

Original article

Carbohydrate-based particles reduce allergic inflammation in a mouse model for cat allergy

Background: Allergen-specific immunotherapy (ASIT) is the only treatment of allergic disease that gives long-lasting relief of symptoms. However, concerns for safety and efficiency have highlighted the need for improvement of the therapy. We have previously suggested carbohydrate-based particles (CBPs) as a novel adjuvant and allergen carrier for ASIT. Our aim of this study was to evaluate the therapeutic potential of CBPs in ASIT, employing a mouse model for cat allergy.

Methods: BALB/c mice were subcutaneously immunized with the recombinant (r) cat allergen Fel d 1 followed by intranasal challenge with cat dander extract (CDE). The sensitized mice were therapeutically treated with rFel d 1 covalently coupled to CBPs (CBP-rFel d 1). Airway hyper-reactivity (AHR), infiltration of leucocytes in bronchoalveolar lavage (BAL) fluid, allergen-specific serum immunoglobulin levels and *in vitro* splenocyte responses were evaluated.

Results: Mice treated with CBP-rFel d 1 showed reduced features of allergic inflammation. They responded with (i) significantly decreased AHR and infiltration of eosinophils in BAL fluid after CDE challenge, (ii) the serum level of rFel d 1-specific IgE was reduced and the level of IgG₂a was more pronounced after CBP-rFel d 1 treatment, and (iii) there was also a tendency of decreased allergen-specific cellular response.

Conclusions: Carbohydrate-based particles are effective tools as adjuvant and allergen carriers for use in ASIT and constitutes a promising strategy to improve allergy treatment.

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The only allergy treatment available today which causes a long-lasting relief of symptoms is allergen-specific immunotherapy (ASIT) (1–3). To generate a slow-release depot and prevent anaphylactic reactions, the allergen extracts are adsorbed to the adjuvant aluminium hydroxide

(alum) (1–3). Despite well documented successful clinical outcome, alum-based ASIT involves a risk of side-effects, such as granuloma formation (4–6). It is therefore a need to make ASIT safer.

Numerous strategies have been suggested to improve ASIT, e.g. usage of recombinant hypoallergenic derivatives (7–9), allergen peptides (10, 11), conjugation of the allergen to inhibitory molecules (12, 13), usage of Th1 inducing agents such as oligonucleotides with CpG motives (14, 15) or monophosphoryl lipid A (16). Another suggested strategy is to couple the allergen to a novel adjuvant and allergen carrier, carbohydrate-based

Abbreviations: AHR, airway hyper-reactivity; Alum, aluminium hydroxide; ASIT, allergen-specific immunotherapy; BAL, bronchoalveolar lavage; CBP, carbohydrate-based particles; CDE, cat dander extract; MCh, methacholine; OVA, ovalbumin; rFel d 1, recombinant *Felis domesticus* 1 allergen; RBL, rat basophil leukaemia.

particles (CBPs). These CBPs have been shown, in the murine system, to induce a mixed Th1/Th2 immune response to the grass pollen allergen Phl p 5 compared with the Th2 skewed response induced by alum (17). In addition, no granulomas were formed at the site of injection (17). In human *in vitro* cultures of monocyte-derived dendritic cells, the major cat allergen Fel d 1 coupled to CBPs resulted in a semi-mature phenotype of the cells, which hypothetically could favour the development of a mixed Th1/Th2 response or a regulatory response (18).

To investigate the allergen-coupled CBPs *in vivo*, we here established a mouse model for cat allergy using the recombinant (r) major cat allergen Fel d 1 (19). The model exhibits the characteristic features of experimental allergic inflammation with elevated allergen-specific serum IgE levels, increased infiltration of eosinophils in the lungs and enhanced airway hyper-reactivity (AHR) to methacholine after allergen challenge. We found that the allergic inflammation was reduced, when therapeutically treating the sensitized mice with rFel d 1 coupled to CBPs. Therefore, we propose CBPs as a promising adjuvant for application in ASIT.

Materials and methods

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

Recombinant Fel d 1 was prepared as previously described (19). Cat dander extract (CDE) was prepared by aqueous extraction from cat dander (Allergon, Ängelholm, Sweden) (20). The Fel d 1 level in the extract was determined to 0.7% using ELISA (Indoor Biotechnologies, Manchester, UK) according to the manufacturer's instruction, with rFel d 1 as standard. Ovalbumin (OVA) was purchased from Sigma-Aldrich (Steinheim, Germany).

rFel d 1 was coupled to CBPs as previously described (17, 18). The amount of coupled rFel d 1 was calculated to 3.2 mg/g CBP by measuring the uncoupled protein in the supernatant using the BCA method (Pierce, Rockford, IL, USA). The rFel d 1 contained 4.5 ng LPS/mg protein, the CDE preparation contained 317 ng LPS/mg protein, the OVA contained 240 ng LPS/mg protein and the level in the CBP-rFel d 1 preparation was 96 pg LPS/mg coupled rFel d 1, measured with the Limulus amoebocyte chromatogenic test (LAC; Charles River Endosafe, Charleston, SC, USA).

