

DR THIERRY BATARD (Orcid ID : 0000-0002-5172-3319)

DR ALEXANDER KETTNER (Orcid ID : 0000-0001-7696-5931)

DR LAURENT MASCARELL (Orcid ID : 0000-0001-7199-0345)

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Bet v 1 contiguous overlapping peptides anchored to virosomes with TLR4 agonist enhance immunotherapy efficacy in mice

Short title: Virosomes-Bet v 1 peptides enhance immunotherapy efficacy

Sabi Airouche^a, MsC, Vanya Beltrami^b, PhD, Sylvain Fleury^c, PhD Thierry Batard^a, PhD, Véronique Bordas-Le Floch^a, PhD, Toon Stegmann^d, PhD, Mario Amacker^{c,e}, PhD, Alexander Kettner^{b§}, PhD, Laurent Mascarell^{a§}, PhD

Affiliations

^a Stallergenes Greer, Antony, France

^b Anergis SA, Epalinges, Switzerland

^c Mymetics SA, Epalinges, Switzerland

^d Mymetics BV, Leiden, the Netherlands

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^e Department of Pulmonary Medicine, Bern University Hospital, University of Bern, Bern, Switzerland

[§] these authors contributed equally to this work

Correspondence: Laurent Mascarell, Innovation & Science Department, Stallergenes Greer, 6 rue Alexis de Tocqueville, 92160 Antony, France, Tel: +33 1 55 59 25 16, e-mail: laurent.mascarell@stallergenesgreer.com

Disclosure

Co-authors (SA, TB, VB and LM) are employees at Stallergenes Greer.

Co-authors (VBe and AK) are employees at Anergis SA.

Co-authors (SF and MA) are employees at Mymetics SA.

Co-author (TS) is an employee at Mymetics BV.

Author contribution

Stallergenes Greer author contribution: LM designed the research. LM, VB and TB supervised the research. SA performed the experiments. LM, VB, SA and TB analysed the data. LM wrote the paper.

Anergis SA author contribution: AK designed the immunogenicity study and analysed the data, VBe contributed to immunogenicity study data analysis. AK wrote the paper.

Mymetics author contribution: MA and TS prepared and optimized virosome production, developed analytical methods and characterized virosomes-Bet v 1 COPs preparations. SF contributed to virosome formulation and immunogenicity study design. MA, TS and SF contributed to analysis of results and writing of the manuscript.

ABSTRACT

Background:

Whereas sublingual allergen immunotherapy (AIT) is routinely performed without any adjuvant or delivery system, there is a strong scientific rationale to better target the allergen(s) to oral dendritic cells known to support regulatory immune responses by using appropriate presentation platforms.

Objective:

To identify a safe presentation platform able to enhance allergen specific tolerance induction.

Methods:

Virosomes with membrane-integrated contiguous overlapping peptides (COPs) of Bet v 1 and TLR4 or TLR2/TLR7 agonists were assessed for induction of Bet v 1-specific IgG1, IgG2a and IgE antibodies, hypersensitivity reactions and body temperature drop following subcutaneous injection in naive CD-1 mice. The most promising candidate, Bet v 1 COPs anchored to virosomes with membrane-incorporated TLR4 agonist (Vir.A-Bet v 1 COPs), was further evaluated by the sublingual route in a therapeutic setting in BALB/c mice with birch pollen-induced allergic asthma. Airway hyperresponsiveness, pro-inflammatory cells in broncho-alveolar lavages and polarization of Th cells in the lungs and spleen were then assessed.

Results:

Both types of adjuvanted virosomes coupled to Bet v 1 COPs triggered a boosted Th1 immunity. Given a more favorable safety profile, Vir.A-Bet v 1 COPs were further evaluated and shown to be able to fully reverse asthma symptoms and lung inflammation in a sublingual therapeutic model of birch pollen allergy.

Conclusions & Clinical Relevance:

We report herein for the first time on the capacity of a novel and safe presentation platform, *i.e.* virosomes with membrane-integrated TLR4 agonist, to improve dramatically sublingual AIT efficacy

in a murine model due to its intrinsic dual properties of targeting and stimulating to further promote anti-allergic immune responses. As such, our study paves the ground for further clinical development of this allergen presentation platform for patients suffering from respiratory allergies.

Key words

Allergen immunotherapy; Bet v 1 contiguous overlapping peptides; Virosomes

INTRODUCTION

Allergen immunotherapy (AIT) by the sublingual route has been well-established for treating type I respiratory allergies to a variety of allergens ^{1, 2}. It consists in daily administration of natural allergen extract in the form of drops or tablets within the oral cavity for inducing tolerance ³. Langerhans cell and macrophages have been identified in the oral mucosa as key target antigen-presenting cells to promote tolerogenic Th1/Treg responses ³⁻⁵.

Sublingual immunotherapy (SLIT) relies on high doses of allergens and expected benefits from combining adjuvants and/or vector systems to AIT products are more simple regimens, earlier onset and longer duration of efficacy ⁶. Various murine allergy models are available and have been used to assess new strategies for sublingual AIT including adjuvant and particulate formulations but most of them have relied on either one or the other approaches ⁶.

Hypoallergenic synthetic contiguous overlapping long peptides (COPs) encompassing the entire Bet v 1 sequence ⁷ represent a good candidate for evaluating the interest of presentation platforms to enhance AIT efficacy. AllerT (*i.e.* a combination of Bet v 1 COPs with alhydrogel adjuvant) has been previously assessed in clinical trials and a clear clinical effect was observed following a short course therapy regimen consisting in 5 subcutaneous administration of 50 µg AllerT ^{8, 9}. The observed increase in Bet v 1-specific IgE after treatment with AllerT suggests that alhydrogel may not be the optimal formulation to enhance clinical efficacy in human ⁹. Displaying antigens as repetitive motifs in organized structure as polypeptides anchored at the surface of adjuvanted virus-like particles like virosomes ¹⁰ is expected to improve and better sustain the wanted anti-allergic responses.

In the present study, we screened next generation AIT candidates by using a novel versatile presentation platform based on H1N1 influenza virosomes with membrane-incorporated agonists of TLR4 (Vir.A) or TLR2/TLR7 (Vir.B) for novel allergy treatment. We report herein on the capacity of this novel platform to enhance sublingual AIT efficacy when coupled to Bet v 1 COPs.

METHODS

Bet v 1 COPs

See supplementary Methods

Virosome formulations

See supplementary Methods

***In vitro* characterization of virosomes-Bet v 1 COPs**

See supplementary Methods

Immunogenicity study

Experimental procedures were performed by Vivotecnia Research (Spain). Animal care and handling was in accordance with Spanish legislation (Spanish Royal Decree 53/2013 BOE) and the guidelines of the European Commission for the accommodation and care of laboratory animals (2010/63/UE). Naive CD-1 (Hsd:ICR; CD-1®; Envigo RMS Spain S.L., Barcelona, Spain) mice were injected subcutaneously either with virosomes with membrane-integrated Bet v 1COPs and TLR4 (Vir.A-Bet v 1 COPs) or TLR2/TLR7 agonists (Vir.B-Bet v 1 COPs), AllerT (Bet v 1 COPs formulated with alhydrogel (Brenntag, Denmark)) or Bet v 1 COPs in saline. Animals received a total of 5 subcutaneous injections of 100 µL per mouse at days 1, 8, 15, 29 and 57. Bet v 1-specific IgG1, IgG2a and IgE antibodies were detected using ELISA in sera collected at day 85. Plates (Maxisorp, Thermo Fisher Inc., Wohlen, Switzerland) were coated with Bet v 1 (2 µg/mL) in carbonate buffer. Sera were serially diluted 1:5 starting with a first dilution of 1:2 000. IgG1 and IgG2a binding to Bet v 1 were detected after sequential incubation with biotinylated anti-mouse IgG1 (clone LO-MG1-2, Lucerna-Chem, Luzern, Switzerland) or anti-mouse IgG2a (clone RMG2a-62, Lucerna-Chem) followed by streptavidin-HRP (BD-Biosciences, San Diego, CA) and TMB substrate. Titer was determined as the highest dilution at which a positive signal was detected. For IgE, sera were diluted 1:20 and IgE antibodies were then detected after sequential incubation with biotinylated anti-mouse IgE (clone RME-1, Lucerna-Chem) followed by streptavidin-HRP (BD-Biosciences, San Diego, CA) and TMB substrate (Sigma, St. Louis, MO). Each of virosomes-Bet v 1 COPs and Bet v 1 COPs groups were compared to AllerT group without adjustment for multiplicity of testing. Data comparing sensitized mice with controls were analysed using Kruskal-Wallis tests, with results considered as statistically significant when the *p*-value was below 0.05.

