

Pru p 3-Glycodendropeptides Based on Mannoses Promote Changes in the Immunological Properties of Dendritic and T-Cells from LTP-Allergic Patients

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Scope: Glycodendropeptides (GDPs) functionalized with mannose can enhance allergen interaction with dendritic cells (DCs) via C-type lectin receptors (CLRs), modulating the immune response. They can present multiple peptides and have potential applications for diagnosis and treatment of food allergy (FA). The immune response induced by GDPs with mannose and Pru p 3 peptides (mono/tetravalent) with ester (D_1 ManPrup3/ D_4 ManPrup3) or ether linkers (D_1 Man_O.Prup3/ D_4 Man_O.Prup3) in lipid-transfer-protein-allergic patients and tolerant controls is analyzed.

Methods and results: The immunological response induced by GDPs is studied by assessing monocyte-derived-DC maturation, lymphocyte proliferation, cytokine production, and basophil response by flow cytometry. D_n ManPrup3 was recognized by DCs via CLRs inducing DC maturation in all subjects. However, CCR7 expression is significantly upregulated in allergic patients compared to tolerant controls. These changes correlate with lymphocyte proliferation and specific production of Th2/Th1 cytokines in allergic patients. Moreover, D_1 ManPrup3 does not induce basophil activation. **Conclusion:** D_n ManPrup3 induces changes in DC maturation and lymphocyte proliferation, indicating specific recognition via CLRs. Prup3-GDPs are recognized by immune cells, inducing a specific immune response and modulating the immunological response in FA patients. The specific geometry of D_1 ManPrup3 in particular makes it a potential candidate for specific immunotherapy development.

1. Introduction

Nonspecific lipid transfer proteins (nsLTPs) are present in multiple edible plants and are frequently responsible for food allergy (FA) in the Mediterranean area. nsLTPs are panallergens that have been associated with sensitization to multiple unrelated plant-derived foods, inducing a complex clinical manifestation defined as LTP-syndrome,^[1] whose management is considered a serious health problem.^[2,3] The peach nsLTP, Pru p 3, is the major allergen in the Mediterranean area.^[4,5] Currently, treatment is based on the avoidance of different foods; however, this does not rule out the possibility of accidental ingestion.^[6] Thus, there is a clear need to develop safe and long-term effective therapies for LTP-syndrome able to modify the nature of the disease.^[7] Allergen-specific immunotherapy in FA is an active area of research and recent studies have demonstrated the safety and effectiveness of sublingual immunotherapy (SLIT) using Pru p 3-enriched extracts for the treatment of FA to nsLTP.^[8,9] It induces an immune response associated with the modulation of allergen-specific effector cells, involving dendritic cell (DC) activity.^[10,11]

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Novel therapeutic approaches use allergen peptides that are recognized by T-cells and induce the specific immune response for the design of vaccine systems.^[12] These peptides offer advantages over conventional or cellular vaccines due to their easy synthesis, low cost, increased stability, and relative safety. Moreover, they should not be able to crosslink IgE and therefore activate effector cells.^[13,14] However, several issues must be overcome for their use in immunotherapy: 1) limitation of single peptide epitopes as vaccine candidates, 2) immune evasion, and 3) failure to elicit a controlled and prolonged immune response. To overcome these limitations, the inclusion of adjuvants can help. Toll-like receptor (TLR) ligands have been included in vaccine design.^[15,16] TLR9 ligands have been effective for treating respiratory symptoms in both patients and murine models.^[17,18] We have previously demonstrated that a system combining Pru p 3 T-cell peptides with oligodeoxyribonucleotides with CpG motifs, which are TLR9 ligands, induces a specific Th1/Treg response that protects from anaphylaxis in a mouse model.^[19] Moreover, DCs have been shown to represent a promising alternative therapeutic target for vaccine design.^[20,21] They recognize antigens via endocytosis, micropinocytosis, and phagocytosis pathways, mediated by surface receptors such as C-type lectin receptors (CLRs).^[22] CLRs are pattern recognition receptors expressed on the surface of DCs that are characterized by a carbohydrate recognition domain that interacts with mannose or fucose in a calcium-dependent manner.^[23] Their engagement not only mediates endocytosis but can modulate the Th1/Treg response, critical for tolerance induction during immunotherapy, as well as antitumor and antiviral therapy.^[24,25] The main CLRs expressed by DCs that recognize sugars are: the mannose receptor (MR; CD206), dendritic-cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN; CD209), and Dectin-2.

These receptors have been shown to take part in the antigen uptake by DCs and the promotion of immune responses. Approaches that target DCs via CLRs have been developed and could be used to treat multiple diseases.^[26] An elegant approach to enhance the DC targeting via CLRs is represented by a new generation of glycosystems. These can be used to present, in a multivalent way, several copies of high-density carbohydrates to CLRs. Glycodendrimers (GDs) functionalized with mannose units represent promising structures for enhancing allergen interaction with antigen presenting cells (APCs) via CLRs, potentially modulating the immune response. Moreover, they can present multiple peptide epitopes in an allergen-specific manner, giving rise to many potential applications for FA vaccine design.

We have combined Pru p 3 peptides with mono- and tetravalent-glycodendrimers and mannose using ester linkers, demonstrating that they induce protection from anaphylaxis in a mouse model. This was confirmed by lack of change in temperature upon challenge, accompanied by a decrease in Pru p 3-sIgE and -sIgG1 antibody levels and secreting cells, and an increase of Treg cells and regulatory cytokines in treated animals compared to anaphylactic animals. Moreover, these changes were maintained for at least 5 weeks after the treatment ended.^[27] However, there are no published data regarding the effects and recognition capacity of these structures on cells from LTP-allergic patients, or how the linkers used for mannose inclusion affect this recognition.

Here, we combined Pru p 3 peptides with GDs including mannose, using ester and ether linkers producing mono- and tetravalent-glycodendropeptides (GDPs), to study the immunological response that they induce in different cell subtypes, specifically monocyte-derived DCs (moDCs), T- and B-lymphocytes, and basophils from LTP-allergic patients and tolerant controls. More specifically, we studied 1) GDP recognition and their ability to induce an immune response in the different groups, 2) the effect of the chemical nature of these structures on their actions, and 3) precise details of their interactions with CLRs on moDCs.

2. Experimental Section

2.1. Allergic Patients and Tolerant Controls

This study included LTP-allergic patients. Inclusion criteria were: clear clinical history of peach allergy, a positive skin prick test (SPT), and specific IgE (sIgE) to the peach LTP (Pru p 3) or a positive oral food challenge (OFC) to unpeeled-peach. OFC was not performed in patients with a history of more than two episodes of anaphylaxis after peach ingestion in the 2 years preceding the study. A tolerant control group of subjects with negative SPT and sIgE results, and good tolerance to unpeeled-peach was also included. The study was conducted at the Allergy Service and Research Laboratory of the Hospital Regional Universitario de Málaga-Instituto de Biomedicina de Málaga (HRUM-IBIMA), Spain, in accordance with the Declaration of Helsinki. The study was approved by the local ethics committee. All participants signed informed consent forms before the study began.

2.2. Methods

Full details of all methods are included in the Supporting Information.