Sensitization protocol

Initial experiments were performed to establish dose and administration routes by immunizing mice ($n = 4$) with 1 or 10 µg rFel d 1 either intraperitoneally (i.p.) or subcutaneously (s.c.). Based on the results, mice (groups of 10) were sensitized s.c. in the neck with 1 µg rFel d 1 adsorbed to 1 mg alum (Sigma) on day 0, 14 and 28.

Table 1. Characterization of the cat allergy model

Group ($n = 10$)	Sensitization (adsorbed to 1 mg alum)	Challenge
A	1 µg rFel d 1	10 µg CDE
B	1 µg rFel d 1	20 µl PBS
C	1 µg rFel d 1	10 µg OVA
D	10 µg OVA	10 µg CDE
E	10 µg OVA	10 µg OVA
F	PBS	10 µg CDE

Alum, aluminium hydroxide; CDE, cat dander extract; OVA, ovalbumin.

Control mice were immunized with 10 µg OVA adsorbed to alum or PBS/alum only. About 1 week after the last immunization, mice were challenged intranasally (i.n.) with 10 µg CDE in a volume of 20 µl, on three consecutive days following light anaesthesia (3.5% isoflurane, Forene; Abbot Scandinavia, Solna, Sweden). Control mice were challenged with 10 µg OVA or 20 µl PBS (Table 1, Fig. 1A).

Therapeutic treatment protocol

Mice were sensitized according to the established protocol, followed by s.c. treatment on day 30, 32 and 34 with 100 µg rFel d 1 coupled to CBPs, CBPs alone, 100 µg rFel d 1 alone, a mixture of 100 µg uncoupled rFel d 1 and CBPs, PBS or left untreated. Subsequently, the mice were challenged with CDE or PBS i.n. on day 39, 40 and 41 (Table 2, Fig. 2A).

Kinetics of immune response

Mice were immunized s.c. with either 100 µg CBP-rFel d 1, CBP alone, 100 µg rFel d 1 alone or CBP plus 100 µg uncoupled rFel d 1, days 0, 7 and 15, to follow the rFel d 1-specific response. Blood was collected from the tail artery preimmunization (PI), and on days 3, 5, 8, 14 and 21. Sera were stored at -20°C for measurement of antibody titres.

Measurement of airway responsiveness

About 1 day after the last i.n. allergen challenge, mice were anaesthetized with an i.p. injection of pentobarbitone (70 mg/kg body weight, BW, from local supplier), tracheostomized and mechanically ventilated with an animal ventilator (FlexiVent®; Scireq, Montreal, PQ, Canada). Bilateral thoracotomies were performed to equalize the pleural pressure to atmospheric pressure. Increasing doses (0, 0.03, 0.1, 0.3, 1 and 3 mg/kg BW) of methacholine (acetyl- β -methylcholine chloride, MCh, Sigma-Aldrich) were given in the tail vein. Respiratory resistance (R_{rs}) was measured by assuming a single-compartment linear model at a sinusoidal frequency of 2.5 Hz every eight breath for 3 min after each injection (21). Dose-response relations for MCh were obtained by plotting peak R_{rs} as a function of dose, and changes in reactivity and sensitivity were assessed using nonlinear regression analysis to calculated maximal responses and effective dose for half maximal response (ED_{50}), respectively.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed as previously described (21). In short, lungs were lavaged with 1 ml PBS containing 0.6 mM EDTA. Erythrocytes were lysed in lysis buffer