Evaluation of hypersensitivity reactions

See supplementary Methods

Body temperature measurement

See supplementary Methods

Sublingual immunotherapy in birch pollen extract-sensitized mice and preclinical outcomes

Female BALB/c mice (6 to 8-week-old) were purchased from Charles River (L'Arbresle, France). Experimental protocols were approved by Stallergenes internal ethical committee for animal experimentation, and animal handling was performed according to international regulations. The experimental protocol (no. 01684.02) was approved by Stallergenes Greer (Antony, France) ethical committee (COREA No. 117). The aqueous birch pollen extract (BPE) was made in ammonium bicarbonate by Stallergenes SA (Antony, France). Mice were sensitized with 2 intraperitoneal (i.p.) injections of BPE (using doses equivalent to 10 µg Bet v 1) on days 0 and 14 in presence of 2 mg aluminum hydroxide (Thermo Scientific, Waltham, MA), then exposed from days 21 to 24 for 20 min per day to aerosolized BPE (equivalent to 1 mg Bet v 1). On day 25, airway hyperresponsiveness (AHR) was evaluated by unrestrained whole-body plethysmography (DATA Science int., St. Paul, MN) and expressed as enhanced pause (Penh) as described elsewhere¹¹. After sensitization, mice were randomized into experimental groups exhibiting similar means of Penh values. For SLIT, sensitized mice (8 mice per group) were treated sublingually twice a week for 8 weeks with either PBS (Life Technologies, Carlsbad, CA), Vir.A, Vir.A-Bet v 1 COPs or BPE (using doses equivalent to 40 µg Bet v 1). Mice were held on their back for 1 minute to prevent immediate swallowing of the allergen. At the end of the treatment, all groups were challenged with BPE aerosols for 2 consecutive days. Plethysmography was performed 24 h after the last aerosol as previously described¹¹. The following day, broncho-alveolar lavage (BAL) fluids were collected under anaesthesia and mice were sacrificed to conduct further analyses.

Analysis of birch pollen specific T cell responses

Lungs and spleen were harvested for T cell response analyses and cell suspension preparations were performed. One lobe of lung was incubated for 1 h in RPMI (Life technologies) supplemented with 75 U/mL of collagenase (Roche, Basel, Switzerland). After blocking residual enzymatic activity with 5 mM EDTA (Life technologies) in PBS, lung tissues were dissociated in PBS. As well, spleen

was dissociated in PBS. Both spleen and lung cells were filtered through a 70- μ m sieve and washed twice with PBS before resuspension in RPMI 1640 medium supplemented with 10% foetal calf serum, 1% L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin (all from Life technologies). Cells were plated at 10^6 cells per well in 96-well plates and stimulated with BPE equivalent to 10 μ g/mL of Bet v 1, or medium alone, in a final volume of 200 μ L. After 72 hours at +37°C in 5% CO₂/95% air, IFN- γ , IL-4, IL-5, IL-10 and IL-13 were quantified in culture supernatants using the Milliplex MAP mouse kit Cytokine Panel and analyzed using a MagPix Luminex xMAP technology according to manufacturer's instructions (Merck Millipore, Darmstadt, Germany).

Detection of birch pollen-specific antibodies following SLIT

See supplementary Methods

Blocking effect of sera samples

See supplementary Methods

Regulatory Foxp3 analysis

See supplementary Methods

Statistical analyses of data for the sublingual therapeutic murine model

Statistical and graphical analyses were performed using the Prism 6 software (GraphPad, La Jolla, CA). Results are presented as mean $\pm$ SEM values. Data comparing sensitized mice with controls were analysed using Kruskal-Wallis tests, with results considered as statistically significant when the *p*-value was below 0.05.

RESULTS

Bet v 1 COPs anchored to virosomes are hypoallergenic

Bet v 1 COPs showed no binding and crosslinking activity with serum IgE from birch pollen-allergic subjects and were already tested in several clinical trials ⁷⁻⁹. To test whether integration of Bet v 1 COPs via lipid linkers into the virosome lipid membrane would influence IgE binding, both virosomes-Bet v 1 COPs formulations were tested in competition ELISA and rat basophil leukemia (RBL) cell-based degranulation assay. No binding to IgE from birch pollen allergic subjects was detected (**Figure 1 and data not shown**).

Administration of Vir.A-Bet v 1 COPs or Vir.B-Bet v 1 COPs in naive animals induces a higher Bet v 1-specific IgG2a/IgG1 ratio than AllerT

Both virosomes-Bet v 1-COPs compositions (Vir.A-Bet v 1 COPs and Vir.B-Bet v 1 COPs) at high and low dose induced a strong IgG2a response to Bet v 1 (**Figure 2A**). A 2-log difference was observed when compared to AllerT at the same dose of Bet v1 COPs. These results indicate that the capacity of Vir.A-Bet v 1 COPs and Vir.B-Bet v 1 COPs to induce Bet v 1-specific IgG2a is strongly enhanced when compared to AllerT. Vir.A-Bet v 1 COPs and Vir.B-Bet v 1 COPs induced the same amount of Bet v 1-specific IgG1 antibodies as AllerT (**Figure 2B**). Differences in IgG1 titers were not statistically significant for these groups. Altogether, the Bet v 1-specific serum IgG2a/IgG1 ratios on day 85 were statistically significantly higher in the groups administered with Vir.A-Bet v 1 COPs and Vir.B-Bet v 1 COPs than in the group administered with AllerT (**Figure 2C**).

Vir.A-Bet v 1 COPs administered to naive animal does not induce Bet v 1-specific IgE antibodies

Induction of Bet v 1-specific serum IgE was monitored after administration of naive CD-1 mice either with Vir.A-Bet v 1 COPs or Vir.B-Bet v 1 COPs at high (40 µg/injection) or low (10 µg/injection) doses of Bet v 1 COPs, Bet v 1 COPs alone or adsorbed to alhydrogel (AllerT). Bet v 1 COPs induced a weak but positive Bet v 1-specific IgE response as compared to AllerT whereas Vir.A-Bet v 1 COPs did not induce detectable IgE antibodies that recognize Bet v 1 (**Figure 2D**). By contrast, animals injected with a low dose of Vir.B-Bet v 1 COPs (*i.e.* with TLR2/7 ligand) exhibited a low yet positive IgE response to Bet v 1 in 2 out of 20 animals.

Vir.A-Bet v 1 COPs have a better safety profile

On administration days, hypersensitivity score and body temperature of animals were recorded under blind conditions. No hypersensitivity symptoms were observed in mice administered with high or low doses of Vir.A-Bet v 1 COPs (**Table 1**). Two mice out of 20 had signs of sensitization at day 57 in the group of mice administered with Vir.B-Bet v 1 COPs at high dose and Bet v 1 COPs. About 50% of mice in the AllerT treated group showed signs of sensitization at treatment days 29 and 57. Two mice died, one at day 29 and one at day 57 about 30 min after receiving AllerT.

Drop in body temperature after treatment can also be used as a surrogate sign of anaphylaxis or sensitization following drug administration. Body temperature measurements on treatment days confirmed that Vir.A-Bet v 1 COPs, both at high and low doses did not sensitize mice as no drop in body temperature was observed in the corresponding groups at any treatment day including day 57 (**Figure 3**). Again, in the group of mice administered with Vir.B-Bet v 1 COPs at high dose and Bet v 1 COPs, 2 mice out of 20 displayed a body temperature drop of more than 2°C compared to group mean at day 57. In the AllerT group, 11 out of 20 mice showed signs of anaphylaxis at day 29, and 50% of mice showed signs of anaphylaxis at day 57 (Figure 2). No change in body temperature was observed for any treatment group at other treatment days.

Vir.A-Bet v 1 COPs enhances efficacy of sublingual immunotherapy in a murine model of birch pollen allergic asthma

The therapeutic effect of Vir.A-Bet v 1 COPs was tested *in vivo* in a murine model of established allergic asthma to BPE according to the administration scheme described in **Figure 4A**. Mice sensitized with BPE and administered sublingually with PBS as negative control displayed a strong airway hyperresponsiveness (AHR), as indicated by the elevated Penh values observed in response to methacholine (**Figure 4B**). As expected, sublingual immunotherapy (SLIT) treatment with BPE used as a positive control resulted in low Penh values, confirming a substantial significant decrease in AHR when compared to sham treatment with PBS ($p < 0.01$). SLIT treatment with Vir.A or Bet v 1 COPs had no significant impact on AHR. By contrast, SLIT treatment with Vir.A-Bet v 1 COPs lead to significantly reduced AHR when compared to PBS control mice ($p < 0.05$; **Figure 4B**).