2.3. Chemical Synthesis and Characterization of GDPs

The chemical synthesis of new compounds is described in the Supporting Information. This includes Pru p 3 T-cell peptides and mannose molecules attached to a base structure, as shown in **Figure 1**, using either ester or ether linkers. The syntheses of the dendropeptides (DPs) without mannose (D₁Prup3 and D₄Prup3) and GDPs with mannose (D₁ManPrup3 or D₄ManPrup3) ester linkers have been published.^[19,27] The sequence of the Pru p 3 peptides was ASSNGIRNVNLLARTPDRQAC, based on the 26–46 region of Pru p 3, with some modifications: Cys residues at positions 27–28 replaced by Ser and with a C-terminal non-native Cys residue to allow conjugation to the mono- and tetravalent systems via a thiol-ene click chemistry reaction. The chemical synthesis and characterization of the compounds with mannose and ether linkers (D₁Man_oPrup3) were performed by NMR and MS (Figures S1–S5, Supporting Information). Moreover, the syntheses of these GDPs using different linkers were based on a convergent approach to prepare the second generation GDs. Then, the insertion in the mannosyl nonavalent dendron of the maleimide moiety (with one or four copies of this functional group) was

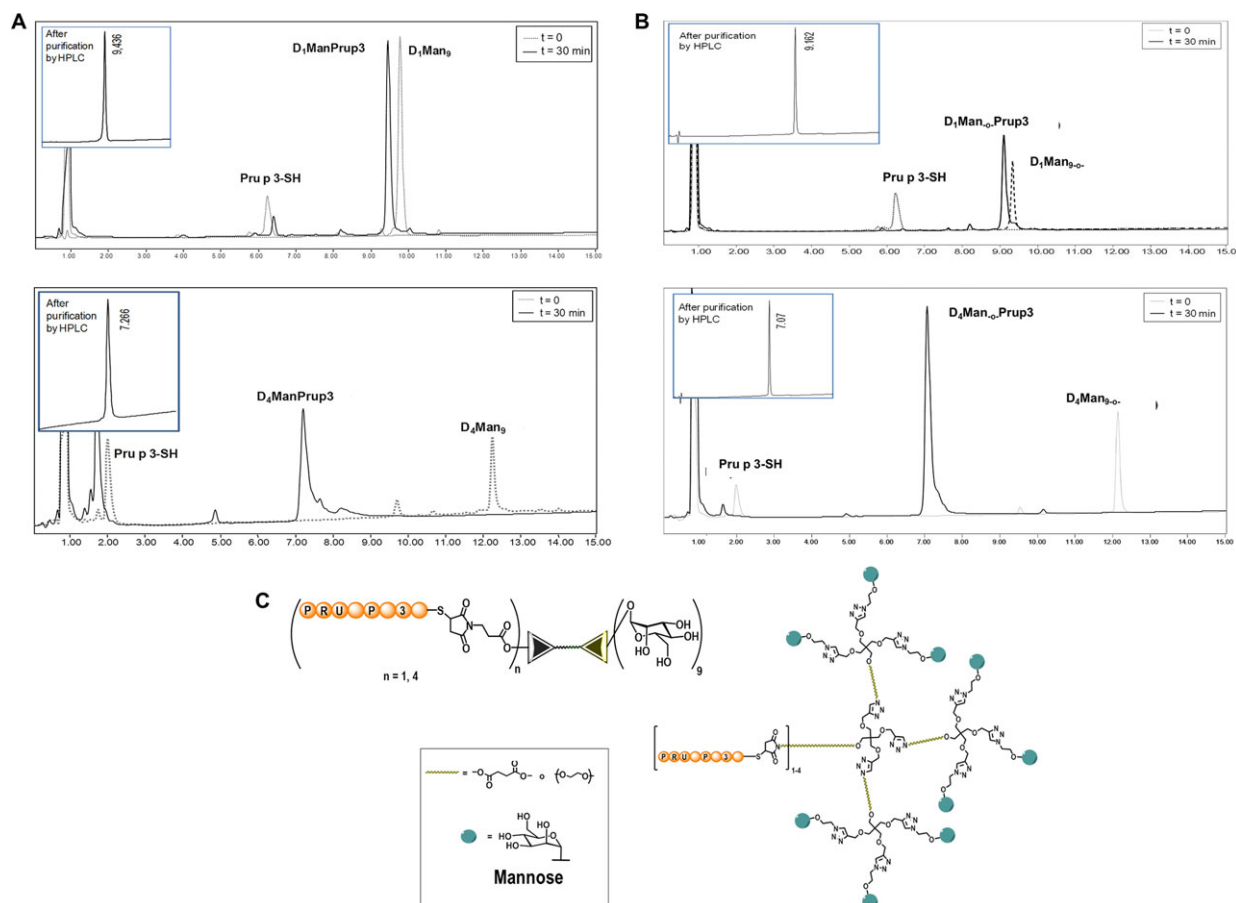


Figure 1. Structure of Pru p 3-glycodendropeptides (Prup3-GDPs). HPLC chromatograms and the peaks corresponding to the same compounds after HPLC purification (left). A) For D₁ManPrup3 and D₄ManPrup3. B) For D₁Man₉.Prup3 and D₄Man₉.Prup3 and C) schematic representation of Prup3-GDPs with different linkers.

conducted via Cu(I) azide–alkyne cycloaddition (CuAAC) click chemistry reaction. The peptides were conjugated to the maleimide-glycodendrons via a thiol–ene reaction to obtain the GDPs.

2.4. Sample Collection and Storing

Peripheral blood was obtained by venopunction. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient and stored in liquid nitrogen. Samples were biobanked according to HRUM-IBIMA Biobank, Andalusian Public Health System Biobank standard procedures.

2.5. Generation of Monocyte-Derived DCs (moDCs) and DC Maturation Studies

Monocytes were purified from PBMCs and differentiated to moDCs using GM-CSF and IL-4. The moDCs were used for maturation studies in the presence of GDPs using ester linkers (D₁ManPrup3 or D₄ManPrup3) or ether linkers (D₁Man₉.Prup3 or D₄Man₉.Prup3) at 10 nM. DP without mannose (D₁Prup3 and D₄Prup3) were used as controls.

2.6. Specific Proliferative Response of Different Cell Subpopulations

The specific proliferation of different lymphocyte subpopulations was evaluated using autologous moDCs pre-stimulated with different Prup3-GDPs, using Prup3-DPs as controls. This proliferative response was determined by 5,6-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) dilution assay, analyzing the expression of the CFSE_{dim} cells.

2.7. Cytokine Determination

The supernatants from the lymphocyte culture were collected and cytokine production was determined using the human ProcartaPlex Multiplex Immunoassays kit.

2.8. Basophil Activation Test

Basophil response to different Prup3-GDPs was determined by analyzing the percentage of CCR3⁺CD203⁺CD63⁺ cells in all subjects.

Table 1. Clinical characteristics and allergological work-up results.

N. Subjects	Sex	Age	Type reaction	Aeroallergens	SPT LTP	sIgE Pru p 3
P1	M	57	Anaphylaxis	—	+	8.48
P2	F	49	Anaphylaxis	+	+	2.88
P3	M	32	Anaphylaxis	—	+	11.3
P4	F	39	Urticaria/AE	—	+	18.0
P5	F	63	Anaphylaxis	+	+	3.45
P6	F	31	Urticaria/AE	+	+	7.09
P7	F	33	OAS	+	+	0.88
P8	F	32	Anaphylaxis	—	+	4.52
P9	M	24	Anaphylaxis	—	+	6.35
P10	F	36	Urticaria/AE	+	+	5.37
P11	F	40	Anaphylaxis	+	+	16.60
P12	M	23	Urticaria/AE	+	+	2.88
P13	F	22	Anaphylaxis	+	+	13.10
C1	F	26	Tolerant	—	—	0.35
C2	F	33	Tolerant	—	—	0.35
C3	F	22	Tolerant	ND	—	0.35
C4	F	29	Tolerant	—	—	0.35
C5	F	24	Tolerant	ND	—	0.35
C6	M	29	Tolerant	—	—	0.35
C7	F	35	Tolerant	ND	—	0.35
C8	M	45	Tolerant	—	—	0.35
C9	F	32	Tolerant	—	—	0.35
C10	F	36	Tolerant	—	—	0.35
C11	M	26	Tolerant	—	—	0.35
C12	M	45	Tolerant	—	—	0.35
C13	F	41	Tolerant	—	—	0.35
C14	F	42	Tolerant	—	—	0.35

OAS, oral allergy syndrome; ND, not determined.

