

Original article

Prolonged antigen-exposure with carbohydrate particle based vaccination prevents allergic immune responses in sensitized mice

Background: Defined particles carrying tightly bound allergens at high density have been suggested as alternatives in allergy vaccination. Carbohydrate based particles (CBP), sized 2 µm, provide a platform for covalent coupling of allergens.

Objective: To investigate the mechanisms of antigen presentation by CBP, as well as cellular and humoral responses after vaccination with the major cat allergen Fel d 1, covalently coupled to CBP.

Methods: Mice ($n = 10/\text{group}$) were subcutaneously vaccinated with CBP-rFel d 1, CBP or phosphate buffer saline (PBS) before sensitization with rFel d 1 and challenged with cat dander extract. Fluorescent and ^{75}Se -radiolabeled tracking of allergens and particles were performed with flow cytometry and whole-body autoradiography. Humoral, cellular and regulatory immune responses were analyzed by ELISA and flow cytometry. Cytokines were measured in bronchoalveolar lavage fluid and splenocyte cultures.

Results: CBP-rFel d 1 prevented induction of airway inflammation and induced allergen-specific T-cell anergy. CBP-rFel d 1 also induced rapid IgM and IgG1-responses compared with soluble rFel d 1. Particles were phagocytosed by antigen-presenting cells and transported to draining lymph nodes and spleen. Moreover, antigen coupled to CBP remained longer at the injection site compared with alum.

Conclusions: Covalent coupling of rFel d 1 to CBP induces rapid antibody production, prevents induction of allergic immune responses and systemic allergen spreading. Thus, CBP comprise several attractive adjuvant features for use in allergy vaccination.

Clinical Implications: Prolonged allergen exposure through covalent coupling to particles suitable for phagocytosis, provides an adjuvant for safer and efficient allergy vaccination.

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Adjuvants were introduced to vaccine formulations about 70 years ago. Apart from being nontoxic and safe with regard to local and systemic side-effects, an adjuvant shall function as an antigen depot, slowly releasing the antigen at the site of injection, which prolongs and enhances antigen presentation, thus eliciting humoral and/or cell-mediated immune responses (1, 2). Aluminum hydroxide

(alum), is by far the most commonly used adjuvant for human vaccination (1, 3). Alum induces high antigen-specific antibody titers and acts as an antigen depot. However, alum is associated with local side-effects such as contact hypersensitivity and granuloma formations (1, 4) as well as being ineffective in inducing cell-mediated immunity (2, 5). Furthermore, the amount of antigen and the stability of the adsorbates vary between preparations (6).

To ensure effective uptake and presentation by antigen presenting cells (APCs), adjuvant compounds should have a size and particulate structure equivalent to that of

Abbreviations: Alum, aluminum hydroxide; BAL, bronchoalveolar lavage; CBP, carbohydrate based particles; CDE, cat dander extract; Cpm, counts per minute; Fel d 1, major cat allergen; MCh, methacholine; Se, selenium; Treg, regulatory T-cell.

pathogens and microbes normally encountered and recognized by the immune system. Virus sized particles (20–200 nm) as well as soluble proteins, are usually taken up by fluid-phase endocytosis (pinocytosis), while larger particles (0.5–5 µm) are phagocytosed by macrophages and dendritic cells (DCs) (7–9). Vidard and colleagues showed that ovalbumin linked to 2–4-µm polystyrene beads are phagocytosed and presented to T-cells 1000–100 000-fold more efficiently compared with soluble ovalbumin, and the means of antigen uptake dictate the immunogenic epitopes generated (8). Thus, coupled antigens are presented more efficiently compared with soluble antigens because of differences in antigen uptake.

New types of adjuvants have been formulated with respect to size and particulate shape and tested *in vitro* and *in vivo* (10–13). We recently introduced 2-µm carbohydrate based particles (CBP), as a novel particulate adjuvant to improve allergy vaccination (14–16). CBP covalently coupled to the timothy grass pollen allergen Phl p 5 exhibited desirable adjuvant properties, compared with alum-adsorbed Phl p 5 when administered to naïve nonsensitized mice. Both adjuvants induced significantly increased allergen-specific antibody responses compared with soluble Phl p 5. Unlike alum, CBP-Phl p 5 did not induce granulomatous tissue reactions (16). Moreover, sensitized mice therapeutically treated with CBP coupled to the major cat allergen Fel d 1 showed reduced features of allergic inflammation compared with controls (15). In this study, we investigated mechanisms of CBP up-take as well as humoral and cellular responses after subcutaneous prophylactic vaccination with CBP coupled to recombinant (r) Fel d 1. We show that CBP-rFel d 1 induce rapid allergen-specific antibody production and prevent induction of allergic immune responses in mice sensitized to rFel d 1. Moreover, in contrast to alum adsorbed rFel d 1, CBP-rFel d 1 remain at the site of injection, thus enabling prolonged antigen exposure and effective phagocytosis by APCs in the skin. The intrinsic features of allergen-coupled CBP highlight carbohydrate particles as a promising adjuvant for human use.