The statistically significant decrease of AHR observed in BPE- and Vir.A-Bet v 1 COPs-treated animals was associated with a lower lung eosinophilia when compared to PBS-treated mice ($p < 0.0001$ and $p < 0.01$, respectively; **Figure 4C**). The percentages of eosinophils in bronchoalveolar lavages (BAL) also declined in Vir.A and Bet v 1 COPs-treated mice when compared to PBS-treated mice, yet the difference was not statistically significant (**Figure 4C**). No major alteration was observed in the percentages of the other immune BAL cells (*i.e.* lymphocytes, neutrophils, macrophages) analyzed (data not shown).

Production of cytokines (*i.e.* IFN- γ , IL-4, IL-5, IL-10, IL-13 and IL-17) by lung and spleen cells after *in vitro* re-stimulation with BPE extract (equivalent to 10 μ g/mL Bet v 1) was analyzed in all groups of mice. A significant decrease of IL-4, IL-5 and IL-10, as well as IFN- γ secretion by lung cells in response to BPE extract stimulation was observed in BPE-treated mice when compared to PBS-treated control mice (**Figure 5**; $p < 0.05$ or $p < 0.01$, depending on the cytokine). Treatment with Vir.A-Bet v 1 COPs induced a tendency to a reduced but not statistically significant production of Th2 cytokines (*i.e.* IL-5 and IL-10) in the lung as compared to PBS-treated mice. A similar trend was observed for spleen cells after administration of Vir.A-Bet v 1 COPs, but not of Vir.A or Bet v 1 COPs alone (**Figure 6**). This decrease was significant for IL-4 ($p < 0.05$) but not for the other cytokines evaluated (*i.e.* IL-5, IL-10 and IL-13). IFN- γ secretion by spleen cells, IL-13 secretion by lung cells, as well as IL-17 secretion by both lung and spleen cells, in response to BPE extract stimulation, were low and often below detection limit in all groups of mice (data not shown).

No significant alteration of frequency of Tregs in spleen, defined as CD3⁺ CD4⁺ CD127⁻ CD25^{high} Foxp3⁺ cells, was observed. (**Supplementary Figure 1**).

BPE-specific antibody responses were assessed by ELISA using sera from all groups of mice. No noticeable changes were observed in BPE-specific serum IgE in most groups of mice (**Supplementary Figure 2**). As an exception, treatment with Vir.A induced a significant decrease of BPE-specific IgE titers ($p < 0.01$). Significant diminution of BPE-specific IgG1 and IgG2a antibodies were observed in both Vir.A ($p < 0.05$) and BPE-treated mice ($p < 0.0001$ and $p < 0.001$, respectively), as compared to PBS-treated control mice (**Supplementary Figure 2**) but no differences in the IgG2a/IgG1 ratio could be observed (data not shown). In contrast, no significant alteration in titers of BPE-specific IgG1 and IgG2a antibodies was observed between Vir.A-Bet v 1 COPs-treated mice and PBS-treated control mice. Interestingly, a significant ($p < 0.05$) blocking effect of sera from BPE-sensitized mice sublingually treated by Vir.A Bet v 1 COPs but not or Bet v 1

COPs alone highlights the interest of this new allergen presentation platform to efficiently enhance neutralizing responses (**Supplementary Figure 3**)

DISCUSSION

Sublingual AIT with drops or tablets is established as a safe and efficacious treatment for type I respiratory allergies ^{1, 2} reorienting allergenic immune responses from a Th2 to a mixed Th1/Treg pattern ³⁻⁵. High cumulated doses of the allergen(s) are needed to reach clinical efficacy, likely due to the fact that sublingual AIT is carried out without any adjuvant or presentation platform ³. Substantial progresses have been made in the last decade regarding our understanding of the peculiar biology of the oral immune system. Particularly, Langerhans cell and macrophages were identified as key target cells to better activate allergen-specific regulatory immune mechanisms ^{3, 12-18}. In this context, there is a clear scientific rationale for combining allergens with appropriate presentation platforms in order to design second generation sublingual AIT products. Such a combination is expected to provide patients with a benefit in terms of better clinical effect, faster onset of action, sustained allergy control over a longer period of time and/or disease-modifying effect.

Innovative strategies based on synthetic allergens have been proposed to replace natural allergen extracts for AIT since they can be produced in large amounts as well-characterized molecules ¹⁹⁻²¹. In the context of birch pollen allergy for which more than 95% of patients exhibit IgE reactivity against the major allergen Bet v 1 ²², synthetic continuous overlapping peptides (COPs) encompassing the entire Bet v 1 sequence represent a good candidate for evaluating the interest of presentation platforms to enhance AIT efficacy ⁷. Bet v 1 COPs recapitulate the core T cell response while withdrawing IgE conformational epitopes of Bet v 1 as shown by using ELISA and basophil degranulation *in vitro* assays as well as skin prick testing in birch pollen-allergic volunteers ⁷. This approach offers the advantage to increase the dose of allergen administered while maintaining a very low allergenicity. Bet v 1 COPs adjuvanted with alhydrogel (AllerT) were previously tested in clinical trials and showed a clear clinical effect following a short course therapy regimen consisting in 5 subcutaneous administration of 50 µg AllerT ^{8, 9}. However, an increase in Bet v 1-specific IgE after treatment with AllerT was observed in human. Alhydrogel may be in part responsible for this increase, thus impeding an optimal efficacy ⁹. Our present data support this hypothesis since the presence of IgE antibodies against Bet v 1 in mice was strongly enhanced with AllerT when compared to non-adjuvanted Bet v 1 COPs. Combining Bet v 1 COPs with TLR adjuvanted virosomes as a delivery vehicle offering organized structure and antigen repetitive motifs was likely to overcome the capacity of Bet v 1 COPs alone or AllerT to sensitize animals. Two virosome compositions with membrane-incorporated contiguous overlapping peptides (COPs) of Bet v 1 and

TLR4 (Vir.A-Bet v 1 COPs) or TLR2/TLR7 (Vir.B-Bet v 1 COPs) agonists, were first tested in naive mice for evaluation of immunogenicity and safety.

This strategy combining specificity, *i.e.* Bet v 1 COPs, targeting and adjuvantation in a single particle resulted in an enhanced Bet v 1-specific antibody production of non-IgE isotype. The stronger IgG2a response compared to AllerT indicates that incorporation of TLR adjuvants directs the immune response towards a Th1 response. Moreover, both virosome formulations showed a significantly lower sensitization potential than AllerT (*i.e.* low development of IgE). Given a better safety profile and lower sensitization potential (*i.e.* induction of IgE) over Vir.B-Bet v 1 COPs, Vir.A-Bet v 1 COPs was subsequently compared by the sublingual route to Bet v 1 COPs and Vir.A alone, in a therapeutic model of allergic asthma to birch pollen. Mice sensitized with birch pollen extract (BPE) exhibit a chronic airway inflammation associated with airway hyperresponsiveness (AHR) and eosinophil infiltrates in broncho-alveolar lavages (BAL). Sensitized animals also mount BPE-specific Th2-biased T cell responses in the lungs and spleen as well as high IgE/IgG1 responses directed mainly to Bet v 1¹¹. Treatment with Vir.A-Bet v 1 COPs fully reversed asthma symptoms as well as lung inflammation (*i.e.* significant reduction in eosinophils in bronchial fluids). Pro-allergic immune responses also decreased upon therapy with Vir.A-Bet v 1 COPs in both lung and spleen cells with a significant decrease of the IL-4, particularly seen in the spleen. Lack of IgE suppression, IL-10 and regulatory T cell induction are likely due to the adjuvant rather than to the virosomes²³.

Previous studies have clearly established that a number of adjuvants or delivery systems as candidate allergen-presentation platforms for the sublingual route were able to improve AIT efficacy⁶. These allergen-presentation platforms includes adjuvants (*e.g.* 1,25-dihydroxy vitamin D3, glucocorticoids, toll-like receptor ligands or selected bacterial probiotic strains) able to stimulate allergen-specific Th1 and/or regulatory CD4⁺ T cell responses as well as allergens encapsulated within particulate delivery systems to foster allergen uptake by tolerogenic oral dendritic cells⁶. In the context of this study, our allergen-presentation platform encompasses both a delivery system and an adjuvant molecule. Moreover, our adjuvant selection was guided by the ability of TLR4 agonists to favour a tolerogenic response in Langerhans cells from human biopsies^{12 13} as well as by the capacity of both TLR4 and TLR2 agonists to enhance AIT efficacy in asthma animal models^{24, 25}. In the context of our study the choice of adjuvant was driven by the fact that 3D-PHAD is a well-defined molecule identical to one of the most active molecules present in 3-desacyl MPLA and that 3D-PHAD

incorporation in virosomes showed promising data for feasibility and stability ²⁶. CL413 was also chosen as results presented by Laino et al. ²⁷ suggested efficacy in allergen immunotherapy.