2.9. Inhibition Assays

The inhibition assays were performed using specific anti-human CD206 mAb (anti-CD206) and anti-human CD209 mAb (anti-CD209) to determine Prup3-GDP-CLR reactions.

3. Results

Thirteen LTP-allergic patients were included, more of whom were female (70%), with an average age of 37 ± 12.6 years. One patient (7.7%) developed oral allergy symptoms, four (30.7%) developed urticaria-angioedema, and eight (61.5%) anaphylaxis. Regarding pollen sensitization, eight patients (61.5%) also showed sensitivity to other aeroallergens, *Cupressus*, *Phleum*, and *Artemisia* pollens being the most frequent ones, as determined by either SPT and/or specific IgE. The tolerant control group comprised 14 individuals (mean age, 33.21 ± 7.7 years) with confirmed tolerance to peach (Table 1).

The immunological effects were studied for the different molecules, GDPs, D₁ManPrup3 or D₄ManPrup3 (ester linkers), D₁Man_oPrup3 or D₄Man_oPrup3 (ether linkers), and DPs (D₁Prup3 or D₄Prup3) in terms of moDC maturation,

lymphocyte proliferation and specific cytokine production, and basophil activation.

3.1. Prup3-GDPs Induce Changes in moDC Maturation

Changes in moDC status were analyzed by detecting the expression of migration (CCR7), activation (CD83), and maturation (CD80 and CD86) cell surface markers (Figure 2).

There was a significant increase of CCR7 in allergic patients compared to tolerant controls when moDCs were stimulated with D₁ManPrup3 and D₄ManPrup3, whilst CD86 only increased significantly when stimulated with D₁ManPrup3 (Figure 2).

In terms of treatment effects, the insertion of mannose led to significantly increased CD86 levels in allergic patients, for D_nManPrup3. However, for tolerant controls, only an effect for D₄ManPrup3 was seen. Increased valence led to higher CD86 expression in allergic patients in the absence of mannose (D₄Prup3 vs. D₁Prup3) and for tolerant controls in the presence of mannose (D₄ManPrup3 vs D₁ManPrup3) (Figure 2).

3.2. Prup3-GDPs Induce Changes in the Specific Proliferative Response

The moDCs were pre-incubated with different structures at 10 nM, washed, and then used as APCs by co-culturing them with autologous lymphocytes. Proliferation of CD3⁺, CD4⁺, and CD8⁺T-cells, and CD19⁺B-cells was determined.

D₄Prup3 led to significantly increased CD4⁺T- and CD19⁺B-cell proliferation in allergic patients compared to tolerant controls (Figure 3A). D_nManPrup3 (independently of the number of peptides but with ester linkers), led to significantly higher proliferation of T-cells and B-cells in allergic patients compared to tolerant controls (Figure 3A). Moreover, the compounds with the ether linker induced an increased CD4⁺T-cell proliferation. More specifically, D₄Man_oPrup3 induced increased CD19⁺B-cell proliferation only in allergic patients with respect to tolerant controls, suggesting that the nature of the Prup3-GDP structure, in terms of how the mannose is anchored, can influence the immune response.

Stimulation with D₄Prup3 in allergic patients led to a significant increase in CD8⁺T-cell proliferation; however, D₁Prup3 did not, indicating that the number of T-cell epitopes of Pru p 3 in the structures induced an antigen-specific cytotoxic immune response. However, the Prup3-GDPs with different linkers did not show this behavior, indicating again that the nature of the Prup3-GDPs is important for the cellular response (Figure 3A).

3.3. Cytokine Production

To evaluate the cytokine production profile, supernatants were collected from the proliferation experiments. The production of inflammatory cytokine IFN- γ tended to increase for all the structures that include mannose via ester linkers, compared to the D_nPrup3 and D_nMan_oPrup3 molecules (Figure 3B), in all subjects, showing the importance of the Prup3-GDP structure in

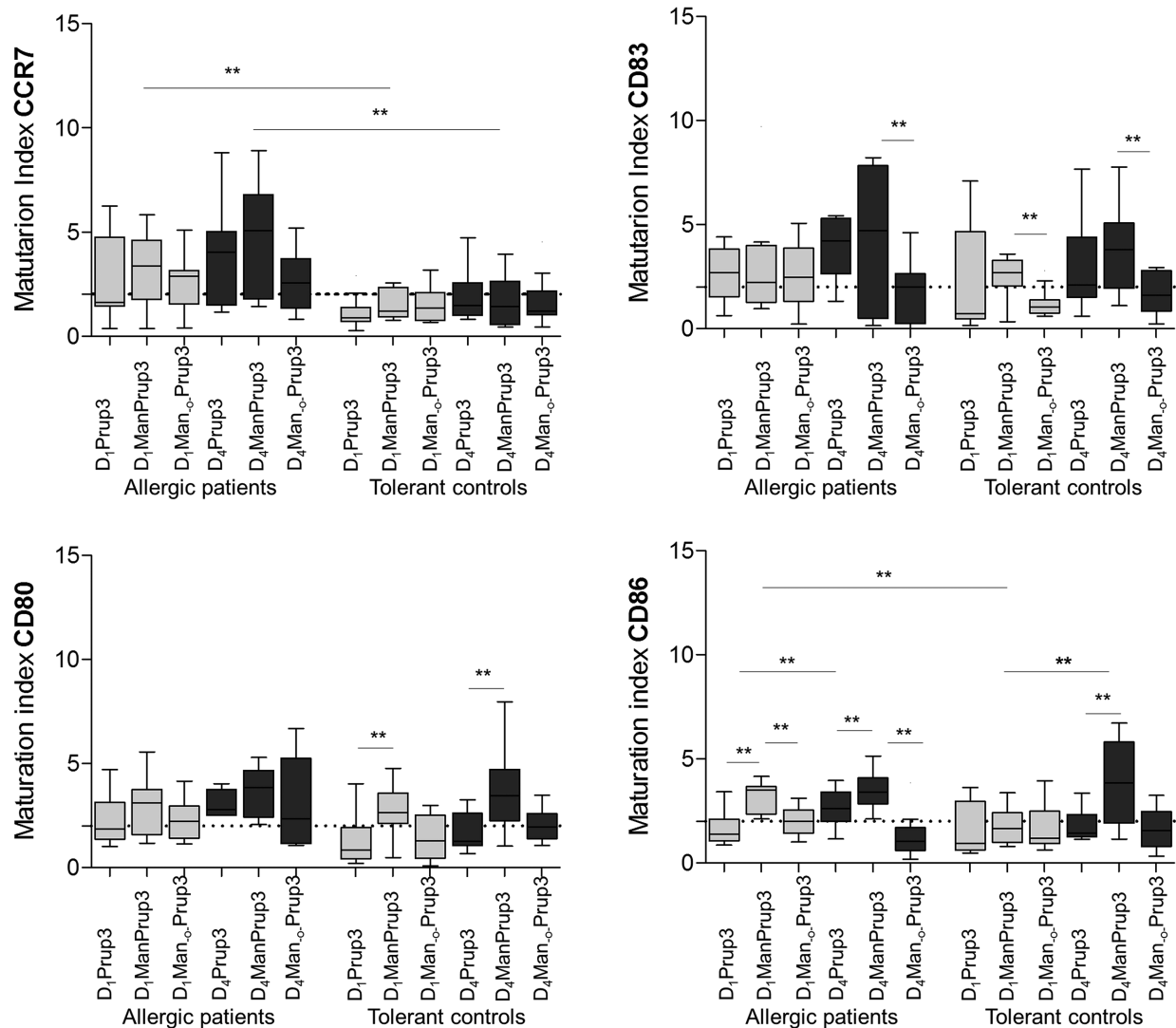


Figure 2. Prup3-GDPs change DC maturation. Box-plots represent median and interquartile range of MI for the different surface markers on moDC for D_nPrup3, D_nManPrup3, and D_nMan_O-Prup3 for allergic patients (*n* = 13) and tolerant controls (*n* = 14). Mann–Whitney *U* test was used for pairwise comparisons between unrelated groups and Wilcoxon signed-rank test was used for pairwise comparisons in related samples, showing significant differences as **, (*p* < 0.0083 and *p* < 0.0071, Bonferroni correction, respectively).

terms of linking the mannose; however, this was only shown to be significant for D₁ManPrup3 versus D₁Prup3 in allergic patients, and for D₁ManPrup3 versus D₁Prup3 and D₁ManPrup3 versus D₁Man_O-Prup3 in tolerant controls (Figure 3B).