Methods

Mice

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

Allergens

The recombinant Fel d 1 protein, produced as a hybrid molecule consisting of chain 2 linked to chain 1, was prepared (17) and covalently coupled to CBP or adsorbed to alum as previously described (14, 16). Cat dander extract (CDE) was prepared from cat

Table 1. Vaccination and sensitization protocol of naïve BALB/c mice

Group (<i>n</i> = 10/group)	Vaccination	Sensitization	Challenge
CBP-rFel d 1	100 µg CBP-rFel d 1	1 µg rFel d 1	10 µg CDE
CBP	CBP	1 µg rFel d 1	10 µg CDE
sham	PBS	1 µg rFel d 1	10 µg CDE
control	no treatment	1 µg rFel d 1	PBS

CBP, carbohydrate based particles; PBS, phosphate buffer saline; CDE, cat dander extract.

dander (Allergon, Ängelholm, Sweden). The Fel d 1 level in the extract was determined to 0.7% using ELISA (Indoor Biotechnologies, Manchester, UK) with rFel d 1 as standard. The endotoxin levels were 4.5 ng LPS/mg rFel d 1, tested with limulus amoebocyte lysate endochrome assay, according to the manufacturer's instructions (Charles River Endosafe, Charleston, USA). The endotoxin level in the extract was measured to 317 ng LPS/mg protein. After coupling, the CBP-rFel d 1 was washed and dissolved in sterile PBS and kept at +4°C. Production of ⁷⁵Se-labeled rFel d 1 was performed using *in situ* labeling of the selenocysteine residue in Sel-tagged rFel d 1, as previously described (18, 19).

Vaccination and sensitization protocol

For kinetics of CBP and CBP-rFel d 1-specific antibody production, mice (*n* = 5/group) were randomly divided into four groups. The mice were bled in the tail artery on days –4 (preimmunization), 3, 5, 8, 14, 21. Mice were subcutaneously (s.c.) immunized (200 µl/injection) thrice with 7-day intervals using preparations consisting of 100 µg rFel d 1 and/or 13 mg CBP. The administered preparations, given days 0, 7, and 15, were rFel d 1 coupled to CBP (CBP-rFel d 1), rFel d 1 alone, CBP mixed with rFel d 1 or CBP alone. In the vaccination study (Table 1), we used an established mouse model for cat allergy (15). Briefly, mice (*n* = 10/group) received s.c. injections, days 0, 7, and 14 with 100 µg CBP-rFel d 1, CBP, PBS (sham) or left un-treated (control). One week after final vaccination, all groups were s.c. sensitized with 1 µg rFel d 1-alum three times on days 21, 35, and 49, before intranasal (i.n.) challenge during three consecutive days on days 55, 56, and 57, with 10 µg CDE (groups rFel d 1-CBP, CBP, and sham) or PBS (control). Measurements of methacholine (MCh) induced airway hyper-reactivity was performed with a small animal ventilator (FlexiVent, Scireq, Montreal, Canada), described in detail by Neimert-Andersson *et al.* (15). Bronchoalveolar lavage (BAL) was performed after i.n. allergen challenge. One milliliter of ice-cold PBS containing 0.6 mM EDTA was used to lavage the lungs three times. BAL fluid supernatants were stored at –80°C before IL-5, IL-13, and IFNγ were measured with cytometric bead array (CBA) Flex according to the manufacturer's instruction (BD Biosciences, San Jose, CA, USA). Total BAL cell numbers were counted with Trypan blue and calculated back to cells/ml BAL. 5×10^4 total cells were cytospun onto microscopic slides and stained with May–Grünwald–Giemsa (Sigma Aldrich, Steinheim, Germany) according to manufacturer's instructions. Between 300 and 500 cells were counted/slide with 1 slide/animal.

