Liposomes as delivery system for allergen-specific immunotherapy

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Chapter 6

Bet v 1 attached to cationic liposomes through coiled coil-forming peptides induces stronger antibody responses than aluminum-adsorbed Bet v 1

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Abstract

Background: Although aluminum hydroxide (alum) has long been used as safe vaccine adjuvant, there is growing concern about its toxicity after chronic exposure via allergen specific immunotherapy (SCIT). Replacing alum with safer alternatives is currently being investigated.

Objective: The aim of this study was to evaluate Bet v 1 bearing cationic liposomes as an alternative vaccine delivery system/adjuvant to replace alum in SCIT.
Methods: Cationic liposomes were functionalized with one peptide of a coiled coil (CC) forming peptide pair. The resulting liposomes were characterized with dynamic light scattering and laser Doppler electrophoresis. IgE binding and cross-linking were studied by ImmunoCAP and rat basophil leukemia cell assays. The immune responses of naïve mice immunized with Bet v 1 bearing liposomes or alum adsorbed Bet v 1 were compared.

Results: Bet v 1 bearing cationic liposomes were 200 nm in size and had a positive zeta potential. The coiled coil attachment between the liposomes and Bet v 1 resulted in approximately 15-fold less allergenic potential than free Bet v 1 and was crucial to induce high Bet v 1-specific IgG1 and IgG2a levels, which were several orders of magnitude higher than alum-adsorbed Bet v 1 immunized mice. This strong humoral response was accompanied by relatively high IL-10 cytokine levels.

Conclusion: The hypoallergenic character and strong humoral immune response of cationic liposomes bearing Bet v 1 via coiled coil attachment are advantageous properties for SCIT adjuvants. Therefore, these liposomes are a promising replacement for alum in SCIT.

Introduction

Subcutaneous allergy immunotherapy (SCIT) has been used to treat allergies for more than 100 years [1]. The treatment commonly consists of monthly subcutaneous injections of allergen extracts for 3 to 5 years to achieve optimal therapeutic effect. Therapy adherence is relatively low because of this long duration and the allergic side-effects that can occur [2]. Often, aluminum hydroxide (alum) is used as adjuvant for SCIT. Although alum has been reported to skew towards T helper (Th) 2 immune responses [3], during SCIT it has been shown to result in a more mixed Th1/Treg cytokine response in combination with production of interleukin (IL)-10 by regulatory T- and B-cells [4, 5]. Most importantly, these regulatory B-cells then also produce the required protective allergen-specific immunoglobulin (Ig) G_4 antibodies. In mouse models, the protective effect of SCIT has been associated with the production of allergen-specific IgG1 and particularly IgG2a antibodies and of IL-10 [6, 7].

Alum has a long history of safe use in vaccines for infectious diseases but also in SCIT [3]. Nevertheless, there is growing concern with respect to the long-term exposure to alum during SCIT, particularly in a pediatric setting [8].Therefore, good alternatives to ultimately replace alum as adjuvant for SCIT are needed. Besides directing the immune response, alum also serves as a depot for adsorption of allergens, shielding them from IgE antibodies and reducing the risk of allergic side-effects [9]. In recent years, different types of nanoparticles have drawn attention to serve as effective vaccine delivery systems [7, 10, 11]. Liposomes are one of the most promising nanoparticles that could replace alum [12, 13].

Liposomes consist of one or more lipid bilayers with an aqueous core and are a versatile delivery system and adjuvant for vaccines [13, 14]. Antigens can be adsorbed to the lipid bilayer [15], incorporated in the lipid bilayer [16], or encapsulated in the aqueous core of the vesicle [17, 18]. Recently, we described a novel antigen attachment method which is based on the interaction between two complementary α-helical peptides that form a coiled coil (CC) structure [19]. Immunization of mice with antigen attached to cationic liposomes via this CC formation resulted in strong $CD4^+$ T-cell proliferation and production of both interferon gamma (IFN-γ) and IL-10. These cytokines are a signature of a Th1 and a regulatory T-cell response, respectively, both of which are reported to be required for effective SCIT [7, 20-22].

The goal of this study was to design a novel, alum free SCIT candidate vaccine using Bet v 1, the major allergen in birch pollen allergy, and liposomes. We produced a fusion protein between Bet v 1 and one of the two CC forming peptides, peptide E (pepE-Bet v 1) and attached this to cationic liposomes

bearing the complimentary CC forming peptide, peptide K (pepK). The resulting liposomes were characterized and compared to alum-adsorbed Bet v 1 with regard to physicochemical and immunological properties.

Material & Methods

Chemicals and reagents

Cholesterol, 1,2-distearoyl-sn-glycero-3-phosphocoline (DSPC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Lipids. Recombinant protein Bet v 1 (isoform Bet v 1.0101) was produced by the Department of Molecular Biology of the University of Salzburg (Salzburg, Austria) [23]. Dimethylformamide (DMF), piperidine, acetic anhydride, pyridine, trifluoroacetic acid (TFA) and acetonitrile (ACN) were purchased from Biosolve (Valkenswaard, Netherlands). N,N-diisopropylethylamine (DIPEA), and ethyl cyanohydroxyiminoacetate (Oxyma) were obtained from Carl Roth (Karlsruhe, Germany). Dicholoromethane (DCM) and diethyl ether were supplied by Honeywell (Landsmeer, Netherlands). Tentagel HL-RAM was obtained from Rapp Polymere (Tübingen, Germany). All amino acids were supplied by NovaBioChem (Darmstadt, Germany). Fmoc-NH-PEG_4-COOH was purchased from Iris Biotech GmbH (Marktredwitz, Germany). Pierce BCA assay and Imject ® Alum were purchased from Thermo Fisher Scientific (Rockford, Ill., USA). Isopropyl β-D-1-thiogalactopyranoside was obtained from Invitrogen (Carlsbad, CA., USA). Fetal calf serum (FCS) was supplied by Thermo Fisher Scientific. Sucrose, HEPES, HATU, Triisopropylsilane (TIPS), sodium azide, Tyrode´s salts, BSA, lysozyme, sodium bicarbonate, 4-methyl umbelliferyl-N-acetyl-beta-D-glucosaminide, Triton X-100 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich. Disodium hydrogen phosphate and sodium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). Ampicillin was obtained from Roche (Basel, Switzerland).

Mice

Six to eight weeks old female BALB/c mice were purchased from ENVIGO (The Netherlands). The animals were housed under specific pathogen-free conditions at the animal facility of the Amsterdam University Medical Centers, location AMC. All experiments were performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament and were approved by the Animal Ethics Committee of the AMC.

Peptide synthesis

Peptides (pepK: CG-KIAALKEKIAALKEKIAALKE, and K4: KIAALKEKIAALKEKIAALKEKIAALKE) were synthesized by standard Fmoc chemistry using solid-phase peptide synthesis with an automated microwave peptide synthesizer (CEM liberty blue).

