

Covalent Coupling of Vitamin D3 to the Major Cat Allergen Fel d 1 Improves the Effects of Allergen-Specific Immunotherapy in a Mouse Model for Cat Allergy

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Key Words

Allergen-specific immunotherapy • Cat allergy • Fel d 1 • Mouse model • Vitamin D3

Abstract

Background: Allergen-specific immunotherapy (SIT) is currently the only curative treatment for allergy but the treatment needs to be improved. We hypothesize that covalent coupling of immunomodulating vitamin D3 to the major cat allergen Fel d 1 can enhance the beneficial effects of SIT to cat allergy. **Methods:** We treated mice sensitized to Fel d 1 with subcutaneous injections of two doses of recombinant Fel d 1 coupled to 1 α ,25-dihydroxyvitamin D3 (rFel d 1:VD3) and compared to treatment with the same doses of rFel d 1 in a mouse model for cat allergy. Airway hyperresponsiveness (AHR), cytokines and cells in bronchoalveolar lavage (BAL), in vitro activation of splenocytes to rFel d 1, and Fel d 1-specific immunoglobulins were evaluated. **Results:** Treatment with both doses of rFel d 1:VD3 decreased AHR, cellular influx and Th2 cytokines in BAL compared to untreated mice. High- and low-dose rFel d 1 treatment also decreased AHR and BAL Th2 cytokines, with less decrease for the low-dose

treatment. Importantly, the total number of cells and eosinophils in BAL was markedly reduced at both high- and low-dose rFel d 1:VD3 compared to treatment with rFel d 1 alone. Finally, treatment with both rFel d 1 and rFel d 1:VD3 induced Fel d 1-specific serum IgG. **Conclusion:** Our results indicate a beneficial therapeutic effect of rFel d 1:VD3 on airway inflammation, AHR and rFel d 1-specific immune responses and thus suggest that this novel immunomodulatory candidate may improve both the efficacy and safety of SIT.

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Introduction

Allergen-specific immunotherapy (SIT) is currently the only curative treatment of allergic diseases that gives long-lasting relief of symptoms [1]. The treatment has now been practiced for a century, but the exact mechanisms of action are still not fully understood. Proposed mechanisms involve both humoral and cell-mediated responses. These include production of blocking IgG antibodies [2–4] and induction of a state of anergy/deletion or immune deviation (that is, a shift from a predominant-

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ly Th2-like to a more Th1-like phenotype) among allergen-specific T cells [5, 6]. Generation of regulatory T cells (Tregs) and IL-10 has also been demonstrated in successful SIT [7–10].

Current SIT involves some drawbacks including a long treatment period (3–5 years) with repeated administration of allergen and a risk of both local and systemic side reactions [11]. Many approaches for how to improve both the efficacy and safety of SIT have been proposed, including targeting of the allergen to Fcγ receptors on antigen-presenting cells (APCs) or effector cells [12, 13], targeting the MHC class II pathway [14], displaying the allergen on virus-like particles [15], linking to CpG-containing oligonucleotides [16, 17] or carbohydrate-based particles [18] and co-administration with immunomodulatory substances [19–21].

In order to improve SIT, it would be desirable to more specifically target allergen-specific regulatory mechanisms. One well-characterized immunomodulatory substance is 1α,25-dihydroxyvitamin D3 (VD3) [22]. Most effects of VD3 are exerted through binding to the vitamin D receptor that dimerizes with the retinoic X receptor, and this dimer binds to vitamin D-responsive elements in the promoter region of VD3-responsive genes. Many of these genes are involved in immunomodulatory mechanisms and the vitamin D receptor is expressed by a variety of immune cells [22, 23]. VD3 is capable of inducing dendritic cells (DCs) with tolerogenic properties by inhibiting differentiation and maturation of the DCs, and by enhancing their production of IL-10 [24–26]. These tolerogenic DCs have the ability to induce Tregs [27] and recent data also suggest that VD3 can act directly on CD4+ T cells downregulating pro-inflammatory cytokines and promoting a Treg phenotype [28, 29]. In this study, we hypothesize that by covalent coupling of VD3 to the recombinant (r) major cat allergen Fel d 1 (rFel d 1:VD3), the efficacy, and thereby also the safety, of SIT to cat allergy can be improved. We have evaluated rFel d 1:VD3 in a mouse model for cat allergy and compared the outcome of rFel d 1:VD3 treatment to treatment with rFel d 1 alone. We observed an effect of rFel d 1:VD3 superior to that of rFel d 1 on airway inflammation, reflected by reduced infiltration of total cells in general and eosinophils in particular, in bronchoalveolar lavage (BAL). Furthermore, rFel d 1:VD3 was more potent than rFel d 1 in reducing airway hyperresponsiveness (AHR). Our data support that VD3 directly linked to rFel d 1 may reduce the allergen-specific airway response, and thus is a promising candidate for use in SIT to cat allergy.

Methods

Animals

Female BALB/c mice (6–8 weeks) were purchased from Charles River Laboratories (Sulzfeld, Germany) and housed under 12-/12-hour light/dark cycle with food and water ad libitum. The experiments were approved by the local ethics committee for animal welfare.

Antigens

Cat dander extract (CDE) [18] and rFel d 1 were prepared as previously described [30]. VD3 was covalently coupled directly to rFel d 1, essentially as described by Jean et al. [31]. Briefly, rFel d 1 was dissolved in 0.1 M 2-(N-morpholino)ethanesulfonic acid and 0.15 M sodium chloride buffer (1.5 mg/ml), and VD3 (99%; Sigma-Aldrich, Steinheim, Germany) was dissolved in 99.5% ethanol (1.8 mg/ml). The VD3 solution was added to the rFel d 1 solution during constant stirring to a concentration of 1.1 mg/ml rFel d 1 and 0.48 mg/ml VD3. This was followed by addition of formaldehyde (>36.5% in water; Sigma-Aldrich) to the reaction mixture (21% of total volume). Ethanol was added to clear the solution (12% of final volume) and the reaction mixture was kept in the dark with constant stirring at room temperature overnight. The solution was stored at 4°C before exchanging the buffer to phosphate-buffered saline (PBS) using an NAP-10 column (GE Healthcare, Uppsala, Sweden).

The rFel d 1 contained 4.5 ng lipopolysaccharide (LPS)/mg protein and the rFel d 1:VD3 contained less than 0.8 ng LPS/mg protein as measured by Limulus amoebocyte lysate endochrome assay (Charles River Endosafe, Charleston, S.C., USA).