Immunoglobulin measurement

Sera were stored at –20°C for measurement of antibody titers using ELISA and added to V-shaped 96-well plates (kinetic study) (Nunc, Roskilde, Denmark) together with 5 µg/ml rFel d 1-CBP or CBP alone. In order to wash the antibody bound particles, ELISA wash

buffer was added and plates were centrifuged twice. Analysis of sera from the vaccination study was performed with flat 96-well plates (Nunc), which were coated overnight with 5 µg/ml rFel d 1 before the addition of sera. In the kinetics experiments, IgM and IgG1 were detected with alkaline phosphatase-conjugated goat anti-mouse IgM (diluted 1 : 3000) or IgG1 (diluted 1 : 1000) (Southern Biotech, Birmingham, AL, USA). In the vaccination study, IgE was detected using a biotinylated rat anti-mouse IgE (diluted 1 : 500, BD Biosciences), while 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). IgG2a was detected using a biotinylated rat anti-mouse IgG2a (diluted 1 : 1000, BD Biosciences). ALP-streptavidine (diluted 1 : 3000, Dako Cytomations, Glostrup, Denmark) and phosphatase substrate (Sigma) were used for detection, and color development was monitored after 60 min at 405 and 620 nm. Single samples from individual mice were measured and all analyses were performed in parallel.

Splenocyte cultures

After disruption and lysis, splenocytes were filtered through a 70-µm nylon mesh strainer and resuspended in complete culture medium (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, UK), 50 µM 2-mercaptoethanol (Sigma) and 10% bovine growth serum (Hyclone, Logan, UT, USA). 2×10^5 /well of splenocytes were cultured in triplicate in 96-well plates and stimulated with 10 µg/ml of rFel d 1, 10 µg/ml CDE or 5 µg/ml Concanavalin A (Con A) (Sigma). The splenocytes were incubated for 5 days in a humidified atmosphere with 5% CO₂ at 37°C and the supernatants were collected before addition of 1 µCi/well [³H] thymidine during the final 18 h. The Th1/Th2 CBA-kit (BD Biosciences) was used to analyze rFel d 1-stimulated production of IL-5 and IFNγ, according to the manufacturer's instruction. The detection limit was set to 2.5 pg/ml.

Fluorescein isothiocyanate-coupled CBP

Fluorescein isothiocyanate (FITC) (Sigma) was coupled to CBP with the same method as rFel d 1 (15, 16) and administered s.c. in the neck (in 200 µl, total dose; 5 µg FITC coupled to 20 mg CBP) of naïve mice ($n = 5$ /time point). Control mice received PBS ($n = 5$). Spleens, inguinal and peritoneal lymph nodes (LNs) were harvested after 6, 24 h and 5 days, for flow cytometry analysis. To analyze cells at the site of injection, neck skin samples were mashed and pooled from five mice at day 5.

Flow cytometry

1×10^6 splenocytes or cells from LNs were surface stained with anti-mouse CD4-FITC, CD11c-FITC, B220-PerCP, CD8a-PECy7, CD25-APC, CD11b-APC or CD3e-pacific blue (BD Pharmingen, San Jose, CA, USA). Intracellular staining of foxp3 was performed with anti-mouse foxp3-PE (clone PCH101) or rat IgG2b-PE isotype control (eBioscience, San Diego, CA, USA). Cell suspensions from PBS or CBP-FITC treated mice were stained with F4/80-PE, B220-PerCP, biotinylated CD11c as well as CD11b. PBS samples determined background auto-fluorescence in the FITC channel. Isotype controls rat IgG1-FITC and rat IgG1-APC were used (BD Pharmingen). Cell acquisition and analysis were performed on a FACSAria flow cytometer with FACSDIVA software (BD Biosciences).

Whole-body autoradiography

In vivo tracking of 100 µg radioactively labeled [⁷⁵Se]rFel d 1 (2 µCi) coupled to CBP or adsorbed to alum was performed as previously described (18, 20). Briefly, mice were s.c. injected with CBP-[⁷⁵Se]rFel d 1 ($n = 2$, 24 h or $n = 1$, 1 week) or alum-[⁷⁵Se]rFel d 1 ($n = 2$, 24 h and 1 week) and killed after 24 h or 1 week. The mice were frozen and processed for tape section autoradiography. The sections (60-µm thick) were pressed against X-ray film (Structurix, Agfa, Mortsel, Belgium) and developed using D19 (Kodak, Rochester, NY, USA).

Statistics

Nonparametric Kruskal–Wallis ANOVA with Dunn's multiple comparisons test (cytokine and immunoglobulin measurements) or one way ANOVA, followed by Bonferroni's multiple comparisons test (splenocyte proliferation) were used to evaluate differences between multiple groups. The Mann–Whitney *U*-test was used when two groups were compared. GRAPHPAD PRISM version 4.03 (Graphpad Software Inc., San Diego, CA, USA) or STATISTICA version 7.0 (Stat Soft, Tulsa, OK, USA) were used for calculations. $P < 0.05$ was considered significant.

