

Treatment with a Fel d 1 hypoallergen reduces allergic responses in a mouse model for cat allergy

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Abstract

Background: A hypoallergen of the major cat allergen Fel d 1, recombinant (r) Fel d 1 (DTE III), was previously shown to have retained T-cell reactivity and strongly reduced IgE-binding capacity compared to unmodified rFel d 1. Here, we evaluated the therapeutic capacity of rFel d 1 (DTE III) in a mouse model for cat allergy.

Methods: Mice were subcutaneously (s.c.) sensitized with rFel d 1 and subsequently treated (s.c.) with 50 or 200 µg rFel d 1 (DTE III), or 50 µg rFel d 1, prior to intranasal challenge with cat dander extract. Airway hyperreactivity (AHR), cells and cytokines in bronchoalveolar lavage fluid, splenocyte *in vitro* response, and serum immunoglobulins were analyzed. Seven cat-allergic patients and ten healthy controls were tested for skin prick test (SPT) reactivity to rFel d 1 (DTE III) and rFel d 1.

Results: Mice treated with 50 and 200 µg rFel d 1 (DTE III), and 50 µg rFel d 1, produced increased serum levels of rFel d 1-specific IgG1 and IgG2a compared to sham-treated mice. IgG from all treatment groups could block binding of patients' IgE to rFel d 1. The 200 µg rFel d 1 (DTE III) treatment tended to reduce AHR. All mice tolerated treatment with rFel d 1 (DTE III), in contrast to only four of ten treated with rFel d 1. Compared to rFel d 1, the hypoallergen showed a tendency of reduced SPT reactivity.

Conclusion: The rFel d 1 (DTE III) hypoallergen might be a promising candidate for application in immunotherapy of cat allergy with improved safety and efficacy.

Allergen-specific immunotherapy (SIT) has been used for almost a century as therapy for allergic disease and represents the only treatment causing long-lasting relief of allergic symptoms (1). Current SIT vaccines, which are based on the use of allergen extracts, have some disadvantages. Besides the risk of causing severe allergic reactions to the disease-eliciting allergen, the extracts commonly contain other allergens that may give rise to new sensitizations (2–4), or nonallergenic components that may induce local and systemic side-effects (5, 6). Many approaches have been suggested to improve the safety and efficacy of SIT, such as alternative routes of administration (7), and the use of different adjuvants and immunomodulators (8–10), recombinant allergens (11, 12), allergen peptides (13) and modified allergens (12, 14). Single allergens can be produced in large amounts as pure and well-defined recombinant proteins. The recombinant allergens should exhibit the same IgE-binding epitopes as their natural counterparts, but can be modified in various ways to reduce

the risk of side-effects caused by IgE-mediated activation of effector cells. One strategy is to make hypoallergens that have a reduced IgE-binding capacity, but a retained T-cell reactivity. We have previously made hypoallergens of the major cat allergen Fel d 1 through a series of genetic modifications on the recombinant (r) Fel d 1 (15, 16). Our approach was to change IgE-binding epitopes of Fel d 1 without disrupting T-cell epitopes by inserting duplications of T-cell epitopes (DTE) on both polypeptide chains of Fel d 1 and by disrupting disulphide bonds linking the two chains (16). These disruptions were made by changing cysteines to serines using site-directed mutagenesis. *In vitro* evaluation showed that three of the modified Fel d 1 derivatives had strongly reduced IgE reactivity, decreased ability to activate basophils and stimulated similar or stronger lymphoproliferation compared to rFel d 1. Based on the *in vitro* results, these derivatives were identified as hypoallergen candidates for use in SIT.

We have now selected the hypoallergen with the most reduced IgE reactivity, rFel d 1 (DTE III), for *in vivo* evaluation in a recently established mouse model for cat allergy (17, 18). The safety and efficacy of rFel d 1 (DTE III) was studied in a therapeutic treatment protocol that was applied to mice sensitized to rFel d 1. Moreover, skin prick testing on cat-allergic patients was used to evaluate the *in vivo* reactivity of the hypoallergen in humans.

Materials and methods

Subjects

A pool of sera from 31 patients sensitized to cat dander (range 10–59 kU_A/l, median 20 kU_A/l) (16) was used for analysis of blocking antibodies. Seven patients with a history of rhinitis and/or asthma because of cat and with positive skin prick test (SPT) to cat dander (Allergopharma, Reinbek, Germany), and 10 healthy controls were tested for SPT reactivity to rFel d 1 and rFel d 1 (DTE III). None of the subjects had undergone any immunotherapy nor received any anti-allergic medication 7 days prior to testing. The human studies were approved by the local Ethics Committees in Stockholm, Sweden and Wroclaw, Poland, respectively.