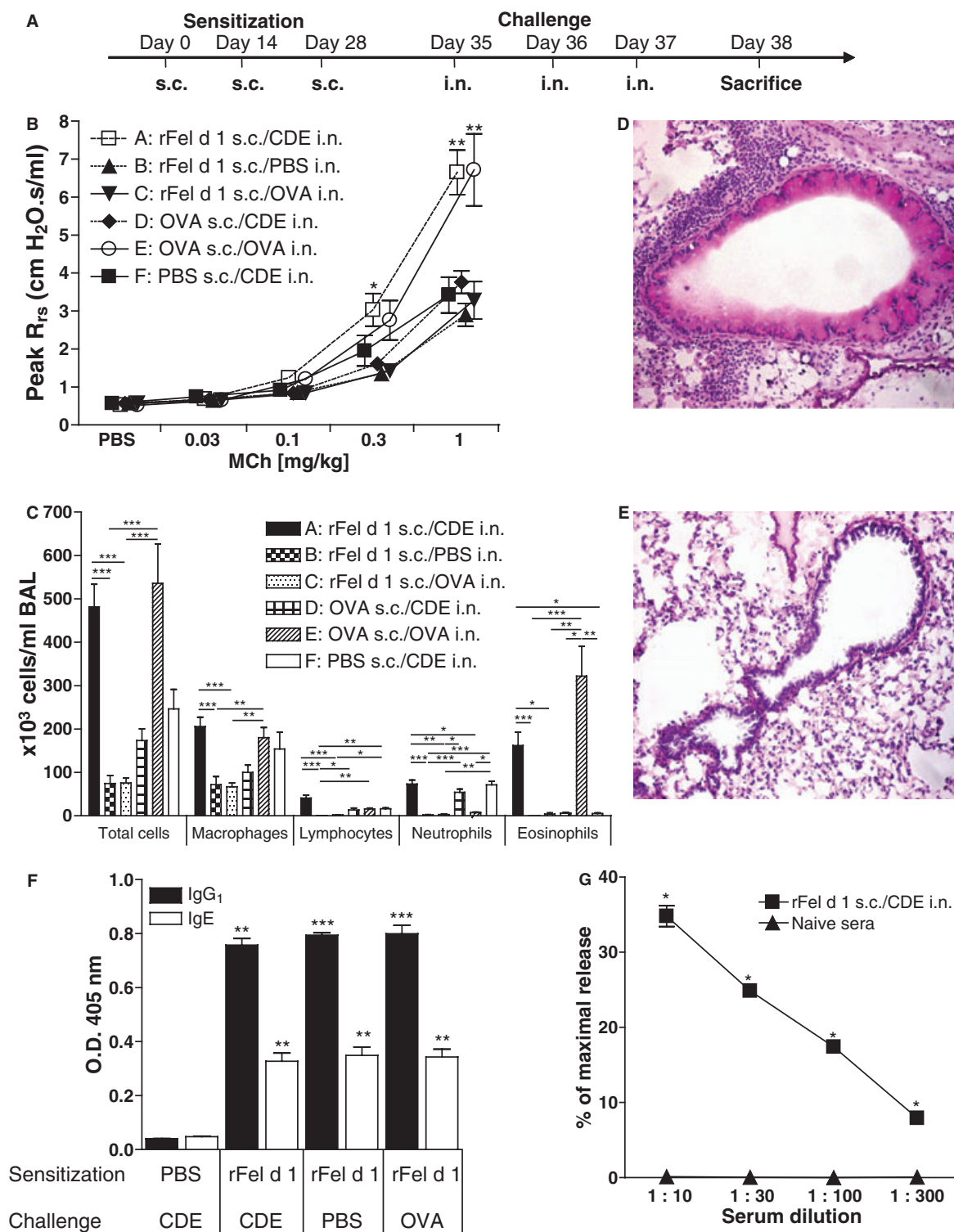


Figure 1. Characterization of the cat allergy model: (A) Sensitization and challenge protocol. (B) Airway hyper-reactivity expressed as peak respiratory resistance (R_{rs}) and plotted against methacholine challenge doses. $n = 7-10$, mean $\pm$ SEM. Statistically significant differences compared with PBS-challenged mice are indicated. (C) Total and differential BAL cell count. $n = 10$, mean $\pm$ SEM. (D) Mucus production in lungs from a CDE challenged mouse and (E) a PBS challenged mouse. (F) Allergen-specific IgE and IgG₁ antibody levels measured in serum and expressed as optic density values. $n = 10$, mean $\pm$ SEM. Statistically significant differences compared with PBS-immunized mice are indicated. (G) Release of β -hexosaminidase from rat basophil leukaemia cells upon rFel d 1-induced IgE receptor crosslinking. $n = 4$, mean $\pm$ SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

Table 2. Treatment groups

Group (<i>n</i> = 10)	Sensitization (adsorbed to 1 mg alum)	Treatment	Challenge
1-treatment group	1 µg rFel d 1	100 µg CBP-rFel d 1*	10 µg CDE
2-treatment control	1 µg rFel d 1	200 µl CBPs	10 µg CDE
3-positive control	1 µg rFel d 1	200 µl PBS	10 µg CDE
4-treatment control	1 µg rFel d 1	100 µg rFel d 1*	10 µg CDE
5-treatment control	1 µg rFel d 1	100 µg rFel d 1 + 200 µl CBPs	10 µg CDE
6-negative control	1 µg rFel d 1	—	20 µl PBS

*In 200 µl.

Alum, aluminium hydroxide; CBP, carbohydrate-based particles; CDE, cat dander extract.

(150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.2). Total number of live cells was counted, cytospun and stained with May–Grünwald–Giemsa (Sigma) according to manufacturer's instructions. For differential cell counts, a minimum of 300 cells were counted per BAL sample.

Lung histology

Histocon® (HistoLab Products, Västra Frölunda, Sweden) was injected into the lungs from two mice per group, removed and snap frozen. The lungs were stored at –80°C until sectioned for 10 µm thick sections in a cryostat. To detect mucus production into the airways, sections were stained with Periodic Acid/Schiff's reagent according to manufacturer's instructions (Sigma).