Cholesterol-PEG-K4 (CPK) was prepared as described elsewhere [10]. In short: Fmoc-NH-PEG_4-COOH was coupled to resin-bound K4 in the presence of DIPEA (5 eq.) and HATU (2.5 eq.) for 2.5 hours. Fmoc was removed with 20% piperidine in DMF before the reactive amine was coupled to 1.05 equivalents amino-cholestene hemisuccinate in the presence of DIPEA (5 eq.) and HATU (2.5 eq.) for 4 hours at room temperature. The peptide was cleaved from the resin with a mixture of TFA:TIPS:water (95:2.5:2.5 v/v/v), precipitated in ice-cold diethyl ether and collected via centrifugation.

Crude peptides were purified using a Shimadzu RP-HPLC system comprising two LC-8A pumps and a SPD-10AVP UV-Vis detector equipped with a Kinetic Evo C18 column. A gradient of 20-80% B, (where B is ACN containing 1% v/v TFA , and A is water with 1% v/v TFA) with a flow rate of 12 mL/min was used. Collected fractions were measured on a LC-MS system (Thermo Scientific TSQ quantum access MAX mass detector connected to a Ultimate 3000 liquid chromatography system fitted with a 50 × 4.6 mm Phenomenex Gemini 3 µm C18 column). The resulting chromatogram and spectrum are shown in Supplementary Figure 1. ACN was removed by rotary evaporation (150 mbar, 50 °C) before lyophilization, leaving dry purified peptide powder which was stored at -20 °C until use.

Design, expression and purification of pepE-Bet v 1.

A detailed description of the manufacturing of pepE-Bet v1 can be found in the supplementary Materials and Methods. In short, the pepE-Bet v 1 gene was produced by GenScript (Piscataway, NJ, USA) and used to transfect *E. coli* BL21 (DE3) cells. Ampicillin resistant clones were grown in a 5 L stirred tank coupled to a BIOSTAT® controller (Sartorius Stedim Biotech) for protein production. Harvested cells were pelleted by centrifugation and frozen for storage. The protein was isolated from the frozen cell pellets by disrupting the cells using sonication. Cellular debris was removed by centrifugation. The supernatant was filtered through 0.2 µm before affinity purification using cross-linked agarose beads functionalized with PepK, the complementary peptide of the pepE/K self-assembling peptide pair. The affinity purification matrix was equilibrated with buffer, loaded with filtered supernatant and washed to remove unbound proteins. Bound pepE-Bet v 1 was eluted by lowering the pH to 2.5 to unfold the pepE/pepK coiled coil. Elution fractions were collected and directly neutralized with 1 mol/L TrisHCl, pH 9. Flow through, wash and elution fractions were analyzed with SDS PAGE. Elution fractions containing pepE-Bet v 1 were pooled and loaded onto a Superdex 75 pg column (GE Healthcare) for polishing. Fractions containing the pure protein were pooled and stored at -20°C until further use.

Preparation of liposomes

Liposomes were prepared by the dehydration-rehydration method as described

elsewhere [15]. In short: lipids (DSPC, DOTAP and cholesterol in a 2:1:1 molar ratio, optionally including 1 mol% CPK) were mixed in the desired ratio. Subsequently, the organic solvent was evaporated in a rotary evaporator, leaving a lipid film. This film was hydrated in the presence of glass beads with a 10 mmol/L HEPES, 280 mmol/L sucrose buffer and lyophilized overnight. The resulting lipid cake was rehydrated with filtered Milli-Q® water to a final volume of 2 mL and homogenized using a LIPEX extruder (Evonik, Canada) over a stacked 400 nm & 200 nm Nuclepore Track-Etch membrane (Whatman, Netherlands). Throughout this manuscript 3 different liposome formulations were used:

1. Cationic liposomes with pepE-Bet v 1 adsorbed (pepE-Bet v 1 liposomes)
2. CPK-functionalized liposomes with Bet v 1 adsorbed (Bet v 1 CPK-liposomes)
3. CPK-functionalized liposomes with pepE-Bet v 1 adsorbed (pepE-Bet v 1 CC-liposomes)

Each of these formulations was prepared by adding 50 µg of either Bet v 1 or pepE-Bet v 1 (as was determined by BCA) to a liposome suspension (1 mg lipids) with a final volume of 1 mL. This mixture was incubated for at least 15 minutes.

Liposome characterization.

Hydrodynamic diameter (Z_{ave}) and polydispersity (PDI) were measured by dynamic light scattering (DLS) using a Zetasizer Nano Zs (Malvern Instruments Ltd., Worcestershire, UK). The zeta potential was measured using laser Doppler electrophoresis (lDe) on the same machine with a Zeta Dip Cell (Malvern Instruments Ltd.). Each sample was diluted 100 fold in 10 mmol/L HEPES buffer (pH 7.4, 0.2 µm filtered) before measurement.

ImmunoCAP IgE inhibition

IgE binding to pepE-Bet v 1 CC-liposomes was determined by ImmunoCap IgE inhibition assay using rBet v 1 ImmunoCAPs (T215). The liposomes and control samples were serially diluted (10-fold dilutions) in 10 mmol/L HEPES, 280 mmol/L sucrose, pH 7.4 and pre-incubated 1:1 (v/v) at room temperature with a serum pool. The pool was composed of 36 birch pollen allergic patient sera, 1:1 (v/v) mixed and was pre-diluted to approximately 14 kU/mL before mixing with sample. Bet v 1, pepE-Bet v 1 and serum without sample were used as controls.

Rat basophil leukemia (RBL) assay

To assess the allergenicity of pepE-Bet v 1-CC-liposomes, their ability to induce mediator release from effector cells was compared to that of soluble wild type rBet v 1.0101 (hereafter designated Bet v 1) [16] and pepE-Bet v 1. To that end, rat basophil leukemia cells (RBL-2H3), transfected with the human high-affinity IgE receptor (FcεRI) [17], were sensitized with serum of Bet v 1 sensitized birch pollen

allergic patients, and a β-hexosaminidase mediator release assay was performed a as previously described [16]. In short, 2 x 10^5 transfected RBL-2H3 cells/well were seeded in flat-bottom 96-well, Nunclon Delta-treated microplates (Thermo Fisher Scientific, Waltham, MA, USA) and passively sensitized overnight with sera derived from birch pollen allergic patients (n=8). To neutralize the complement system, the sera were incubated with P3X63Ag8.653 cells ((ATCC CRL-1580™), Manassas, VA, USA) prior to the sensitization step. For β-hexosaminidase release, the cells were stimulated with the samples in eight 15-fold dilution steps ranging from 10 µg/mL to 0.06 pg/mL Bet v 1 concentration. Samples were diluted in Tyrode´s buffer containing 9.5 g/L Tyrode´s salts, 0.1% (w/v) BSA, 0.5 g/L sodium bicarbonate. The cells were stimulated with the samples for one hour at 37 °C, 7% CO_2 before the cell supernatant was incubated with the β-hexosaminidase substrate, 4-methyl umbelliferyl-N-acetyl-beta-D-glucosaminide, diluted in 0.1 M citric acid (pH 4.5) for another hour at 37 °C and then quenched with 0.2 M of glycine buffer (pH 10.7). The fluorescence was measured with an Infinite 200Pro spectrophotometer (Tecan, Switzerland) at an excitation and emission wavelength of 360 nm and 465 nm, respectively. The data are presented as percentage of cell release normalized to the maximal enzyme release caused by cell lysis (10% Triton X-100, Sigma-Aldrich, Inc.), which was firstly corrected for spontaneous release (no serum sensitization). Cell viability was confirmed by performing a MTT assay.