Regarding the regulatory cytokine IL-10, increased production was observed in tolerant controls compared to allergic patients, being statistically significant for the D₁Man_O-Prup3 and D₄ManPrup3 groups. D₄ManPrup3, led to significantly increased production compared to D₁ManPrup3 for tolerant controls. Moreover, D₁ManPrup3 led to significantly increased IL-10 production compared to D₁Prup3 only in allergic patients (Figure 3B).

The Th2 cytokine levels were significantly higher in allergic patients compared to tolerant controls when cells were stimulated with D_nPrup3 for IL-13 and D_nManPrup3 for IL-13 and IL-4 (Figure 3B).

3.4. Analysis of Basophil Response to Prup3-GDPs

Basophil response was determined by analyzing the percentage of CCR3⁺CD203⁺CD63⁺ cells in all subjects (Figure 4A). Results showed that only D₄ManPrup3 was able to induce a higher percentage of CCR3⁺CD203⁺CD63⁺ cells, suggesting positive basophil activation in allergic patients; D₁ManPrup3 did not lead to an increase in CCR3⁺CD203⁺CD63⁺ cells, suggesting it was not able to crosslink sIgE to Pru p 3 in allergic patients (Figure 4B). Moreover, basophil activation results showed that, in the presence of the complete protein Pru p 3, the percentage of CCR3⁺CD203⁺CD63⁺ cells was high in allergic patients and negative in tolerant controls (Figure 4B).

All these data indicate that Prup3-GDP with ester linkers are specifically recognized by both DCs and lymphocytes from LTP-allergic patients, showing a Th2 pattern of response that agrees

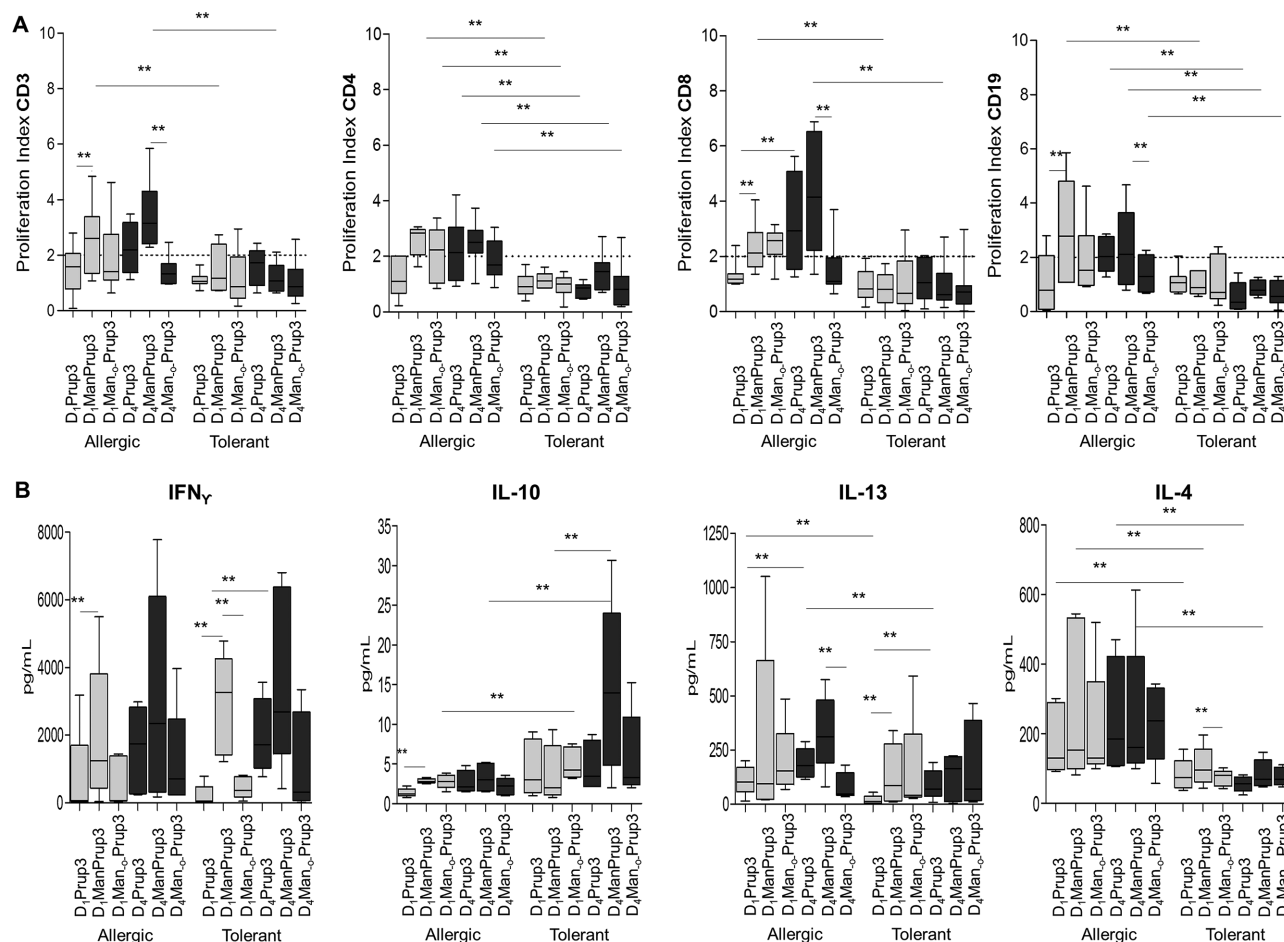


Figure 3. Specific lymphocyte proliferation in the presence of Prup3-GDPs. A) PI of T- and B-cells for D_n Prup3, D_n ManPrup3, and D_n Man $_O$.Prup3 and B) cytokine production in supernatants from lymphocyte proliferation assays, with D_n Prup3, D_n ManPrup3, and D_n Man $_O$.Prup3 in allergic patients ($n = 13$) and tolerant controls ($n = 14$). Box-plots represent median and interquartile range. Mann–Whitney U test was used for pairwise comparisons between unrelated groups and Wilcoxon signed-rank test was used for pairwise comparisons in related samples, representing significant differences as **, ($p < 0.0083$ and $p < 0.0071$, Bonferroni correction, respectively).

with the immunological response induced by Pru p 3 (Figure 5). This response to Pru p 3 led to increased DC markers except for CD86 (Figure 5A) and positive T-cells proliferation (Figure 5B) with increased IL-13 production (Figure 5C).

3.5. Prup3-GDP Interaction with CLR

We investigated the interaction of Prup3-GDPs with DC receptors by analyzing the expression of MR (CD206) and DC-SIGN (CD209) on moDCs from allergic patients and tolerant controls. There was a significant decrease in CD206 and in CD209 expression when moDCs were stimulated with D_n ManPrup3 in both allergic patients and tolerant controls, compared to unstimulated cells (Figure 6A). These results suggest that D_n ManPrup3 with ester linkers interacts with both receptors. No significant decrease in CD206 or CD209 expression was observed when stimulating with either Pru p 3, D_n Prup3, or D_n Man $_O$.Prup3.

