

Title: Pru p 3-Epitope-based sublingual immunotherapy in a murine model for the treatment of peach allergy

Authors: Rodriguez Maria J, BSc^{1#}, Mascaraque Ainhoa, PhD^{2#} Ramos-Soriano Francisco J, BSc^{2#}, Torres Maria J MD, PhD³, Perkins James R, PhD¹, Gomez Francisca, MD, PhD³, Garrido Maria, PhD⁴, Cubells Nuria, BSc⁴, Andreu David, PhD⁵, Diaz-Perales Araceli, PhD^{4*}, Rojo Javier, PhD^{2*}, Mayorga Cristobalina PhD^{1,3*}

Affiliation: ¹Research Laboratory, IBIMA, Regional University Hospital of Malaga, UMA, Malaga, Spain, ²Glycosystems Laboratory, Instituto de Investigaciones Químicas (IIQ), CSIC - Universidad de Sevilla, Sevilla, Spain. ³Allergy Unit, IBIMA, Regional University Hospital of Malaga, UMA, Malaga, Spain, ⁴Center for Plant Biotechnology and Genomic (UPM-INIA), Madrid, Spain, ⁵Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain.

and * These authors contributed equally in this manuscript

Corresponding author:

PhD. Cristobalina Mayorga Mayorga

Research laboratory-Allergy Service, Pavilion 5, basement

Regional University Hospital of Malaga. Plz Hospital Civil. Malaga, Spain

Tel: +34 951290224. FAX: +34 951290302; E-mail: lina.mayorga@ibima.eu

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Keywords.

Anaphylaxis, food-allergy, lipid transfer protein, mouse model, sublingual immunotherapy.

Abbreviations

APCs: Antigen-presenting cells *ELISpot*: Enzyme-Linked ImmunoSpot

IT: Immunotherapy

LTP: Lipid transfer protein

ODN: Oligodeoxyribonucleotide

OIT: Oral immunotherapy

SIT: Specific immunotherapy

SLIT: Sublingual immunotherapy

Th: Helper T lymphocyte

TLRs: Toll-like receptors

Treg: Regulatory T cell

CFSE: 5,6-carboxyfluorescein diacetate *N*-succinimidyl ester

ABSTRACT

Scope: Food-specific immunotherapy (SIT) is a promising treatment for LTP-syndrome. We propose a novel sublingual-SIT (SLIT) that combines a Pru p 3 T-cell peptide and an oligodeoxyribonucleotide (ODN) with CpG motifs (ODN-CpG) as adjuvants to induce a specific Th1/Treg response.

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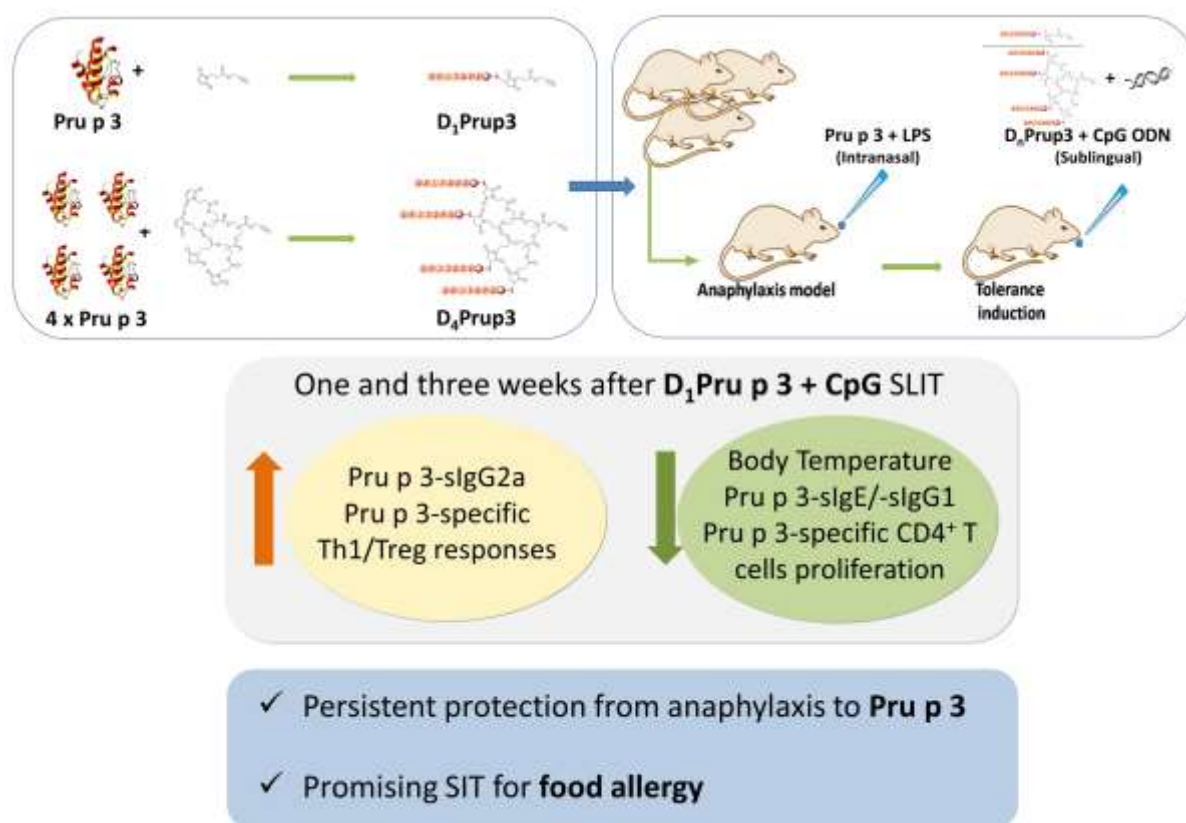
Methods and results: LTP-peach allergic mice were treated sublingually with a combination of a CpG sequence and mono- or tetravalent systems including a Pru p 3 peptide, D₁(Prup3) or D₄(Prup3). Mice were challenged intraperitoneally with Pru p 3 one/three weeks after SLIT and tolerance was assessed.

Mice treated with D₁(Prup3)+CpG were protected from anaphylaxis after Pru p 3 challenge. They showed no change in body temperature, lower levels of Pru p 3-specific IgE and IgG1 antibodies and higher levels of sIgG2a compared to the untreated group. They had fewer IgE and IgG1 secreting cells and more sIgG2a secreting cells. Moreover, a significantly lower number of Pru p 3-specific CD4⁺T-cells and a higher number of Treg cells were found, alongside a Th1 cytokine pattern. These changes were maintained for three weeks after stopping treatment.

Conclusion: D₁Prup3+CpG represents a promising SIT for food allergy. It is easily synthesized and induces protection from anaphylaxis to Pru p 3 that is maintained for at least three weeks.

Graphical Abstract Text

In this study LTP-peach anaphylactic mice were treated sublingually with a combination of a CpG sequence and mono- or tetravalent systems including a Pru p 3 peptide. We found that the monomeric compound administered sublingually represents a promising specific immunotherapy for food allergy since it is easily synthesized, safe, induces a persistent protection from anaphylaxis to Pru p 3.



INTRODUCTION

Food allergy is defined as an immunologically mediated adverse reaction that occurs following ingestion of certain foods. It represents an important public health problem, affecting 2-3% of adults and 6-8% of children, and can cause life-threatening symptoms such as anaphylaxis [1], moreover even in milder forms it can greatly impact quality of life, create a burden for health systems and adversely affect the economy [2].

Current protocols to manage food allergy include strict avoidance of the causal food as well as related ones. This strategy can have a negative impact on quality of life, leading to reduced growth in children as well as severe allergic reactions through accidental exposure to hidden allergens [4]. Consequently, there is a clear and unmet need for the development of food allergy treatments that modify the natural course of the disease.

Food-specific immunotherapy (SIT) is a promising treatment that involves administering increasing doses of the allergenic protein(s) [5]. Oral immunotherapy (OIT) and SLIT are probably the most commonly applied method for treating food allergy, although there are significant concerns related to its safety and capacity to induce permanent tolerance [6-8]. The efficacy of SIT could be enhanced by increasing the allergen dose. However it has limitations, for example, in SLIT, the amount of extract available and the maximum volume that can be placed sublingually are usually restricted [9]. An even more serious limitation of SIT is the difficulty of obtaining homogeneous extracts from natural sources in order to guarantee reproducible, effective and safe dosage [9, 10].

Specific T and B epitopes from relevant allergens, as small peptides, can stimulate antigen-presenting cells (APCs), leading to immune tolerance through the production of IgG4 and IL-10, and Th1/Treg [11]. Importantly, they are not able to cross-link IgE and therefore activate effector cells [12]. Synthetic peptides have been evaluated in both animal models and human clinical trials and represent a promising therapy to treat reactions to aeroallergens [13]. However, a key concern relating to the use of small peptides in SIT is their weak immunogenicity; this may be overcome by including adjuvants. To this end, Toll-like receptors (TLRs), whose activation modulates the adaptive immune response, have been proposed as interesting targets for the design of vaccine adjuvants [14, 15]. Among TLRs, TLR9 ligands have been shown to be both safe and effective in reducing respiratory symptoms in

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both patients with allergic rhinitis and murine model of respiratory diseases [7, 16-18]. For food allergies, however, only a small number of studies have been made with SIT [19-21].

Dendrimers are monodisperse, biocompatible, biodegradable and non-toxic particles [22]. Their modular architecture allows their functionalization with various units of either repeated or different allergenic peptides. Such epitope multivalency allows increasing the number of T-cell peptides interacting simultaneously with immune cells. In food allergies, different nanoparticles loaded with peanut proteins have shown promise for immunotherapy by promoting a Th1 response in experimental models [20].