Animal study

Mice were immunized subcutaneously on day 0, 7 and 14 with pepE-Bet v 1-CC-liposomes or alum-adsorbed Bet v 1 (1 mg alum per dose) containing 10 µg Bet v 1. Control groups received buffer (10 mmol/L HEPES, 280 mmol/L sucrose, pH 7.4), pepE-Bet v 1 liposomes or Bet v 1 CPK-liposomes. Serum for antibody detection was collected on days -1, 6, 13 and 20. On day 27, 28 and 29 the animals received an intranasal challenge under 3% (v/v) isoflurane anesthesia with 100 µg/mL birch pollen extract (BPE) in PBS to induce lung inflammation. On day 31, the mice were sacrificed and blood and lung draining lymph nodes were collected to analyze Bet v 1 specific levels of IgG1, IgG2a and IgE in serum and determine the production of cytokines (IL-4, IL-5, IL-13, IL-10 and IFN-γ) after stimulation of lymphocytes in the lymph nodes with Bet v 1.

Determination of Bet v 1 specific antibodies

Serum was analyzed for the level of Bet v 1-specific IgG1 and IgG2a by ELISA (IgG1: Opteia, BD, San Diego, CA, USA, IgG2a: eBioscience) as previously described [6]. In short, Maxisorp plates were coated overnight with Bet v 1. After blocking with FCS (10%), serum samples were incubated for 2 hours and followed by an HRP-conjugated anti IgG1 or IgG2a detection step, according to the manufacturer's instructions. Serum samples were diluted 10,000-fold, unless stated otherwise.

Ex vivo re-stimulation of lung draining lymph node cells.

Lung draining lymph node cell suspensions were plated in a 96-well round bottom plate at a density of 2 x 10^5 cells per well in RPMI supplemented with gentamicin, 10% FCS and β-mercaptoethanol. The cells were re-stimulated for 4 days with 10 µg/mL Bet v 1. Expression levels of cytokines (IL-4, IL-5, IL-10, IL-13, IFN-γ and IL-17A) were determined in the supernatant by ELISA (eBioscience).

Statistics

Data was processed and statistically analyzed in GraphPad v8 (Prism) for Windows. The statistical method is indicated in the figure legends.

Results

All liposomes were cationic and approximately 200 nm in size.

To determine the size and charge, all formulations were characterized by DLS and laser Doppler electrophoresis. All liposome formulations had a hydrodynamic diameter of approximately 200 nm and a positive zeta potential. The pepE-Bet v 1 CC-liposomes were slightly larger than the other formulations. The zeta potential was lower after coiled-coil mediated antigen adsorption. In contrast to liposomes, alum-adsorbed Bet v 1 showed a slightly negative zeta potential and the Z-average diameter was larger than 1000 nm (Table 1).

Table 1. Overview of all formulations and their physicochemical characteristics (mean values ± SD, n = 3-5). All formulations contained the same buffer composed of 10 mmol/L HEPES, 280 mmol/L sucrose, pH 7.4.

Formulation	Protein/carrier ratio (w/w)	Z-average diameter (nm)	PDI	Zeta potential (mV)
Liposomes (non-functionalized)	n.a.	179.3 ± 13.8	0.073 ± 0.039	48.1 ± 3.3
CPK-liposomes	n.a.	176.7 ± 14.4	0.071 ± 0.047	44.9 ± 6.2
pepE-Bet v 1 CC-liposomes	1/10	207.2 ± 10.7	0.159 ± 0.064	29.4 ± 4.2
Bet v 1 CPK-liposomes	1/10	189.3 ± 17.6	0.165 ± 0.029	40.0 ± 1.7
pepE-Bet v 1 liposomes	1/10	176.8 ± 16.4	0.145 ± 0.043	38.1 ± 3.9
Alum-adsorbed Bet v 1	1/100	1245.1 ± 131.9	0.318 ± 0.036	-6.3 ± 1.0

PepE-Bet v 1 CC-liposomes are hypoallergenic compared to Bet v 1.

Next, we characterized the IgE binding capacity of pepE-Bet v 1 CC-liposomes by testing its potential to inhibit IgE binding to rBet v 1 caps. Compared to Bet v 1, the IgE inhibition curves of pepE-Bet v 1 and pepE-Bet v 1 CC-liposomes revealed a higher inhibitor concentrations which indicated reduced IgE binding capacity (Figure 1A). Subsequently, we tested the IgE cross-linking capacity of pepE-Bet v 1 CC-liposomes by RBL mediator release assay. Testing a broad concentration

range yielded typical bell-shaped mediator release curves [24]. Based on the ascending part of the bell shaped curve, pepE-Bet v 1 appeared hypoallergenic compared to Bet v 1. PepE-Bet v 1 CC-liposomes induced approximately 15-fold less mediator release than recombinant Bet v 1 (Figure 1B). This was observed in all individual donors. Moreover, except for 1 donor, pepE-Bet v 1 CC-liposomes were more hypoallergenic than pepE-Bet v 1 (Supplementary Figure 3).

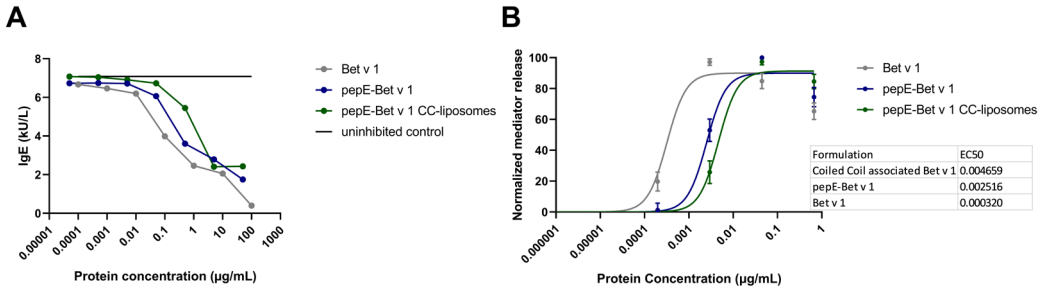

Figure 1. ImmunoCAP IgE inhibition assay and rat basophil lymphocyte assay. (A) The amount of IgE binding to serially diluted Bet v 1, pepE-Bet v 1 and Bet v 1 CC-liposomes was determined by immunoCAP. (B) Basophils loaded with IgE from serum of Bet v 1-sensitized subjects were exposed to a titration of different allergen formulations. Mediator release was measured and normalized based on positive and negative controls. Each data point is the mean of 8 experiments. A non-linear regression (variable slope, 4 parameters) fit was used to extract the EC50.

