BP-allergic donors stimulated *in vitro* with *apo*- or *holo*-Bet v 1 or VD3, z-normalization of pg/mL for each cytokine was performed as described in material and methods (ANOVA followed by Newman-Keuls Multiple Comparison Test); (B) MFI (z-normalization) of HLA-DR+CD86+ expression on CD14+ monocytic cells (left), relative number of regulatory CD3+CD4+CD25+CD127− T cells (middle), relative number of CD3+CD4+CRTh2 cells (right) (ANOVA followed by Newman-Keuls multiple comparison test); (C) relative number of CD19+ B cells (left) (ANOVA followed by Newman-Keuls multiple comparison test), IgE levels in sera of 10 BP-allergic individuals (middle) (ELISA, paired samples *t* test) and ß-hexosaminidase release from human mast cells sensitized with serum pools from non-allergic and BP-allergic donors (right) (ANOVA followed by Tukey's multiple comparison test) against *apo*- or *holo*-Bet v 1; means +/− SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$

luciferase activity was dose-dependently activated by VD3 (Figure S1), ensuring the functionality of the assay. Incubation of IZ-VDRE cells with *holo*-Bet v 1 (preincubated with 1 μmol or 0.1 μmol VD3) induced luciferase expression comparable to the signal from the positive control VD3 alone, while empty *apo*-Bet v 1 did not elicit luciferase activity (Figure 1B). We hypothesized that shuttling of VD3 via Bet v 1 induced the luciferase activity, although we cannot exclude intracellular dissociation of VD3 from Bet v 1. Nevertheless, our results so far proposed that Bet v 1 is able to bind VD3 into its intramolecular cavity *in vitro*.

We then investigated the influence of VD3 in *holo*-Bet v 1 on the cellular immune response. Stimulation of PBMCs from birch pollen (BP)-allergic donors with *apo*-Bet v 1 induced significant upregulation of Th1 (IFNγ) and Th2 (IL-10, IL-13) cytokines in supernatants compared to medium control (Figure 2A). In contrast, *holo*-Bet v 1-treated cells displayed significantly decreased IFNγ,

IL-10 and IL-13 levels in comparison with *apo*-Bet v 1 stimulated cells, similar to treatment with VD3 alone (Figure 2A). When transported in *holo*-Bet v 1, VD3 more than VD3 alone, reduced the expression of co-stimulatory molecules HLA-DR+ and CD86+ on CD14+ monocytes, indicating their reduced activation status (Figure 2B left). Remarkably, in the T-cell compartment, only *holo*-Bet v 1 treatment led to significant reduction of CD3+CD4+ cells expressing the Th2 marker CRTh2 (Figure 2B right). Prostaglandin D2 receptor CRTh2 is expressed by Th2 lymphocytes, eosinophils, basophils and innate lymphoid cells (ILC2s) and involved in recruitment of these cell types and Th2 cytokine production during allergic response.[5] The CD3+CD4+CD25+CD127- regulatory T cells were significantly increased by both *apo*- and *holo*-Bet v 1 treatments (Figure 2B middle). In the B-cell compartment, we found significantly reduced numbers of CD19+ B cells after treatment with the *holo*- but not with the *apo*-form of Bet v 1 (Figure 2C left). Reduction of IgE committed B cells may be one mechanism why VD3 previously inhibited IgE synthesis *in vitro*.[6]

The *holo*-Bet v 1 also showed significantly lower IgE-binding capacity, at least in some patients, compared to the *apo*-allergen (Figure 2C middle), and it induced significantly less mediator release from primary human mast cells pre-sensitized with serum IgE of BP-allergic donors (Figure 2C right; Figures S2 and S3). Based on our *in silico* structural analyses, the IgE-specific effects may be due to epitope masking by the VD3 ligand of two previously described IgE-binding B-cell epitope regions in the molecular surface of Bet v 1 (Figure 1C).

Thus, our data for the first time revealed VD3 as a novel ligand in the pocket of the major birch pollen allergen Bet v 1, and that VD3 is able to down-regulate the allergic immune response to Bet v 1. We evidence that *holo*-Bet v 1 shuttles VD3 into immune cells, prevents Th2 responses to Bet v 1 *in vitro* by reducing CRTh2+ Th2- and CD19+ B cells, inhibits antigen presentation and reduces production of inflammatory cytokines. The present data are in line with our findings that loading of Bet v 1 allergen with vitamin A metabolite induces immune tolerance.[1] The principle of vitamin spiking of allergens could be of use to improve immunotherapeutic approaches in future, and at much lower doses than recently suggested.[7]

KEYWORDS

Bet v 1, birch pollen allergy, immunomodulation, lipocalin-like, vitamin D

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Sublingual immunotherapy reduces reaction threshold in three patients with wheat-dependent exercise-induced anaphylaxis

To the Editor,

Wheat-dependent exercise-induced anaphylaxis (WDEIA) is a rare IgE-mediated systemic hypersensitivity reaction caused by the combination of wheat product ingestion and cofactors, such as physical exercise, acetylsalicylic acid and alcohol.[1–3] For diagnosis, an appropriate patient's history has to be associated with a sensitization to wheat flour and gluten proteins as detected by skin prick test (SPT), specific IgE and /or basophil activation test (BAT). Confirmation of the diagnosis by oral challenge test (OCT) with gluten and cofactors allows to determine the patient's individual threshold.[4] It has been shown that using high amounts of pure gluten for a challenge, a reaction can even be elicited even without cofactors.[5] Dietary recommendations range from total avoidance of gluten to temporal separation of wheat ingestion from cofactors. Gluten avoidance has been associated with lower reaction thresholds.[6] Currently, no curative treatment such as immunotherapy (IT) has been developed. We describe three patients with WDEIA, who have been treated with a sublingual gluten IT (SLIT) over a course of 3 years. The clinical study was approved by local ethics committee.

For this exploratory study, three female patients with a history of several reactions only to a combination of wheat products plus cofactors and positive challenge with pure gluten were diagnosed with WDEIA and gave their informed consent for SLIT. Titrated SPT with pure wheat gluten and wheat protein hydrolysates (Solpro

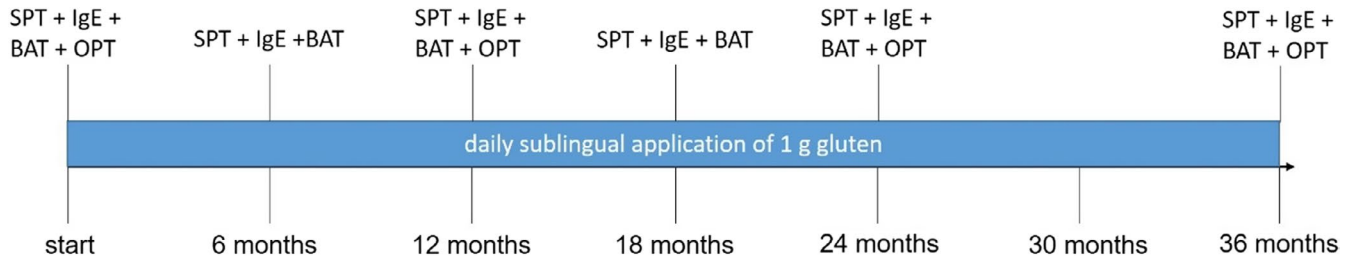

FIGURE 1 Timetable of the study