Results

Covalently coupled rFel d 1-CBP induces rapid and enhanced antibody responses compared to un-coupled rFel d 1

In order to follow the kinetics of early humoral responses induced by CBP, groups of mice were immunized with CBP-rFel d 1, CBP, rFel d 1 or CBP plus uncoupled rFel d 1. CBP-rFel d 1 specific IgG1 and IgG2a as well as CBP-specific IgM, IgG1 and IgG2a were analyzed with ELISA. CBP-rFel d 1 specific IgG1 was detected in sera from CBP-rFel d 1 immunized mice at day 8, which further increased at days 14 and 21, compared with mice immunized with soluble rFel d 1. CBP-rFel d 1 specific IgG1 was also detected in sera from mice immunized with CBP plus rFel d 1, but the response appeared later and was weaker compared with CBP-rFel d 1 immunized mice (Fig. 1A). Detected CBP-rFel d 1 specific IgG2a was also elevated compared with soluble rFel d 1. The difference was significant only at day 21 (Fig. 1B). Out of the three groups immunized with CBP, only mice immunized with CBP-rFel d 1 developed CBP-specific IgM antibodies, which peaked at day 5 ($P = 0.03$, compared to CBP). The CBP-specific IgG1 peaked at day 14 ($P = 0.009$), after which it started to decline (Fig. 1C, IgM not shown). Thus, the CBP-specific antibody responses appeared later with a transient character and with low titer, compared with allergen-specific antibody responses.

Vaccination with rFel d 1-coupled CBP induces IgG2a and protects against allergen sensitization and provocation

We sought to test the protective effects of CBP-rFel d 1 in a mouse allergy model. Mice were vaccinated with CBP-rFel d 1, CBP, PBS (sham) or untreated (control) and

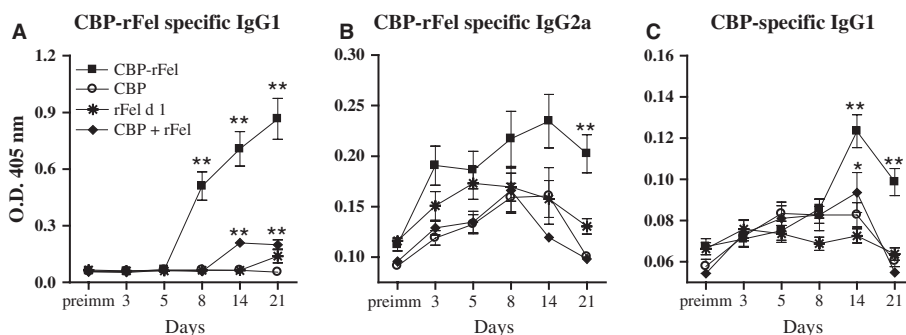


Figure 1. Vaccination with CBP-rFel d 1 induces rapid CBP-rFel d 1 specific IgG1. Mice ($n = 5/\text{group}$) were vaccinated three times at days 0, 7, and 15 with CBP-rFel d 1 (■), Carbohydrate based particles (CBP) (○), rFel d 1 (*) or CBP mixed with rFel d 1 (◆). Comparisons were made between CBP-rFel d 1 and soluble rFel d 1 (A) and CBP-rFel d 1 and CBP (B). Mean O.D. $\pm$ SE measured at 405 nm is shown. * $P < 0.05$, ** $P < 0.01$.

subsequently sensitized with rFel d 1 and challenged i.n. with CDE (groups CBP-rFel d 1, CBP and sham) or PBS (control) (Table 1). Mice treated with CBP-rFel d 1 had a reduced total cell number in BAL ($P = 0.0001$),

reflecting decreased infiltration of lymphocytes, macrophages and especially eosinophils compared with sham vaccinated mice (Fig. 2A). BAL fluid was also analyzed for cytokine content. The levels of IL-5 and IL-13 were