We analyzed then the effect of blocking the interaction of Prup3-GDPs with CLR on moDCs state for allergic patients (Figure 6B) and tolerant controls (Figure S6A, Supporting

Information), using specific blocking antibodies of the mannose receptor (anti-CD206) and DC-SIGN (anti-CD209). We focused on structures that interacted specifically with the CLR (D $_n$ ManPrup3). For allergic patients, blocking MR (anti-CD206) significantly decreased the upregulation of all surface markers except CD80 in D $_1$ ManPrup3-stimulated DCs. No changes were observed for DCs stimulated with tetravalent compounds (Figure 6B). Blocking DC-SIGN (anti-CD209) significantly impaired the upregulation of CCR7 and CD86 markers in D $_1$ ManPrup3-stimulated DC, and the CD86 marker in D $_4$ ManPrup3-stimulated DC (Figure 6B). These results suggest that D $_4$ ManPrup3 presents a specific geometry that modulates the maturation of DCs via the CD209 receptor but not DC migration, imparting an intermediate immune phenotype in DCs without the ability to present the antigen to T-cells via this receptor. In the tolerant control group, CD80 and CD83 expression induced by D_n ManPrup3 was significantly reduced in the presence of both anti-CD206 and anti-CD209 (Figure S6A, Supporting Information).

We analyzed the effect of these DC receptor blockages on cellular proliferation, observing that blocking MR (anti-CD206)

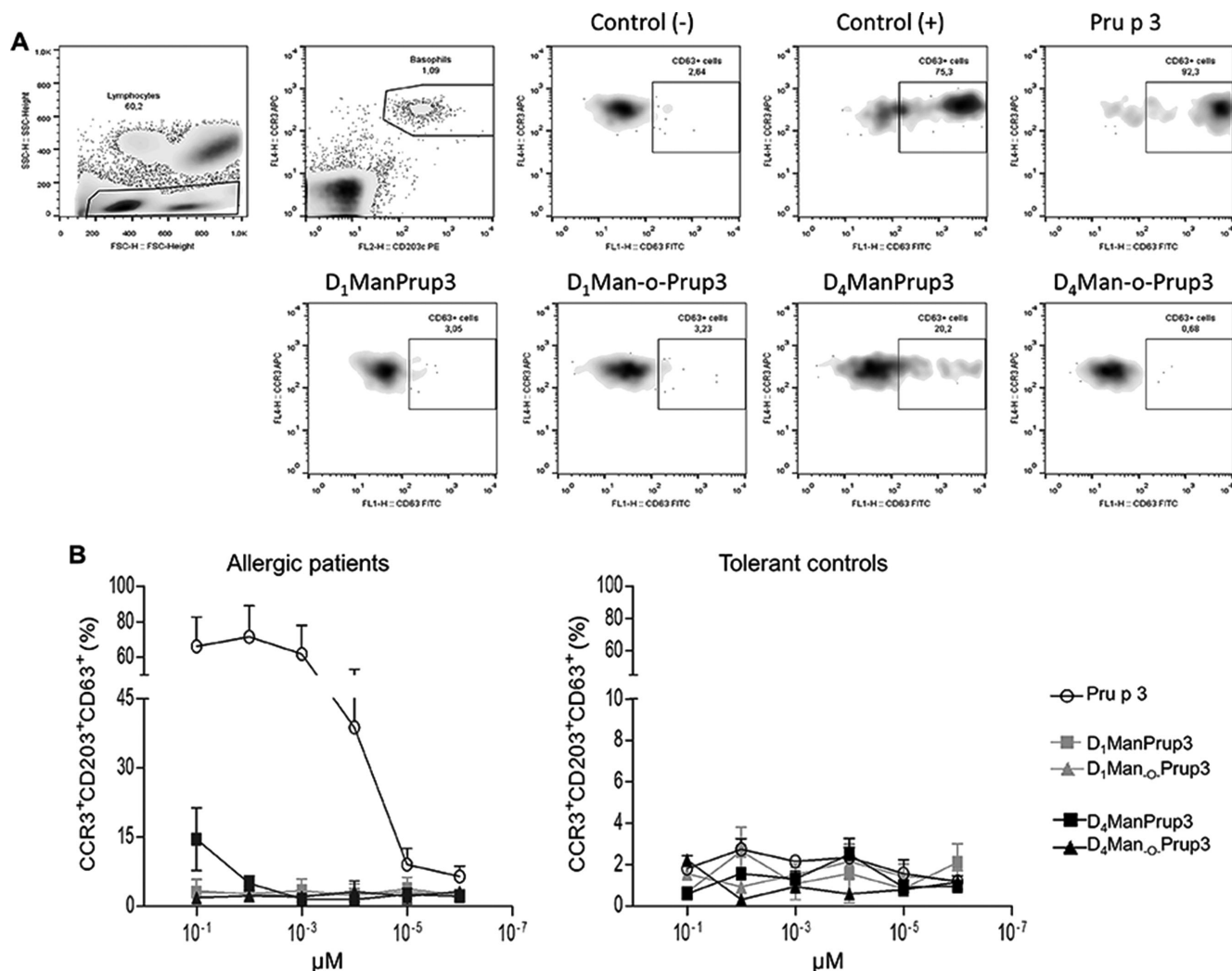


Figure 4. Specific basophil activation in the presence of Prup3-GDPs. A) Basophil activation strategy of the flow cytometry gating for cells expressing CCR3⁺CD203⁺CD63⁺ from LTP-allergic patients. CCR3⁺CD203⁺CD63⁺ expression in cells using phosphate-buffered saline as negative control, and anti-IgE and Pru p 3 as positive control (upper). CCR3⁺CD203⁺CD63⁺ expression in cells in the presence of the different Prup3-GDPs (lower). B) Lines represent means $\pm$ SEMs of % of basophil activation after stimulation with different concentrations of Pru p 3, D₁ManPrup3, and D₁Man-o-Prup3 in allergic patients ($n = 13$) and tolerant controls ($n = 14$).

decreases T- and B-cell proliferation in D₁ManPrup3-stimulated DCs (Figure 6C). The blockage of DC-SIGN using anti-CD209 significantly impaired the proliferation of all cell subsets in D₁ManPrup3-stimulated DC in allergic patients (Figure 6C). For the tolerant control group, blocking MR and DC-SIGN receptors on DCs led to an absence of T- and B-cell proliferation (Figure S6B, Supporting Information).

4. Discussion

The use of nanostructures for biomedical purposes has increased markedly in recent years.^[28,29] In allergy, their use has been proposed for diagnosis and therapy, as carriers of specific allergenic structures.^[30,31] These structures are potential candidates for safer vaccines, by carrying only allergenic peptides that can be recognized specifically without causing sIgE crosslinking on

the surface of effector cells. However, nanostructures coupled with T-cell peptides for specific recognition by the immunological system may show low immunogenicity, requiring the inclusion of adjuvants to improve the immune response.^[32,33] Previous approaches have used ligands that interact with TLR9, such as CpG.^[34,35] In one study of allergic rhinitis, the adjuvant was included as part of the nanostructure, alongside the specific peptides.^[36] We have demonstrated that the combination of nanostructures containing Pru p 3 T-cell peptides and CpG generates a tolerant response to Pru p 3 in anaphylactic mice, persisting after the treatment ends.^[19] Similar results have been obtained with peanut extract.^[37] Recently, carbohydrates such as mannose have been proposed as adjuvants, acting by increasing DC uptake.^[22,38]

Inspired by these findings, we have synthesized similar structures including Pru p 3 T-cell peptides and mannose in dendrimeric structures, using two different linkers, ester and ether.