In the Mediterranean area, lipid transfer proteins (LTPs) from the fruits of several Rosaceae plants are frequently responsible for allergic reactions. Of these LTPs, the peach allergen *Pru p 3* constitutes the major allergen and the primary sensitizer [23], and its main T and B-cell epitopes have been mapped [24-26], although little is known about their role in SIT. In this work we present a novel approach for SIT that combines a peptide-induced, *Pru p 3* T-cell-specific response with amplification and/or modulation of the immune response towards a Th1/Tregs pattern through the use of a modified oligodeoxyribonucleotide (ODN) containing CpG motifs. To this end, we prepared mono- and repeated tetravalent systems and used them to administer SLIT to *Pru p 3*-allergic mice. The induction of immune tolerance was assessed using different *in vivo* and *in vitro* parameters.

MATERIALS AND METHODS

1. Synthesis of dendrimeric Pru p3 immunogens

Preparation of Pru p 3-functionalized systems D_n Pru p 3, with $n = 1$ and $n = 4$ (Fig. 1A), required the prior synthesis and purification of several intermediate building blocks as described [27]. For the monovalent presentation ($n=1$), a linker provided with both maleimide and alkyne functionalities (D_1 -Alk) was selected as scaffold. D_1 -Alk was prepared in 69% yield by standard esterification of propargyl alcohol and 3-maleimido propionic acid. The tetravalent maleimide dendron (D_4 -Alk), with an alkyne group at its focal position, was readily prepared as described previously [27]. A peptide with the sequence ASSNGIRNVNNLARTPDRQAC was prepared, based on the 26-46 region of Pru p 3, but with the Cys residues in positions 27-28 replaced by Ser to avoid cross-linking problems, and with a C-terminal non-native Cys residue to allow conjugation to the mono- and tetravalent systems. All new compounds described in the work were perfectly purified by HPLC and characterized by Mass spectrometry (MS) (chromatograms and MS spectra are provided as supplementary information).

2. Mice, sensitization, immunotherapy and challenge

Four to five week old BALB/c female mice (Janvier Lab, Le Genest-Saint-Isle, France) were used. All experimental animal procedures were conducted according to international standards of animal welfare and approved by the Animal Experimentation Ethics Committee of BIONAND, Malaga, Spain.

Mice were divided into 6 groups: 1) Non-Sensitized (N=10); 2) Sensitized, Non-Treated (N=20); 3) Sensitized, Treated with D_1 Prup3+CpG (N=10); 4) Sensitized, Treated with D_4 Prup3+CpG (N=10); 5) Sensitized, Treated with D_1 Prup3+CpG (3 weeks after treatment) (N=10) and 6) Sensitized, Treated with D_4 Prup3+CpG (3 weeks after treatment) (N=10).

Sensitization. The procedure was carried out using Pru p 3 in combination with lipopolysaccharide (LPS) as described [28]. Mice from Groups 2-6 were anaesthetized and sensitized with intranasal administrations of 20 μ g of natural Pru p 3 (Bial Laboratory, Zamudio, Spain) and 20 ng of LPS (InvivoGen, San Diego, CA) in a final volume of 12 μ l. The same schedule was followed but with 12 μ l of PBS in mice of group 1. Sensitization procedure is shown in Fig. 1B.

Immunotherapy. Once sensitized, at week 6, mice from Groups 3-6 were treated with 1 nmol of D₁Pru p 3 or D₄Pru p 3 and 50 µg of adjuvant CpG (a modified ODN phosphorothioate with the sequence 5'-TGACTGTGAACGTTTCGAGATGA-3') (Fig. 1B) [29] by sublingual administration (10 µl), once a week for eight weeks. The same schedule was followed but with 10 µl of PBS in mice of group 2. Mice were anaesthetized to assure the compound was maintained sublingually.

Challenge. To assess anaphylaxis 10 mice from Group 2 (Sensitized) were challenged one week after the last sensitization dose (week 6) with one intraperitoneal dose of Pru p 3 (100 µg). To assess tolerance, mice were challenged with one intraperitoneal dose of Pru p 3 (100 µg) either one or three weeks after the last doses of SLIT (weeks 14 or 16 respectively) (Fig. 1C).

3. In vivo evaluation of the response

The appearance of systemic anaphylaxis was evaluated at weeks 14 and 16 within 30-40 min after challenge with one intraperitoneal dose of Pru p 3 by measuring change in body temperature with a rectal probe, and by assessing physical and behavioural symptoms. Symptoms were established according to a scoring system [30].

Mice were bled from the retro-orbital plexus on day 0 (basal) and week 14 and 16 after challenge, and sera were stored at -20°C for following humoral studies. After the challenge and *in vivo* evaluation, mice were sacrificed by cervical dislocation and the spleen was removed aseptically and teased to prepare a single-cell suspension for cellular analysis.

4. Determination of Pru p 3-sIgE, -sIgG1 and -sIgG2a and study of Pru p 3-sIgE, -sIgG1 and -sIgG2a secreting spleenocytes (ELISpot Assay)

Pru p 3-specific serum antibody levels (sIgE, sIgG1 and sIgG2a) were measured at 0 and week 14 and 16 after challenge in mice sera by ELISA using 5 µg/ml of Pru p 3 for coating microtiter plates (Corning, Corning, NY). The Pru p 3-specific antibodies were revealed with biotinylated labelled rat anti-mouse IgE, IgG1 or IgG2a (all from BD Biosciences Pharmingen, San Diego, CA). The ELISA results were expressed as optical density.

The number of antibody-secreting cells was measured using a ELISpot assay as described [31]. ELISpot Multiscreen HTS plates (Millipore, Darmstadt, Germany) were coated with Pru p 3 at 5 µg/mL. A total of 1.5×10^5 splenocytes were seeded and incubated for 48 hours at 37°C and with 5% of CO₂. After washing, 50 µl of alkaline phosphatase labelled-anti IgE, -IgG1, -IgG2a and -IgM (positive control) (SouthernBiotech, Birmingham, AL) were added. The number of Ig secreting cells was determined by counting the formed spots using an ELISpot Bioreader(R) 6000 (BioSys, Karben, Germany).

5. Studies of cellular proliferative response and cytokine production

Proliferation analysis was performed by flow cytometry. Spleen cells were harvested and labelled with 5,6-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) using CellTrace™ CFSE Cell Proliferation Kit (Life Technologies-Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Cells were incubated in the absence (negative control) or presence of Pru p 3 at 5 µg/mL for 4 days at 37°C and 5% of CO₂.

After 72h, cell culture supernatants were collected for cytokine analysis with specific fluorochrome-conjugated mAbs, antiCD4-PE and antiCD8-PE-Cy7A (BD Biosciences Pharmingen), phenotyped with a BD™ FACSCanto II flow cytometer and analyzed using FlowJo® software (Tree Star, Inc., St Ashland, OR). Results were expressed as proliferation index (PI) measured as the ratio: %CFSE^{dim} in stimulated sample / %CFSE^{dim} in non-stimulated sample and considered positive when higher than 2.

For intracellular cytokine detection, cells were fixed with BD Cytofix/CytoPerm Fixation/Permeabilization solution kit (BD Biosciences Pharmingen) and stained with specific fluorochrome-conjugated mAbs, antiIFNγ-APCCy7 and antiIL-4-PE-Cy7A (BD Biosciences Pharmingen). Regulatory cells were defined as CD3⁺CD4⁺CD25⁺FoxP3⁺ using antiCD25 and FoxP3 (BD Biosciences Pharmingen). Results were expressed as percentage of CD4⁺CD25⁺FoxP3⁺ over CD3⁺ cells.

Secreted IL-10 was determined from the 72 h culture supernatants using sandwich ELISA kits (BD Biosciences Pharmingen) according to the manufacturer's instructions. Results were expressed as pg/ml.

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6. Statistical analysis

Data are presented as individual data values, mean and standard deviation (S.D.) or median, as indicated. Quantitative non-related variables were analyzed using the Mann-Whitney U test, Kruskal-Wallis test and χ^2 -test. Comparison of related samples was carried out by Wilcoxon signed-rank test. Correlation analysis was performed using Pearson's correlation coefficient. P-values lower than 0.05 were considered statistically significant.

RESULTS

Mice receiving SLIT with D₁Prup3+CpG were protected from anaphylaxis

We confirmed the mice were anaphylactic after the 5 week sensitization period by performing an intraperitoneal challenge with Pru p 3, observing similar *in vivo* and *in vitro* data to those previously reported [28] (Data not shown). The *in vivo* evaluation showed that Sensitized, Treated with D₁(Prup3)+CpG mice maintained a body temperature comparable to basal conditions and to that of the Non-Sensitized group. In contrast, Sensitized, Treated with D₄(Prup3)+CpG group mice showed consistently lower temperature after Pru p 3 challenge, compared to both basal and Non-Sensitized ($p=0.0059$ and $p=0.0002$, respectively), and similar to those in the Sensitized, Non-Treated group (Fig. 2A). As expected, no changes in the body temperature were observed in the Non-Sensitized group after challenge with Pru p 3.

Only two mice (20%) in the Sensitized, Treated with D₁(Prup3)+CpG group showed a drop in body temperature with systemic symptoms, compared to 7 mice (70%) in the Sensitized, Treated with D₄(Prup3)+CpG group. The most relevant and characteristic symptoms were inactivity, isolation from other animals, difficulty breathing and convulsions.