Cationic liposomes with coiled-coil associated Bet v 1 triggered strong antibody responses in naïve mice.

To evaluate the immune response induced by pepE-Bet v 1 CC-liposomes, naïve mice were immunized 3 times at weekly intervals followed by intranasal birch pollen extract challenge. Bet v 1-specific antibody levels were measured before each injection and at the end of the experiment. Mice that received pepE-Bet v 1 CC-liposomes had 77 fold higher IgG1 and 220 fold higher IgG2a levels (Figure 2A and B, respectively) than alum-adsorbed Bet v 1 at the endpoint. The IgE levels in all the liposome receiving groups were higher than the Bet v 1 + alum group but this was not significant (Figure 2C, 2F). In fact, the IgG1/IgE and IgG2a/IgE ratios in the pepE-Bet v 1 CC-liposomes group was much more favorable compared to the alum-adsorbed Bet v 1 (11.2 vs. 1.72 and 3.78 vs. 0.25 respectively).

To evaluate whether the CC formation was crucial for the strong humoral response, Bet v 1 CC-liposomes were compared to pepE-Bet v 1 liposomes (non-functionalized liposomes) and Bet v 1 CPK liposomes (non-functionalized Bet v 1). IgG1 and IgG2a induction was already observed 6 days after the second injection of the pepE-Bet v 1 CC-liposomes, but not after injection of the other liposome groups (Figure 3A and B, respectively). At the endpoint, all liposome formulations induced stronger antibody responses than allergen alone, but significantly more IgG1 and IgG2a was detected in mice immunized with pepE-

Bet v 1 CC-liposomes (Figure 2D and E), while the level of IgE was similar (Figure 2F). A serial dilution of pooled serum confirmed that pepE-Bet v 1 CC-liposomes induced the strongest immune response (Supplementary Figure 4). This also revealed that pepE-Bet v 1 induced a higher level of IgG1 than alum-adsorbed Bet v 1. Moreover, Bet v 1 attached to liposomes without CC induced a stronger humoral response than alum-adsorbed Bet v 1 or antigen without adjuvant.

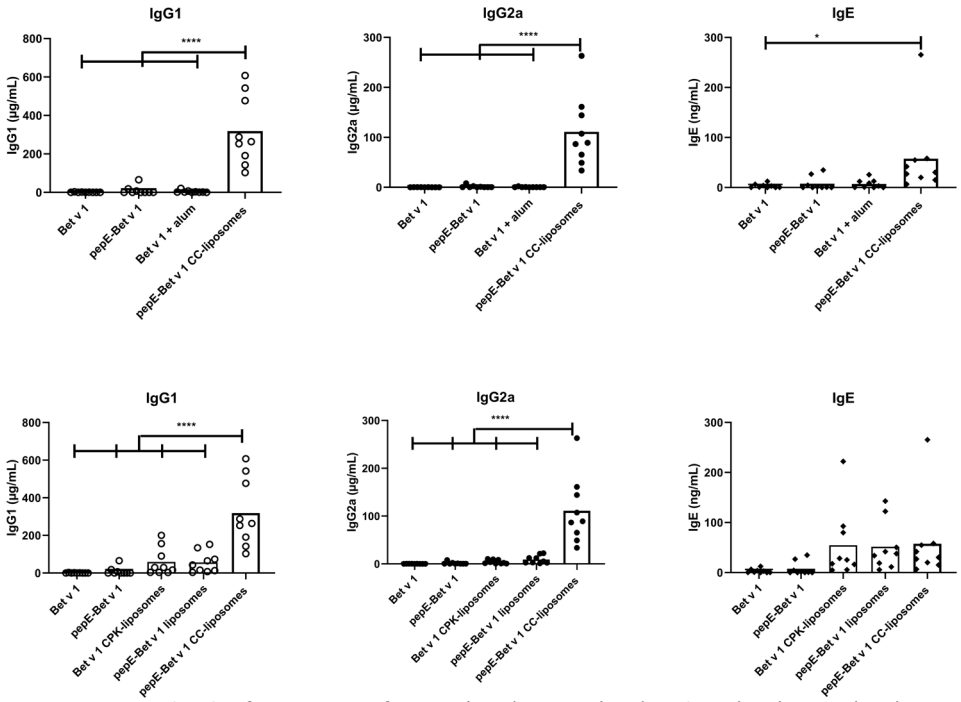

Figure 2. Serum levels of Bet v 1-specific IgG1 (A, D), IgG2a (B, E) and IgE (C, F). Mice (n=9) were immunized with various formulations on day 0, 7 and 14 and received 3 intranasal challenges with birch pollen extract for 3 consecutive days prior to the sacrifice. Group means were compared with a one-way ANOVA and subsequent Tukey's multiple comparison test (= $p < 0.05$, **** = $p < 0.0001$).*

PepE-Bet v 1 CC-liposomes induced a strong, regulatory skewed immune response.

To evaluate the cellular immune response, cells were isolated from lung-draining lymph nodes and stimulated with Bet v 1. Mice immunized with alum-adsorbed Bet v 1 showed induction of Th2 related cytokines IL-4, IL-5, IL-13, T regulatory (Treg) associated IL-10, Th1 associated IFN-γ and Th17 associated IL-17a. Immunization with pepE-Bet v 1 CC-liposomes, however, resulted in significantly higher IL-4, IL-5, IL-13 and IL-10 production compared to the other groups (Supplementary Figure 5). Remarkably, when ratios of different cytokines were calculated pepE-Bet v 1 CC-liposomes had a significantly higher IL-10/IL-4

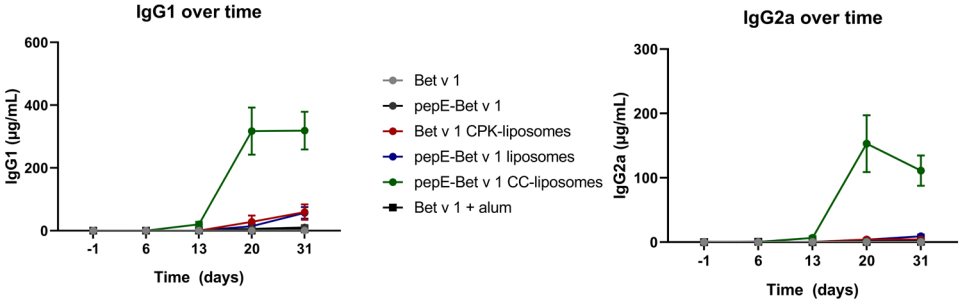

Figure 3. Bet v 1-specific levels (mean ± SEM) of IgG1 (A) and IgG2a (B) over time. Mice (n=9) were immunized with various formulations on day 0, 7 and 14 and received 3 intranasal challenges with birch pollen extract for 3 consecutive days prior to the sacrifice.