It is now well established that a birch pollen natural extracts can be substituted by a single recombinant natural-like rBet v 1 for AIT ^{6, 28, 29}. However, synthetic Bet v 1 alone, either in the form of a natural-like or a hypoallergenic molecule as a single or multiple polypeptides, is unlikely to demonstrate a superior efficacy to natural birch pollen extract ⁶. Our present data support the attractiveness of formulations combining such synthetic peptides with a tolerance-inducing and disease modifying-platform.

The methodology for selecting the most appropriate adjuvant to be associated with virosomes allowed us to choose a TLR4 agonist and to report herein on the capacity of Vir.A influenza virosomes with membrane-incorporated TLR4 agonist as a novel and safe allergen-presentation platform able to dramatically increase efficacy of sublingual AIT. The uniqueness and versatility of this platform relies on the combination of both a delivery system and an adjuvant to better (i) target and (ii) activate immune responses for enhancing tolerance induction. Our study paves the way for further clinical development of adjuvanted virosomes based treatment e.g. Vir.A-Bet v 1 COPs as a promising approach for patients suffering from respiratory allergies.

Figure legends

Figure 1. Vir.A-Bet v 1 COPs display no IgE binding. (A) IgE binding to Bet v 1 in a competition ELISA (n=4). (B) Degranulation in RBL test (n=19). Results are expressed as mean $\pm$ SD.

Figure 2. Vir.A-Bet v 1 COPs administration in naive mice increases Bet v 1-specific IgG2a/IgG1 ratio without promoting IgE responses when compared to AllerT. Mice were administered with either Vir.A-Bet v 1 COPs, Vir.B-Bet v 1 COPs (high dose 40 μ g/mL, n=20; low dose 10 μ g/mL, n=10), Bet v 1 COPs (n=20) or AllerT (n=20). Naive mice (n = 5) were used as controls (A) Serum Bet v 1-specific IgG2a and (B) IgG1 responses were measured by ELISA. Responses are expressed as mean $\log_{10}$ transformed titer $\pm$ standard deviation (SD). (C) Log ratios of IgG2a/IgG1. Ratios are represented as mean $\pm$ SD. (D) Serum Bet v 1-specific IgE were measured by ELISA. IgE responses are expressed as arbitrary units (A.U.) and presented as box and whiskers plots in the style of Tukey with median and interquartile. Statistical significance was determined using Kruskal-Wallis test and Dunn's multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ and ns (non-significant)).

Figure 3. Vir.A-Bet v 1 COPs display a favorable safety profile. Body temperature was measured before (0 min) and 30 minutes after the last injection at day 57. Results are presented as box and whiskers plots in the style of Tukey with median and interquartile. Statistical significance was determined using the 2-tailed Mann-Whitney test (* $p < 0.05$).

Figure 4. Treatment with Vir.A-Bet v 1 COPs significantly reduces airway inflammation in birch pollen allergic mice. (A) Study design. (B) AHR was determined by measuring the Penh index in response to methacholine (50 mg/mL). (C) Percentages of eosinophils were determined in BALs by using flow cytometry. Results are expressed as mean $\pm$ SEM, with n = 8 mice per group. Statistical significance was determined by the Kruskal-Wallis test for comparison between groups of mice (* $p < 0.05$; ** $p < 0.01$, **** $p < 0.0001$).

Figure 5. Sublingual immunotherapy with Vir.A-Bet v 1 COPs decreases the production of Th2 cytokines by lung cells. Levels of IFN- γ , IL-4, IL-5 and IL-10 were assessed in culture supernatants of lung cells restimulated with BPE extract equivalent to 10 μ g/mL of Bet v 1 by using multiplex cytokine quantification assay. Background levels of cytokines produced by non-stimulated cells were subtracted. Results are expressed as mean $\pm$ SEM, with n = 8 mice per group. Statistical significance

was determined by the Kruskal-Wallis test for comparison between groups of mice (* $p < 0.05$; ** $p < 0.01$).

Figure 6. Sublingual immunotherapy with Vir.A-Bet v 1 COPs decreases the production of Th2 cytokines, mainly IL-4, by spleen cells. Levels of IL-4, IL-5, IL-10 and IL-13 were assessed in culture supernatants of spleen cells restimulated with BPE extract equivalent to 10 $\mu\text{g/mL}$ of Bet v 1 by using multiplex cytokine quantification assay. Background levels of cytokines produced by non-stimulated cells were subtracted. Results are expressed as mean $\pm$ SEM, with $n = 8$ mice per group. Statistical significance was determined by the Kruskal-Wallis test for comparison between groups of mice (* $p < 0.05$).

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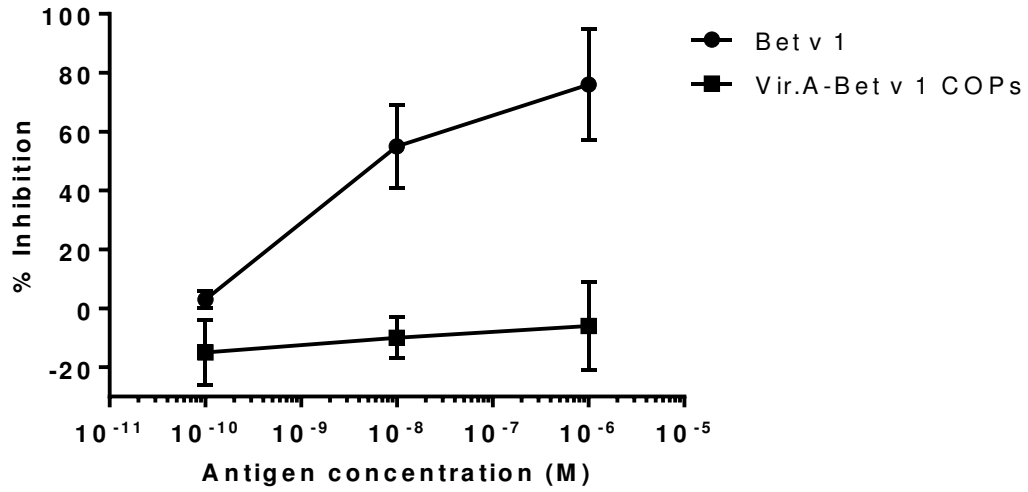
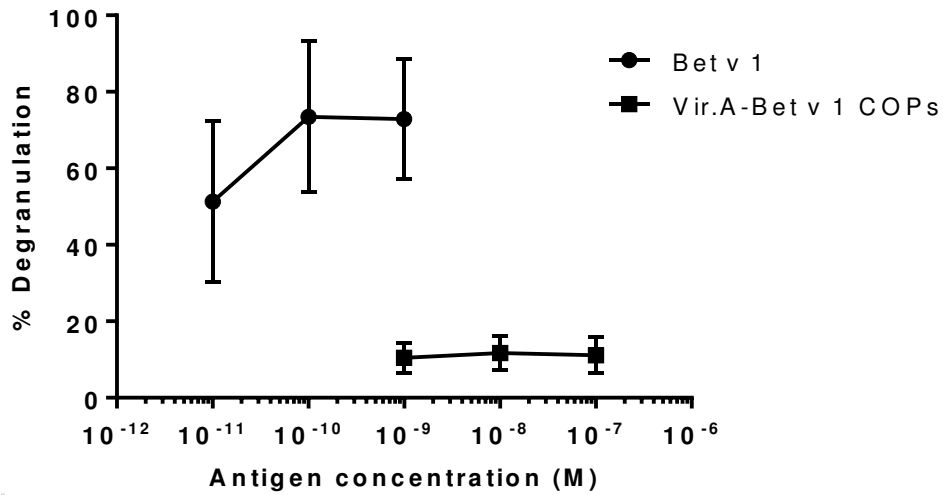
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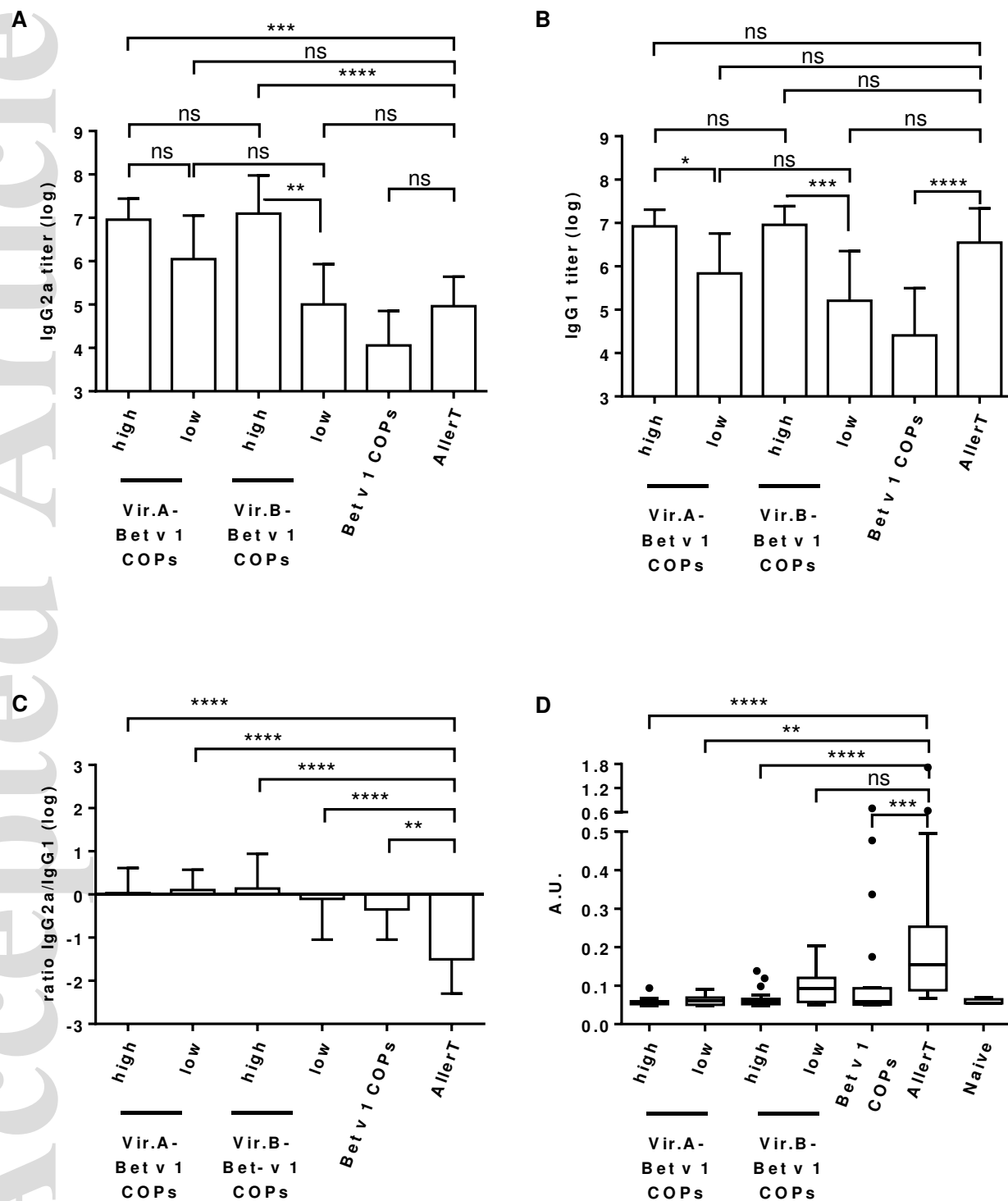
Table 1. Hypersensitivity scores observed at treatment days.