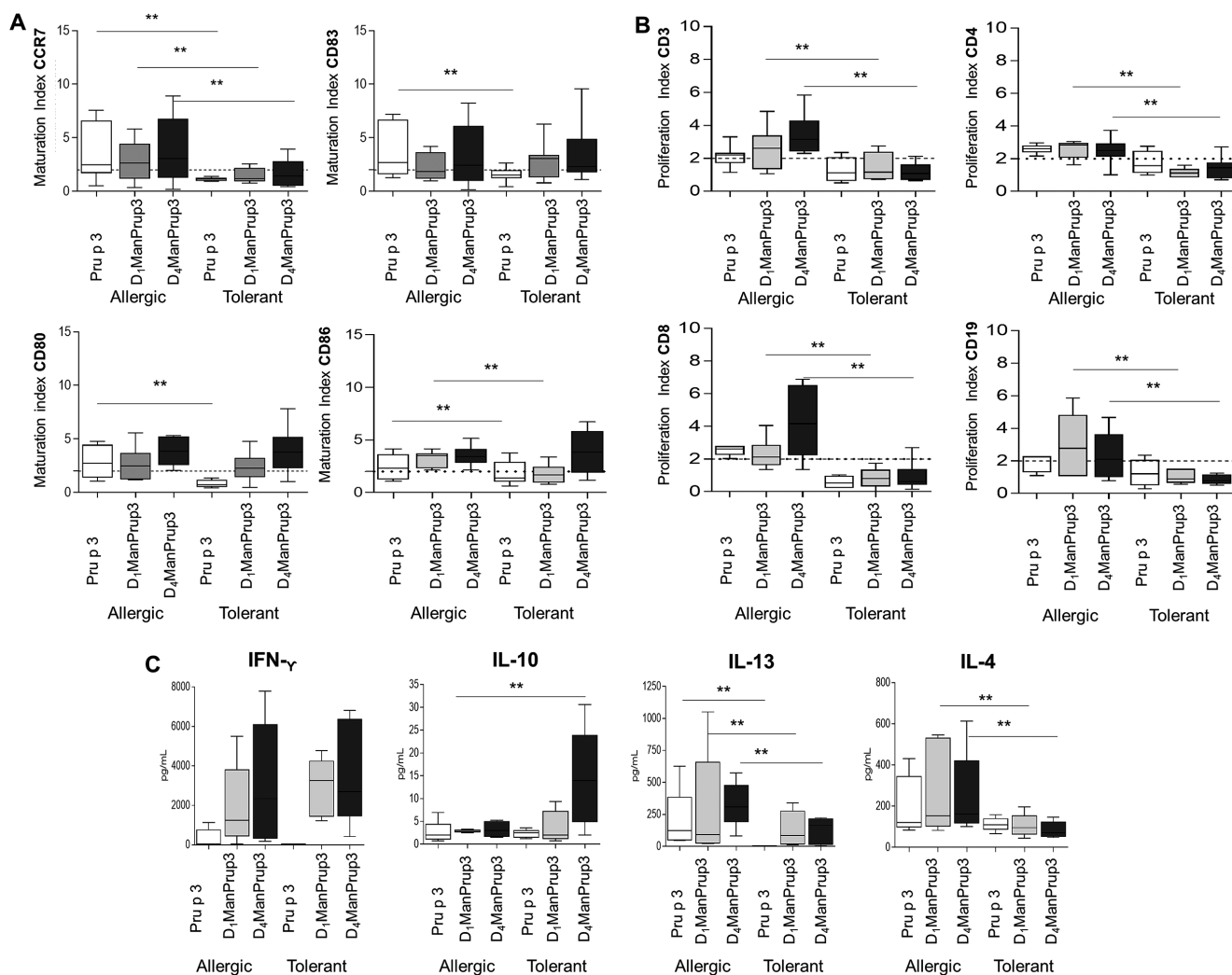


Figure 5. Specific immune response in the presence of Prup3-GDPs and Pru p 3. Box-plots represent median and interquartile range of A) MI of DC markers, B) PI of T- and B-cells, and C) cytokine production in supernatants from lymphocyte proliferation assays for Pru p 3 and D_n ManPrup3 in allergic patients ($n = 13$) and tolerant controls ($n = 14$). Mann–Whitney U test was used for pairwise comparisons between unrelated groups, representing significant differences as **, ($p < 0.0166$, Bonferroni correction).

The synthesis strategy is based on a convergent approach to prepare the second generation GD. The mannosylated dendron with nine copies of mannose was functionalized with the maleimide moiety (with one or four copies of this functional group) on the focal position of the GD using a Cu(I) azide–alkyne cycloaddition (CuAAC) click chemistry reaction. The conjugation of the peptide epitope in the last step was achieved via a thiol–ene click chemistry reaction between the thiol of the terminal Cys and the maleimides to obtain the corresponding GDPs in good yields after purification using HPLC.

These monomeric and tetrameric structures with ester linkers have already been investigated in an anaphylactic mouse model. The group treated with D_1 ManPrup3, was protected from anaphylaxis, as determined by both in vivo (no temperature changes or symptoms) and in vitro parameters (decreased Prup3-sIgE and -sIgG1 antibody levels/secreting cells; increased Treg cells and IFN- γ /IL-10 cytokines). These changes were persisted during 5 weeks after stopping treatment.^[27] Whether these results

can be produced in humans must be evaluated further; however, before this it is necessary to assess the immunological response to these structures using immune cells from LTP-allergic patients.

Here, we showed that Prup3-GDPs can present multiple epitopes and induce an allergen-specific immunological response. GDPs functionalized with mannose and different numbers of Pru p 3 T-cell epitopes are able to interact with DCs via CLR, induce their maturation, and promote the specific proliferative response inducing specific Th2 cytokine production.

Regarding DC maturation, we observed that the Prup3-GDPs functionalized with mannose, independently of the peptide valence, can stimulate DC maturation compared to DP (without mannoses), for both LTP-allergic patients and tolerant controls. This effect is higher with D_4 ManPrup3, in agreement with previous work.^[39] When we compared the LTP-allergic patients and tolerant controls, significant CCR7 upregulation was found for LTP-allergic patients only. The differential expression