Mass Spectrometry

Coupling of VD3 to rFel d 1 was analyzed by electrospray ionization mass spectrometry (ESI-MS). Before application to ESI-MS, the sample was desalted using a ZipTip C18 10 μl pipette tip (Millipore, Billerica, Mass., USA). Data were acquired on a Q-TOF Ultima API mass spectrometer (Waters Corp., Milford, Mass., USA) equipped with a standard Z-spray API source in positive mode. The sample was introduced from a metal-coated borosilicate glass capillary needle (Proxeon Biosystems A/S, Odense, Denmark). The capillary voltage was 1.6 kV. Data were sampled over a mass range between 300 and 2,000 m/z with a scan time of 2 s for about 5 min. The spectra were combined and deconvoluted to zero charged ions (average mass) with MaxEnt 1 in Masslynx 4.0 software (Waters Corp.).

VD3 Activity Analysis

Upregulation of CD14 on human monocyte-derived dendritic cells (MDDCs) was used as a measure of VD3 activity in order to confirm that the VD3 coupled to rFel d 1 was still active. Peripheral blood mononuclear cells were separated from buffy coats from three healthy donors and MDDCs generated as previously described [32, 33]. On day 6, the MDDCs were harvested and CD14 as well as other surface markers were analyzed by flow cytometry before starting stimulation. The MDDCs were stimulated with PBS, 10 μg/ml rFel d 1, 10 μg/ml rFel d 1:VD3 or 5×10^{-8} M VD3 and after 48 h stimulation the cells were analyzed using flow cytometry as on day 6. In the stimulation of MDDCs from one donor, 0.045 ng/ml LPS was added to the rFel d 1:VD3 and VD3 stimulations in order to match the LPS content of the rFel d 1 stimulation.

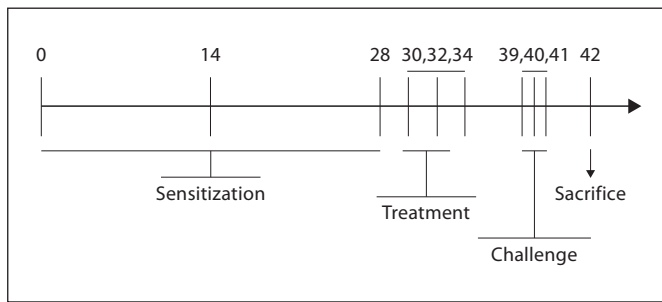


Fig. 1. Therapeutic treatment protocol. Groups of mice were sensitized with 1 μ g rFel d 1/alum s.c. on days 0, 14 and 28, treated with 5 or 10 μ g rFel d 1, 5 or 10 μ g rFel d 1:VD3, VD3 or PBS s.c. on days 30, 32 and 34 and challenged with 10 μ g CDE or PBS i.n. on days 39, 40 and 41 before sacrifice on day 42. $n = 8-10$.

Flow cytometry was performed on a BD FACSaria instrument (BD Biosciences, San Diego, Calif., USA) with FITC conjugated CD209 (clone MR-1; AbD Serotec, Oxford, UK) and CD40 (clone 5C3; BD Biosciences), PE conjugated CD83 and CD80 (clone HB15e and L307.4, respectively; BD Biosciences), PerCP conjugated HLA-DR (clone L243, G46-6; BD Biosciences) and PE-cyanine7 conjugated CD14 (clone HCD14; Biolegend, San Diego, Calif., USA) with corresponding isotype controls [IgG1 conjugated to FITC (clone X40; BD Biosciences), PE and PE-Cyanine7 (both clone MOPC-21; BD Biosciences) and IgG2a conjugated to PerCP (clone MOPC-173; Biolegend)]. Data were analyzed using FlowJo 7.6 software (Tree Star Inc., Ashland, Oreg., USA).

Therapeutic Treatment Protocol

Mice were sensitized subcutaneously (s.c.) with 1 μ g rFel d 1 adsorbed to 1 mg alum (Sigma-Aldrich) in 200 μ l PBS on days 0, 14 and 28. Groups of mice ($n = 8-10$) were treated s.c. with 5 or 10 μ g rFel d 1, 5 or 10 μ g rFel d 1:VD3, 20 ng VD3 (equal to the amount VD3 in the 10 μ g rFel d 1:VD3 treatment) in 200 μ l PBS or 200 μ l PBS alone (sham) on days 30, 32 and 34. VD3 was dissolved in dimethyl sulfoxide for storage and freshly diluted 38.5 times in PBS before injection. On days 39, 40 and 41, mice were challenged intranasally (i.n.) with 10 μ g CDE in a total volume of 20 μ l. Control mice were treated with 200 μ l PBS and challenged with 20 μ l PBS according to the same protocol (fig. 1).

Measurement of Airway Responsiveness

One day after the last i.n. challenge of the treatment protocol, intravenous metacholine (MCh)-induced AHR was measured with a small animal ventilator (Flexivent®; Scireq, Montreal, Que., Canada) as previously described [18]. Data are presented as peak respiratory resistance (R_{rs}) in response to 1 mg/kg MCh.

Bronchoalveolar Lavage

Lungs were lavaged with 1 ml PBS and total number of live cells was counted. The BAL fluid was collected and stored at -80°C until cytokine and Fel d 1-specific IgA analysis. BAL cells were resuspended in PBS, cytopun and stained with May-Grünwald-Giemsa (Sigma-Aldrich). For differential cell counts, a minimum of 300 cells/BAL sample were counted.

Lung Histology

Histocon® (HistoLab Products, Västra Frölunda, Sweden) was injected in the lungs of two mice per group. The lungs were removed and frozen in cryostat embedding medium (Bio-Optica, Milano, Italy). The lung specimens were stored at -80°C until preparation of 10- μ m cryostat sections. The sections were stained for mucus production with Periodic Acid/Schiff's reagent (Sigma-Aldrich) according to the manufacturer's instructions.