SLIT with D₁Prup3+CpG and D₄Prup3+CpG induces changes in Pru p 3-sIgE, -sIgG1 and -sIgG2a in both serum and secreting cells

Assessment of serum specific antibodies (Fig. 2B) revealed lower levels of Pru p 3-sIgE in treated mice compared to the Sensitized, Non-Treated group. Regarding sIgG1, the decrease was only observed for Sensitized, Treated with D₁(Prup3)+CpG mice compared with the Sensitized, Non-Treated group (p=0.002), with no changes in Sensitized, Treated with D₄(Prup3)+CpG mice. We observed significantly higher sIgG2a levels in Sensitized, Treated with D₁(Prup3)+CpG and Sensitized, Treated with D₄(Prup3)+CpG mice compared to mice in the Non-Sensitized group (p=0.0016 and p=0.0179, respectively).

The determination of Pru p 3 specific-Ig-secreting cells by ELISpot (Fig. 2C) showed similar results with significantly lower number of sIgE and sIgG1 secreting cells and higher number of sIgG2a secreting cells in Sensitized, Treated with D₁(Prup3)+CpG mice compared to the Sensitized, Non-Treated group (p=0.0027, p=0.001 and p=0.0317, respectively). Regarding mice from the Sensitized, Treated with D₄(Prup3)+CpG group, these showed a significantly lower number of sIgE secreting cells and an higher number of sIgG2a secreting cells, compared to the Sensitized, Non-Treated group (p=0.0043 in both cases). No change was observed for sIgG1 secreting cells.

SLIT with D₁Prup3+CpG induces a decrease in Pru p 3-specific CD4⁺-T-cell proliferation and an increased number of Tregs

The analysis of splenocyte proliferative responses from the different groups (Fig. 3A) showed a significantly lower level of Pru p 3-specific CD4⁺T-cells in Sensitized, Treated with D₁(Prup3)+CpG and Sensitized, Treated with D₄(Prup3)+CpG mice, compared to the Sensitized, Non-Treated group (p=0.0002 and p=0.0068, respectively). Moreover, there was a significant increase in the percentage of Tregs (CD4⁺CD25⁺FoxP3⁺) over CD3⁺, in mice treated with D₁(Prup3)+CpG and mice treated with D₄(Prup3)+CpG, compared to the Sensitized, Non-Treated group (p=0.0002 for both groups) (Fig. 3B). No changes in Pru p 3-specific CD8⁺T-cells were observed in any of the treated groups (data not shown).

SLIT with D₁Prup3 +CpG shifts cytokine production from a Th2 to a Th1 pattern

In order to characterize the Th response pattern resulting from treatment we evaluated the production of different cytokines in response to Pru p 3 using cultured splenocytes (Fig. 3C). higher level of IL-4 producing CD4⁺T-cells was observed for Sensitized, Non-Treated mice compared to Sensitized, Treated with D₁(Prup3)+CpG and Sensitized, Treated with D₄(Prup3)+CpG mice (p=0.038 and p=0.032, respectively). In contrast, higher levels of IFN- γ producing CD4⁺T-cells were only observed for Sensitized, Treated with D₁(Prup3)+CpG mice; this difference was not significant. Moreover, significantly higher levels of IL-10 were detected in the supernatant of mice from groups Sensitized, Treated with D₁(Prup3)+CpG and Sensitized, Treated with D₄(Prup3)+CpG compared to Non-Sensitized (p=0.0095 for both). However, although we found higher levels of this cytokine in Sensitized, Treated with D₁(Prup3)+CpG compared to the Sensitized, Non-Treated group, these were not significant (Fig. 3C).

Mice with SLIT with D₁Prup3+CpG maintained tolerance three weeks after stopping the treatment

We analyzed tolerance maintenance in order to assess whether SLIT could continue to give beneficial effects after stopping treatment. For this, three weeks after treatment ended Sensitized, Treated with D₁(Prup3)+CpG mice were challenged with Pru p 3 and *in vivo* and *in vitro* parameters were assessed. Since mice from the Sensitized, Treated with D₄(Prup3)+CpG group did not show tolerance immediately after the SLIT protocol, we could not test the maintenance of tolerance with D₄Prup3+CpG.

In vivo data showed no significant changes in body temperature (Fig. 4A) and no systemic symptoms in Sensitized, Treated with D₁(Prup3)+CpG mice after challenge with Pru p 3 three weeks after ending the IT. Immunoglobulin production was similar to that observed after only one week of SLIT with mice showing significantly lower levels of IgE and sIgG1 compared to the Sensitized, Non-Treated group (p=0.001 and p=0.009, respectively). Moreover, sIgE levels were lower compared to those obtained

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for the Sensitized, Treated with D₁(Prup3)+CpG mice after challenge with Pru p 3 one week after ending the IT ($p=0.0008$). We also observed lower levels of Pru p 3 sIgG2a three weeks after stopping SLIT ($p=0.04$) (Fig. 4B).

Regarding Ig-secreting cells, data showed a significantly lower number of sIgE and sIgG1 in Sensitized, Treated with D₁(Prup3)+CpG mice ($p=0.0145$ and $p=0.0019$ respectively). Moreover, we observed a higher number of sIgG2a secreting cells, although this was not significant compared to the Sensitized, Non-Treated group (Fig. 4C).

Analysis of the proliferative response to Pru p 3 showed a significant decrease in both CD4⁺ and CD8⁺T-cells compared to the Sensitized, Non-Treated group ($p<0.0001$ and $p=0.0002$, respectively) (Fig. 5A). Moreover, we observed a significantly lower proliferative response of CD8⁺T-cells after three weeks of SLIT compared to mice that were challenged after one week of stopping the SLIT ($p=0.0035$). The levels of CD4⁺T-cells producing IL-4 and IFN- γ maintained their decreased and increased levels, respectively, compared to the Sensitized, Non-Treated group, with no changes compared to Sensitized, Treated with D₁(Prup3)+CpG mice one week after SLIT (Fig. 5C).

The percentage of Treg cells showed an important decrease after three weeks of stopping SLIT ($p<0.0001$) (Fig. 5B) although levels of IL-10 remained higher than in the Sensitized, Non-Treated group ($p=0.032$) (Fig. 5D).

DISCUSSION

Food allergy represents a growing health problem that adversely affects the quality of life of children and adults [1]. SIT to Pru p 3, the most prevalent plant-food allergen in the Mediterranean area, represents a promising strategy for treating these patients. Various studies have demonstrated the safe and beneficial effects of SLIT with a Pru p 3-enriched peach extract, including a reduction in systemic allergic reactions [32, 33].

In this study we have assessed the immunomodulation of anaphylaxis to the peach LTP, Pru p 3, using a novel system that includes T-cell peptides presented in a monovalent or tetravalent manner and an adjuvant molecule (ODN-CpG) that binds TLR9. Assessment of the *in vivo* and *in vitro* responses suggests that the immune protection offered by SIT is produced by a decrease in the Th2 effector response with a reduction of Pru p 3-specific IgE and IgG1, a decreased proliferative response of specific CD4⁺T-cells, the production of IL-4, a simultaneous increase of Treg cells, and the production of IFN- γ and IL-10. These data indicate that this strategy shows promise for food allergy immunotherapy.

The development of vaccines for large-scale, low cost immunotherapy, using natural protein extracts as the allergen source, is strongly limited by difficulties relating to purification, variations in concentration and differences in immunogenicity arising from different preparation processes. These problems can lead to differences between batches of the final product; however they can be overcome using synthesized T-cell peptides that can be produced efficiently and reproducibly, thus avoiding batch differences and other systematic problems. However, these peptides may only induce weak immune responses that need to be boosted by adding an adjuvant. In this regard, previous work has shown ODN-CpG, a TLR9 ligand, to be useful in shifting the Th2 immune response towards a Th1/Treg balance in the treatment of allergic rhinitis [18, 34] as well as food allergy [21].

We have shown that SLIT with D₁ can induce a specific shift towards a Th1/Treg immune response. These observations are consistent with those previously observed for peanut-allergic mice, that were co-administered ODN-CpG orally with peanut proteins [19, 21, 34, 35].

IT using nanoparticles has previously been evaluated in food allergy, using peanut OIT to induce protection from anaphylaxis [6, 20]. In this work we have used both monomeric and tetravalent dendrimeric structures in order to assess whether the inclusion of repeated epitopes in the same structure increased the immune response. Our mono- and tetravalent immunogens are monodisperse and homogeneous compounds, which have been perfectly characterized by mass spectrometry. They do not show any adverse effects in this study, although a more comprehensive study would be required in order to determine safety. Results show that, although immunological changes can be observed after SLIT with either D₁ or D₄, immunoprotection against anaphylaxis was achieved in 80%

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of mice treated with monovalent D₁, but only 30% of mice treated with tetravalent D₄. Lack of effective immunotherapeutic effects in the latter group could be related to the smaller decrease in specific IgE, with no changes in Pru p 3-specific IgG1 and IFN- γ and IL-10 production compared to the Sensitized, Non-Treated group. We propose several explanations for these differences. Firstly, as shown by others, the size of the nanostructure can influence the immune response. As we have shown previously, smaller fluorescent gold nanoclusters are better internalized by dendritic cells and enhance immunosuppressive Th1/Treg responses [36]. Similar data have been found for nanoparticles in a mouse model of allergic airway inflammation [37]. Secondly, the ability to induce IgE cross-linking may also be important. We estimated the maximal distance between two Pru p 3 epitopes in nanostructure D₄Prup3+CpG to be approx. 3 nm (30 Å), close to the distance judged as optimal (44-51 Å) for effective cross-linking of specific IgE on rat basophils to stimulate degranulation [38]. This may lead our nanostructure to interact with effector cells such as mast cells and basophils (albeit suboptimally), maintaining a residual Th2 response during SLIT, which may counteract the increase in Th1/Treg response. In this sense, we have confirmed that only D₄Prup3 but not D₁Prup3 was able to induce basophil activation from peach allergic patients indicating its ability to cross-link IgE bound to the basophil surface. Third, the ability to cross the sublingual mucosa is related to the size of compounds. Transport of proteins across mucosa depends on size (influenced by aggregation), polarity, and shape. In this sense it is important to mention that nanoparticles smaller than 200nm can go through lymphatic barriers and be taken up by dendritic cells in lymph nodes inducing Th1 responses, as demonstrated by Silva *et al.* using similar particles functionalized with ODN-CpG [39].