Animals

Female BALB/c mice (6–8 weeks of age) were obtained from Charles River (Sulzfeld, Germany) and housed with food and water *ad libitum*. The experiments were approved by the Swedish local ethics committee for animal welfare.

Antigens

Cat dander extract (CDE), rFel d 1 and rFel d 1 (DTE III) were prepared as previously described (15–17). Protein concentrations were determined by BCATM protein assay (Pierce, Rockford, IL, USA). Recombinant protein preparations contained less than 110 ng lipopolysaccharide (LPS)/mg protein, as measured by the Limulus amoebocyte lysate EndochromeTM assay (Charles River Endosafe, Charleston, SC, USA).

Immunization protocol

Groups of mice ($n = 4$) were subcutaneously (s.c.) immunized (200 µl/injection) with 10 µg rFel d 1 or rFel d 1 (DTE III) adsorbed to 1 mg alum (Sigma-Aldrich, Steinheim, Germany) on day 0, 14, 28, 42 and 56. Blood and spleen were collected from all mice on day 59.

Treatment protocol

Mice were s.c. sensitized with 1 µg rFel d 1 adsorbed to 1 mg alum (Sigma-Aldrich) on day 0, 14 and 28. Groups of sensitized mice ($n = 8$ –11) were therapeutically treated (s.c.) with 50 µg rFel d 1, 50 or 200 µg rFel d 1 (DTE III) in phosphate buffered saline (PBS), or PBS only (sham treatment) on day

30, 32 and 34. The mice were subsequently challenged intranasally (i.n.) with 10 µg CDE in a volume of 20 µl on day 39, 40 and 41, following light anesthesia (3.5% isoflurane, Forene[®]; Abbot Scandinavia, Solna, Sweden). Control mice were challenged with 20 µl PBS. On day 42, airway hyperreactivity (AHR) measurements were performed, prior to collection of Bronchoalveolar lavage (BAL) fluid, cardiac blood and spleen.

Measurement of airway responsiveness

At day 42 of the treatment protocol, intravenous (i.v.) methacholine (MCh)-induced AHR was measured with a small animal ventilator (FlexiVent[®]; Scireq, Montreal, PQ, Canada) and expressed as peak respiratory resistance (17).

Bronchoalveolar lavage

Bronchoalveolar lavage was performed as previously described (19). Total numbers of live BAL cells were counted with Trypan blue, before cytospin of 5×10^4 cells that were stained with May-Grünwald-Giemsa (Sigma-Aldrich) according to the manufacturer's instructions. For differentiated cell counts, a minimum of 300 cells were counted per BAL sample. BAL fluid was stored at -80°C until cytokine analysis.

Splenocyte *in vitro* proliferation

Cell suspensions were prepared from spleens and cultured as described earlier (17). Briefly, splenocytes were cultured in triplicates (2×10^5 cells per well) with either complete RPMI medium, 10 µg/ml rFel d 1 or rFel d 1 (DTE III), or 5 µg/ml concanavalin A (Sigma-Aldrich). After 5 days of culture, subsamples of supernatants were frozen at -80°C , before addition of 1 µCi [³H]-thymidine per well and incubation for another 18 h. Stimulation index (SI) was calculated as mean cpm of triplicates for antigen-stimulated splenocytes divided by mean cpm for splenocytes cultured in medium alone. $\text{SI} \geq 2.0$ was considered positive.

Determination of allergen-specific antibody levels

Blood was collected by cardiac puncture and sera were stored at -20°C . Ninety-six-well plates (Nunc, Roskilde, Denmark) were coated with 5 µg/ml rFel d 1 or rFel d 1 (DTE III), or with 2 µg/ml rat anti-mouse IgE (BD Biosciences, San Diego, CA, USA) for IgE analysis. Sera were diluted 1 : 20 for IgE, 1 : 1000 for IgG2a, and 1 : 50000 or 1 : 250000 for IgG1. Bound immunoglobulins were detected as described previously with some modifications (17). Bound IgE was detected using 2 µg/ml biotinylated rFel d 1 or rFel d 1 (DTE III) followed by alkaline phosphatase (AP)-conjugated streptavidine (Sigma-Aldrich, diluted 1 : 3000).

For rFel d 1- and rFel d 1 (DTE III)-immunized mice, sera were analyzed for IgG1 specific for the respective antigen. Because of great variations in titers, serial dilutions of each serum from rFel d 1 (DTE III)-immunized mice were made to normalize IgG1 titers to the hypoallergen. The dilution obtained for hypoallergen-specific IgG1 (ranging 1 : 600–

1 : 4000 for different sera) was then used to analyze rFel d 1-specific IgG1.