Treatment	Total Score ¹⁾		
	Day 1, 8, 15	Day 29	Day 57
Vir.A-Bet v 1COPs high	0	0	0
Vir.A-Bet v 1COPs low	0	0	0
Vir.B-Bet v 1COPs high	0	0	5 (2/20) ²⁾
Vir.B-Bet v 1COPs low	0	0	0
No adjuvant	0	0	9 (2/20) ²⁾
AllerT	0	67 (13/20) ²⁾	66 (10/20) ²⁾

1) Sum of scores for all mice per group observed at each time point (15, 30, 60, 120 and 180 min post treatment)

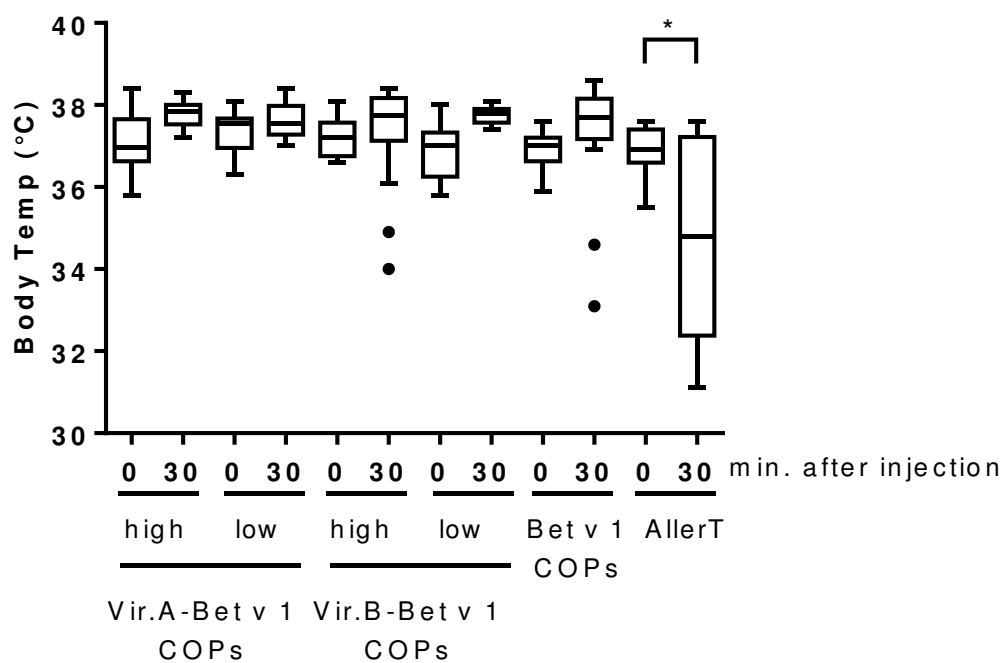
2) Proportion of mice showing signs of hypersensitivity

A**B**

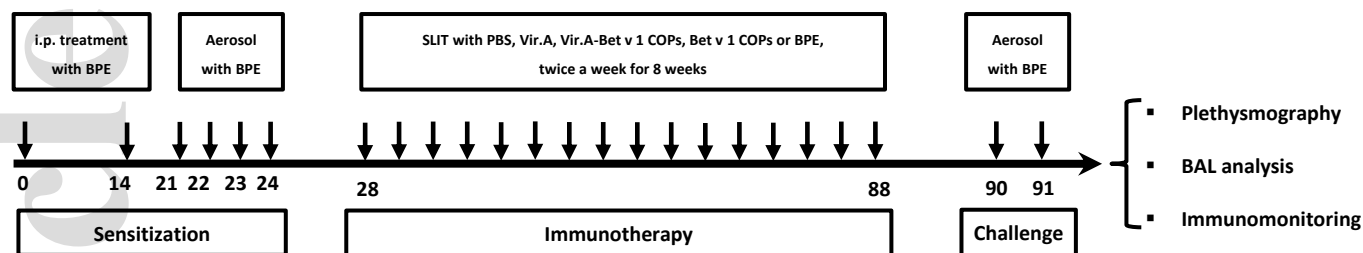


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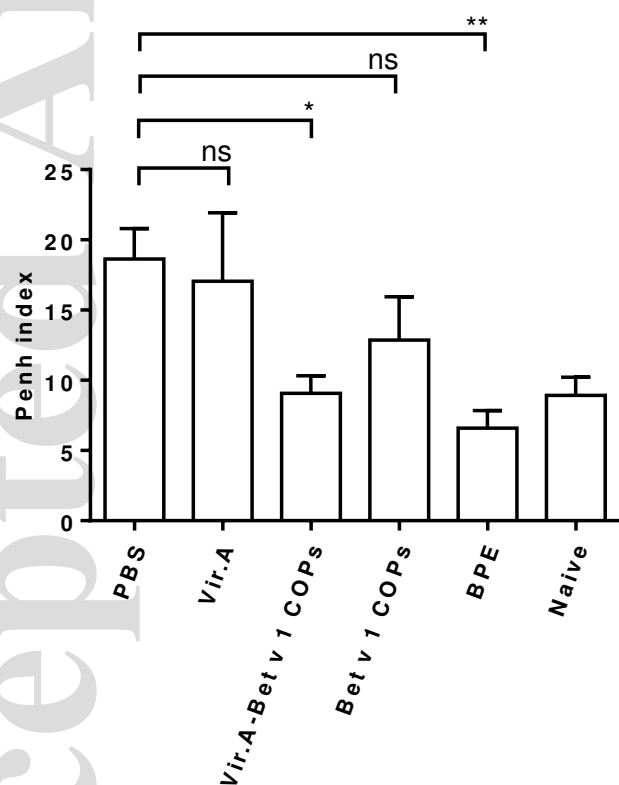
Figure 2