Determination of total and allergen-specific antibody levels

Blood was collected by cardiac puncture and sera were stored at –20°C (22). Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with 5 µg/ml rFel d 1 or 2 µg/ml rat anti-mouse IgE (BD Biosciences, San Diego, CA, USA). Sera were diluted 1 : 20 for IgE, 1 : 1000 for IgG_{2a}, 1 : 5000 for IgG₁ or 1 : 1000 for IgM. Bound IgE was detected using a biotinylated rat anti-mouse IgE (diluted 1 : 500, BD Biosciences). IgG₁ was detected using rat anti-mouse IgG₁ (diluted 1 : 1000, BD Biosciences) followed by a biotinylated rabbit anti-rat IgG (diluted 1 : 2000, Vector Laboratories, Burlingame, CA, USA). IgG_{2a} was detected using a biotinylated rat anti-mouse IgG_{2a} (diluted 1 : 1000, BD Biosciences). For kinetic experiments IgM, IgG₁ and IgG_{2a} were detected with alkaline phosphatase (ALP)-conjugated goat anti-mouse IgM (diluted 1 : 3000), IgG₁ (diluted 1 : 1000) or IgG_{2a} (diluted 1 : 500) (Southern Biotech, Birmingham, AL, USA). ALP-conjugated streptavidine (diluted 1 : 3000, Dako Cytomations, Glostrup, Denmark) was used together with phosphatase substrate (Sigma), and colour development was monitored after 60 min at 405 nm. Results are presented as optic density (OD) values. The samples from the different groups were always measured on the same plate for each isotype to allow comparisons between the groups.

Rat basophil leukaemia cell mediator release assay

Rat basophil leukaemia (RBL)-H2H3 cells were incubated with mouse sera (diluted 1 : 10, 1 : 30, 1 : 100 and 1 : 300). Degranulation of RBL cells was induced by adding 0.03 µg of rFel d 1 in Tyrode's buffer. Supernatants were analysed for β-hexosaminidase activity as previously described (23). Results are reported as percentages of total β-hexosaminidase released after addition of 1% Triton X-100.

Splenocyte *in vitro* proliferation and cytokine release

Cell suspensions were prepared from spleens and erythrocytes were lysed with lysis buffer. Cells were resuspended in complete RPMI (cRPMI), i.e. RPMI 1640 medium supplemented with 25 µg/ml gentamicin, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine (Gibco, Invitrogen Corporation, Paisley, UK), 50 µM 2-mercapto-ethanol (Sigma-Aldrich) and 10% (v/v) heat-inactivated bovine growth serum (Hyclone, Logan, UT, USA), and 2 × 10⁵ splenocytes were cultured in triplicates in flat-bottomed 96-well plates (BD) with either medium, 10 µg/ml rFel d 1 or 5 µg/ml concanavalin A (con A; Sigma). After 5 days of culture, 1 µCi [³H]-thymidine was added to each well for 18 h. Background c.p.m. values obtained by medium stimulation were subtracted from the allergen-specific c.p.m. values generating delta c.p.m. Samples from the culture supernatants were collected before addition of [³H]-thymidine, and cytometric bead array (CBA) flex (BD) was used according to the manufacturer's instructions to measure the levels of released IL-5, IL-13, IL-10 and IFN-γ. The detection limit was based on the standard curve and set to 5 pg/ml. When the levels of the cytokines were below the detection limit of the assay, the value of the detection limit was used for statistical calculations.

Statistical analysis

Results are expressed as mean ± SEM or median. The nonparametric Kruskal–Wallis with Dunn's multiple comparison test was used to evaluate differences between multiple groups using GRAPH-PAD PRISM version 4.03 for Windows (Graphpad Software Inc., San Diego, CA, USA). Mann–Whitney *U*-test was used when two groups were compared. *P* < 0.05 was considered significant.

Results

Establishment of a mouse model for cat allergy

A mouse model for cat allergic asthma was first established (Fig. 1A). Groups of mice were sensitized and challenged according to Table 1. Measurements of AHR demonstrated that rFel d 1 sensitized and CDE challenged mice had similar increase in the maximal airway reactivity as in OVA sensitized and OVA challenged control mice (Fig. 1B). Compared with PBS challenged mice, both rFel d 1 and OVA sensitized and challenged mice had significantly increased maximal airway reactivity to MCh injection (*P* < 0.01). For rFel d 1 sensitized mice, significant differences could be