One of the major concerns related to SIT for food allergy is its ability to induce long-term protection or tolerance [40]. It is well documented that patients can be desensitized to food allergens with OIT or SLIT, however it is difficult to induce long-lasting tolerance [41]. Few studies have addressed protection after stopping OIT [42]; however one study in mouse showed that the protective effects were lost after 2 weeks [43]. In our study, we confirmed the maintenance of tolerance three weeks after stopping SLIT and moreover found the immunological changes to be similar to those obtained one week after the treatment.

Tregs are thought to be essential in tolerance induction, although their full role is still not completely understood. It is important to note that increased IL-10 production has been shown to be involved in the suppressive activity of Treg induced by peanut SLIT in mice [44]; this could be the cause of the lower levels of Pru p 3-specific IgE and IgG1 and reduction in effector response of mast cells and basophils. Lower levels of IgE, IgG1 and IgG2a have also been observed following peanut specific OIT [20]. Specific SLIT with a T-cell peptide from Pru p 3 represents a good approach for inducing tolerance in peach allergic mice, due to the low amount of antigen necessary. Further studies will be necessary for human applications.

In conclusion, we have shown using an animal study that a monomeric compound functionalized with a T-cell epitope of Pru p 3, the major peach allergen, administered sublingually has potential as a safe and effective immunotherapy. It has many desirable characteristics, inducing protection from anaphylaxis that persists after stopping the treatment. Moreover, it only requires relatively few doses and has an easy application (sublingual) that could reduce potential adverse side effects. Importantly, it can be produced relatively easily and at a low cost, and can potentially be adapted to a wide range of allergens.

AUTHOR CONTRIBUTIONS

CM, JR and ADP conceived and designed the study and were involved in analysis and interpretation and manuscript production. MJR, AM and JRS performed the experiments, data analysis and drafted the article. MJT and DA helped write and revise the article. NCB, FG and MG were involved in conducting the experiments and data analysis; JRP was involved in data analysis and final manuscript revision. All authors have read and approved the manuscript.

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CONFLICT OF INTEREST STATEMENT

None of the authors has any conflict of interest. None received money for the present study. Research is part of their daily activities. All authors had full access to all the data (including statistical reports) and can take responsibility for the integrity of the data and the accuracy of the data analysis.

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FIGURE LEGENDS

Figure 1.- A) Preparation of D₁Prup3 and D₄Prup3 by conjugation of a Pru p 3 epitope and the mono- and tetravalent maleimide scaffolds D1-Alk and D4-Alk, respectively B) Structure of CpG-ODN; C) Experimental procedures. At day 0, blood samples were collected from each mouse. Seven sensitizations were performed intranasally. SLIT with either D₁Prup3+CpG or D₄Prup3+CpG beginning at week 6 (for 8 weeks). At week 14 or 16 mice were intraperitoneally challenged with Pru p 3, following this *in vivo* parameters were measured and blood and spleen were collected.

Figure 1

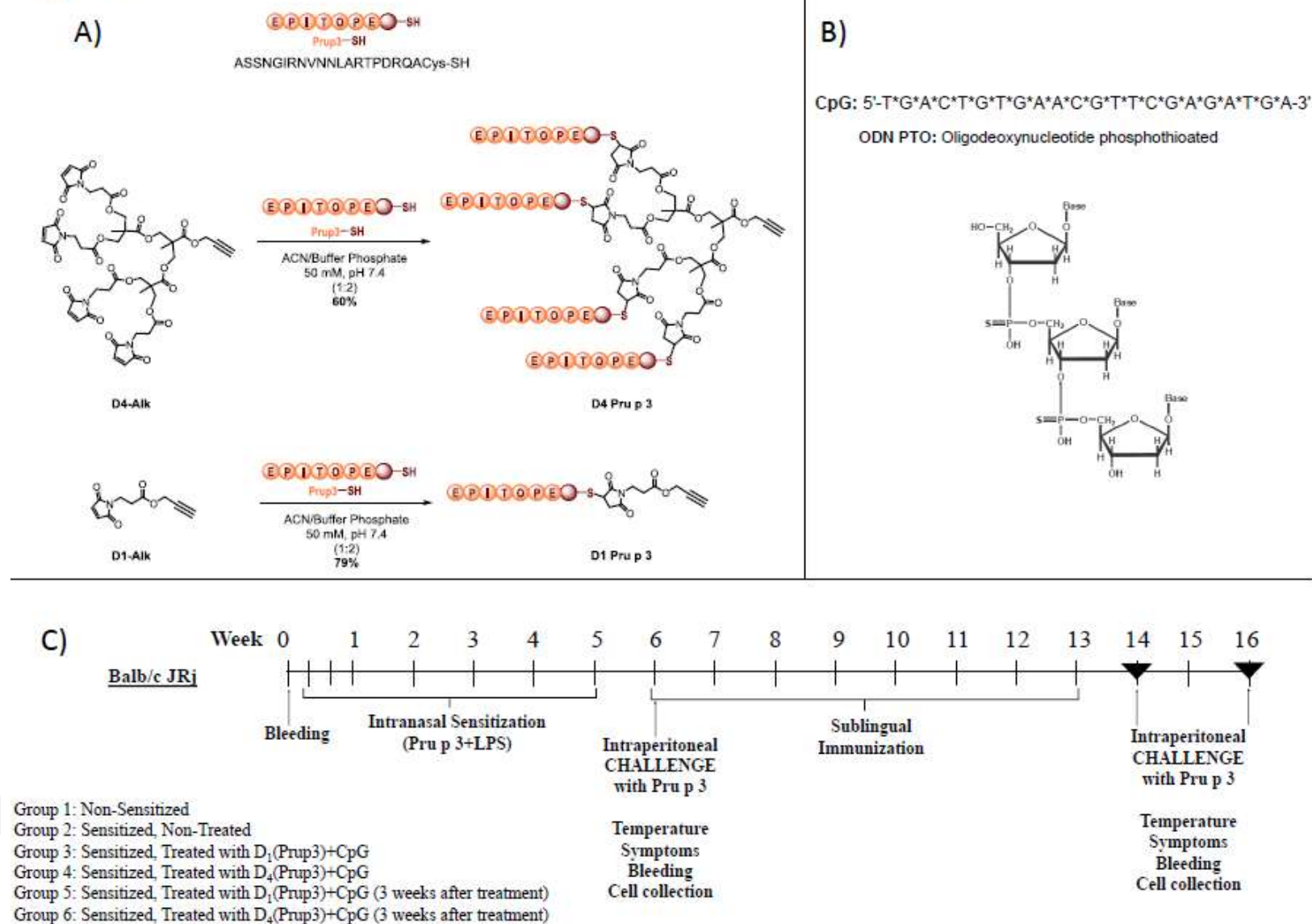


Figure 2.- A) Measurement of the appearance of systemic anaphylactic symptoms was evaluated 30-40 min after challenge (AC) with Pru p 3 by measuring change in body temperature (°C). B) Bars represent the mean serum level of Pru p 3-sIgE, IgG1 and IgG2a by ELISA in different groups. C) Box plots represent the number of Pru p 3-sIgE, IgG1 and IgG2a secreting cells by ELISpot assay. Horizontal lines represent the median values for each group. Samples were obtained 30-40 minutes after challenge, one week after finishing SIT. **Group 1:** Non-Sensitized; **Group 2:** Sensitized, Non-Treated; **Group 3:** Sensitized, Treated with D₁(Prup3)+CpG; **Group 4:** Sensitized, Treated with D₄(Prup3)+CpG