For blocking antibody analysis, rFel d 1-coated wells were preincubated with sera from treated mice for 1 h, followed by 1 h incubation with a pool of sera from cat-allergic patients diluted 1 : 32. The dilution of mouse sera used in the blocking experiments was determined to 1 : 80 by titration of pools of mouse sera from the respective groups of treated and sham-treated mice. A pool of sera from naïve mice was diluted 1 : 20. Bound human IgE was detected essentially as described previously (15), with rabbit anti-human IgE antibody (MIAB, Uppsala, Sweden), followed by AP-conjugated goat anti-rabbit IgG (H+L) antibody (Jackson Immuno-Research Laboratories Inc., West Grove, PA, USA; diluted 1 : 1000) and substrate. Percent blocking was calculated as $100 \times (1 - A/B)$, where A and B represent the optical density (OD) after preincubation with serum from a treated mouse and a pool of sera from naïve mice ($n = 3$), respectively.

To allow comparisons between the groups, the samples from the different groups were measured on the same plate for each isotype. Results are presented as OD values for IgE and IgG2a, whereas the IgG1 results are calculated from a standard curve and presented in arbitrary units/ml (AU/ml).

Cytokine analysis

Cytometric bead array (CBA) mouse soluble protein flex set assay (BD) was used according to the manufacturer's instructions to measure the levels of released IL-5, IL-6, IL-10, IL-13 and IFN- γ in BAL fluid and splenocyte culture supernatants. The detection limit was set to 2.5 pg/ml based on the standard curve. The presented concentrations for splenocytes represent the cytokine concentration in supernatants of rFel d 1-stimulated splenocytes minus the concentration in unstimulated splenocytes.

Skin prick testing

SPT was performed on the volar region of the forearms by a trained physician. rFel d 1 (50 μ g/ml) and rFel d 1 (DTE III) (50 μ g/ml) were pricked with a sterile lancet (Allergopharma) for each SPT. Twenty minutes after testing, the ballpoint pen-surrounded wheal area was transferred with scotch tape to a paper. The wheal areas were calculated with the image processing program Image (Technical University, Wroclaw, Poland).

Table 1 Sensitization and treatment protocol for naïve BALB/c mice

Group	Sensitization	Treatment	Challenge
50 μ g rFel d 1	1 μ g rFel d 1/alum	50 μ g rFel d 1	10 μ g CDE
50 μ g rFel d 1 (DTE III)	1 μ g rFel d 1/alum	50 μ g rFel d 1 (DTE III)	10 μ g CDE
200 μ g rFel d 1 (DTE III)	1 μ g rFel d 1/alum	200 μ g rFel d 1 (DTE III)	10 μ g CDE
Sham	1 μ g rFel d 1/alum	PBS	10 μ g CDE
Control	1 μ g rFel d 1/alum	PBS	PBS

Sensitization (s.c.) on day 0, 14 and 28. Treatment (s.c.) on day 30, 32 and 34. Challenge (i.n.) on day 39, 40 and 41.

PBS, phosphate buffer saline; CDE, cat dander extract.

Statistical analysis

The nonparametric Kruskal-Wallis with Dunn's multiple comparison test was used to evaluate differences between multiple groups. The Wilcoxon signed rank test was used to compare rFel d 1 and rFel d 1 (DTE III) in SPT. The analyses were performed using GRAPHPAD PRISM version 4.03 for Windows (GRAPHPAD Software Inc., San Diego, CA, USA, www.graphpad.com). $P < 0.05$ was considered significant.

Results

Immune responses to rFel d 1 and rFel d 1 (DTE III) in immunized mice

To evaluate whether both the allergen and hypoallergen raise humoral responses and to ensure that rFel d 1 (DTE III) stimulates lymphoproliferation in BALB/c mice, groups of mice were immunized with rFel d 1 or rFel d 1 (DTE III) according to the immunization protocol. All sera had IgG1 to the respective antigen, with variations in titers. Sera from hypoallergen-immunized mice were diluted to normalize the levels of IgG1-binding to rFel d 1 (DTE III) and were thereafter analyzed for rFel d 1-specific IgG1. IgG1 antibodies specific for both rFel d 1 and rFel d 1 (DTE III) were detected in serum from the rFel d 1 (DTE III)-immunized mice (data not shown). Splenocytes from rFel d 1 and rFel d 1 (DTE III)-immunized mice proliferated equally well in response to both antigens (data not shown).

Tolerability to rFel d 1 and rFel d 1 (DTE III) in treated mice

A mouse model for cat allergy was used to study the therapeutic effect of rFel d 1 (DTE III) hypoallergen (Table 1). All mice from the groups treated with rFel d 1 (DTE III) tolerated the treatments without any observed complications, while only four of ten mice survived the three treatment injections with rFel d 1 and could be characterized after treatment and challenge.

Serum immunoglobulins in treated mice

The levels of rFel d 1-specific IgE showed a tendency of reduction in all groups receiving treatment with rFel d 1 or rFel d 1 (DTE III) compared to sham-treated or control mice (Fig. 1A).