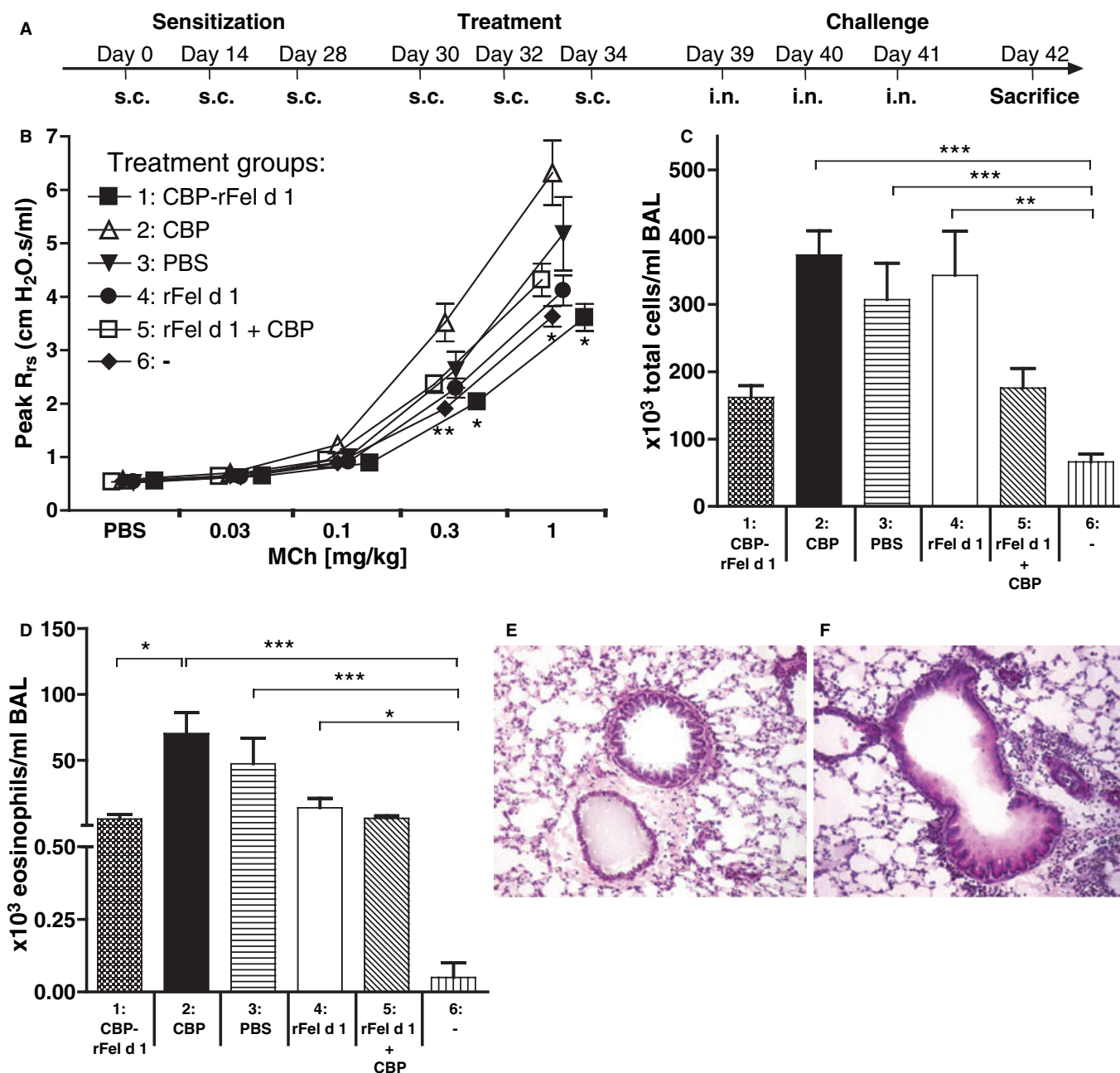


Figure 2. Effect of CBP-rFel d 1 treatment on the airway inflammation: (A) Sensitization, treatment and challenge protocol. (B) Airway hyper-reactivity expressed as peak respiratory resistance (R_{rs}) and plotted against methacholine challenge doses. $n = 7-10$, mean $\pm$ SEM. Statistically significant differences compared with CBP-treated control mice are indicated. (C) Total BAL cell count and (D) BAL cell count of eosinophils, $n = 8$, mean $\pm$ SEM. Mucus production in lung sections from (E) one CBP-rFel d 1 or (F) one CBP-treated mouse. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

detected at even lower MCh concentrations than for the OVA group (0.3 mg/kg, $P < 0.05$). Significant changes in AHR was also observed after injections of MCh for the rFel d 1 sensitized and extract challenged mice compared with rFel d 1 immunized and OVA challenged control mice (1 and 0.3 mg MCh/kg, $P < 0.01$). For OVA sensitized and challenged mice the AHR was also

significantly increased compared with rFel d 1 sensitized and OVA challenged mice (1 mg MCh/kg, $P < 0.05$). There were no differences in airway sensitivity to MCh, i.e. ED₅₀-values, between the different groups ($P > 0.05$, data not shown).

rFel d 1 immunized and CDE challenged mice, demonstrated a significantly ($P < 0.001$) increased

infiltration of cells in the BAL fluid compared with the PBS challenged mice (Fig. 1C). Differential count of May–Grünwald–Giemsa stained cells indicated an allergic inflammation with a significant increase of infiltrating eosinophils ($P < 0.001$) (Fig. 1C) compared with the PBS challenged mice. The OVA immunized and challenged mice had, as expected, a significantly enhanced infiltration of cells, mainly eosinophils ($P < 0.001$) (Fig. 1C). Furthermore, for rFel d 1 sensitized mice, secreted mucus could only be detected in PAS stained lung sections from CDE challenged mice, and not from PBS challenged mice (Fig. 1D and E).

Serum was collected to evaluate the humoral response to rFel d 1. All groups immunized with rFel d 1 had significantly ($P < 0.01$) elevated levels of rFel d 1-specific IgE and IgG₁ (Fig. 1F) compared with PBS immunized mice. In addition, the levels of total serum IgE were significantly ($P < 0.01$) elevated after rFel d 1 sensitization compared with PBS immunized mice (data not shown). OVA sensitized mice had significantly elevated levels of OVA-specific IgE and IgG₁ compared with PBS immunized mice (data not shown). An RBL assay was performed with serum from rFel d 1 sensitized mice, demonstrating that the rFel d 1-specific serum IgE induced basophil degranulation (Fig. 1G).