Splenocyte in vitro Proliferation

Cell suspensions were prepared from spleens and cultured as described earlier [18]. Briefly, spleens were passed through a 70- μ m cell strainer and splenocytes cultured in triplicates (2×10^5 cells/well), either unstimulated or stimulated with 10 μ g/ml rFel d 1 or 5 μ g/ml concanavalin A (Sigma-Aldrich) as positive control. All splenocyte cultures were able to proliferate in response to concanavalin A. After 5 days of culture, 20 μ l supernatant was removed and pooled for each triplicate and stored at -80°C until cytokine analysis. To each well, 1 μCi ^3H -thymidine in 10 μ l RPMI 1640 was added and the cells were cultured for an additional 18 h. Stimulation index (SI) values were calculated as triplicate mean counts per minute of rFel d 1-stimulated cells/triplicate mean counts per minute of unstimulated cells. $\text{SI} \geq 2$ was considered as positive.

Cytokine Analysis

Cytokine content in splenocyte in vitro culture supernatants and BAL fluid was analyzed by cytometric bead array (CBA; BD Biosciences) for IL-13, IL-5, IL-10 and IFN- γ according to the manufacturer's instructions. Results were calculated using FCAP Array™ software (BD Biosciences) and the detection limit was set according to the standard curves to 2.5 pg/ml for all cytokines except IL-10, for which the detection limit was set to 10 pg/ml. Stimulated cytokine levels in splenocyte culture supernatants were calculated by subtracting the amount in un-stimulated culture from the amount in rFel d 1-stimulated culture. If the cytokine amount in a sample was below the detection limit, it was set to the detection limit.

Determination of Fel d 1-Specific Antibodies

Blood was collected by cardiac puncture and serum was separated and stored at -80°C until analysis. ELISA was used for analysis of serum levels of Fel d 1-specific IgE, IgG1 and IgG2a, and BAL levels of Fel d 1-specific IgA. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 2 μ g/ml rat anti-mouse IgE (BD Biosciences) for IgE analysis, or 5 μ g/ml rFel d 1 for IgG1, IgG2a and IgA analysis. Sera were diluted 1:20 for IgE, 1:1,000 for IgG2a, and 1:25,000, 1:100,000 or 1:1,000,000 for IgG1. The amounts of IgG1 in the samples were pre-tested in order to determine the dilution where concentrations were within the detection range. BAL samples were used undiluted. Bound IgE was detected using 2 μ g/ml biotinylated rFel d 1 followed by incubation with alkaline phosphatase (AP)-conjugated streptavidine (diluted 1:3,000; Sigma-Aldrich). IgG2a, IgG1 and IgA were detected using goat anti-mouse IgG2a, IgG1 and IgA, respectively, conjugated to AP (diluted 1:1,000 for IgG2a, 1:5,000 for IgG1 and 1:1,000 for IgA; SouthernBiotech, Birmingham, Ala., USA). Phosphatase substrate (Sigma-Aldrich) was added and color development was monitored after 45 min for serum ELISA and 75 min for the BAL ELISA at 405 nm. Results are presented as OD values for

IgE, IgG2a and IgA, whereas the IgG1 results are presented as AU/ml calculated from a standard curve. All samples from the different groups were measured on the same plate for each isotype to allow comparisons between the groups. For analysis of antibodies capable of blocking binding of human Fel d 1-specific IgE, a blocking ELISA was performed essentially as described previously [34]. Briefly, a microtiter plate was coated with rFel d 1 as described earlier and pre-incubated with mouse sera diluted 1:80 for immunized/treated mice or 1:10 for a pool of sera from 8 naïve mice. This was followed by incubation with a pool of sera from cat-allergic patients diluted 1:32. The patients had a median IgE to cat of 24.5 kU/l (range 16–53) as determined by ImmunoCAP™ (Phadia AB, Uppsala, Sweden). Binding of human IgE was detected with rabbit anti-human IgE (diluted 1:2,000; MIA, Uppsala, Sweden) followed by AP-conjugated goat anti-rabbit IgG (diluted 1:1,000; Jackson ImmunoResearch Laboratories Inc., West Grove, Pa., USA) and phosphatase substrate. The blocking capacity was calculated and expressed in percent by $100 \times (1 - A/B)$ where A and B represent adjusted OD values for immunized/treated (A) and naïve (B) mice.

Statistical Analysis

Results are presented as median values. Differences between the treatment groups were evaluated by Kruskal-Wallis with Dunn's multiple comparisons test. When indicated, pairwise comparisons between rFel d 1 and rFel d 1:VD3 at the different treatment doses were evaluated using the Mann-Whitney U test. The analyses were performed using Graphpad Prism 5 software (Graphpad Software Inc., San Diego, Calif., USA). $p < 0.05$ was considered significant.

Results

Coupling of VD3 to rFel d 1

Analysis by ESI-MS revealed that the rFel d 1:VD3 preparation consisted of approximately 90% unmodified rFel d 1 and 10% of the rFel d 1 coupled to VD3 at a 1:1 ratio (fig. 2a, b). Thus, the peak at 19,593 Da, corresponding to the molecular weight of rFel d 1 + VD3 (fig. 2b), was approximately 1/10 the height of the peak at 19,176 Da, corresponding to free rFel d 1 (including a six-histidine tag; fig. 2a). No protein aggregates could be detected when analyzing rFel d 1:VD3 by SDS-PAGE (data not shown).

Activity of rFel d 1:VD3

Several studies have shown that addition of VD3 during differentiation or maturation of human MDDCs leads to upregulation of CD14 [25, 35]. Stimulation for 48 h with rFel d 1:VD3 and VD3 alone upregulated CD14 in MDDCs at least 40% compared to rFel d 1 stimulation. Expression of other DC markers (CD40, CD80, CD83, CD209 and HLA-DR) differed only marginally between the different stimulations (fig. 2c). To rule out that differ-