A



B



C

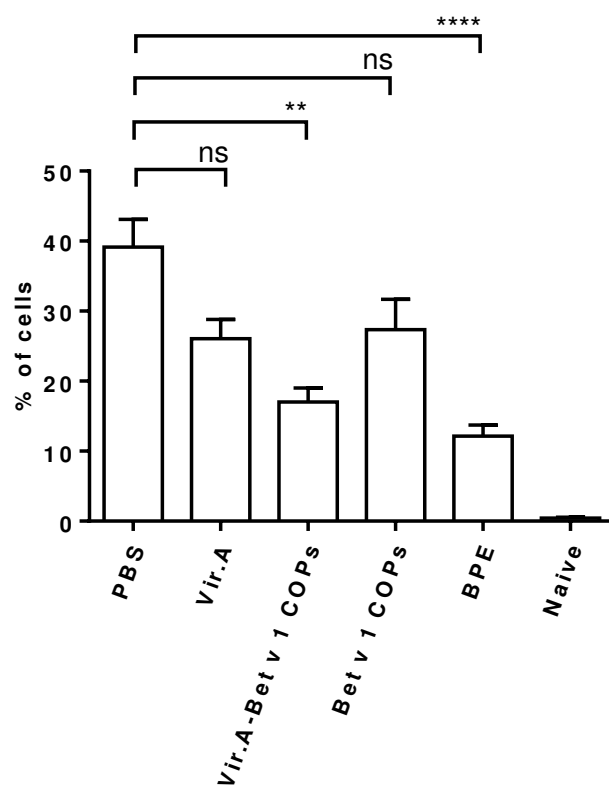


Figure 5

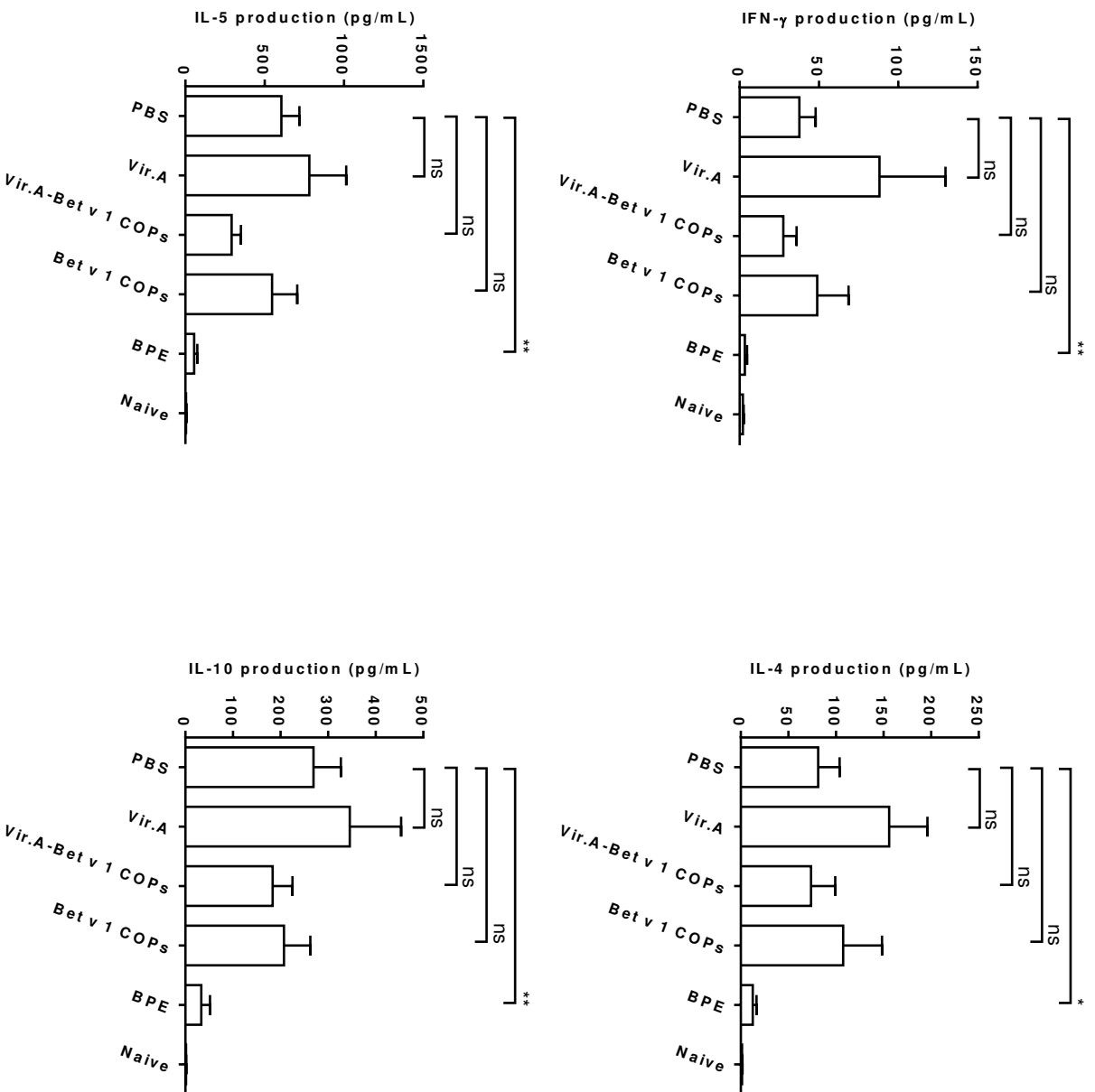
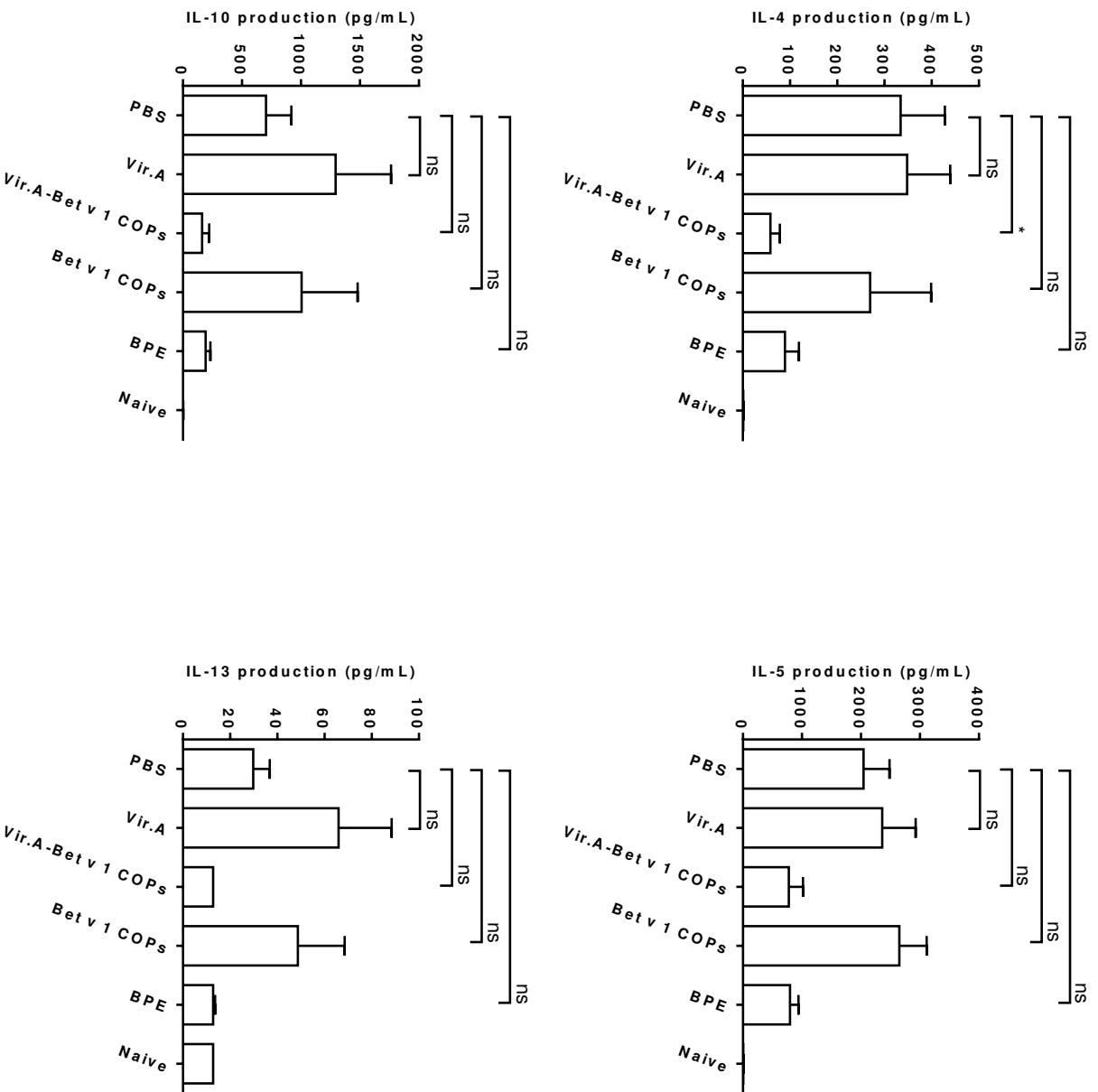