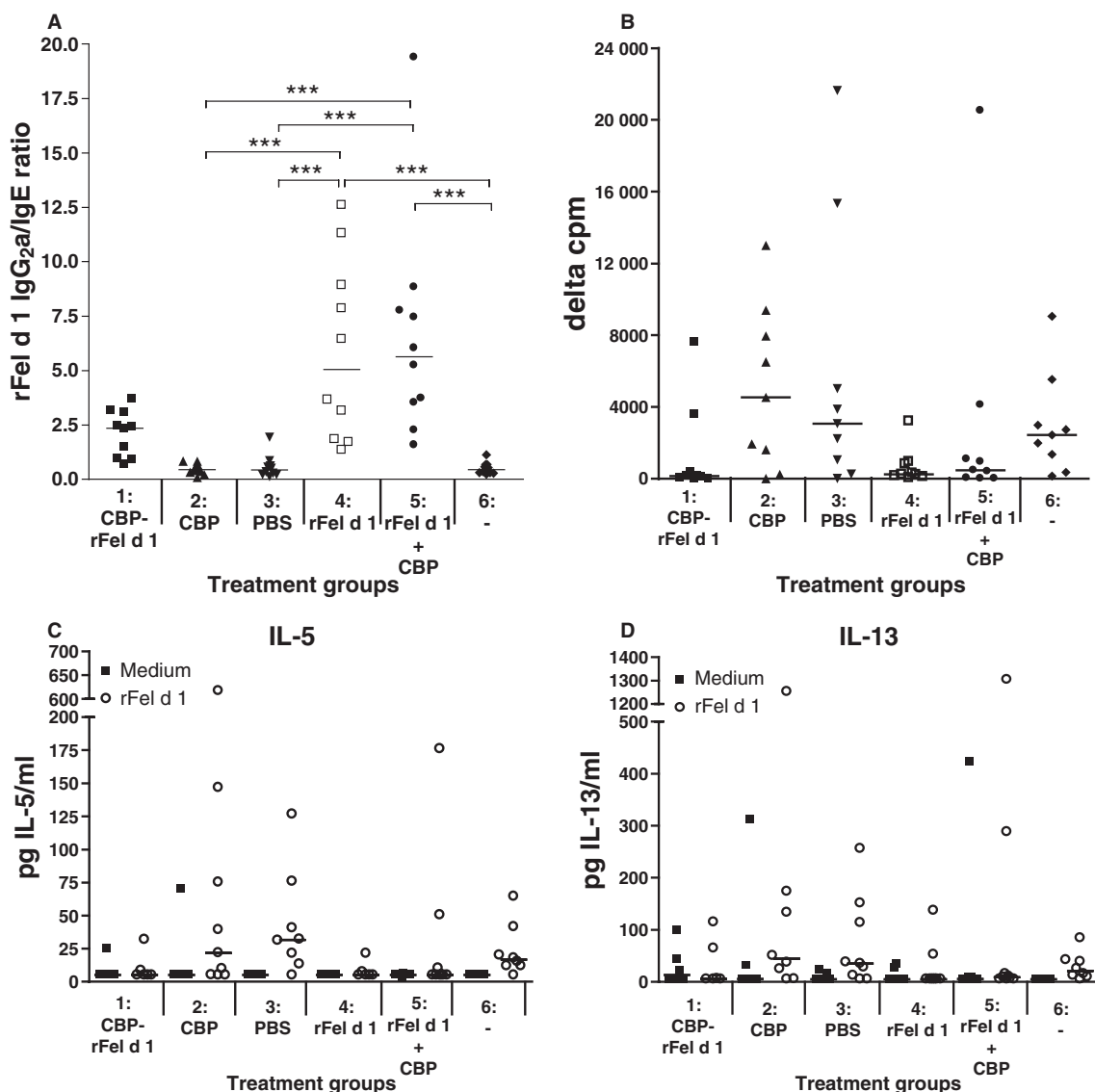


Figure 3. Effect of CBP-rFel d 1 treatment on immunoglobulin levels and splenocyte proliferation: (A) Ratio of allergen-specific IgG_{2a} to IgE calculated from optical density values, $n = 10$. *** $P < 0.001$. (B) Allergen-specific splenocyte proliferation. Background proliferation induced by medium alone was subtracted from the allergen-induced c.p.m.-values. Levels of released (C) IL-5 and (D) IL-13 in culture supernatants, $n = 9$. Median values are indicated.

Effect of CBP-rFel d 1 treatment on allergic inflammation

Having established the model for cat allergy, we next wanted to evaluate the therapeutic effect of CBP-rFel d 1 in these mice (Table 2 and Fig. 2A). Mice treated with CBP-rFel d 1 revealed a significantly reduced maximal lung resistance compared with CBP-treated control mice (Fig. 2B, $P < 0.05$). In fact, CBP-rFel d 1-treated mice responded in the same fashion as negative control mice challenged with PBS. Mice treated with rFel d 1 alone and mice treated with uncoupled rFel d 1 together with CBP had lower maximal airway resistance values than untreated animals, but the difference was not statistically significant (Fig. 2B, $P > 0.05$).