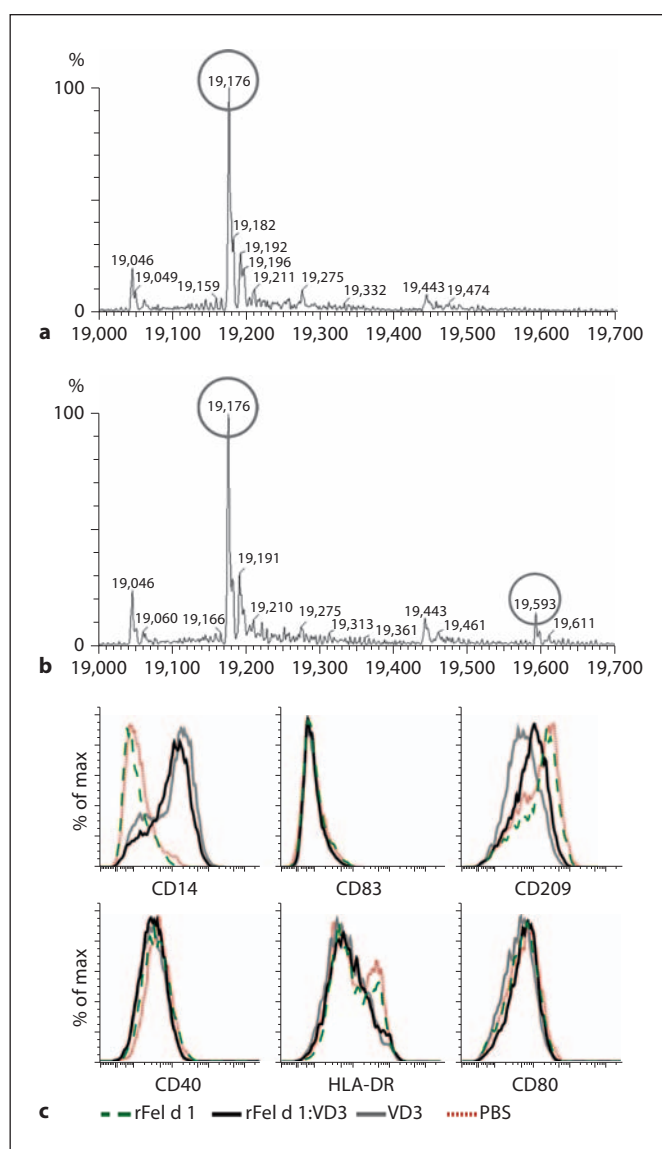


Fig. 2. Analysis of vitamin D3 coupling to rFel d 1. Electrospray ionization mass spectrometry was used to analyze the coupling of VD3 to rFel d 1. rFel d 1 (a) and rFel d 1:VD3 (b) spectrum. Expression of surface markers on MDDCs stimulated for 48 h with rFel d 1, rFel d 1:VD3, VD3 and PBS (c).

ences in LPS contamination in the MDDC stimulation cultures affected CD14 expression, the LPS content in the rFel d 1:VD3 and VD3 preparations was adjusted to the same amount as present in rFel d 1. The addition of LPS to rFel d 1:VD3 and VD3 had no effect on expression of CD14 or other markers. Thus, the rFel d 1:VD3 had the same effect on CD14 expression as VD3 alone, supporting the preserved activity of VD3 when coupled to rFel d 1.

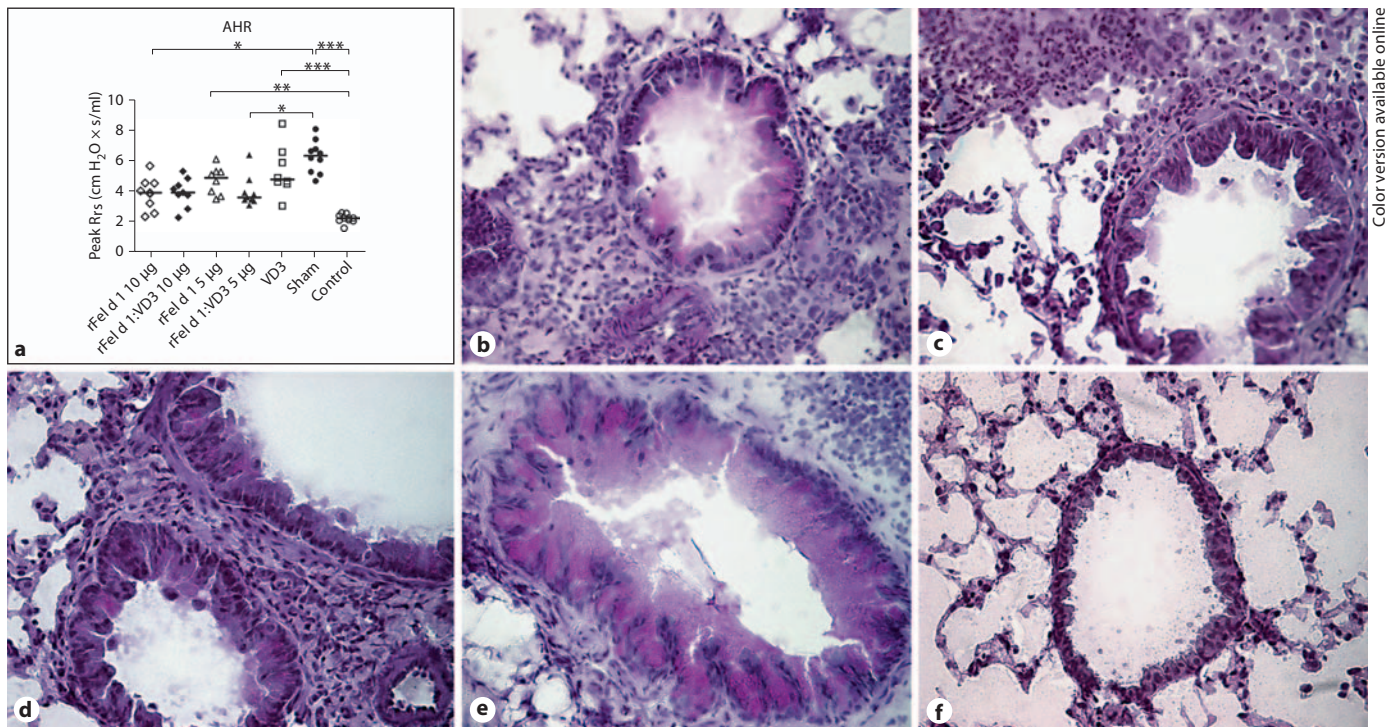


Fig. 3. Airway responses. AHR and mucus production was determined in treated and challenged mice. **a** AHR was measured with a small animal ventilator after challenge with 1 mg/kg MCh and is expressed as peak respiratory resistance (R_{rs}). $n = 8-10$, median, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **b-f** Periodic acid/Schiff's reagent staining of 10 µm representative lung sections from mice in 5 µg rFel d 1 (**b**), 5 µg rFel d 1:VD3 (**c**), VD3 (**d**), sham (**e**) and control (**f**) groups.