Figure 6



Supplementary Methods

Bet v 1 COPs

Three virosomes-Bet v 1 COPs compositions were prepared comprising either Bet v 1 COP 1 (amino acid (aa) 2-50 of Bet v 1.0101), Bet v 1 COP 2 (aa 48-118 of Bet v 1.0101) or Bet v 1 COP 3 (aa 106-160 Bet v 1.0101) ¹. For mouse immunogenicity studies, Bet v 1 COPs were lipidated at the N-terminal lysine residues to allow integration of Bet v 1 COPs into the virosome lipid membranes. Bet v 1 COPs used in the therapeutic model were synthesized with a maleimide group at the N-terminus (PolyPeptide Laboratoires, Strasbourg, France) that allowed for direct conjugation to a single phospholipid molecule with a free thiol group (1, 2-dipalmitoyl-sn-glycero-3-phosphothioethanol, Avanti Polar Lipids, Alabaster, AL) group at the N-terminus after reacting the maleimide group with a thiol group containing phospholipid. Bet v 1 COP 2 used in the therapeutic model corresponds to aa 49-118 of Bet v 1.0101, without the asparagine residue that is prone to deamidation.

For Bet v 1 COPs in saline and AllerT (Bet v 1 COPs formulated with alhydrogel (Brenntag, Denmark), peptides were used in the unlipidated form. Bet v 1 COPs were prepared as 0.8 mg/mL solution in saline (0.9% w/v NaCl) with an equimolar content of Bet v 1 COP 1, Bet v 1 COP 2 and Bet v 1 COP 3. Bet v 1 COPs in saline were mixed with equal volume of either alhydrogel (4 mg/ml) or saline solution to reach a concentration of 0.4 mg/mL resulting in a delivery of 40 µg/injection/mouse.

Virosome formulations

The general principle of virosome manufacturing process was previously described ². Virosomes were prepared by Mymetics from inactivated and purified influenza virus (A/Brisbane/59/2007 (H1N1), Seqirus, Australia) with membrane-integrated 3D-PHAD TLR4 agonist (Monophosphoryl 3-deacyl Lipid A (Avanti Polar Lipids, Alabaster, AL) or CL413 TLR2/TLR7 agonist ((S-(2,3-bis(palmitoyloxy)- (2RS)propyl)- (R)-cysteinyl-(S)-seryl-(S)- lysyl-(S)-lysyl- (S)-lysyl- (S)-lysyl 4 -((6-amino-2- (butylamino)-8- hydroxy-9H- purin-9-yl) methyl) also named Adilipoline™ (Invivogen, Toulouse, France). Bet v 1 COPs bearing lipid tails were integrated into virosomes membranes. Virosome formulations were a mixture consisting of equivalent amounts of virosomes-Bet v 1 COP 1, virosomes-Bet v 1 COP 2 and virosomes-Bet v 1 COP 3 at 0.4 mg/mL resulting in a delivery of 40 µg/injection/mouse for the high dose and were further diluted 1:4 in HN buffer (50 mM HEPES pH 7.4, 145 mM NaCl buffer containing 100 mM octaethyleneglycol-mono-(n-dodecyl)ether) (OEG, Sigma, St. Louis, MO) for the low dose (10 µg/injection/mouse). 3D-PHAD concentration was 0.044 mg/mL for preparations used in immunogenicity study and 0.15 mg/mL for preparations used in the murine model. CL413 concentration was 0.04 mg/mL. Concentrations of each Bet v 1 COP

and of CL413 in the virosomal formulations were determined by reversed phase Ultra-Performance Liquid Chromatography (RP-UPLC), and 3D-PHAD concentration was determined by thin layer chromatography (TLC).

***In vitro* characterization of virosomes-Bet v 1 COPs**

IgE binding to virosomes-Bet v 1 COPs was assessed by competition ELISA. Maxisorp plates (Thermo Fisher Inc., Wohlen, Switzerland) were coated with recombinant Bet v 1 (Bet v 1.0101, Biomay, Vienna, Austria) at 2 µg/mL in carbonate buffer (pH 9.6) overnight at 4°C. Plates were then washed and unspecific binding sites were blocked by incubation with BSA (2 hours at RT). Sera from 20 birch allergic subjects with Bet v1 specific IgE (dilution was adjusted for IgE titers) were pre-incubated with either virosomes-Bet v 1 COPs formulations or Bet v 1 allergen for 15 min on ice prior to addition to the wells. After incubation for 2 hours at RT, plates were washed and biotinylated anti-human IgE (1:1000, Lucerna-Chem, Luzern, Switzerland) was added for one hour at RT. Plates were washed and streptavidin coupled horse radish peroxidase (1:1000, BD-Biosciences) was added for 30 min at RT. Specific IgE binding was revealed with TMB (Sigma) as substrate. Plates were read at 450 nm (with a reference filter set to 630 nm). Results are expressed as percent (%) of IgE binding to Bet v 1.

RBL degranulation assay was performed as described elsewhere ³. RBL-703/21 cells transfected with the human FcεRI receptor were plated in 96-well tissue culture plates (10⁵ cells/well) overnight. Passive sensitization of RBL-703/21 cells was carried out with sera from patients with birch pollen allergy diluted 1:20 overnight. Unbound antibodies were removed by washing the cell layer twice with Hanks' balanced salt solution (Gibco, Life technologies) the next day. Degranulation of RBL cells was induced for 1h at 37°C by adding virosomes-Bet v 1 COPs formulations or Bet v 1 allergen serially diluted in Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES pH7.4, 0.1% BSA). β-hexosaminidase activity was analyzed by incubating 30 µL of cell supernatant with 50 µL of 1.3 mg/mL 4-Nitrophenyl N-acetyl-β-D-glucosaminide (Sigma) in citrate buffer (0.1 M, pH 4.5) for 1 hour at 37°C. The reaction was stopped by addition of 100 µL glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and optical densities were measured at 405 nm. Results are expressed as percent (%) of degranulation observed after anti-IgE crosslinking. Crosslinking with anti-IgE antibody gave maximal degranulation mediated through the IgE- FcεRI receptor complex.

Evaluation of hypersensitivity reactions

Animal handling was performed according to international regulations. On administration days, treated animals were checked under blind conditions prior to and approximately 15, 30, 60, 120 and 180 minutes after treatment. The behavior of the animals was monitored for

approximately 1 min and reactions were graded according to Supplementary Table 1 modified from Sade et al.⁴. **Body temperature measurement**

Rectal temperature was measured using a digital recording system coupled to a probe that was inserted approximately 1.5 cm into the rectum of each animal. The probe was dipped in lubricating oil before each measurement to facilitate insertion and wiped down with 75% ethanol between each measurement. On administration days, rectal temperature was recorded twice before administration, and approx. 30 min after treatment in all animals, except for in those from the non-treated group, in which only the basal record was taken.

Detection of birch pollen-specific antibodies following SLIT

Blood was collected by cardiac puncture under anaesthesia and sera stored at -20°C. Specific antibody responses were evaluated by ELISA in sera samples incubated for 24 hours at 37°C in 96-well plates coated overnight with BPE (at doses equivalent to 10 µg/mL Bet v 1). Plates were washed by using an automate strip washer for 96-well plates Bio Tek ELx405 (Bio Tek, Winooski, VT) and incubated with HRP-conjugated antibodies to mouse IgG1 (Bio-Rad laboratories, Hercules, CA), IgG2a (BD Biosciences) or IgE (Fisher scientific, Hampton, NH) for 5 hours at +37°C. After washing, specific antibody responses were detected with OPD (Sigma) and ABTS substrate (Roche, Basel, Switzerland), respectively, for IgG1/IgG2a and IgE. Plate reading was performed at 492 nm (for OPD) or at 405 nm (for ABTS) using a spectrophotometer (Thermo Labsystems, Waltham, MA). Antibody titres were defined as the reverse of the last dilution for which the absorbance was 2-fold higher than the mean value obtained for control mice.