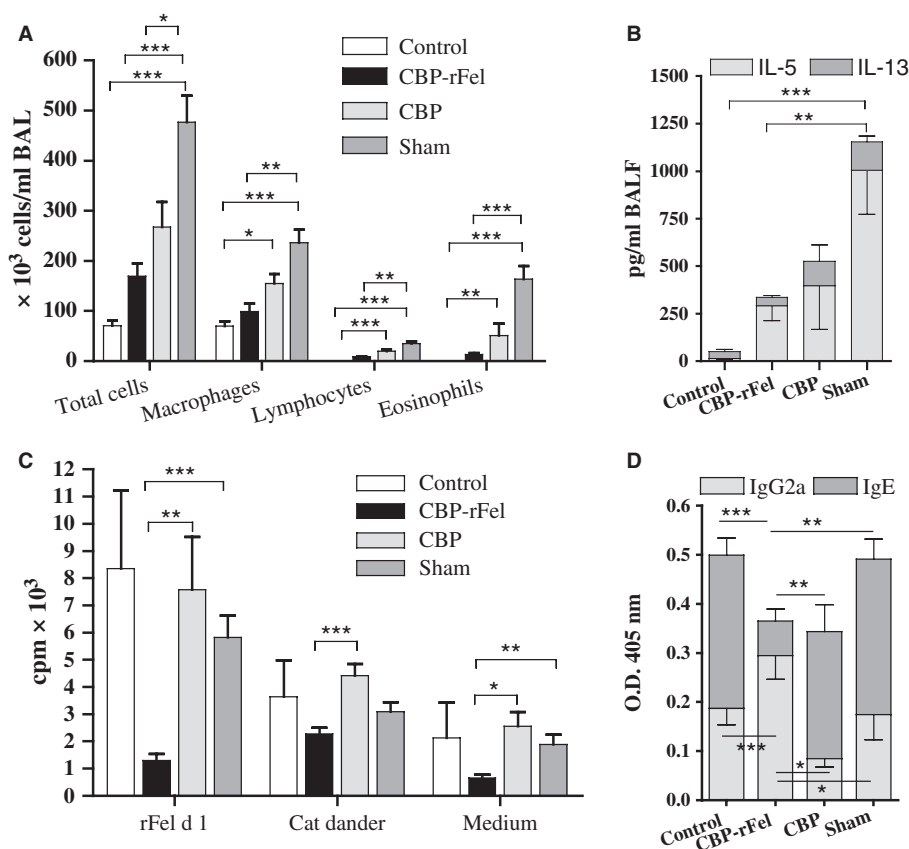


Figure 2. CBP-rFel d 1 vaccination prevents cell infiltration and induces IgG2a. Mice ($n = 10/\text{group}$) were subcutaneously vaccinated with CBP-rFel d 1, CBP, PBS (sham) or un-treated (control) with subsequent rFel d 1-sensitization and airway challenge with cat dander extract (groups rFel d 1-CBP, CBP and sham) or PBS (control). (A) Total and differentiated BAL cell counts. Bars represent mean $\pm$ SE cells/ml BALF. (B) IL-5 and IL-13 in BALF. Data represent mean $\pm$ SE pg/ml. (C) *In vitro* proliferation of allergen-specific splenocytes measured by [^3H] thymidine incorporation. Bars represent mean $\pm$ SE cpm. (D) IgE and IgG2a measured in sera from vaccinated mice. P -values represent increased IgE- and decreased IgG2a levels, compared with CBP-rFel d 1 vaccinated mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

reduced for the CBP-rFel d 1 injected group, compared with sham ($P = 0.009$ and 0.03 , respectively) (Fig. 2B), while no significant difference in the levels of IFN γ was detected (data not shown). Hence, prophylactic vaccination with CBP-rFel d 1 prevents induction of airway inflammation and secretion of IL-5 and IL-13.

To examine the allergen-specific T-cell response, splenocytes were stimulated with rFel d 1 or CDE. Splenocytes from mice pretreated with CBP-rFel d 1 did not proliferate upon stimulation with rFel d 1, while splenocytes from the CBP and sham pretreated groups proliferated ($P = 0.005$ and $P = 0.0001$, respectively) (Fig. 2C). Furthermore, the IL-5 production in response to rFel d 1 was significantly higher in sham ($P = 0.0005$) compared with the CBP-rFel d 1 group. There was no significant difference between groups in the production of IFN γ (not shown). Proliferation to CDE was comparable, and splenocytes from CBP and sham pretreated mice showed enhanced proliferation compared with CBP-rFel d 1 vaccinated mice ($P = 0.0003$). However, the difference in proliferation between sham and CBP-rFel d 1 was not significant ($P = 0.07$). The background proliferation of nonstimulated splenocytes was lower in the group vaccinated with CBP-rFel d 1 than CBP or sham vaccinated groups. However, all cells proliferated equally well to stimulation with positive control Con A. Within the CBP-rFel d 1 vaccinated group, splenocytes stimulated with CDE proliferated significantly more ($P = 0.007$) compared with rFel d 1 stimulated splenocytes indicating that the splenocyte T-cell population is nonresponsive to rFel d 1, but capable of responding to other antigens within the extract (Fig. 2C). CBP-rFel d 1 was also analyzed for its ability to shift production of antibody isotype. After sensitization and allergen challenge, mice vaccinated with CBP-rFel d 1 displayed significantly reduced levels of rFel d 1-specific IgE, compared with mice vaccinated with CBP alone, sham ($P = 0.006$ and 0.002 , respectively) as well as control mice ($P = 0.0001$). In addition, vaccination with CBP-rFel d 1 increased rFel d 1-specific IgG2a levels, compared with vaccination with CBP ($P = 0.001$), sham or control ($P = 0.03$ and 0.02). CBP alone did not show any differences in IgE or IgG2a levels compared with sham vaccinated mice (Fig. 2D). Thus, the results show that CBP-rFel d 1 was able to increase IgG2a and decrease IgE-production as well as induce allergen-