Treatment with rFel d 1:VD3 Reduces AHR

AHR to MCh was measured in rFel d 1-sensitized mice treated with either 5 or 10 µg rFel d 1, 5 or 10 µg rFel d 1:VD3, VD3 alone or PBS (sham) before i.n. challenge with CDE or PBS (control). All treatments where rFel d 1 was given (rFel d 1:VD3 or rFel d 1 alone) reduced AHR compared to sham treatment (fig. 3a). This reduction was significant for the 5-µg rFel d 1:VD3 and 10-µg rFel d 1 treatment ($p < 0.05$). Noteworthy is that although the reduction of AHR for the 10-µg rFel d 1:VD3 treatment did not reach significance, the distribution and median appeared almost identical to those of the 10-µg rFel d 1 treatment (fig. 3a). In contrast, groups treated with 5 µg rFel d 1 or VD3 had a significantly higher AHR than the control group ($p < 0.01$; fig. 3a). Treatment with rFel d 1:VD3 at the 5-µg dose decreased the AHR compared to treatment with 5 µg rFel d 1 alone (fig. 3a; not significant, $p = 0.07$), by pairwise comparison.

To investigate if increased AHR was associated with enhanced mucus production in lung tissue, lung sections from two mice per group were analyzed by PAS staining.

Figure 3b-f shows representative micrographs revealing that the mucus production corresponded to the AHR results, with increased mucus staining in mice from groups with higher AHR.

Infiltration of Inflammatory Cells, IL-5 and IgA levels in BAL Are Decreased by rFel d 1:VD3 Treatment

To evaluate the effect of rFel d 1:VD3 treatment on airway inflammation, total cells and eosinophils in BAL were counted. Only treatment with rFel d 1:VD3 clearly limited cell infiltration to the airways at both high- and low-dose (10 and 5 µg) treatment. Accordingly, in contrast to rFel d 1, VD3 or sham treatment, the rFel d 1:VD3 treatment did not result in significant increases in either total cells (fig. 4a) or eosinophils (fig. 4b) in BAL after CDE challenge when compared to control mice. There were significantly less total cell numbers in rFel d 1:VD3 5-µg-treated compared to rFel d 1 5-µg-treated mice, and of eosinophils in rFel d 1:VD3 10-µg-treated compared to rFel d 1 10-µg-treated mice by pairwise comparison (fig. 4; $p < 0.05$).

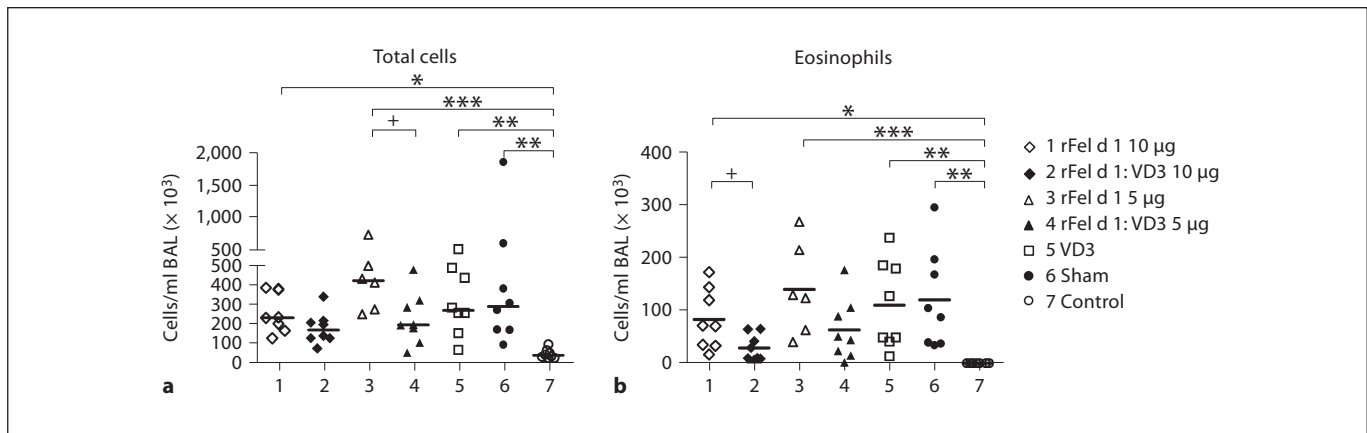


Fig. 4. Cellular infiltration in bronchoalveolar lavage. BAL cells from treated and challenged mice were stained with May-Grünwald-Giemsa and analyzed for total number of infiltrating cells (a) and the number of eosinophils (b). $n = 6-8$, median, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. + $p < 0.05$ by pairwise comparison.

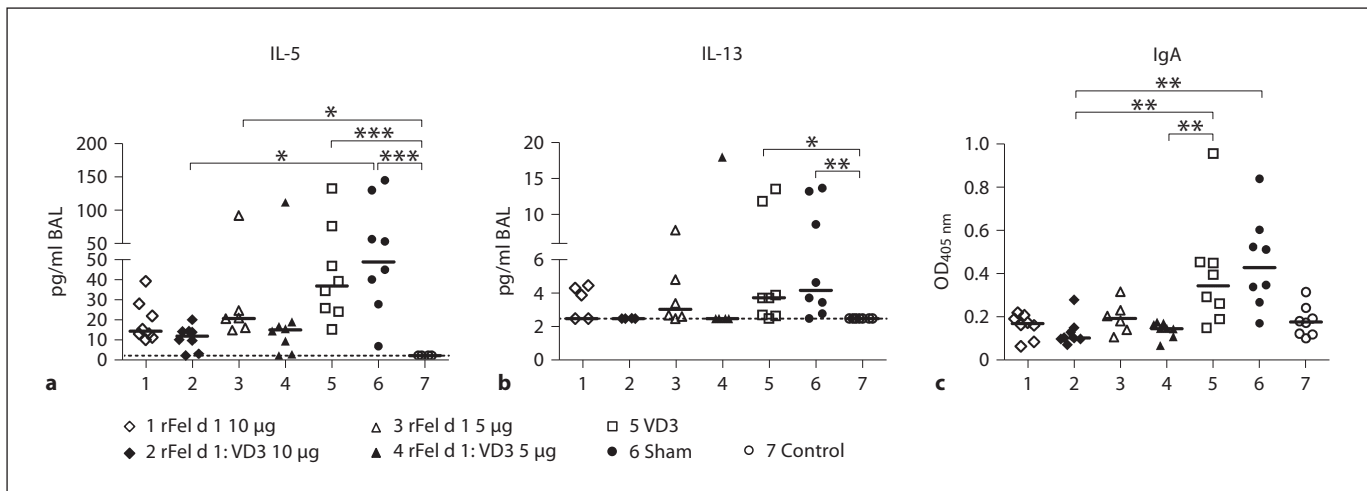


Fig. 5. Th2 cytokine and Fel d 1-specific IgA levels in bronchoalveolar lavage fluid. Cytokine levels and Fel d 1-specific IgA in BAL fluid of treated and challenged mice were determined by CBA analysis and ELISA, respectively. IL-5 (a), IL-13 (b), and Fel d 1-specific IgA (c) are shown. $n = 6-8$, median, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