When assessing the number of leucocytes in the BAL fluid, we found that CBP-rFel d 1 treatment tended to result in fewer total infiltrating cells after CDE challenge (Fig. 2C). In particular, the amount of infiltrating eosinophils was reduced compared with CBP-treated mice (Fig. 2D, $P < 0.05$). CBP-rFel d 1-treated mice had also less mucus secreted into the bronchi, compared with CBP-treated control mice (Figs 2E and F).

Mice treated with CBP-rFel d 1 tended to have higher levels of rFel d 1-specific IgG₂a and lower IgE levels, resulting in an elevated IgG₂a/IgE ratio, compared with CBP- or PBS-treated mice (Fig. 3A). Mice treated with rFel d 1 or uncoupled rFel d 1 mixed with CBPs respectively, both exhibited increased ratios of IgG₂a to IgE ($P < 0.001$), which could be explained by the high doses of free rFel d 1 they received.

Splenocytes from the sensitized mice were stimulated with rFel d 1 *in vitro*, and proliferation and cytokines were measured. Splenocytes from CBP- or PBS-treated mice showed a more pronounced proliferation in response to *in vitro* restimulation compared with splenocytes from CBP-rFel d 1-treated mice (Fig. 3B). The levels of released IL-5 and IL-13 in the culture supernatants of the stimulated splenocytes on day 5 showed a tendency to be lower in mice treated with CBP-rFel d 1 compared with both PBS and uncoupled CBP-treated mice (Figs 3C and D). There was no difference in the production of IFN- γ or IL-10 between the various groups (data not shown).

Kinetics of the anti-rFel d 1 humoral immune response

Next, mice were immunized with CBP-rFel d 1, rFel d 1 alone, rFel d 1 mixed with CBPs or CBPs alone days 0, 7 and 15, and the humoral anti-rFel d 1 response was monitored. Only when rFel d 1 was covalently coupled to the CBPs, the rFel d 1-specific IgM response peaked 8 days after the primary immunization (Fig. 4A, $P < 0.01$ compared with rFel d 1 mixed with CBPs). The levels of IgG₁ (Fig. 4B) and IgG₂a (Fig. 4C) were also significantly higher, and appeared earlier, when rFel d 1 was given coupled to CBPs compared with free

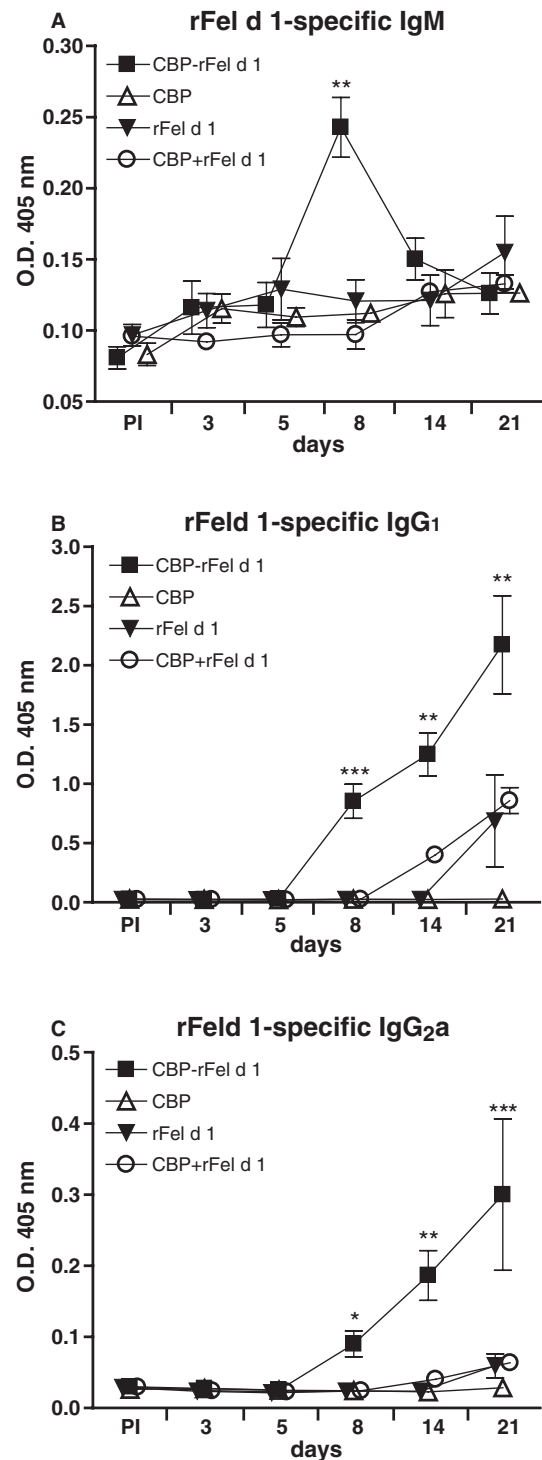


Figure 4. Kinetics of the CBP-rFel d 1 immune response. Serum levels of rFel d 1-specific (A) IgM, (B) IgG₁ or (C) IgG₂a were measured after immunization with CBP-rFel d 1, CBP, rFel d 1 or a mixture of CBP and rFel d 1 on day 0, 7 and 14. Statistical significances indicated, represent differences between mice immunized with CBP-rFel d 1 and rFel d 1 mixed with CBPs (A, day 8), rFel d 1 (B and C, day 8 and 14) and CBPs (B and C, day 14 and 21) $n = 5$, mean $\pm$ SEM. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

rFel d 1 ($P < 0.01$ for days 8 and 14) and CBPs alone ($P < 0.01$ for days 14 and 21).