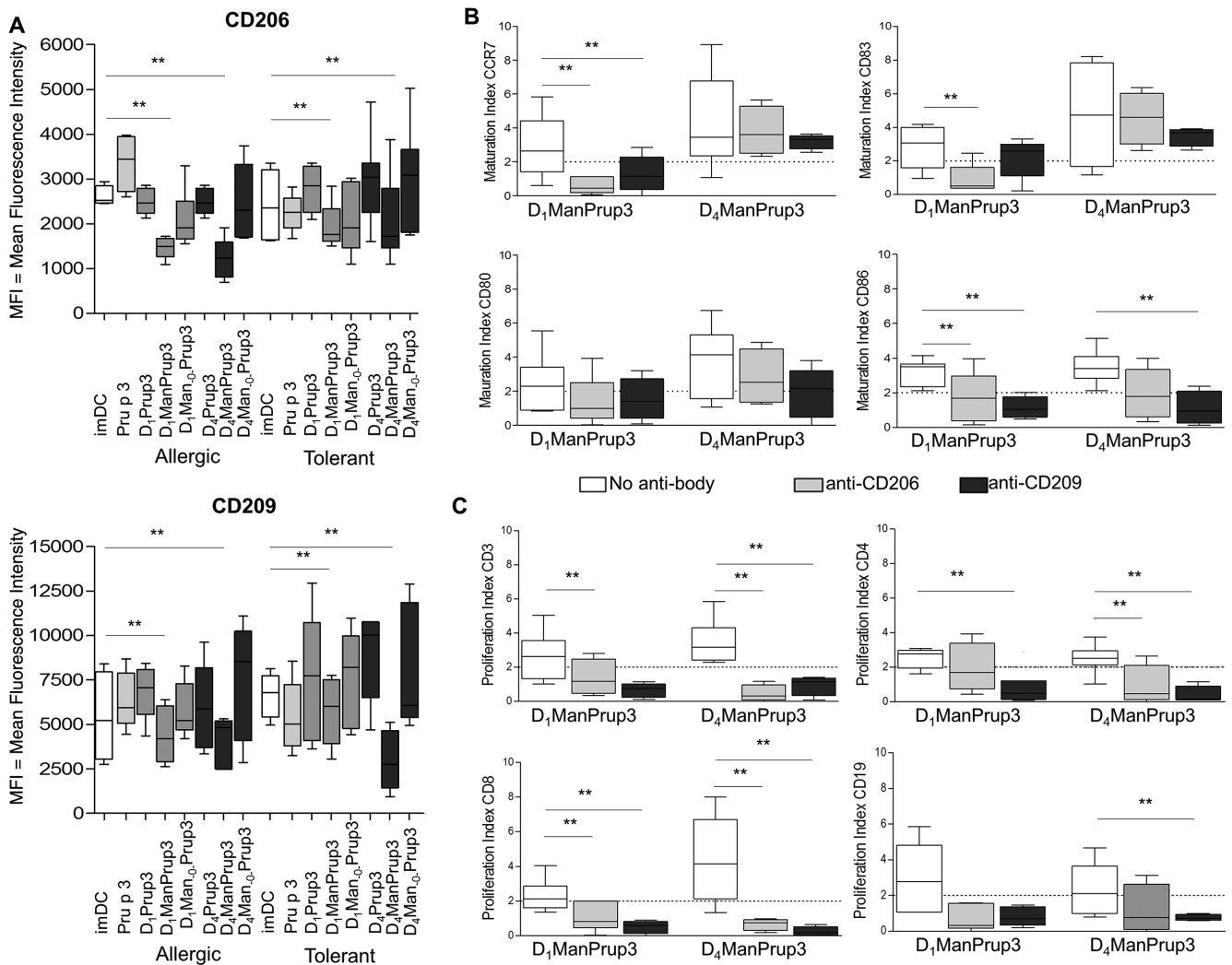


Figure 6. MR and DC-SIGN expression for Pru p 3 and Prup3-GDPs. A) MFI of CD206 or CD209 in the presence of Pru p 3 and Prup3-GDPs for all subjects. Wilcoxon signed-rank test was used for pairwise comparisons in related samples, showing significant differences as **, ($p < 0.0071$, Bonferroni correction). B) MFI for D_n ManPrup3-stimulated DCs, without or with blocking antibodies (anti-DC206 or anti-CD209) and C) PI of different lymphocyte subpopulations after being incubated with D_n ManPrup3-stimulated DCs, without or with blocking antibodies (anti-DC206 or anti-CD209) in allergic patient ($n = 13$). Box-plots represent the median and interquartile range. Wilcoxon signed-rank test was used for pairwise comparisons between related samples, showing significant differences as **, ($p < 0.025$, Bonferroni correction). MFI: mean fluorescence intensity

of CCR7 between groups may be associated with the capacity of D_n ManPrup3 to stimulate complete DC maturation, activation and DC survival,^[40,41] leading to interactions with other immune cells and the differentiation of naive T-cells.^[42] Our results also show that the presence of D_n ManPrup3 induces specific T- and B-cell proliferation with a Th2 response pattern (significant increases in IL-13 and IL-4, with low levels of IL-10) as reported previously.^[10,43,44] These results suggest that the T-cell epitope of Pru p 3 in the GDPs stimulates DC maturation and allergen-specific T-cell proliferation, inducing an immune response similar to that of the native allergen.

We analyzed maturation and proliferation data obtained for Prup3-GDPs with ester linkers in LTP-allergic patients versus tolerant controls, observing important differences, as although maturation was induced in both groups, proliferation was only found in allergic patients. These differences may be explained by the

lack of upregulation of CCR7 in moDCs from tolerant controls, which will impair DC maturation and therefore no presentation to lymphocytes will occur. Our data suggest that the upregulation of CCR7 is necessary for full DC maturation, which will induce specific T-cell proliferation, as has been demonstrated in other in vitro studies.^[45,46] Moreover, these results are associated with specific patterns of Th1/Th2 cytokine production, in agreement with a previous study from our group.^[27]

We only observed a basophil response with the tetravalent GDP with ester linkers (D_4 ManPrup3) in LTP-allergic patients, suggesting that the size of the compound and the valence confer the ability to crosslink at least two IgE antibodies on the basophil surface, producing an effector response. On the contrary, D_1 ManPrup3 does not show this capacity, inducing instead a reduced allergic response. This is in accordance with our previous studies using D_4 Prup3 and D_4 ManPrup3.^[19,27]

The nature of these structures clearly influences the immunological response. The use of nanostructures functionalized with multivalent mannose ligands results in greatly enhanced human DC targeting, presumably due to increased diffusion and target cell binding.^[22] We show that the presence of mannose in the dendrimeric structure confers important immunologic properties, as previously reported.^[47,48] More specifically, our results suggest that Prup3-GDPs with ester linkers interact with CLRs, leading to their internalization, as shown by decreased MR and DC-SIGN in the presence of D_nManPrup3. Thus, they can induce DC maturation and enhance the presentation of antigen to T- and B-cells, stimulating their proliferation.^[49,50]

Recognition by different CLRs is highly dependent on carbohydrate branching, spacing, and multivalency.^[51] Our data show that D_nManPrup3 can target both MR and DC-SIGN, inducing the immune response, since specific blocking of CLRs impaired the CD4⁺T and CD8⁺T-cell and B-cell proliferation induced by D_nManPrup3 in LTP-allergic patients but not in tolerant controls. The specific B-cell proliferation induced by D_nManPrup3 suggests that the structures could be involved in the modulation of the humoral response. In fact, these structures have been associated with the modulation of Pru p 3-sIgE and IgG production in previous studies from our group in an experimental model of Pru p 3 anaphylaxis.^[19,27] However, for determining their specific induction and elucidating their full role, it would be necessary to increase the length of the study, including stimulation time. Nevertheless, if these structures are to be used in clinical studies, it will be likely necessary to change manufacturing conditions and therefore the effects of changing the synthesis procedures should be assessed.

In conclusion, we have demonstrated that GDPs including Pru p 3 T-cell peptides are recognized by LTP-allergic patients. The nature of the Prup3-GDP structure with mannose and ester linkers influences the immune response, inducing moDCs maturation. This suggests that DCs have the capacity to present Pru p 3 to immune cells inducing a specific T- and B-cell proliferation. Although these structures mainly induce Th2 cytokines, they also induce the production of IFN- γ , a Th1 cytokine^[27] in all subjects, suggesting that they may also modulate the immune response. D₁ManPrup3, although being specifically recognized, did not activate the basophil response in LTP-allergic patients, suggesting that it leads to a reduction in the effector response. The interaction of these Prup3-GDPs with moDCs (APCs) is produced via CLRs, both MR and DC-SIGN, although other pathways should be analyzed further.