specific nonresponsiveness of T cells. To further evaluate vaccination with CBP-rFel d 1, mice were subjected to *in vivo* MCh provocation after allergen challenge. As shown in Table 2, sham vaccinated mice reached the highest peak resistance compared with PBS challenged control mice. Vaccination with CBP-rFel d 1 resulted in reduced airway hyper-reactivity to 0.3 and 1 mg/kg MCh, compared with mice in the sham vaccinated group ($P = 0.04$ and 0.02 , $n = 10$, respectively). Contrary to data from humoral and cellular analysis, vaccination with CBP was protective against airway hyper-reactivity in comparison with sham vaccinated mice (Table 2).

Flow cytometry was used to determine whether the allergen-specific T-cell nonresponsiveness was because of induction of regulatory T cells (Treg). Analysis of fresh splenocytes showed similar percentage of CD4⁺CD25^{bright} cells in all groups, but a higher percentage of CD4⁺foxp3⁺ Treg cells was detected in splenocytes from the sham pretreated group compared with CBP-rFel d 1 ($P = 0.003$). There was no significant difference in foxp3-expression between CBP-rFel d 1-vaccinated mice compared with CBP vaccinated mice and control mice (Fig. 3).

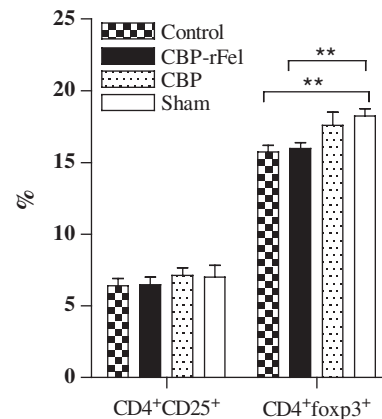


Figure 3. Increased number of foxp3⁺ regulatory T-cells in splenocytes from sham vaccinated mice. Fresh splenocytes were obtained from mice ($n = 10$ /group) vaccinated with CBP-rFel d 1, sham, CBP, and control and analyzed with flow cytometry. Bars represent percent CD4⁺CD25^{bright} and CD4⁺foxp3⁺ of CD4⁺ (mean $\pm$ SE). ** $P < 0.01$.

Table 2. Methacholine induced airway hyper-reactivity expressed as median peak respiratory resistance after i.n. allergen challenge

MCh (mg/kg)	CBP-rFel d 1	CBP	Sham	Control (PBS challenge)
0	0.56 (0.53–0.72)	0.60 (0.48–0.68)	0.56 (0.52–0.62)	0.56 (0.42–0.88)
0.03	0.65 (0.52–0.81)	0.67 (0.54–0.75)	0.72 (0.57–0.81)	0.64 (0.48–0.95)
0.1	0.95 (0.79–1.55)	1.05 (0.89–1.26)	1.46 (0.87–2.13)	0.86 (0.81–1.1)
0.3	2.44 (1.63–3.26)*	2.30 (1.52–3.41)*	4.02 (1.98–5.76)	1.75 (1.27–2.05)***
1	4.39 (3.09–5.55)*	3.97 (2.36–5.97)**	5.91 (4.01–7.99)	2.86 (2.42–3.57)***

MCh, methacholine; CBP, carbohydrate based particles; PBS, phosphate buffer saline.

* $P = 0.04$; ** $P = 0.02$; *** $P < 0.001$, compared with sham treated mice.

CD11c⁺ and F4/80⁺ phagocytes transport CBP to draining lymph nodes and spleen

We sought to investigate where CBP were located after injection and the cell types responsible for its uptake. For this purpose, the particles were labeled with the fluorescent marker FITC, injected s.c. and analyzed after 6, 24 h or 5 days with flow cytometry. A 5.2% (3.2–7.9) of FITC-positive cells, with phagocytosed FITC-labeled CBP, were localized in the inguinal lymph node (LN) 6 h ($P = 0.008$, compared with PBS-injected control mice) after injection, followed by 6.4% (4.7–9.8) after 24 h ($P = 0.008$) and 6.6% (2.7–11) after 5 days ($P = 0.02$). No FITC-positive cells were detected in the peritoneal LN at any time point or in the spleen 6 h after injection. However, 1.8% (1.4–2.2) positive cells were detected in the spleen after 24 h ($P = 0.008$), which further increased to 3.6% (3.2–4.2) after 5 days ($P = 0.008$) (Fig. 4A). The FITC-positive cells in spleen and LN expressed CD11c and F4/80 (a marker for macrophages and Langerhans cells in mice (21)), but not CD11b. However, most FITC-labeled particles ($\approx 92\%$ of total cells obtained from skin samples) still resided at the site of injection as long as 5 days after injection. Unlike FITC-positive cells in LN and spleen, the positive cells in the skin showed high expression of CD11b and intermediate expression of CD11c (Fig. 4B).