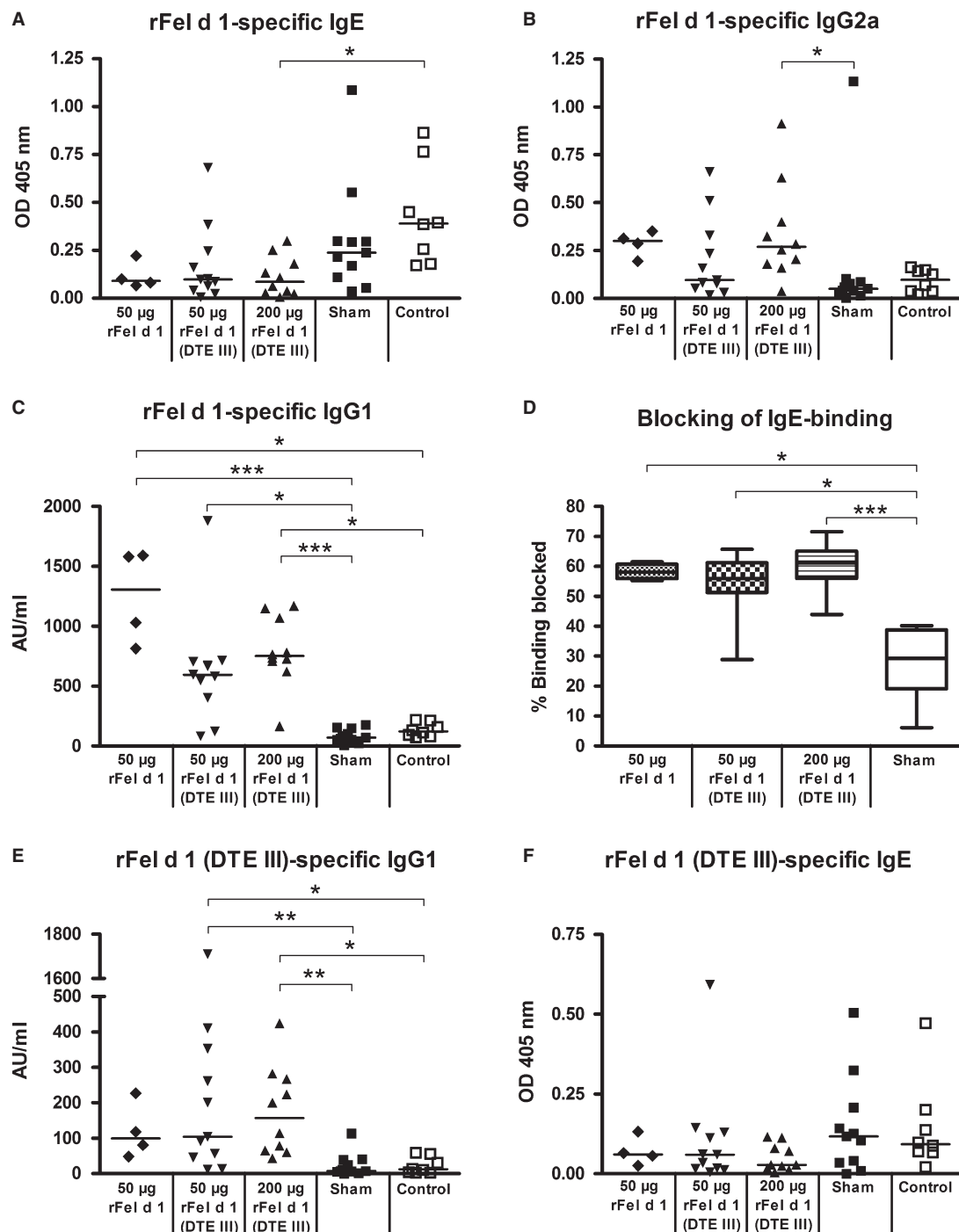


Figure 1 Immunoglobulin responses in treated mice. Mice were sensitized and treated as described in Table 1. Serum levels of rFel d 1-specific (A) IgE, (B) IgG2a, and (C) IgG1. (D) Human rFel d 1-specific IgE-binding blocked by immunoglobulins from treated

and sham-treated mice. Serum levels of rFel d 1 (DTE III)-specific (E) IgG1 and (F) IgE. $n = 4-11$, median. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The levels of rFel d 1-specific IgG2a were significantly higher in mice treated with 200 µg rFel d 1 (DTE III) compared to sham-treated mice ($P < 0.05$; Fig. 1B). Also, the rFel d 1-treated mice had slightly elevated levels of rFel d 1-specific IgG2a.