IL-5 was significantly reduced in BAL fluid of mice treated with 10 µg rFel d 1:VD3 compared to the sham treatment group ($p < 0.05$). For 5 µg rFel d 1-, VD3- and sham-treated mice, the IL-5 levels were significantly increased compared to control mice (fig. 5a; $p < 0.05$ and $p < 0.001$, respectively). A similar pattern was found for IL-13 levels in BAL when comparing the different groups of mice, although the amounts of IL-13 were generally lower and differences less pronounced than for IL-5 (fig. 5b). Concentrations of IFN- γ and IL-10 in BAL were

below the detection limits for both cytokines in most samples (data not shown).

Treatment with rFel d 1:VD3 led to significantly reduced levels of Fel d 1-specific IgA in BAL compared to VD3- and sham-treatment (fig. 5c; $p < 0.01$). The Fel d 1-specific IgA levels in BAL of control mice and both groups treated with rFel d 1 were similar to those detected in the rFel d 1:VD3 groups, although significant differences compared to VD3 and sham were not detected for these groups (fig. 5c; $p > 0.05$).

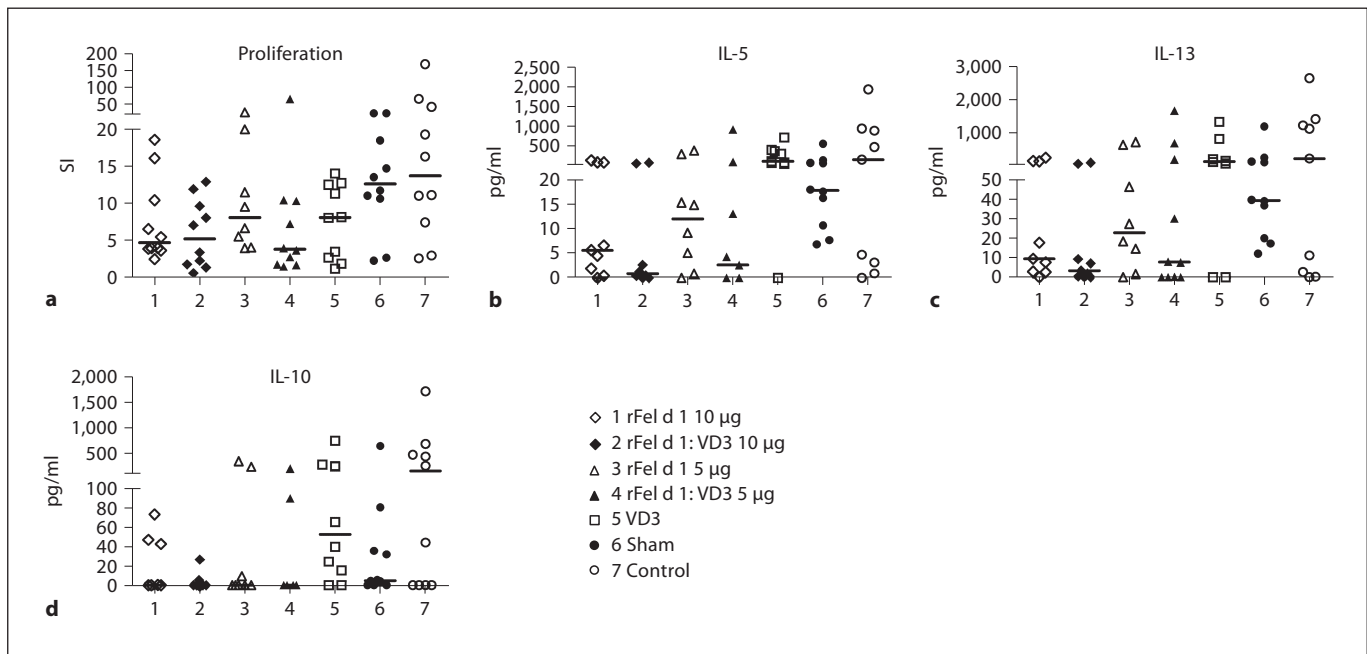


Fig. 6. Allergen-stimulated responses in splenocytes. **a** In vitro proliferation of splenocytes in response to rFel d 1 was measured by incorporation of ^3H -thymidine and results expressed as SI. **b–d** Cytokine levels in in vitro stimulated splenocyte culture supernatants were measured by CBA analysis and the results are expressed as the difference of cytokine levels in rFel d 1-stimulated versus unstimulated culture; IL-5 (**b**), IL-13 (**c**), IL-10 (**d**). $n = 8\text{--}10$, median.

Reduced rFel d 1 Stimulated Activation of Splenocytes from rFel d 1:VD3-Treated Mice

As a measure of treatment-induced T cell activation, splenocyte incorporation of ^3H -thymidine and production of cytokines in response to rFel d 1 stimulation was analyzed. Treatment with either dose of rFel d 1:VD3 or 10 µg of rFel d 1 tended to reduce splenocyte activation compared to sham treatment and control (fig. 6a). This activation was reduced to a lesser extent by the 5-µg rFel d 1 treatment, resulting in similar SI values as for VD3 treatment. There was a tendency that the levels of IL-5 and IL-13 in culture supernatants from both rFel d 1:VD3 treatment groups were reduced compared to rFel d 1 (fig. 6b, c), whereas no induction of IL-10 could be detected in any of these groups (fig. 6d). Due to large individual variation in all treatment groups, the splenocyte stimulation experiments did, however, not reveal any significant differences between the groups.