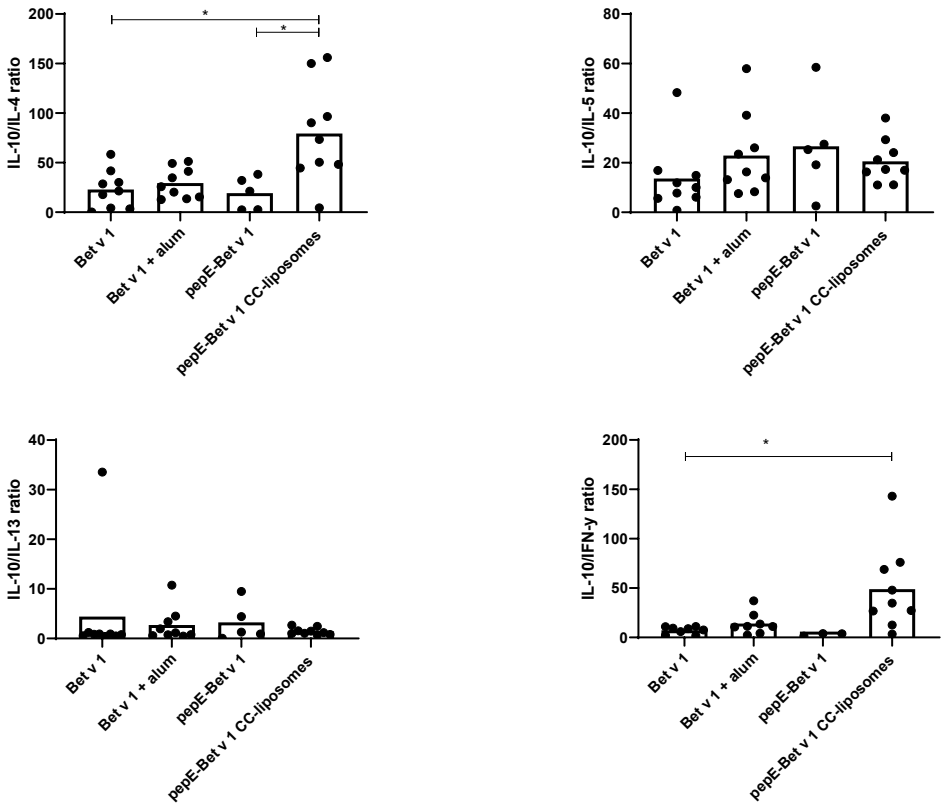

*Figure 4. Ratios between cytokines that were detected in supernatants of lung draining lymph node cells after ex vivo stimulation with Bet v 1 as measured by ELISA. Mice were immunized with various formulations on day 0, 7 and 14 and received 3 intranasal challenges with birch pollen extract for 3 consecutive days prior to the sacrifice. Bars represent the mean ratio (n=6-9). Group means were compared with a Kruskal Wallis test and Dunn's multiple comparison test. * = $p < 0.05$.*

ratio, which indicates a more regulatory skewed response compared to Bet v 1 or alum-adsorbed Bet v 1 (Figure 4).

Discussion

Previously we described the functionalization of cationic liposomes with a CC-forming peptide, which enhanced the immune response to model antigen OVA323 [19]. In the current study, the CC functionalized cationic liposomes were used to design a new birch pollen SCIT vaccine. In our vaccine design, Bet v 1 was attached to the functionalized liposome surface via peptide E fused to the N-terminus of Bet v 1 which already appeared to interfere with IgE binding. More importantly, the reduced IgE cross-linking capacity of pepE-Bet v 1 CC-liposomes compared to pepE-Bet v 1 indicates a hypoallergenic phenomenon that has also been reported by others. For example, despite being recognized by IgE, intact Fel d 1 displayed on the surface of VLPs failed to cross-link IgE on mast cells [25, 26]. Similarly, trimeric Der p 2 displayed on engineered bioparticles also showed reduced IgE cross-linking potential although the effect of trimerization on IgE cross-linking/binding capacity was not reported [27]. How this hypoallergenic effect is established is not completely clear. Allergens packed on the particle surface could sterically hide IgE epitopes from IgE binding, thereby preventing efficient IgE cross-linking and downstream induced mediator release [27]. Moreover, compared to allergens in buffer, particulate allergy vaccines might be taken up more efficiently by DCs or macrophages before they enable IgE cross-linking on the surface of basophils or mast cells which could also reduce the risk of IgE mediated side effects. Alternatively, combined with a lower diffusion coefficient, the effective free allergen concentration of allergen bearing nanoparticles is strongly reduced which provides another physical explanation for the reduced IgE cross-linking capacity [26]. Nevertheless, more studies are needed to elucidate how allergen bearing nanoparticles show reduced IgE cross-linking capacity compared to free allergen.

Subcutaneously administered alum-adsorbed Bet v 1 induced low antibody levels which is in line with previous observations [28]. In contrast, all tested liposomes in this study were more potent antibody inducers compared to alum-adsorbed Bet v 1. More importantly, the humoral response was strongest when Bet v 1 was associated to cationic liposomes via CC rather than electrostatic adsorption. This might be explained by a relatively stable, multivalent display of Bet v 1 on the liposome surface after CC formation. A repetitive antigenic surface organization has been associated with efficient B-cell receptor cross-linking and subsequent antibody production [29]. This highlights and confirms the potency of cationic liposomes [14, 17] and the added advantage of CC attachment to induce strong antibody responses.

Together with the hypoallergenic character, the strong humoral response of pepE-Bet v 1 CC-liposomes could be advantageous in SCIT. In humans, allergic symptom relief after SCIT is correlated to increased levels of allergen specific IgG_4 and allergen specific IL-10 production [5, 30, 31]. It is hypothesized that IgG_4 blocks IgE binding to allergens which reduces IgE mediated clinical symptoms. In mice, both IgG1 and IgG2a have been associated with relief of clinical symptoms [6, 25]. pepE-Bet v 1 CC-liposomes induced both antibody isotypes strongly which could reduce treatment frequency and duration to achieve early onset and long lasting therapeutic effect.