The levels of rFel d 1-specific IgG1 were significantly higher in mice treated with rFel d 1 ($P < 0.001$), 50 µg rFel d 1 (DTE III) ($P < 0.05$) and 200 µg rFel d 1 (DTE III) ($P < 0.001$) compared to sham-treated mice (Fig. 1C). Therefore, we

analyzed whether the IgG1 antibodies could act as blocking antibodies. Sera from mice treated with 50 µg rFel d 1, 50 µg rFel d 1 (DTE III) and 200 µg rFel d 1 (DTE III) blocked the binding of rFel d 1-specific human IgE to a significantly higher degree than sera from sham-treated mice (Fig. 1D). Compared to 6.2–40.2% (median 29.3%) blocking by sera from the sham-treated mice, the degree of blocking for the respective treated groups were 55.3–61.5% (median 58.0%, $P < 0.05$), 37.3–65.7% (median 55.9%, $P < 0.05$) and 43.9–71.6% (median 61.2%, $P < 0.001$).

Furthermore, significantly higher levels of IgG1 to rFel d 1 (DTE III) were detected in mice treated with 50 µg and 200 µg rFel d 1 (DTE III) compared to sham-treated mice ($P < 0.01$; Fig. 1E). Only low levels of rFel d 1 (DTE III)-specific IgE were detected, and there were no significant differences in the levels between any of the groups in the treatment protocol ($P > 0.05$; Fig. 1F).

Airway hyperreactivity in treated mice

Airway hyperreactivity was measured after MCh challenge. A tendency of reduced maximal AHR was observed in the group treated with the 200 µg dose of rFel d 1 (DTE III) compared to sham-treated mice and mice treated with 50 µg rFel d 1 (DTE III) or 50 µg rFel d 1 (1 mg MCh/kg, $P > 0.05$) (Fig. 2). Only the groups with rFel d 1- and sham-treated mice had significantly higher maximal level of AHR than the control mice (1 mg MCh/kg, $P < 0.05$).

Airway inflammation in treated mice

Total BAL cell numbers and the differentiated counts of BAL cells did not differ between the sham-treated mice and the mice treated with rFel d 1 (DTE III) or rFel d 1

($P > 0.05$; Fig. 3A). Compared to control mice, the cell numbers were in general higher in all mice that had received i.n. challenge with CDE. Cytokine levels were measured in BAL fluid and showed that IL-5 tended to be lower, although not significantly, in rFel d 1- and rFel d 1 (DTE III)-treated mice than in sham-treated mice ($P > 0.05$; Fig. 3B). The differences between the groups were not as obvious for IL-13 (Fig. 3C). The levels of IL-6, IL-10 and IFN- γ were low in general (< 40 pg/ml) and did not differ between any group of treated and sham-treated mice (data not shown).

Cellular responses in treated mice

rFel d 1 stimulated proliferation of splenocytes from all groups of mice, but no significant difference was seen between treated and sham-treated mice (Fig. 4A). The proliferation was highest in the control mice, while a significantly lower proliferation was seen in the groups treated with 50 µg rFel d 1 ($P < 0.05$) and 50 µg rFel d 1 (DTE III) ($P < 0.01$; Fig. 4A). Cytokine levels were measured in the supernatants of splenocytes. Splenocytes from mice treated with 50 µg and 200 µg rFel d 1 (DTE III) produced significantly lower levels of IL-5 ($P < 0.01$ and $P < 0.05$, respectively) and IL-13 ($P < 0.05$) than splenocytes from sham-treated mice (Fig. 4B, C). The levels of IL-10 were also significantly lower in splenocytes from the rFel d 1 (DTE III)-treated mice compared to sham-treated mice ($P < 0.01$; Fig. 4D). The levels of IFN- γ and IL-6 were low (< 40 pg/ml), and no differences were seen between any group of treated and sham-treated mice (data not shown).

Skin prick test reactivity

All the seven cat-allergic patients had an SPT reaction to rFel d 1, while five had an SPT reaction to rFel d 1 (DTE III). The wheal areas induced by rFel d 1 (DTE III) were smaller, although not significantly, than those induced by rFel d 1 ($P > 0.05$; Fig. 5). None of the healthy controls displayed a positive SPT to the allergens.

Discussion

Hypoallergens have been proposed as one approach to improve SIT. Here, we present the first *in vivo* study of the hypoallergen rFel d 1 (DTE III) showing a beneficial effect of treatment in a mouse model of cat allergy.