Enhanced depot effect by [⁷⁵Se]rFel d 1-coupled CBP compared with alum

Distribution of rFel d 1, rather than CBP, was followed with a novel methodology for carboxyl terminal residue-specific ⁷⁵Se-labeling (18, 19). [⁷⁵Se]-labeled rFel d 1 was coupled to CBP or adsorbed to alum and tracked at 24 h and 1 week after injection with whole-body autoradiography.

The radioactivity in mice receiving [⁷⁵Se]rFel d 1 adsorbed to alum was spread throughout the animals after 24 h and was still detected after 1 week. In contrast, the radioactivity in CBP-[⁷⁵Se]rFel d 1 immunized mice was located almost entirely at the injection site after 24 h, where it was still detectable 1 week after injection (Fig. 5). Thus, the radioactivity derived from [⁷⁵Se]rFel d 1 coupled to CBP remains at the injection site for a longer period of time, compared with [⁷⁵Se]rFel d 1 adsorbed to alum.

Discussion

The use of alum as an adjuvant in allergy vaccination has limitations (1, 2, 5, 22). The rationale for particulate adjuvants is to mimic pathogens and enable effective phagocytosis and antigen presentation (10–13). Here we show that vaccination with rFel d 1 coupled to the particulate adjuvant CBP prevents eosinophilic infiltration and airway inflammation after allergen challenge in rFel d 1-sensitized mice. *In vivo* and *in vitro* experiments suggest that both serological and cellular components contribute to the observed protective effects. CBP-rFel d 1 vaccination induced rapid antibody production already after one injection, compared with un-coupled rFel d 1, particles alone or a mixture of rFel d 1 and particles. The rapid induction of allergen-specific IgG antibodies suggests a very efficacious treatment. In accordance, it was recently shown that healthy volunteers vaccinated with 30-nm polypeptide particles covalently coupled to Der p 1 mounted rapid Der p 1-specific antibody responses (23).

Vaccination with CBP-rFel d 1 reduced the levels of IL-5 and IL-13 in BAL fluid, while potentially neutralizing rFel d 1-specific Th1-associated IgG2a antibodies were induced (24). Collectively, it seems that vaccination

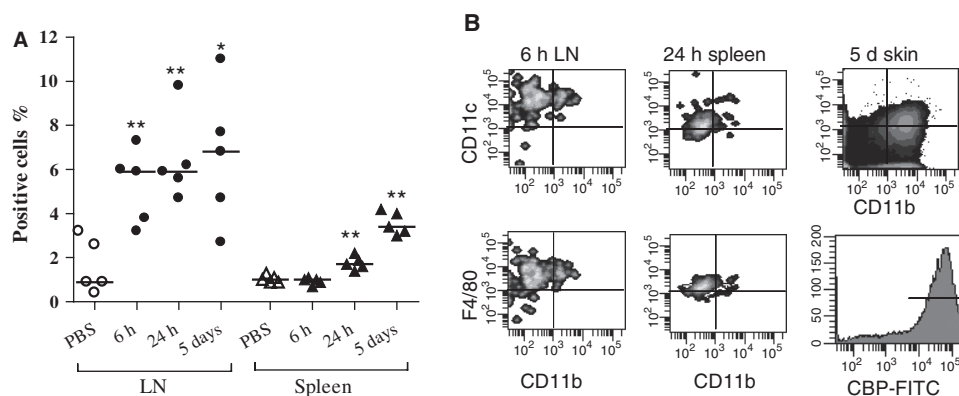


Figure 4. Migration of FITC-labeled carbohydrate based particles (CBP) after subcutaneous injections in mice ($n = 5$ /group). Draining inguinal lymph node (LN) and spleen were harvested after 6, 24 h or 5 days. Skin was collected at the site of injection at day 5. (A) Percent FITC-positive cells in draining LN and spleen in mice injected with CBP-FITC. PBS controls determined background auto-fluorescence in the FITC channel. Horizontal lines represent mean. (B) CD11c⁺, F4/80⁺ and CD11b⁺ phagocytes analyzed in LN and spleen and pooled CD11b⁺ cells in the skin at the site of injection. Dot plots for LN and spleen represents one out of five independent experiments. * $P < 0.05$, ** $P < 0.01$.