Figure 2

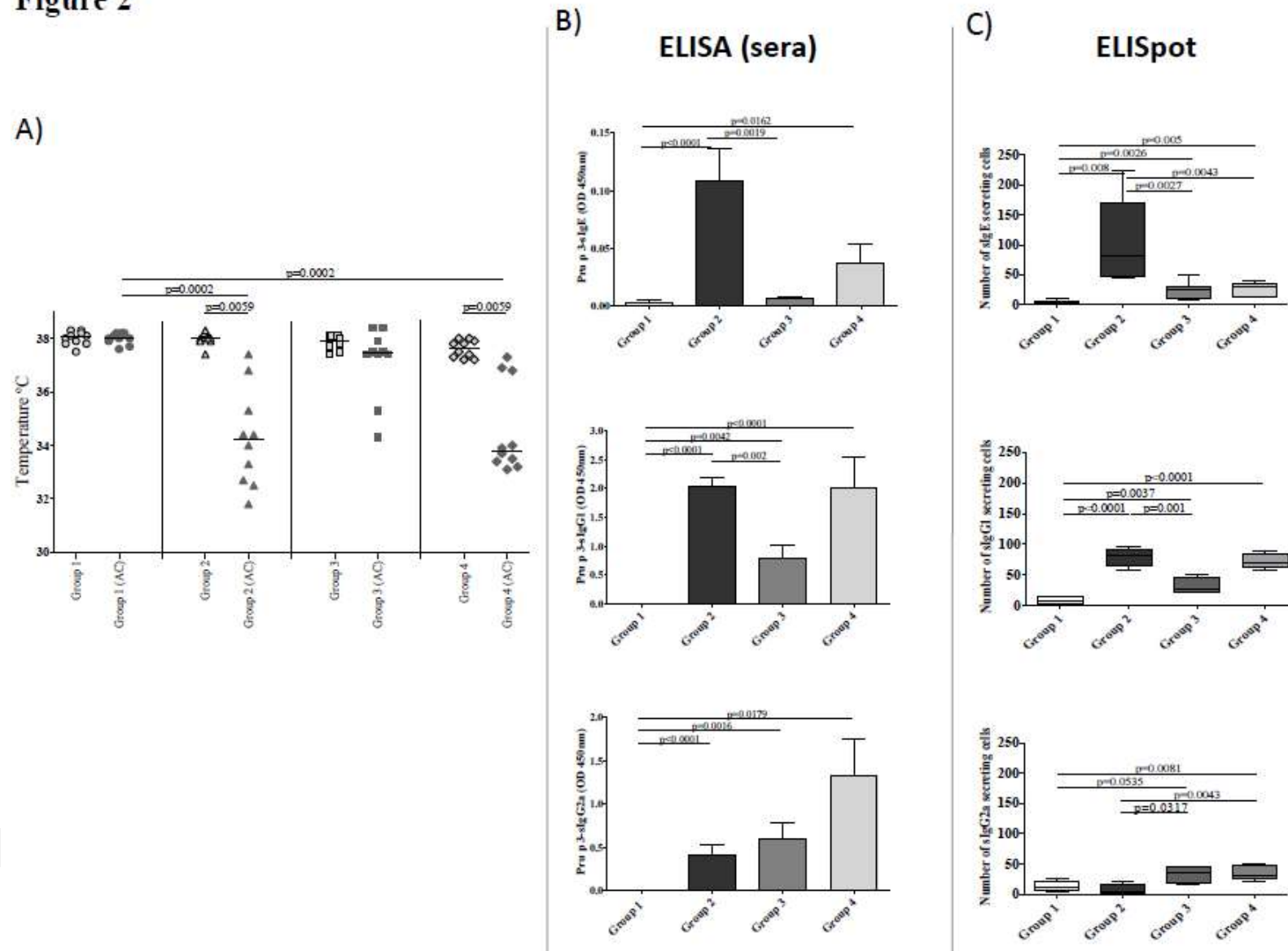


Figure 3.- A and B, Upper: Gate selection strategy for Prup3-proliferating splenocytes (A) and Tregs (B). A and B, Lower: Dots represent CD4⁺-cells proliferation index (A) and Treg percentage (B) after Prup3 stimulation. C: Bars represent mean and standard deviation for percentages of CD4-cells producing intracellular IL-4 or IFN- γ (Upper) and production of IL-10 (Lower) after Prup3 stimulation. **Group 1:** Non-Sensitized; **Group 2:** Sensitized, Non-Treated; **Group 3:** Sensitized, Treated with D₁(Prup3)+CpG; **Group 4:** Sensitized, Treated with D₄(Prup3)+CpG

Figure 3

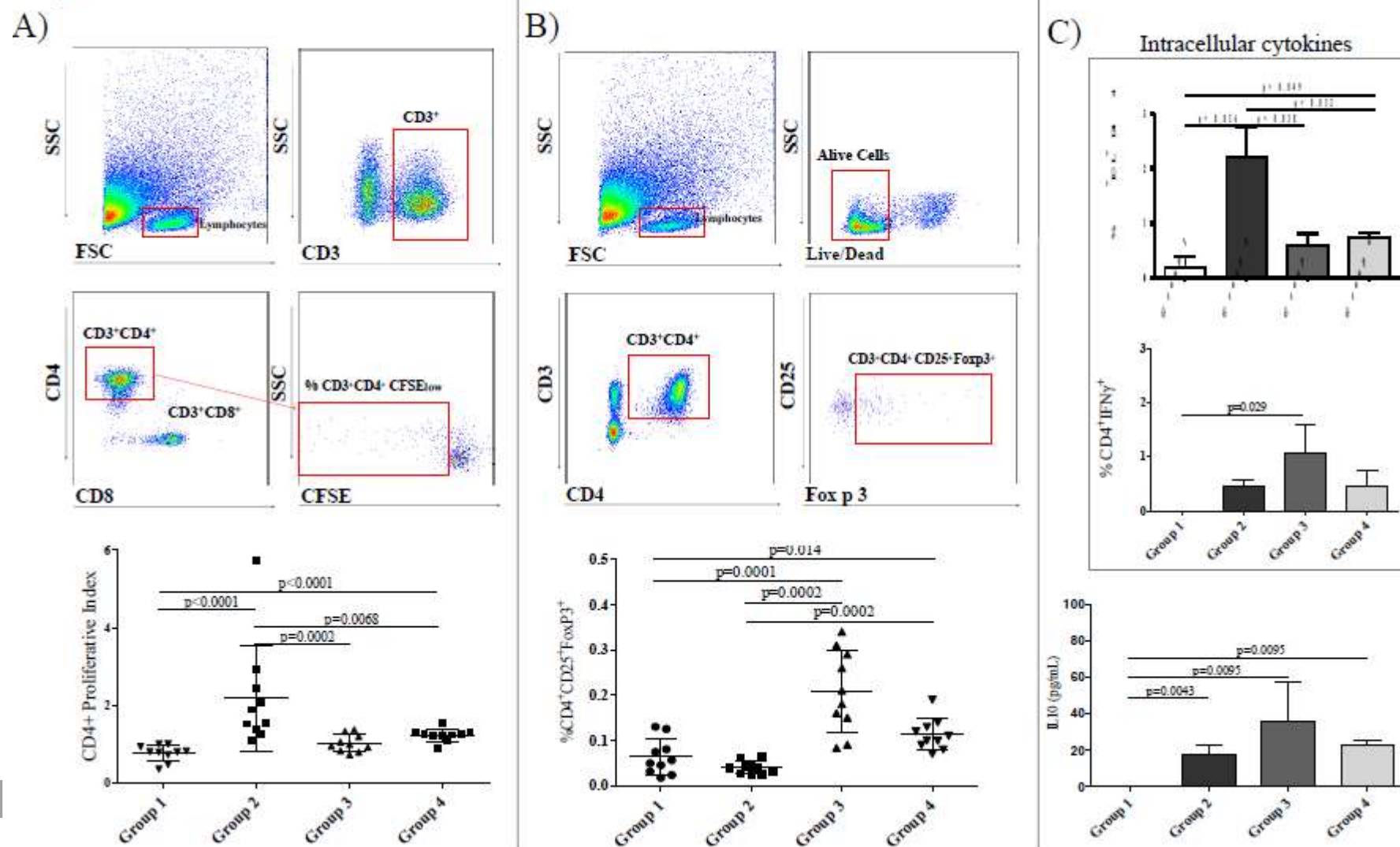


Figure 4.- A) Measurement of the appearance of systemic anaphylactic symptoms was evaluated 30-40 min after challenge (AC) with Pru p 3, 1 and 3 weeks after stopping D₁Prup3+CpG SLIT by measuring change in body temperature (°C). B) Bars represent the mean serum level of Pru p 3-sIgE, IgG1 and IgG2a by ELISA measured 1 and 3 weeks after stopping D₁Prup3+CpG SLIT. C) Box plots represent the number of Pru p 3-sIgE, IgG1 and IgG2a secreting cells, measured 1 and 3 weeks after stopping D₁Prup3+CpG SLIT.

Group 2: Sensitized, Non-Treated; **Group 3:** Sensitized, Treated with D₁(Prup3)+CpG; **Group 5:** Sensitized, Treated with D₁(Prup3)+CpG (3 weeks after treatment).