The strong induction of Th1 associated IgG2a, is in line with the previously observed induction of Th1 $CD4^+$ T-cells [19]. These cells were found to produce high levels of IFN-γ and IL-10 [19]. Both cytokines are beneficial for SCIT: IFN-γ is able to suppress Th2 related IL-4 and Treg related IL-10 is able to suppress both IFN-γ and IL-4. In fact, the ratio of IL-10/IL-4 ratio was the highest after immunization with pepE-Bet v 1 CC-liposomes. This indicates a more regulatory skewed immune response which could aid the suppression of the ongoing Th2 based, pro-inflammatory immune response in allergic subjects during SCIT. However, these murine results should be confirmed with human derived immune cell studies. For instance, T-cell polarization studies using human monocyte-derived dendritic cells isolated from peripheral blood mononuclear cells co-cultured with T-cells could shed more light on the T-cell subset differentiation pattern of pepE-Bet v 1 CC-liposomes.

In summary, we have developed a nanoparticle based delivery system using functionalized, cationic liposomes bearing Bet v 1 via CC formation. The hypoallergenic character, strong humoral as well as regulatory skewed T-cell responses induced by this formulation are advantageous properties for a novel, safe and highly efficacious SCIT vaccines. As such, our functionalized cationic liposomes could present a promising replacement of alum.

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Supplementary information

Supplementary Material and Methods

Design, expression and purification of pepE-Bet v 1.

The gene of pepE-Bet v 1 was optimized for expression in *E. coli* and ligated into the p19b expression vector by GenScript (Piscataway, NJ, USA):

ATGTATGGAGAAATCGCAGCCCTTGAAAAAGAGATTGCCGCCTTAGAAAAAGAAATTGCCGCACT
GGAAAAGGGTGTTTTCAATTACGAAACTGAGACCACCTCTGTTATCCCAGCAGCTCGACTGTTCA
AGGCCTTTATCCTTGATGGCGATAATCTCTTTCCAAAGGTTGCACCCCAAGCCATTAGCAGTTTGA
AAACATTGAAGGAAATGGAGGGCCTGGAACCATTAAGAAGATCAGCTTTCCCGAAGGCTTCCCTT
TCAAGTACGTGAAGGACAGAGTTGATGAGGTGGACCACACAAACTTCAAATACAATTACAGCGTG
ATCGAGGGCGGTCCCATAGGCGACACATTGGAGAAGATCTCCAACGAGATAAAGATAGTGGCAA
CCCCTGATGGAGGATCCATCTTGAAGATCAGCAACAAGTACCACACCAAAGGTGACCATGAGGT
GAAGGCAGAGCAGGTTAAGGCAAGTAAAGAAATGGGCGAGACACTTTTGAGGGCCGTTGAGAGC
TACCTCTTGGCACACTCCGATGCCTACAACTAA.

The underscored base pairs denote the DNA sequence coding for EIAALEKEIAALEKEIAALEK, the pepE amino acid sequence.

The pepE-Bet v 1 fusion protein was expressed in competent *E. coli* BL21 (DE3) cells (Novagen, USA) by transfecting the cells with 5 ng DNA followed by heat shock treatment at 42 °C. Subsequently, the transformed cells were grown on antibiotic selective LB plates containing ampicillin (50 µg/mL). Protein expression was induced in 5 mL Difco™ Terrific Broth (BD, Europe) cultures with 1 mmol/L IPTG at OD600 nm 0.6 for 3 hours at 37°C. The highest producing clones were used for protein production by inoculating 4 L TB medium in a 5 L stirred tank coupled to a BIOSTAT® controller (Sartorius Stedim Biotech). Protein production was induced for 3 hours with 1 mmol/L IPTG when the cells reached an OD 600 nm of 0.6. Finally, the cells were pelleted by centrifugation at 4600 rpm and stored at -20 °C until further use.

Frozen cell pellets were thawed on ice and re-suspended in ice cold lysis buffer (100 mmol/L sodium phosphate, 100 µg/mL lysozyme, pH 7.0) for 1 hour. The cells were disrupted by sonicating at 15 µm amplitude at 5 x 30 second bursts with 30 second intervals. Cellular debris was spun down at 20,000 g and 8°C for 20 minutes. The supernatant was collected. Bacterial DNA was precipitated with 0.4% (v/v) polyethyleneimine and spun down again at 12.000 g and 8 °C for 20 minutes. The supernatant was filtered through 0.2 µm before loading onto the affinity column.

An affinity purification column was prepared by coupling PepK (CGKIAALKEKIAALKEKIAALKE) to a supporting matrix. PepK was synthesized

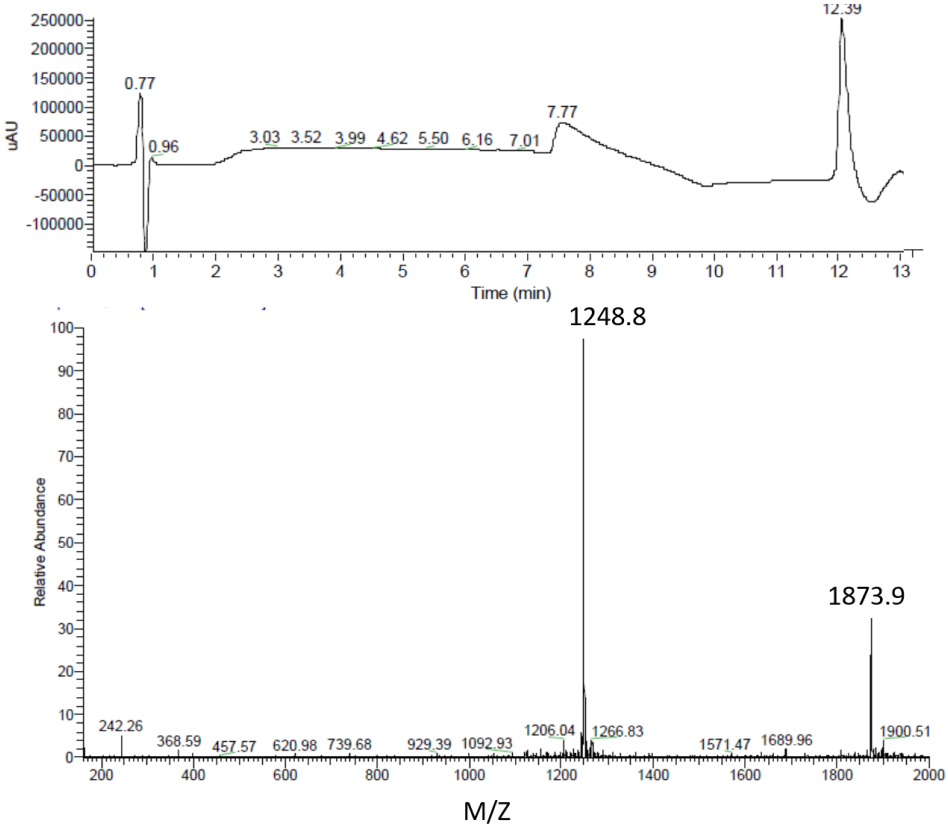
as described in the main article. The peptide was coupled to 6% cross-linked agarose beads functionalized with iodoacetyl groups (Sulfolink, Thermo Scientific) according to the manufacturer's instructions [32].