We have previously constructed hypoallergens of the major cat allergen Fel d 1 through a series of genetic modifications (16). Based on *in vitro* evaluation of the hypoallergens, we selected the variant with lowest IgE-binding capacity, rFel d 1 (DTE III), for further evaluation *in vivo* in a mouse model for cat allergy (17, 18). After sensitization with rFel d 1, groups of mice were therapeutically treated with two doses (50 µg or 200 µg) of rFel d 1 (DTE III), or with rFel d 1 (50 µg) at three occasions, followed by i.n. challenge with CDE to provoke airway inflammation. Compared to only sensitized control mice, sham-treated, CDE-challenged mice obtained a significant increase in AHR. There was a tendency

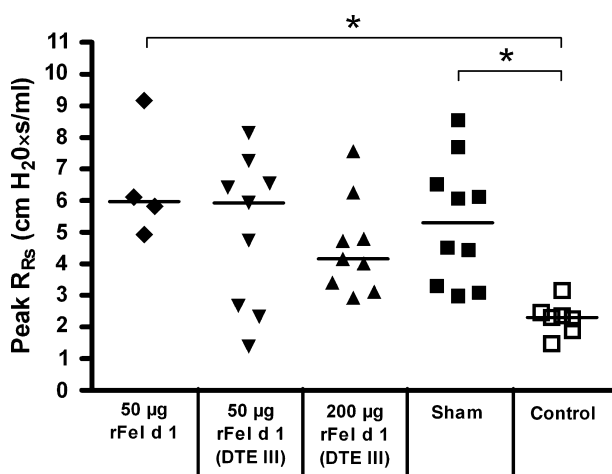


Figure 2 Airway hyperreactivity in treated mice. Mice were sensitized and treated as described in Table 1. Airway hyperreactivity expressed as peak respiratory resistance (R_{RS}) after challenge with 1 mg/kg methacholine at day 42 of the treatment protocol. $n = 4$ –10, median. * $P < 0.05$.

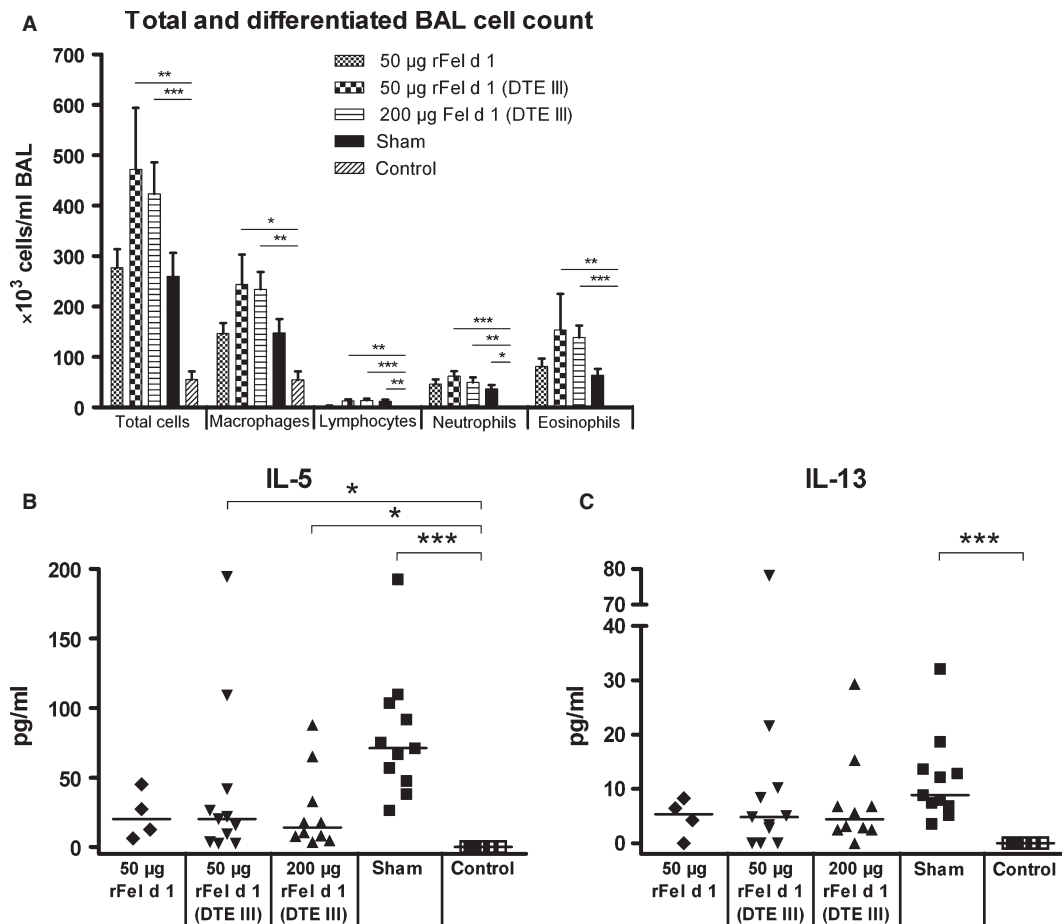


Figure 3 Airway inflammation in treated mice. Mice were sensitized and treated as described in Table 1. (A) Total and differentiated