Figure 4

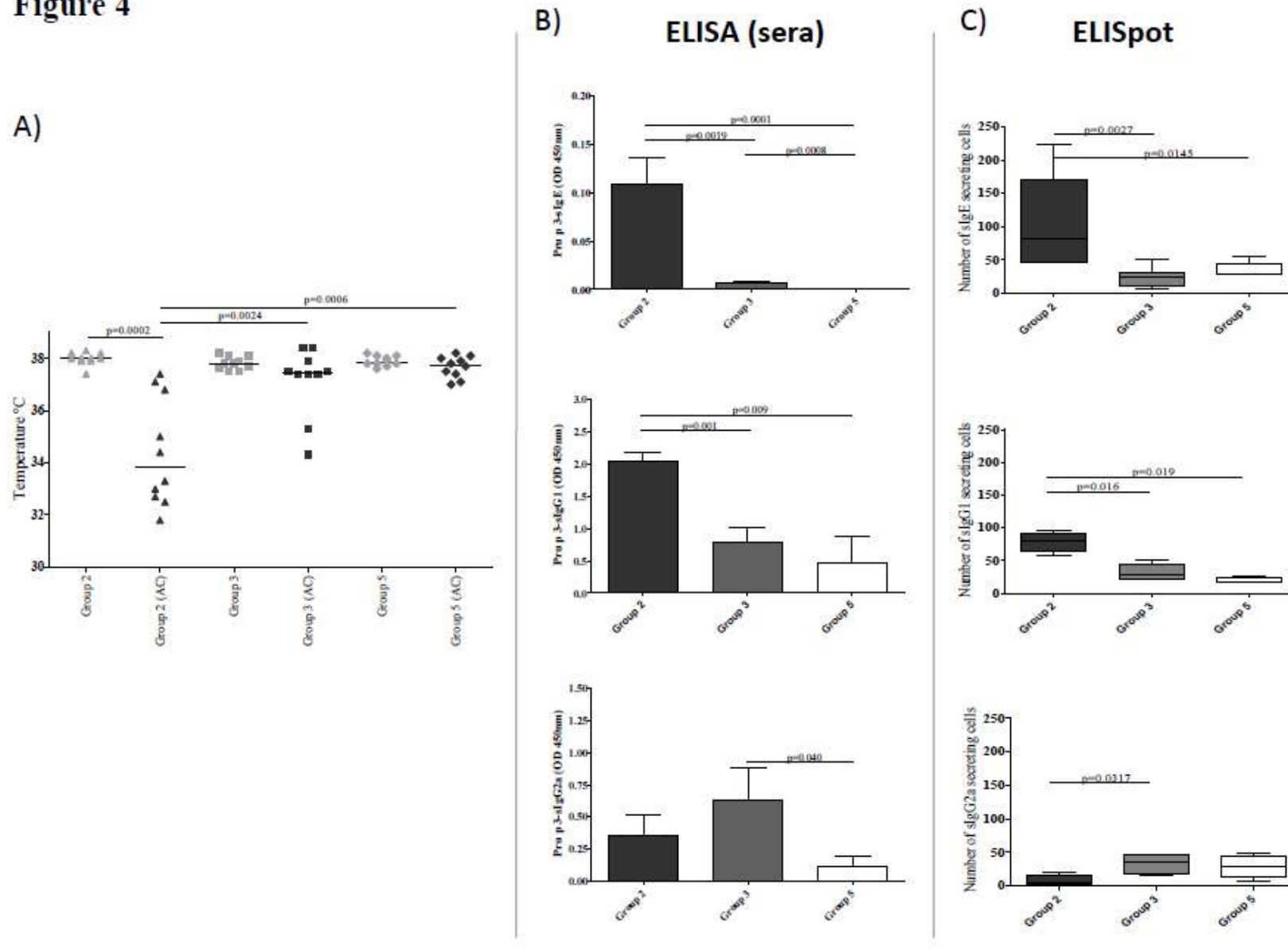
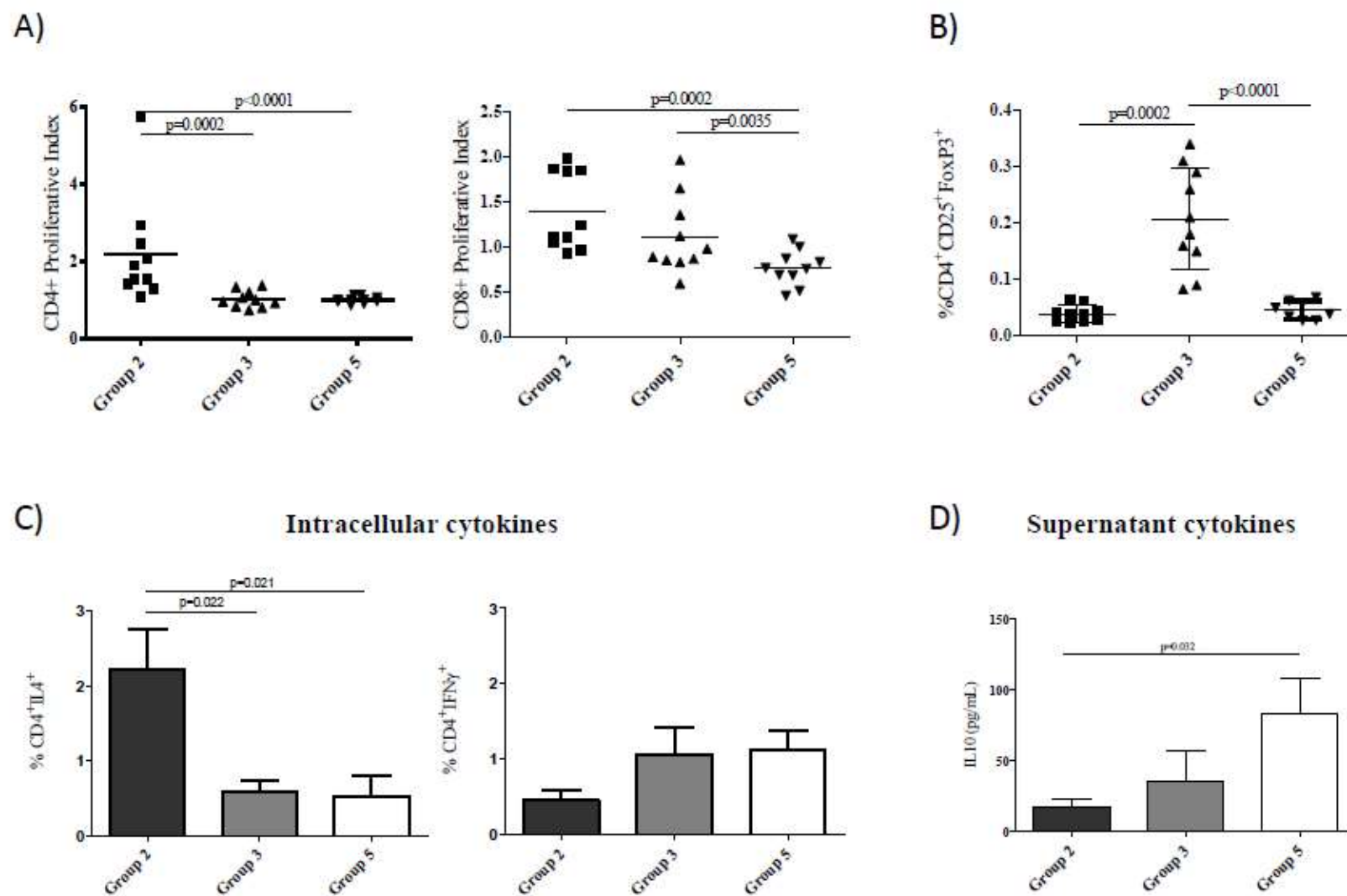


Figure 5.- Dots represent proliferation index for CD4⁺ and CD8⁺ subpopulations (A) and Treg percentage (B) after Pru p 3 stimulation, 1 and 3 weeks after stopping D₁Prup3+CpG SLIT. Bars represent the mean and standard deviation of CD4-cells producing IL-4 or IFN-γ (C) and IL-10 concentration in supernatant (D) after Pru p 3 stimulation, 1 and 3 weeks after stopping D₁Prup3+CpG SLIT. **Group 2:** Sensitized, Non-Treated; **Group 3:** Sensitized, Treated with D₁(Prup3)+CpG; **Group 5:** Sensitized, Treated with D₁(Prup3)+CpG (3 weeks after treatment).

Figure 5

SUPPORTING INFORMATION

Title: Pru p 3-Epitope-based sublingual immunotherapy in a murine model for the treatment of peach allergy

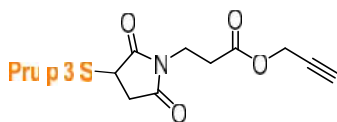
Authors: Rodriguez Maria J, BSc^{1#}, Mascaraque Ainhoa, PhD^{2#} Ramos-Soriano Francisco J, BSc^{2#}, Torres Maria J MD, PhD³, Perkins James R, PhD¹, Gomez Francisca, MD, PhD³, Garrido Maria, PhD⁴, Cubells Nuria, BSc⁴, Andreu David, PhD⁵, Diaz-Perales Araceli, PhD^{4*}, Rojo Javier, PhD^{2*}, Mayorga Cristobalina PhD^{1,3*}

Affiliation: ¹Research Laboratory, IBIMA, Regional University Hospital of Malaga, UMA, Malaga, Spain, ²Glycosystems Laboratory, Instituto de Investigaciones Químicas (IIQ), CSIC - Universidad de Sevilla, Sevilla, Spain. ³Allergy Unit, IBIMA, Regional University Hospital of Malaga, UMA, Malaga, Spain, ⁴Center for Plant Biotechnology and Genomic (UPM-INIA), Madrid, Spain, ⁵Department of Experimental and Health Sciences, Universitat Pompeu Fabra, Barcelona, Spain.

and * These authors contributed equally in this manuscript

Synthesis, HPLC and MS characterization of compounds Da Pru p 3 and D4 Pru p 3

Compound D1 Pru p 3.



A solution of Pru p 3 SH (6.6 mg, 2.91 μ mol) in 50 mM phosphate buffer pH 7.4 (1020 μ L) was added to a maleimide compound **D1-Alk** (540 μ g, 2.67 μ mol) solution in CH₃CN (510 μ L) and the reaction mixture was stirred at room temperature. The reaction progress was monitored by analytical HPLC. After 20 minutes reaction time, the complete conversion to the

desired compound was observed. The crude of the reaction was then purified by semipreparative RP-HPLC, giving the compound **D1 Pru p 3** (5.20 mg, 79%) as a white solid.

ESI-HRMS: calc. for $C_{99}H_{163}N_{37}O_{35}S$ (m/z): 2463.6770; found: 2463.22 $[1]^+$, 1232.6096 $[M+2H]^{+2}$, 822.0800 $[M+3H]^{+3}$, 616.8131 $[M+4H]^{+4}$; HPLC: t_R = 7.10 min (Column: Phenomenex C_{18} ; A: H_2O (0.05 % TFA), B: Acetonitrile (0.1 % TFA), 15 to 50% linear gradient of B into A over 30 min, F = 1 mL/min).