Blocking effect of sera samples

ELISA microtiter plates were coated with rat antibodies to mouse IgE, blocked with bovine serum albumin, and incubated with a pool of sera from the BPE-sensitized mice sublingually administered with PBS (n = 8), so as to capture anti-Bet v 1 IgE. Then, the serum of each BPE-sensitized mouse administered with a test treatment was preincubated at a 1/5 final dilution with an equivalent volume of BPE at a final protein concentration of 10 µg/ml. Each preincubated mixture was then incubated for 2 h in duplicate in the wells of the ELISA plate to allow blocking antibodies to compete with anti-Bet v 1 captured IgE for the binding to Bet v 1. Bet v 1 molecules that remained bound to the captured IgE were detected using a labelled monoclonal antibody to Bet v 1. The percentage of inhibition of the binding to Bet v 1 of captured IgE was calculated as follows: $(DO_{PBS} - DO_{mouse}) / DO_{PBS}$, where “ DO_{PBS} ” is the mean absorbance value obtained for the BPE preincubated with PBS, and “ DO_{mouse} ” is the mean absorbance value obtained with the serum of a given mouse preincubated with that same BPE. Results are expressed as mean $\pm$ SEM, with n = 8 mice per group. Statistical significance was

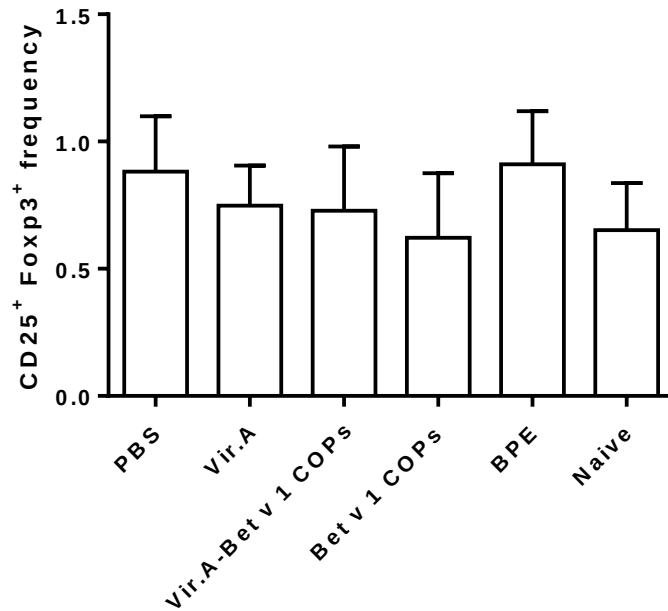
determined by the Kruskal-Wallis test for comparison between groups of mice (comparison of the mean rank of each group with the mean rank of Vir. A group. (* $p < 0.05$; ns: not significant).

Regulatory Fox p 3 analysis

The analysis of Foxp3 expression by spleen T cells was performed using a flow cytometer (Attune NxT, Life Technologies). Data were analysed with the FlowJo software (TreeStar, Ashland, OR). Cells were isolated and stained with anti-CD4 FITC, anti-CD25 APC and anti-CD127 PE-Cy7 mAbs (BD bioscience). After 15 min, cells were fixed, permeabilized and stained intracellularly with anti-Foxp3 PE mAbs using the Foxp3 staining kit (Fisher Scientific, Hampton, NH) according to the manufacturer's instructions.

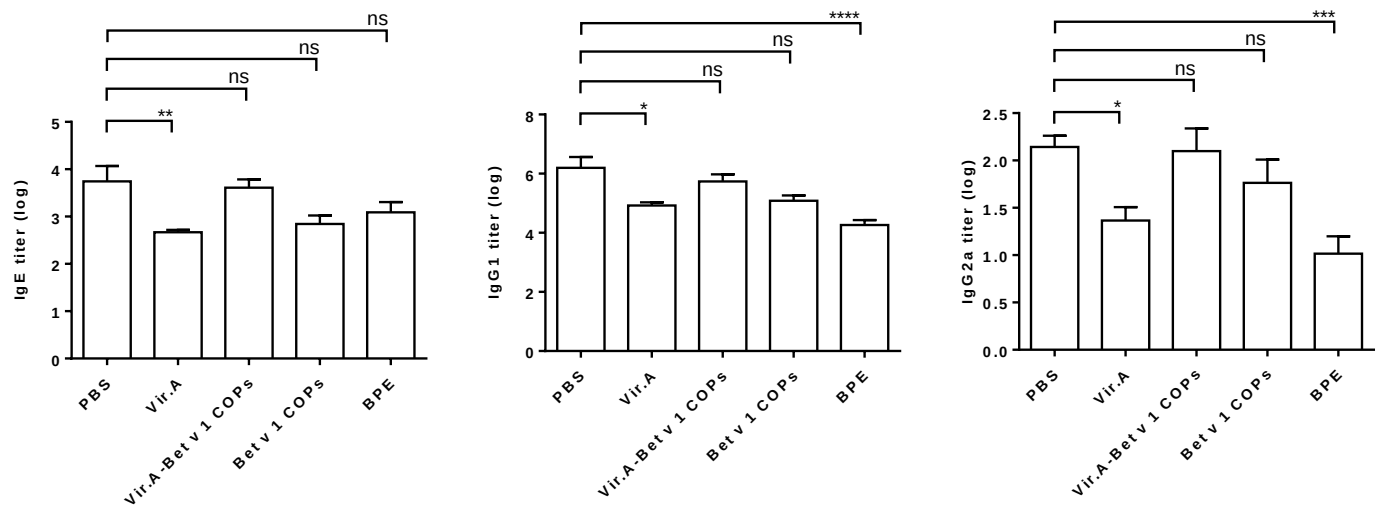
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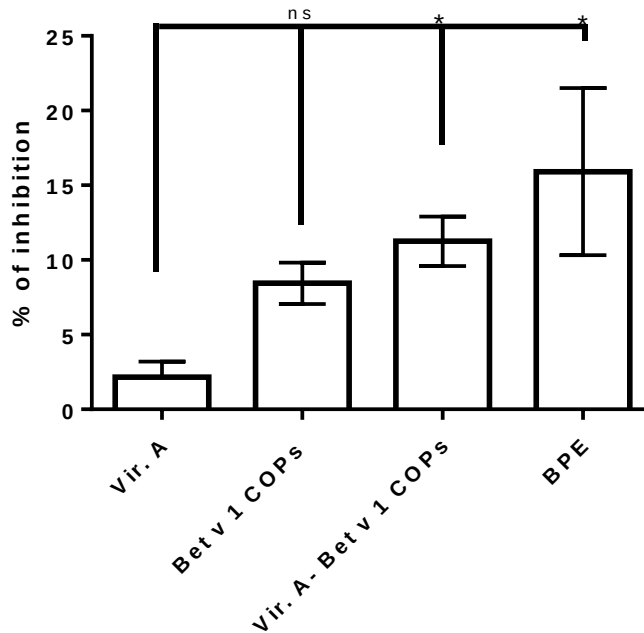
Supplementary Figure 1: No change in regulatory T cell frequencies is observed in spleen from BPE sensitized mice after a 8-week SLIT.

The frequency of Tregs was assessed in spleen for each mouse by using flow cytometry analyses. Results are expressed as mean $\pm$ SEM, with n = 8 mice per group. Statistical significance was determined by the Kruskal-Wallis test for comparison between groups of mice.



Supplementary Figure 2: No major changes were seen in BPE-specific serum antibody responses.

Levels of BPE-specific IgE, IgG1 and IgG2a antibodies were measured in sera by using ELISA. Antibody titers were defined as the reverse of the last dilution for which the absorbance was 2-fold higher than the mean value obtained for control mice. Results are expressed as mean $\pm$ SEM, with $n = 8$ mice per group. Statistical significance was determined by the Kruskal-Wallis test for comparison between groups of mice (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$).



Supplementary Figure 3: Significant blocking effect of sera from BPE-sensitized mice sublingually treated by Vir. A-Bet v 1 COPs or BPE

Blocking effect expressed as percentage of inhibition of a pool of sera from the BPE-sensitized mice by test sera at a 1/10 final dilution with an equivalent volume of BPE at a final protein concentration of 10 µg/ml were measured by using ELISA to allow blocking antibodies to compete with monoclonal anti-Bet v 1 captured IgE for the binding to Bet v 1 (see supplementary Methods). The percentage of inhibition of the binding to Bet v 1 of captured IgE was calculated as follows: $(DO_{PBS} - DO_{mouse}) / DO_{PBS}$, where “ DO_{PBS} ” is the mean absorbance value obtained for the BPE preincubated with PBS, and “ DO_{mouse} ” is the mean absorbance value obtained with the serum of a given mouse preincubated with that same BPE. Results are expressed as mean ± SEM, with n = 8 mice per group. Statistical significance was determined by the Kruskal-Wallis test for comparison between groups of mice (comparison of the mean rank of each group with the mean rank of Vir. A group. (* $p < 0.05$; ns: not significant).

Supplementary Table 1. Hypersensitivity scores modified from Sade et al. (2007) ¹.

Reaction/Sign	Grade
No symptoms	0
Scratching and rubbing around the nose and head	1
Puffiness around the eyes and mouth, diarrhea (if any observed), piloerection (strong), reduced activity and/or decreased activity with increased respiratory rate	2
Wheezing, labors respiration, cyanosis around the mouth, tongue or tail	3
No activity after prodding or tremor and convulsion	4
Death	5

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