[32]. The affinity matrix was equilibrated with 5 column volumes (CV) 100 mmol/L sodium phosphate, 500 mmol/L sodium chloride, pH 7.0. Filtered supernatant was loaded onto the column and unbound proteins were washed out with 10 CV equilibration buffer. Bound pepE-Bet v 1 was eluted by lowering the pH with 5 CV of 100 mmol/L glycine HCl, pH 2.5 to unfold the pepE/pepK coiled coil. Elution fractions were collected and directly neutralized with (1:4, v/v) 1 mol/L TrisHCl, pH 9. Flow through, wash and elution fractions were analyzed with SDS PAGE (supplementary figure 2A and 2B). Elution fractions containing pepE-Bet v 1 were pooled and concentrated before gel filtration. The concentrate was loaded at 1 mL/minute onto a Superdex 75 pg column (GE Healthcare) equilibrated with 10 mmol/L HEPES, 280 mmol/L sucrose, pH 7.4 as a final step. Fractions containing the pure protein were pooled and stored at -20°C until further use.

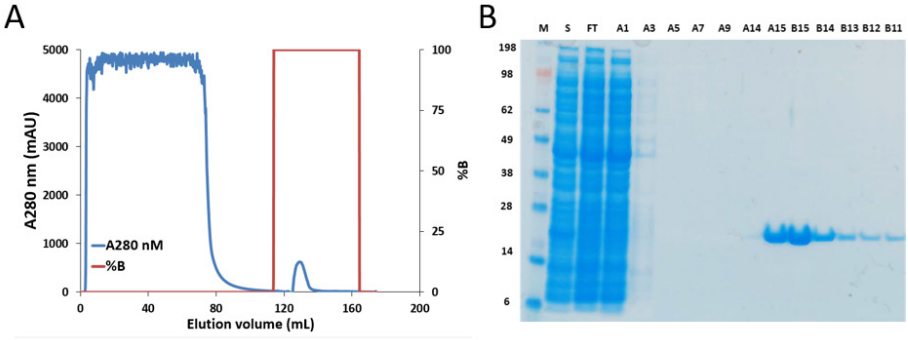
SDS-PAGE

Purification fractions were analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4-12% Bis-Tris gels (GE Healthcare) according to manufacturer's instructions. Gels were stained with PageBlue Coomassie and de-stained overnight in water.