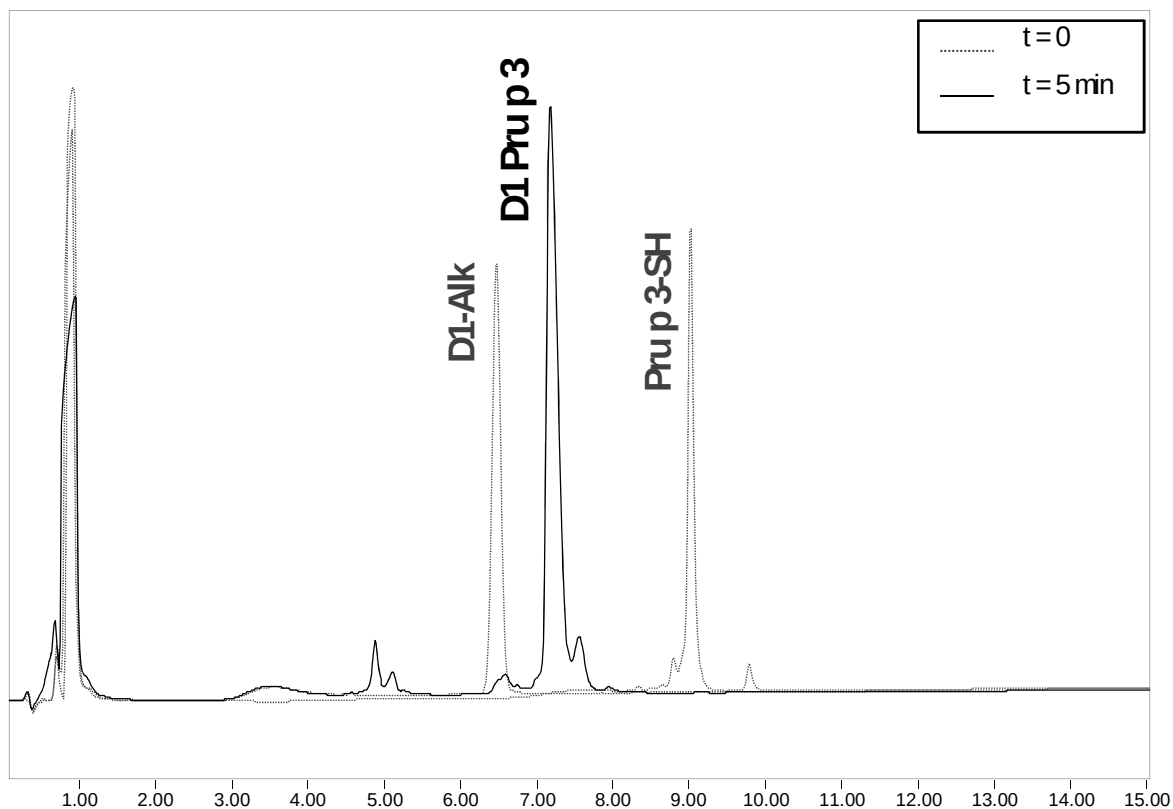


Figure S-1. HPLC monitoring of the thiol-ene reaction leading to 5 min. Peaks corresponding to D1-Alk (t_R = 6.52 min), the compound **D1 Pru p 3**. (t_R = 7.10 min), an impurity (t_R = 7.62 min) and Pru p 3-SH (t_R = 9.02 min)., Elution by 15 to 50% linear gradient of B into A over 30 min, at 1 mL/min flow rate, with UV detection at 214 nm.

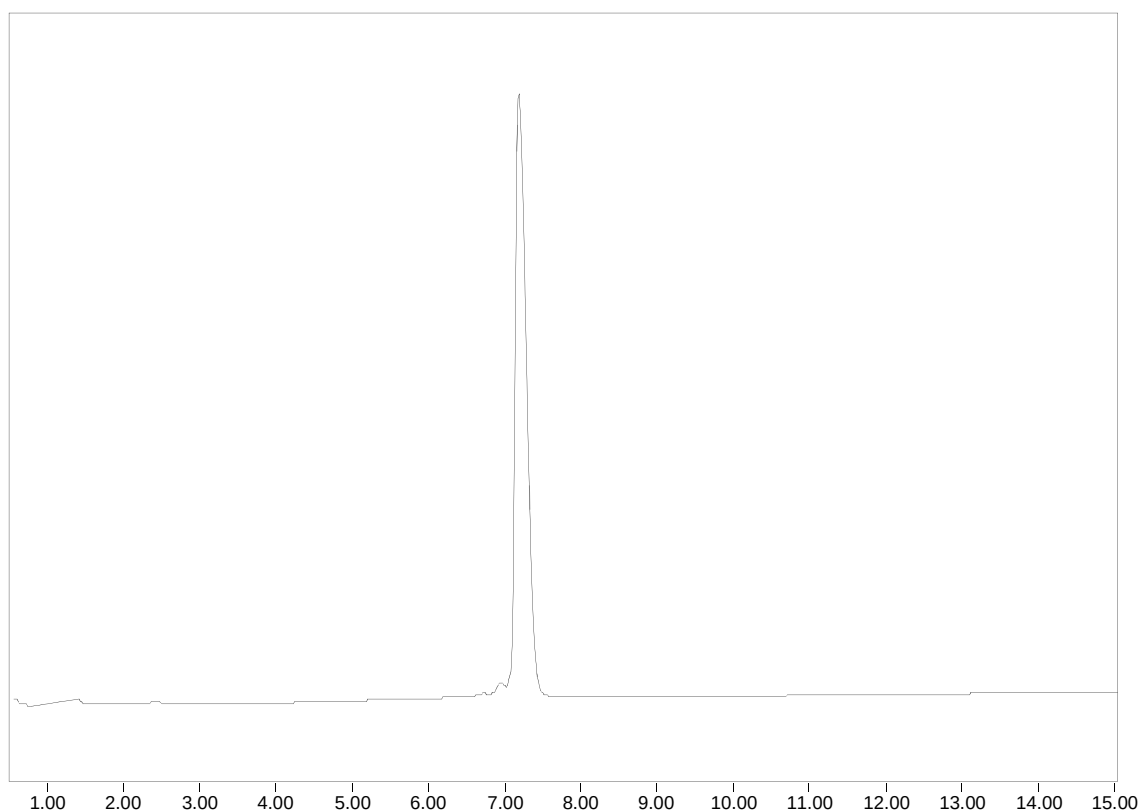


Figure S-2. HPLC chromatogram of the compound **D1 Pru p 3** after purification on HPLC. Elution by 15 to 50% linear gradient of B into A over 30 min, at 1 mL/min flow rate, with UV detection at 214 nm.

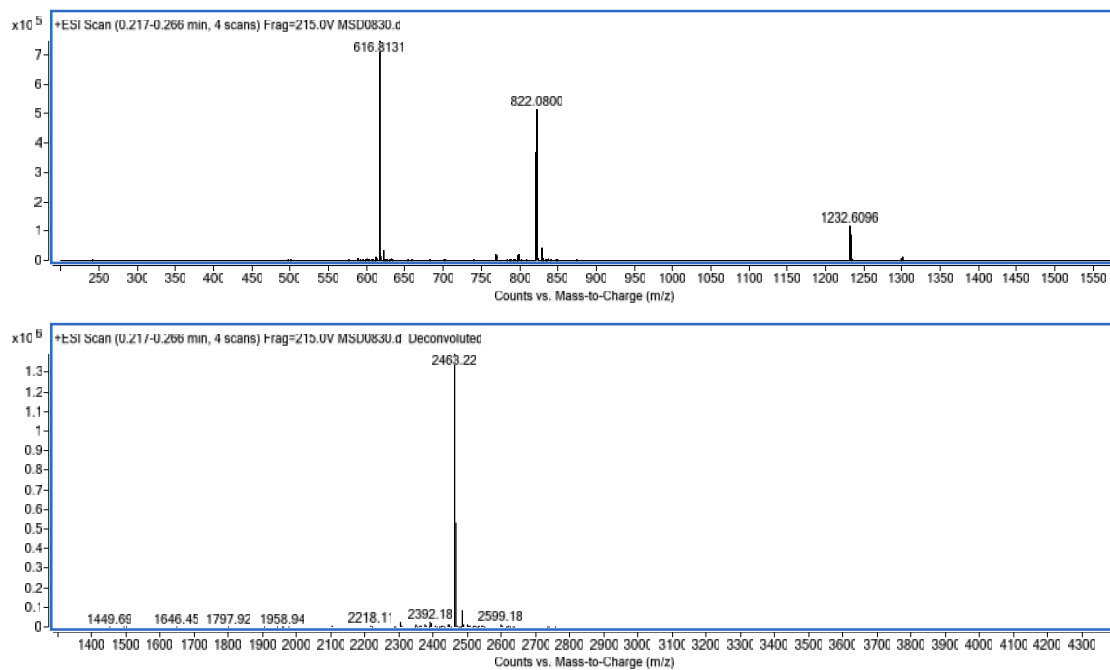
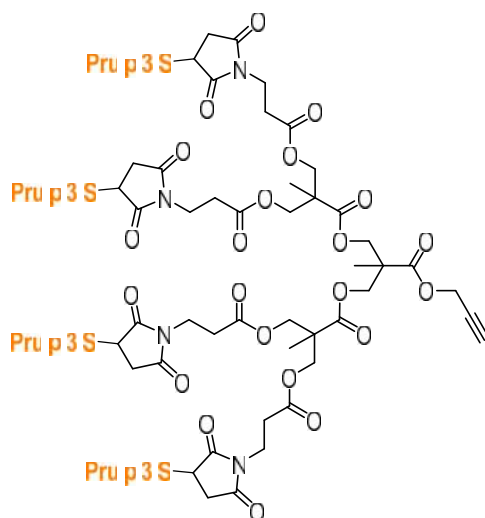


Figure S-3. ESI-MS, deconvoluted, of the compound **D1 Pru p 3**.

Compound D4 Pru p 3.



To a solution of maleimide dendron **D4-Alk** [1] (202 μg , 0.2 μmol) in CH_3CN (170 μL), a solution of Pru p 3 SH (2.4 mg, 0.96 μmol) in 50 mM phosphate buffer pH 7.4 (340 μL) was added and the mixture reaction was stirred at room temperature. The reaction progress was monitored by analytical HPLC. After 5 min reaction time, the complete conversion to the desired compound was observed. The crude of the reaction was then purified by semipreparative RP-HPLC, to give the dendropeptide **D4 Pru p 3** (1.48 mg, 60%) as white solid. ESI-HRMS: calc. for $\text{C}_{402}\text{H}_{664}\text{N}_{148}\text{O}_{146}\text{S}_4$ (m/z): 10034.86; found: 10035.02 $[\text{M}]^+$; HPLC: t_R = 7.05 min (Column: Phenomenex C_{18} ; A: H_2O (0.05 % TFA), B: Acetonitrile (0.1 % TFA), 15 to 50% linear gradient of B into A over 15 min, F = 1 mL/min).