Induction of Fel d 1-Specific Serum IgG and Blocking Antibodies in rFel d 1- and rFel d 1:VD3-Treated Mice

The levels of Fel d 1-specific IgG1, IgG2a and IgE in serum were analyzed. Any treatment that involved rFel d

1 (rFel d 1 alone and rFel d 1:VD3) induced both IgG1 and IgG2a (fig. 7a, b). Treatment with rFel d 1 led to higher levels of IgG2a than the rFel d 1:VD3 treatment (fig. 7b), and by pairwise comparison, the difference was significant at the 10-µg treatment dose ($p < 0.05$). There were no significant differences in the levels of IgE between any of the treatment groups, including sham and control (fig. 7c). Treatment with rFel d 1:VD3 induced antibodies capable of blocking the binding of Fel d 1-specific human IgE at least to the same extent as the rFel d 1 treatment (fig. 7d).

Discussion

There is an increasing body of evidence in support of the immunoregulatory effect of VD3. Successful SIT has in several reports been linked to a Treg response [7, 8, 10, 36], and therefore we set out to investigate if the efficacy of SIT could be improved by linking VD3 to rFel d 1. In this study, rFel d 1 with covalently coupled VD3 was used to treat mice in a model for cat allergy. The covalent coupling ensures that any immunomodulatory effect exerted

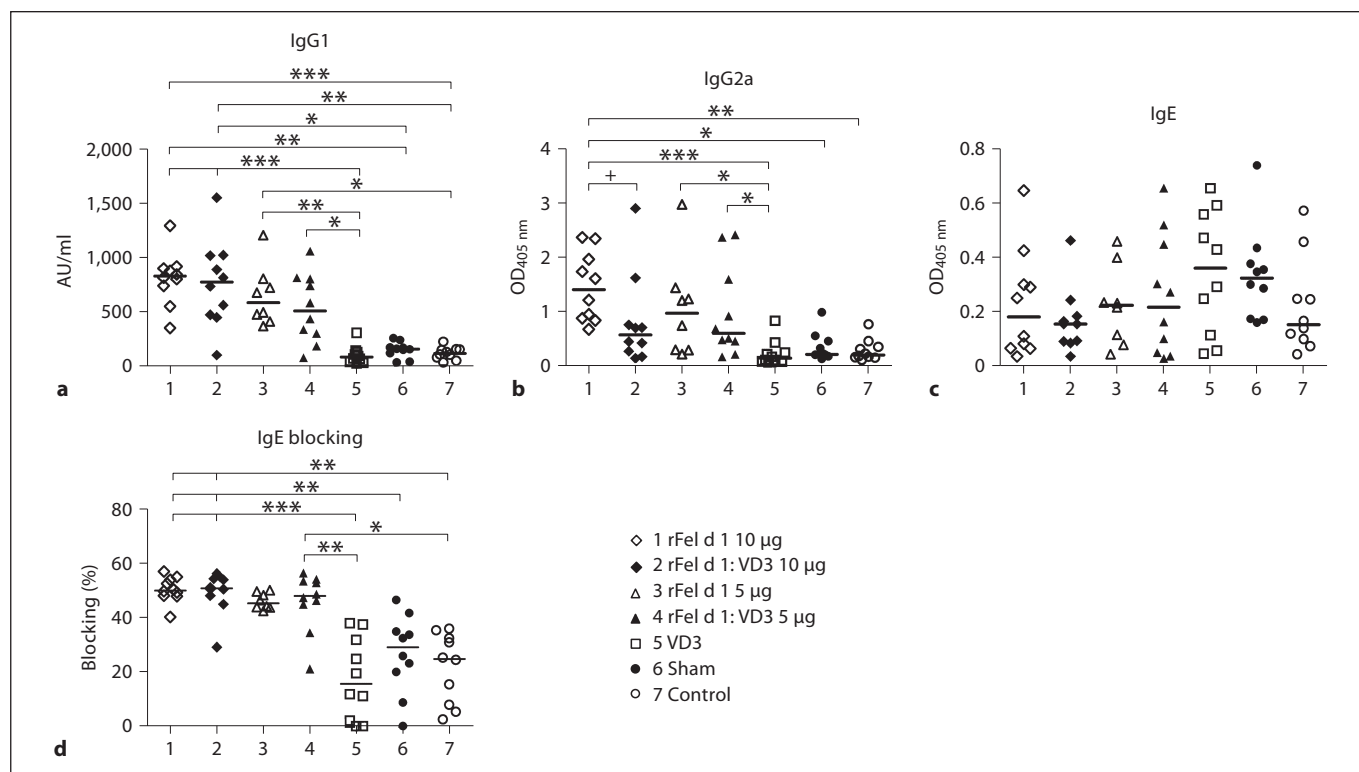


Fig. 7. Serum levels of Fel d 1-specific immunoglobulins and blocking antibodies. Immunoglobulin levels in sera of treated and challenged mice were measured by ELISA. IgG1 (**a**), IgG2a (**b**), IgE (**c**). **d** Capacity of mouse sera to block binding of Fel d 1-specific human IgE. $n = 8-10$, median, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. + $p < 0.05$ by pairwise comparison.

by VD3 affects the same APC that will present Fel d 1 to allergen-specific T cells. With this approach, allergen-specific Tregs may be more precisely induced than if VD3 is co-administered with the allergen, a strategy that was recently shown to potentiate the beneficial effects of SIT in an experimental model [20].

As VD3 was expected to affect allergen-specific T cells, the effect of rFel d 1:VD3 treatment on the allergen-specific T cell responses was analyzed. Treatment with rFel d 1:VD3 tended to reduce production of IL-5 and IL-13 in allergen-stimulated splenocyte cultures compared to rFel d 1 treatment. This was not linked to an induction of IFN- γ , which could neither be detected in supernatants from in vitro splenocyte cultures nor in BAL after allergen challenge. Thus, our results suggest that treatment with rFel d 1:VD3 reduces the allergen-specific Th2 response by a mechanism not involving Th1 skewing of the response. Involvement of IL-10 in the desirable down-regulation of Th2 cytokines could not be supported as no induction, but rather a reduction, of IL-10 production was

observed. This is in contrast to the report by Taher et al. [20] showing that the potentiating effect of VD3 in an OVA SIT model was dependent on both IL-10 and TGF- β . IL-10 has been linked to successful subcutaneously administered SIT [7, 9] and TGF- β has been associated with tolerance to allergens administered over the oral mucosa and suggested to play a role in sublingual allergen specific immunotherapy [37, 38]. Other mechanisms reducing the Th2 response have also been proposed, such as induction of anergy or apoptosis of allergen-specific Th2 cells [39, 40] and of Tregs not producing IL-10 [41]. Such IL-10-independent regulatory mechanisms may be activated by rFel d 1:VD3 treatment, as the treatment effects were not linked to an induction of IL-10 either in allergen-stimulated splenocytes or in BAL of treated animals. Vitamin D3 receptors are expressed in several immune cells, for example, APCs and T cells [22, 23], in addition to nonimmune cells like keratinocytes. Thus the covalent link between VD3 and rFel d 1 may lead to VD3 modulation of a distinct set of immune cells by rFel d 1:VD3 com-

pared to treatments involving free VD3, which will not require protein uptake and degradation.