ated BAL cell counts, mean $\pm$ SEM. (B, C) IL-5 and IL-13 in BAL fluid, median. $n = 4-11$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of reduced AHR, although not significant, in mice treated with the higher dose of hypoallergen compared to the sham-treated mice. The treatments with unmodified rFel d 1 or the lower dose of hypoallergen did not have any impact on AHR. Furthermore, treatment with both doses of rFel d 1 (DTE III) and with rFel d 1 had a beneficial effect on the antibody responses. Accordingly, only baseline levels of rFel d 1-specific IgE were found in all treated mice, while the levels of rFel d 1-specific IgG1 and IgG2a were increased compared to sham-treated and control mice. In sera from mice immunized with rFel d 1 (DTE III), detectable levels of IgG1 specific for both rFel d 1 and for rFel d 1 (DTE III) were found, which indicates some common IgG epitopes in the hypoallergen and the wild-type allergen. This was further supported by the fact that the IgG antibodies from both hypoallergen- and rFel d 1-treated mice in addition to rFel d 1 recognized rFel d 1 (DTE III). We could also show that IgG in sera from all groups of treated mice could act as blocking antibodies, as they inhibited binding of IgE from cat-allergic patients to rFel d 1. Importantly, IgG from the hypoallergen-treated mice blocked the IgE-binding to the same extent as IgG from rFel d 1-treated mice and to a significantly higher

degree than IgG from sham-treated mice. Blocking IgG antibodies are formed during the course of successful SIT (20) and are induced by high doses of antigen, as shown in a mouse model (21). Immunizations of mice with various hypoallergens have also resulted in the induction of blocking IgG antibodies (22, 23). One suggested mechanism for blocking IgG antibodies is that the IgG produced during immunotherapy prevents IgE from recognizing the allergen by direct competition (24). Other explanations for the inhibitory effect of IgG are that Fc ϵ RI and Fc γ RIIb receptors are co-cross-linked and downregulate effector cell activation (25) or that IgE-facilitated antigen presentation is inhibited (26, 27).

The i.n. challenge with CDE established an inflammation in the airways of rFel d 1-sensitized mice, which was marked by infiltration of macrophages, lymphocytes, neutrophils and eosinophils in BAL fluid. Total cell numbers and differential cell counts in BAL fluid showed that all groups of mice had an ongoing inflammation in the airways. The inflammation was apparently so strong that it could not be decreased, neither by treatment with rFel d 1 nor rFel d 1 (DTE III). Furthermore, splenocytes from all groups of mice had a strong ability to proliferate in response to rFel d 1. However,