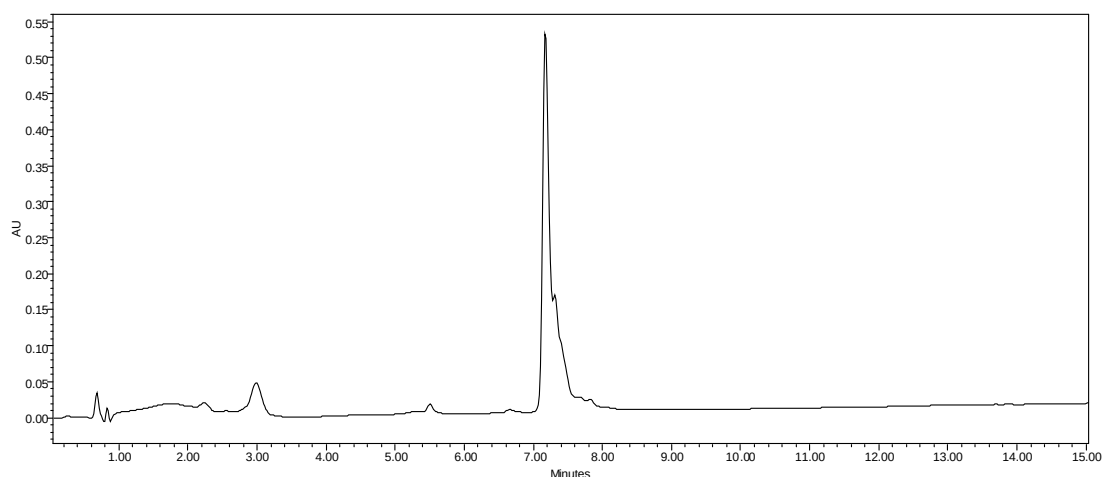


Figure S-4. HPLC monitoring of the thiol-ene reaction leading to 5 min. Peaks corresponding to Prup3SH (t_R = 2.98 min), the compound **D4 Pru p 3** (t_R = 7.05 min) and an impurity (t_R = 7.33 min). Elution by 15 to 50% linear gradient of B into A over 15 min, at 1 mL/min flow rate, with UV detection at 214 nm.

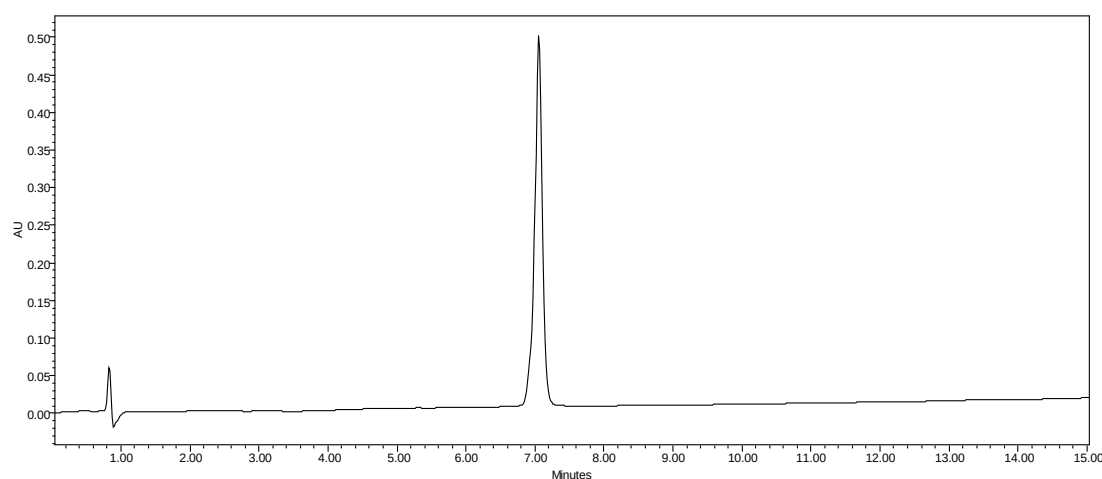


Figure S-5. HPLC chromatogram of the compound **D4 Pru p 3** after purification on HPLC. Elution by 15 to 50% linear gradient of B into A over 15 min, at 1 mL/min flow rate, with UV detection at 214 nm.

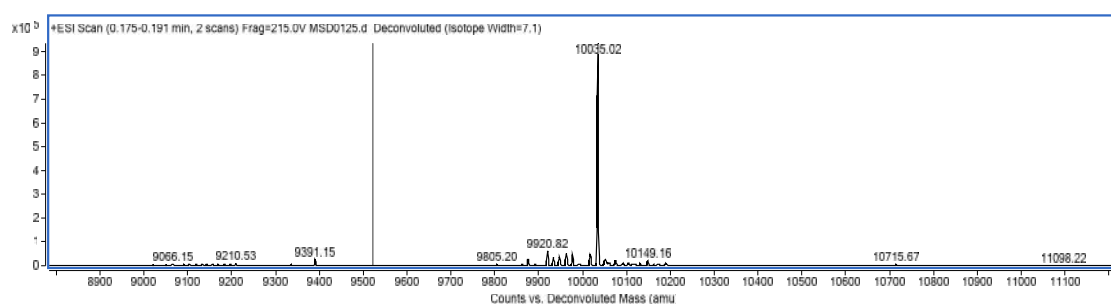


Figure S-6. ESI-MS, deconvoluted, of the compound **D4 Pru p 3**.

Cross-linking experiment with basophil activation test

In order to confirm the ability to induce IgE cross-linking of either D₁Prup3 or D₄Prup3 we have performed the basophil activation test. This was performed as described [2, 3] using whole blood from three peach allergic patients and stimulated with

This test was performed as described [4]. Briefly, heparinized whole blood was incubated with 20 μ L of stimulation buffer (1 M HEPES buffer containing 0.78% NaCl; 0.037% KCl(w/v); 0.078% CaCl₂ (w/v); 0.033% MgCl₂ (w/v); 0.1% HSA (w/v); and 10 μ L/mL IL-3) and 1 μ L of monoclonal antibody (moAb) CCR3-APC (BioLegend Inc, San Diego, CA) for 10 min at 37°C. Then, 100 μ L of Pru p 3 at concentrations from 0.1-0.0001 μ g/mL or D₁Prup3 or D₄Prup3 at concentrations from 50-0.0001 μ g/mL were added and incubated for 30 min at 37°C. PBS was used as a negative control, and anti-human IgE (0.5 mg/mL) (BD Biosciences Pharmingen, San Diego, CA) as a positive control. After 5 min on ice, to stop the degranulation process, samples were incubated with moAb anti-CD203c-PE and CD63-FITC (BioLegend Inc) for 15-20 min at 4°C. Finally, lysing solution (BD Biosciences Pharmingen) was used for red cell disruption. Cells

were analyzed in a FACSCalibur flow cytometer (BD Biosciences Pharmingen), acquiring at least 500 basophils per sample. Results were presented as the percentage of activated basophils ($CD63^+CD203c^+CCR3^+$).

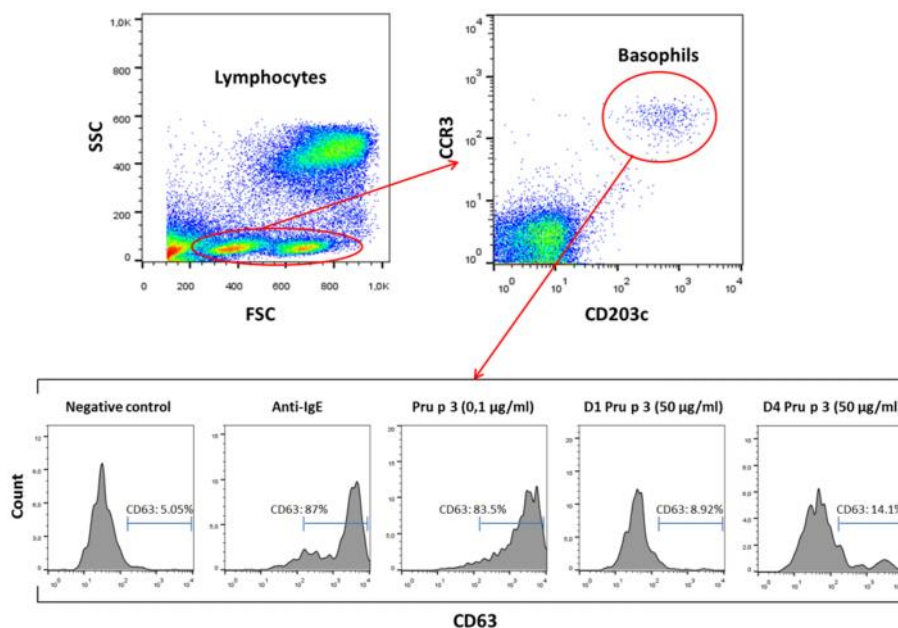


Figure S-7. Gate strategy description on a representative patient allergic to peach.

The results show no activation with D₁Prup3 indicating no crosslinking. On the other hand a slight positive activation was found when stimulating the basophils with D₄Prup3 at the higher concentration although in a lower level compared to the Pru p 3 induced activation. These results confirm that only the compound with a minimum size is able to cross-link at least two IgE molecules on basophil surface.

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