Discussion

Currently, alum is used as an adjuvant and carrier to reduce systemic allergen release during ASIT. This is crucial since systemic distribution of allergen could lead to adverse side-effects such as anaphylactic reactions. However, there are drawbacks associated with alum as an adjuvant (4, 6, 24, 25), and therefore the use of CBP as a means to improve the allergen formulation for ASIT was assessed.

First, a new mouse model for cat allergy was developed, as this was considered a relevant allergen to use in a model to assess ASIT. Almost all mouse models of allergic inflammation use ovalbumin as an allergen. There are a few reports on inducing cat allergy in mice (12, 26). In our model however, we measured AHR with a complete dose–response relation for MCh, which allows for detailed assessment of the effects of interventions on distinct components of the airway physiology. We could show that rFel d 1 coupled to the particles efficiently reduced the infiltration of eosinophils in the BAL fluid, and reduced the AHR after methacholine challenge in sensitized mice. These results suggest that ASIT performed with CBP-coupled allergens will prove efficient for reducing allergen-induced airway symptoms.

The mechanisms behind successful ASIT are not clear but believed to involve both modulation of the B-cell and T-cell responses to the allergens (1–3). The allergen-specific T-cell response may either change to a Th1 skewed or a T-regulatory response during treatment, which can counteract or suppress the established Th2 polarization. Moreover, during ASIT, allergen-specific IgG is produced, which subsequently could interfere with the interaction between allergen and mast cell bound IgE thus preventing cross linking of IgE receptors. We could show that treatment with CBP-rFel d 1 results in increased levels of rFel d 1-specific IgG_{2a} in serum, which indicates a more Th1-skewed response to the allergen and presumably the presence of antibodies which could function in a blocking manner.

We speculate that the allergen-specific nonresponsiveness induced when allergens are linked to CBPs in our system depends on the size (2 µm) of the particles. Proteins, such as allergens, can be coupled to the CBPs with a high density. Moreover, the size is optimal for phagocytosis by antigen-presenting cells (27, 28), and we have previously shown that monocyte-derived dendritic cells are capable of ingesting the particles (18). These features would therefore result in a high antigen uptake by the APC, which is advantageous for the development of allergen-specific nonresponsiveness or tolerance (29, 30). In a recent clinical study, the mite allergen Der p 1 was coupled to virus-sized particles, and significantly

more IgG was induced with a higher dose of Der p 1 compared with a lower dose (31).

Recently, we demonstrated that rFel d 1 is retained in the skin near the site of injection for a longer time period when coupled to CBPs, than when adsorbed to alum (S. Thunberg et al., manuscript in preparation). This feature may also contribute to the beneficial effect of CBPs by allowing a prolonged allergen exposure and phagocytosis by APC in the skin. Moreover, by immunizing naïve mice and following the serum immunoglobulin levels during several weeks, we could show that rFel d 1 coupled to CBPs is much more efficient in inducing an allergen-specific immune response, compared with free allergen or allergen mixed with CBPs (Fig. 4). The levels of IgM were more rapidly upregulated, and the IgG₁ and IgG_{2a} titres were higher when rFel d 1 was given coupled to CBPs. For sensitized mice, treatment with free rFel d 1 or rFel d 1 mixed with CBPs also reduced the allergic inflammation. However, in the human system, it is not an option to treat patients with high doses of free allergen because of the risk of severe anaphylactic shock. We observed that mice which received uncoupled allergen reacted with treatment side-effects, showing piloerection and an affected breathing rate, presumably because of the high allergen amounts given. In contrast, mice treated with rFel d 1 covalently coupled to CBPs did not display these symptoms. Collectively, this supports that rFel d 1 covalently coupled to CBPs is a favourable adjuvant for ASIT, since it allows the administration of high doses of allergen to sensitized mice, as well as induce strong antibody responses in naïve mice.

In conclusion, we show for the first time that CBPs modulate the immune response, allergic inflammation and AHR when used in the treatment of rFel d 1 sensitized mice. We speculate that this novel approach to improve ASIT would be beneficial due to CBPs' ability to deliver high doses of allergen without the risk of systemic spreading and its potency to induce immune responses. Based on the advantageous effects demonstrated in this study, we consider CBPs as a promising candidate for application in ASIT to treat allergic patients. We also propose that our mouse model for cat allergy will be useful in future studies to further develop the ASIT strategy.

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