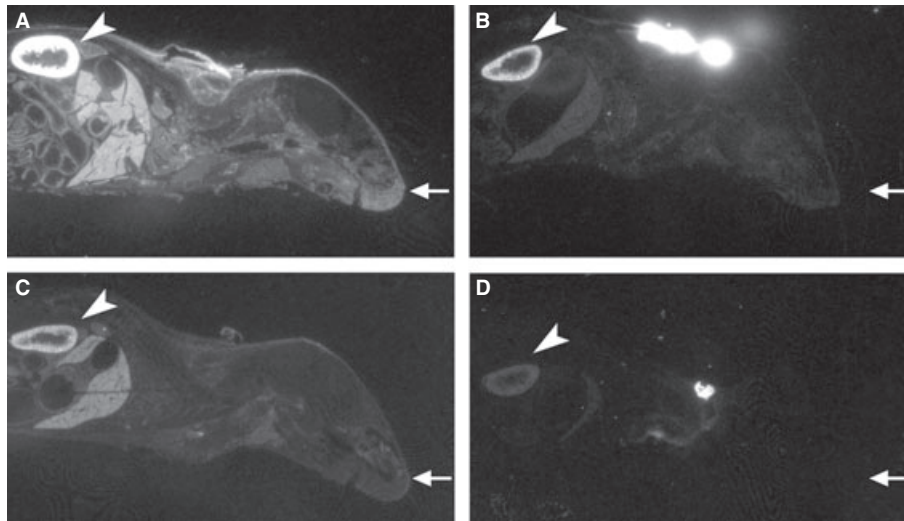


Figure 5. Whole-body autoradiography tracking of [^{75}Se]rFel d 1. Radioactivity was detectable in mice injected with [^{75}Se]rFel d 1-alum (A and C) or [^{75}Se]rFel d 1-CBP (B and D) after 24 h (upper panel) and after 1 week (lower panel). For anatomical orientation, arrows show the kidney cortex and the nose on each sagittal section. White areas represent sites containing radioactivity. The mice received the same amount of radioactivity and representative 60- μm thick sections were identically processed.

with allergen-coupled CBP induces immunological non-responsiveness on one hand and activation of immune responses on the other. Hypothetically, this may be the underlying mechanism behind successful allergy vaccination in humans, where first anergy of Th2 cytokine producing allergen-specific T-cells prepare for induction of de novo synthesized blocking antibodies (16).

Allergen-specific stimulation *in vitro* resulted in significantly increased proliferation of splenocytes from mice pretreated with CBP alone or sham, compared with splenocytes from CBP-rFel d 1 pretreated mice. The CBP-rFel d 1 induced T-cell anergy, which was observed at both allergen-stimulated and unstimulated conditions, was likely not because of induction of Treg cells, because more foxp3 $^{+}$ Treg cells were detected in sham vaccinated mice compared with CBP-rFel d 1 vaccinated mice. The foxp3-expression in the latter group was the same as in PBS challenged control mice, indicating that CBP-rFel d 1 vaccinated mice maintain T-cell homeostasis despite sensitization and allergen challenge. The increased foxp3-expression, observed in sham vaccinated mice may correspond to activated CD4 $^{+}$ CD25 $^{+}$ allergen-specific effector cells transiently expressing foxp3 (25). Interestingly, persistent long-term exposure to antigens has been shown to induce transplant tolerance and anergic T-cells as well as prevent allergic airway inflammation independently of foxp3 $^{+}$ Treg cells (26, 27). Our experiments with CBP coupled to the fluorescent marker FITC suggests that CBP remain at the site of injection at least 5 days after subcutaneous administration, indicating that allergen coupled to CBP is subjected to phagocytosis and antigen-presentation for a long period of time. Following injection, FITC-labeled CBP were phagocytosed by

CD11b $^{+}$ tissue macrophages as well as CD11c $^{+}$ and F4/80 $^{+}$ CD11b $^{-}$ DCs, demonstrating that tissue macrophages remain at the injection site, whereas DCs and Langerhans cells migrate to draining LN and the spleen for antigen presentation within 24 h after injection. Whole-body autoradiography further supported these data, showing that radioactivity in [^{75}Se]rFel d 1-coupled CBP remained at the injection site, while it was dispersed throughout the body when [^{75}Se]rFel d 1 was adsorbed to aluminum hydroxide. Consequently, prolonged allergen exposure, because of CBP-coupling, prevents allergen spreading, and thus contributing to a safer allergy vaccine.

In this study, we demonstrate that vaccination with CBP-rFel d 1 prevents induction of airway inflammation after allergen challenge, induces allergen-specific T-cell tolerance, and shifts immunoglobulin production in mice sensitized to rFel d 1. Moreover, antigen-coupled CBP demonstrate improved antigen-depot effects compared with alum as well as encompass immuno-stimulatory and inhibitory properties *in vivo*. Consequently, CBP show important features as an adjuvant candidate for allergy vaccination, both in a prophylactic and therapeutic setting.

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