Induction of Fel d 1-specific IgG is of importance when considering rFel d 1:VD3 as a candidate for SIT. Increased levels of allergen-specific IgG, especially IgG4, have in several clinical studies been linked to successful SIT [3, 42] by mechanisms involving blocking of IgE binding to allergens, thus preventing IgE receptor cross-linking-induced effector functions [43] and IgE receptor-mediated allergen uptake by APCs [3]. Another proposed mechanism is downregulation of mast cell and basophil activity through binding of IgG to inhibitory FcγRIIB receptors [44]. Our data clearly demonstrate that, equally to rFel d 1 SIT, treatment with rFel d 1:VD3 is able to induce Fel d 1-specific IgG1 and IgG2a in mice. In accordance with the induction of blocking antibodies in successful SIT, we find an induction of blocking antibodies of similar capacity with both rFel d 1:VD3 and rFel d 1 treatment.

IgA is considered to be a noninflammatory antibody isotype. However, in humans, IgA acting through FcαRI has a dual effect and may be either activating or inhibitory in receptor-expressing cells, such as mast cells [45]. Human eosinophils may also be activated by IgA [46], and the level of allergen-specific IgA in human BAL has been shown to correlate with eosinophil cationic protein in allergic patients subjected to allergen bronchoprovocation [47]. Moreover, a recent report showed that suppression of airway inflammation in a mouse model of immunotherapy is independent of B cell responses and induction of IgA [48]. Our results revealed the highest levels of IgA in BAL from mice with the strongest airway inflammation, while the lowest levels were detected in rFel d 1:VD3-treated mice. In contrast, Taher et al. [20] detected an increase in IgA in serum of mice treated with allergen combined with VD3 compared to sham. As mice do not express FcαRI [49] and induction of IgA seems to be redundant in a model of immunotherapy [48], the reduced IgA levels in BAL of rFel d 1:VD3-treated mice are probably secondary to the reduction of inflammation in these mice. At least, the anti-inflammatory effect observed in rFel d 1:VD3-treated mice does not seem to be mediated by locally induced allergen-specific IgA.

When comparing the treatment with rFel d 1:VD3 to treatment with the unmodified rFel d 1, the clearest effect was seen on cellular infiltration in BAL. The beneficial effect of rFel d 1:VD3 was superior to that of rFel d 1 alone and the difference was most obvious when comparing influx of eosinophils. Thus, rFel d 1:VD3 was

more potent in preventing pulmonary eosinophilia. In line with these data, IL-5 levels in BAL were significantly decreased compared to the sham-treated group only for the 10 µg rFel d 1:VD3-treated group. Eosinophilic inflammation is a hallmark for allergic inflammation, and IL-5 stimulates the activation and recruitment of eosinophils [50]. Vitamin D3 possesses anti-inflammatory effects and has been suggested as a potentially interesting compound for clinical use to treat inflammatory diseases [22, 51], including asthma [52]. The anti-inflammatory effect of the rFel d 1:VD3 treatment may be of particular relevance for treating cat-allergic patients with asthma. Importantly, in addition to improvement of the allergen-induced airway inflammation, treatment with rFel d 1:VD3 also led to decreased AHR compared to sham treatment. There was no significant difference between the rFel d 1:VD3 and rFel d 1 treatments, though again rFel d 1:VD3 tended to be slightly more potent than rFel d 1 alone.

The coupling chemistry used for coupling of VD3 to rFel d 1 is based on a Mannich formaldehyde reaction. This type of chemistry has previously been used for coupling of small molecules to proteins with good yields [53], including covalent coupling of VD3 to a BSA derivative [31]. The rFel d 1:VD3 conjugate used in this study consisted of a preparation where only approximately 10% rFel d 1 was linked to VD3 at a 1:1 ratio. The beneficial effects of rFel d 1:VD3 could possibly be enhanced by further optimizing the coupling efficiency to produce a more highly substituted rFel d 1:VD3 conjugate, as well as increasing the proportion of rFel d 1 carrying VD3. High doses of VD3 are, however, toxic, mainly due to effects on calcium metabolism, causing for example hypercalcemia, hypercalciuria, renal calcification and increased bone resorption [54, 55]. Therefore, increasing the amount of VD3 coupled to rFel d 1 has to be carefully balanced. If the current rFel d 1:VD3 would be used in SIT, a maintenance dose of 13–15 µg rFel d 1:VD3 would correspond to a VD3 dose of about 30 ng. This dose is well below doses reported not to induce hypercalcemia if given s.c. every other day [56]. On the other hand, the immunomodulatory effects of VD3 were only observed at concentrations of 10^{-10} M and higher in vitro [55]. In this regard, the concept of covalent linking of VD3 to the allergen is of particular importance for overcoming the problem of achieving effective doses of VD3 but avoiding its toxic effects.

In conclusion, this study suggests that covalent linking of VD3 to rFel d 1 may improve the beneficial effects of SIT for treatment of cat allergy. We demonstrate that in a

mouse model of cat allergy, treatment with rFel d 1:VD3 is more potent than rFel d 1 treatment in affecting airway inflammation and may thus be particularly efficient in treatment for allergic asthma. In addition, at least equally positive effects were observed on AHR and the immune response to Fel d 1 with both treatments. Importantly, the improved effects may allow lower allergen doses in SIT and thus improve both the efficacy and safety of the treatment. This leads us to suggest that rFel d 1:VD3 is a promising candidate for use in SIT for cat allergy. Moreover, the concept of direct linking of VD3 to allergens can be extended to SIT with any kind of defined allergen.

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