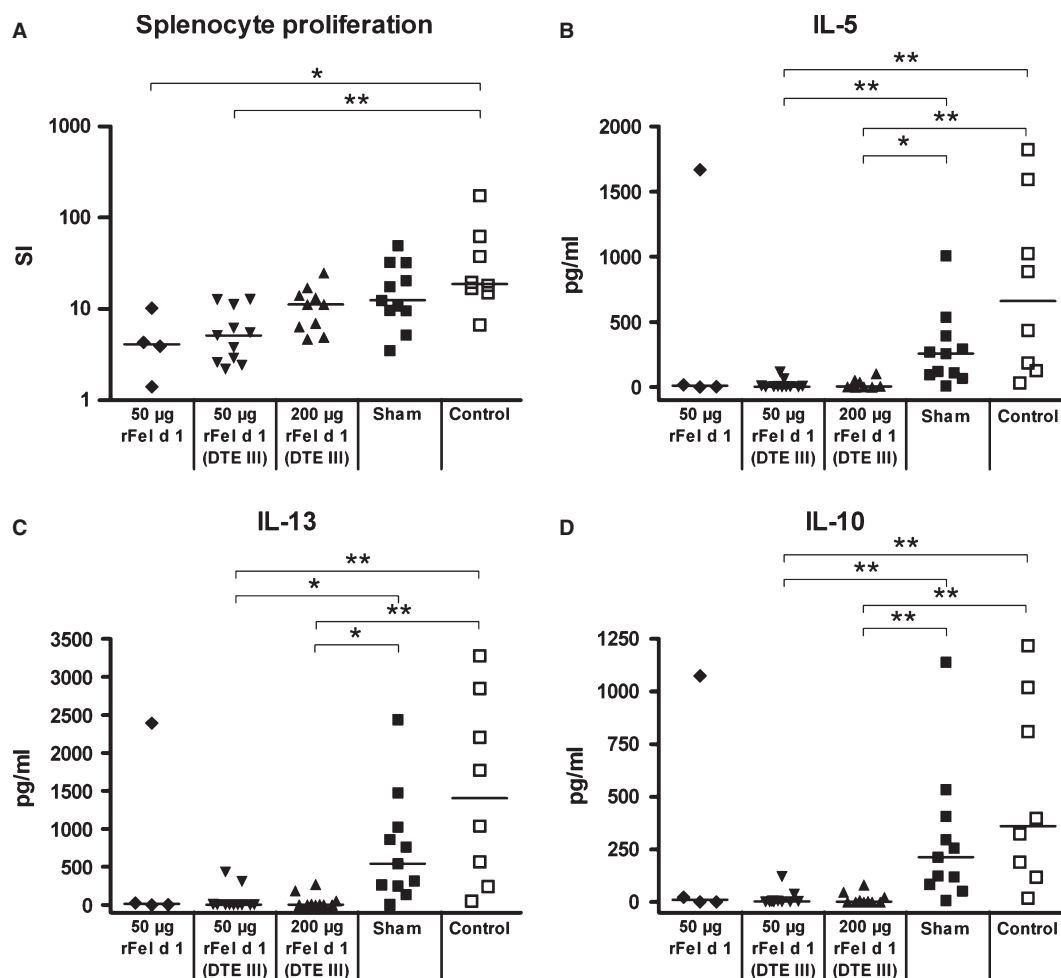


Figure 4 Splenocyte proliferation and cytokines of treated mice. Mice were sensitized and treated as described in Table 1. (A) *In vitro* proliferation of rFel d 1-stimulated splenocytes, expressed as stimulation index (SI). (B, C and D) IL-5, IL-13 and IL-10 in supernatants of

rFel d 1-specific splenocytes corrected for background concentrations of cytokines from unstimulated splenocytes. $n = 4-11$, median. * $P < 0.05$, ** $P < 0.01$.

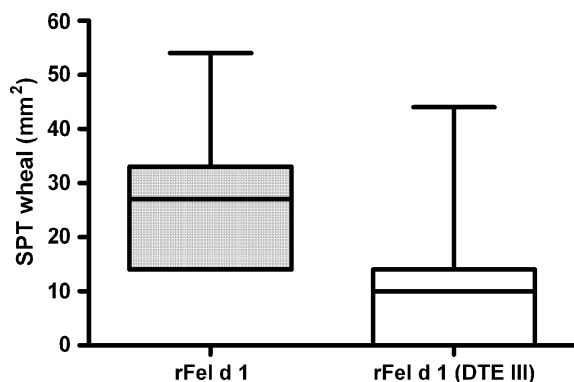


Figure 5 Skin prick test reactivity. The area of SPT wheals (mm²) induced by rFel d 1 and rFel d 1 (DTE III) in cat-allergic patients. $n = 7$, median.

sham-treated mice clearly had higher levels of IL-5 and IL-13 than rFel d 1- or rFel d 1 (DTE III)-treated mice in splenocytes cultures. The cytokine profiles thus showed a somewhat decreased Th2 response in treated compared to sham-treated mice. We could however not detect any differences between treated and sham-treated mice in the levels of e.g., the Th1-driving cytokine IFN- γ or other pro-inflammatory cytokines, like IL-6. Furthermore, allergen-stimulated splenocytes from treated mice produced less IL-10 than sham-treated mice. Thus, despite the presence of duplicated T-cell epitopes, no clear change to T-cell tolerance or anergy could be detected. This apparent lack of induction of a counter-acting cellular immune response, Th1 or a regulatory response, may explain the inability to reduce the Th2-driven airway inflammation. Our mouse model reflects an acute allergic disease, and it is possible that repeated application of the Fel d 1 hypoallergen in a chronic allergy model (or in human allergic disease) would have a more beneficial effect on the T-cell driven inflammation.

The most obvious effect of the therapeutic treatment reported here was the induction of rFel d 1-specific IgG with blocking capacity. This was observed in mice treated with hypoallergen as well as unmodified rFel d 1. A tendency of decreased AHR was observed only in mice treated with the higher dose of rFel d 1 (DTE III), indicating that high treatment doses are required to reduce the AHR. This underlines the importance of the observed tolerability of the hypoallergen at a high treatment dose without any side-effects, in contrast to unmodified rFel d 1. Throughout the study, the rFel d 1-treated group is represented by the minority of mice that survived the treatment with rFel d 1. This raises the question whether these mice thus respond with higher IgG titers and lower airway inflammation than those mice that did not tolerate the treatment. Consequently, the beneficial effects of treatment observed for unmodified rFel d 1 may have been overestimated.

In agreement with the reduced IgE reactivity to rFel d 1 (DTE III), as earlier demonstrated *in vitro* (16), we here report that rFel d 1 (DTE III) induced less SPT reactivity compared to rFel d 1 in cat-allergic patients. These data

indicate that rFel d 1 (DTE III) acts as a hypoallergen also *in vivo* in humans. In conclusion, the first *in vivo* evaluations of treatment in mouse together with SPT reactivity in humans confirm the hypoallergenic nature of rFel d 1 (DTE III). Based on the safety and efficacy, it might be a promising candidate for application in SIT of cat-allergic patients.

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