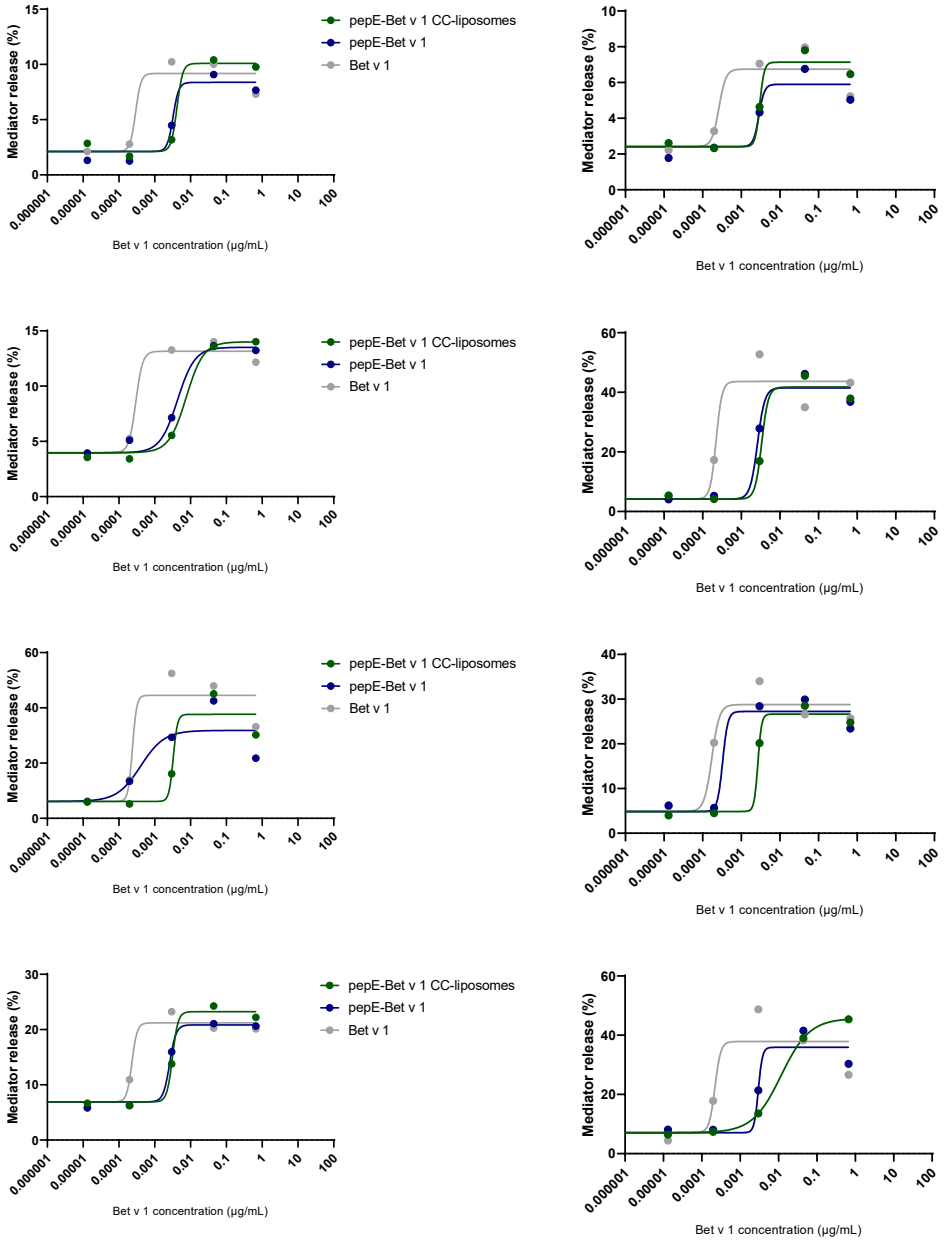
Supplementary figures



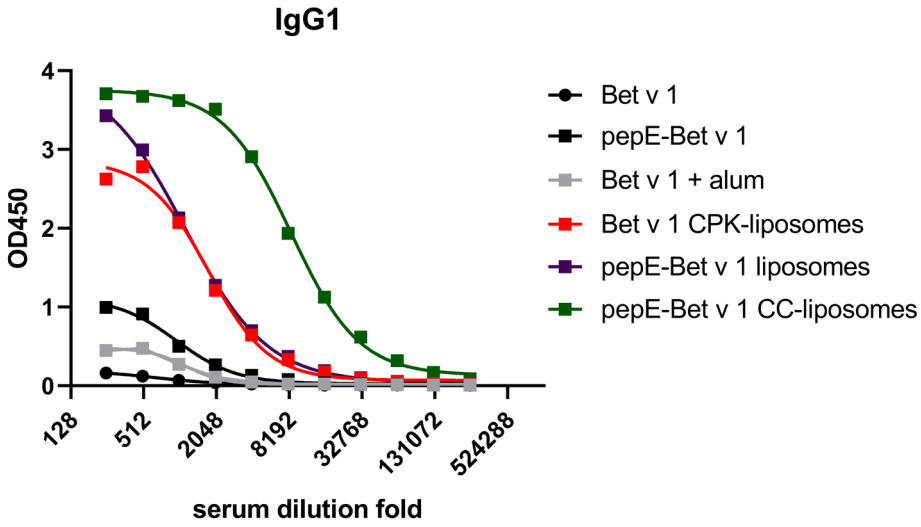
Supplementary Figure 1. LC (top) chromatogram and MS (bottom) spectrum of the CPK peak eluting at 7.77 minutes. The sequence of CPK is cholesterol-PEG4-KIAALKEKIAALKEKIAALKEKIAALKE, which has a theoretical molecular mass of 3747.2. The expected m/z values are: 1874.6 and 1250.1 for 2+ and 3+, respectively.



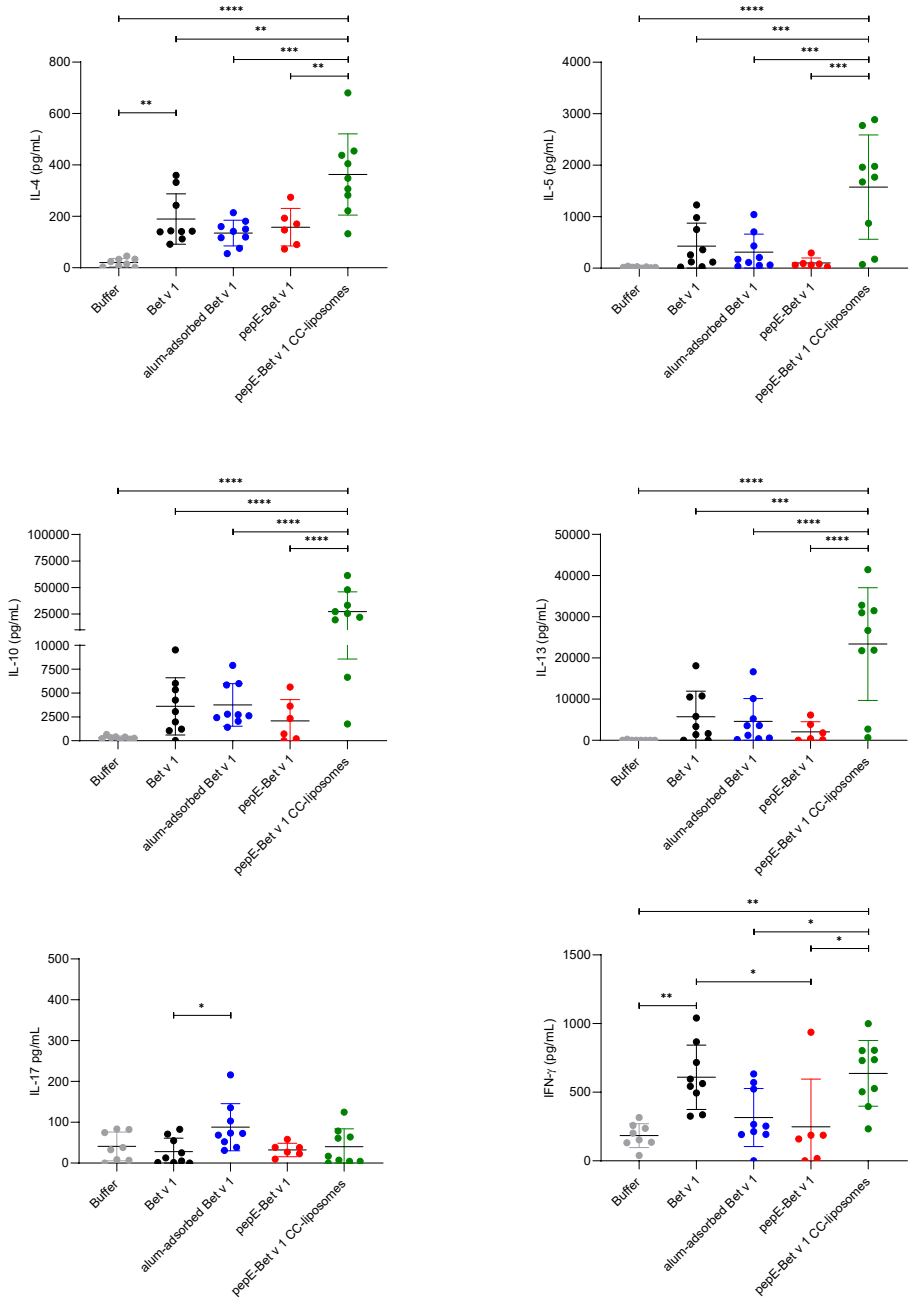
Supplementary Figure 2. Affinity purification and analysis of pepE-Bet v 1. A) elution from pepK-affinity column shows one peak, in which only a protein with the correct molecular weight is found on SDS-PAGE (B).



Supplementary Figure 3. Results of rat basophil leukemia assay. Basophils were loaded with IgE from serum of Bet v 1-sensitized subjects and subsequently exposed to 15-fold dilutions of different allergen formulations. Mediator release was measured and normalized based on positive and negative controls. A non-linear regression (variable slope, 4 parameters) fit was used to extract the EC50. Each graph represents the mediator release response of one patient. This data is summarized in Figure 1.



Supplementary Figure 4. Detection of Bet v 1-specific IgG1 in a 10-fold serial dilution of pooled serum samples of mice that were immunized with various formulations on day 0, 7 and 14 and received 3 intranasal challenges with birch pollen extract for 3 consecutive days prior to the sacrifice.



*Supplementary Figure 5. Cytokine production in supernatants of lung draining lymph node cells after ex vivo stimulation with Bet v 1. Bars represent the mean cytokine concentration and data points represent the signals for each individual mouse (n=6-9). Group means were compared with a one-way ANOVA and subsequent Tukey's multiple comparison test. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.*