These findings indicate that these nanostructures are recognized by immune cells and can stimulate a similar immunological response to the native allergen in LTP-allergic patients. D₁ManPrup3 is particularly promising as a vehicle for specific IT in FA, it is recognized by LTP-allergic patients and its specific geometry and chemical nature confer it the ability to interact with DCs via CLRs, stimulating complete DC maturation and promoting the allergen-specific proliferative response. However, it does not activate the effector response, which we suspect is why it leads to a reduction in the allergic response compared to D₄ManPrup3. These results are in concordance with previous in vivo studies from our group,^[19,27] indicating the relevance of D₁ManPrup3 as a possible candidate for developing new therapeutic tools. However, this study must be complemented with further ones to fully

analyze their capacity to modulate the immunological response and induce a regulatory/tolerance response in FA.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

C-type lectin receptors, dendritic cells, food allergies, lymphocytes, mannose

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Figure S11

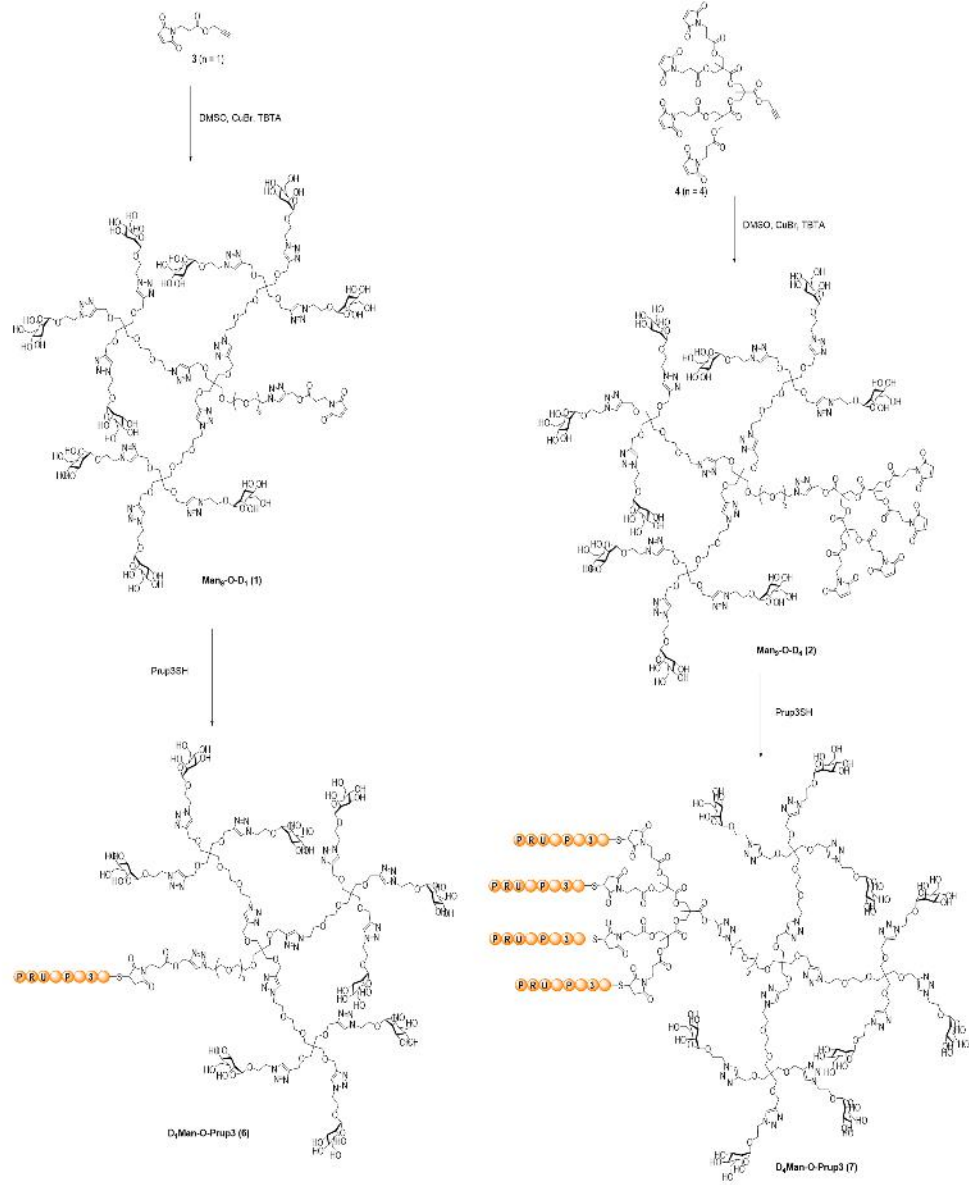
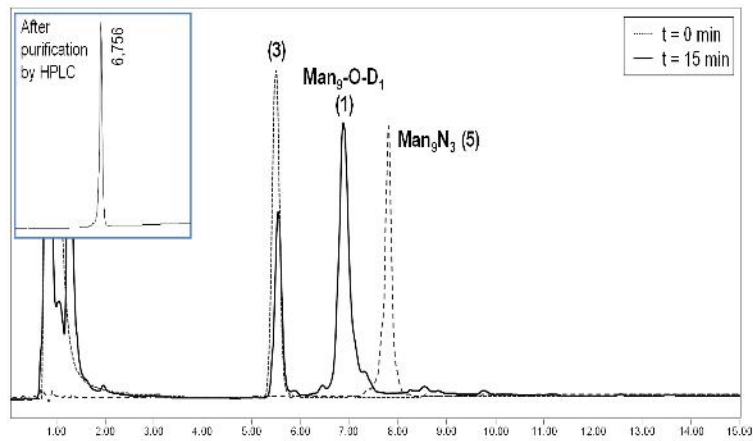
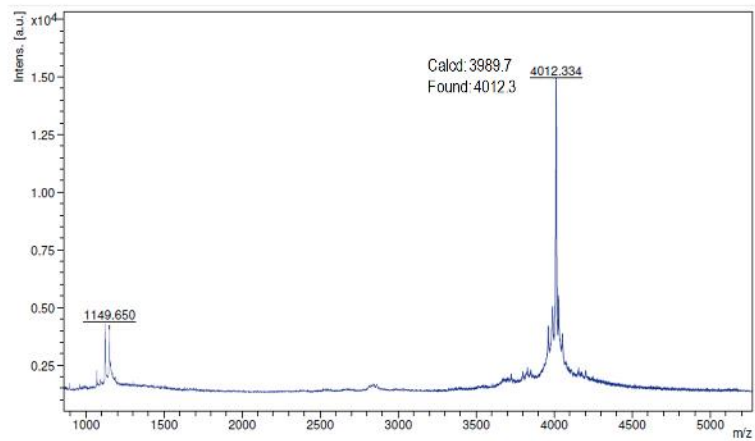


Figure S12

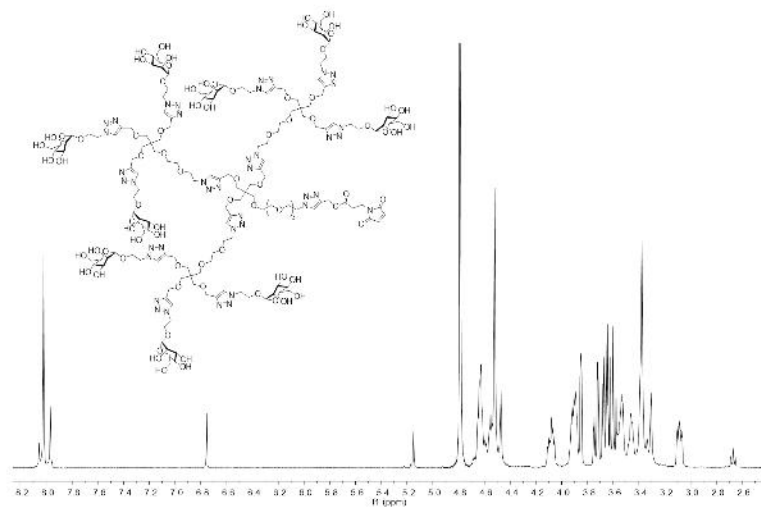
A) HPLC chromatogram



B) MALDI-ToF



C) $^1\text{H-NMR}$ (400 MHz, D_2O)



D) $^{13}\text{C-NMR}$ (100 MHz, D_2O)

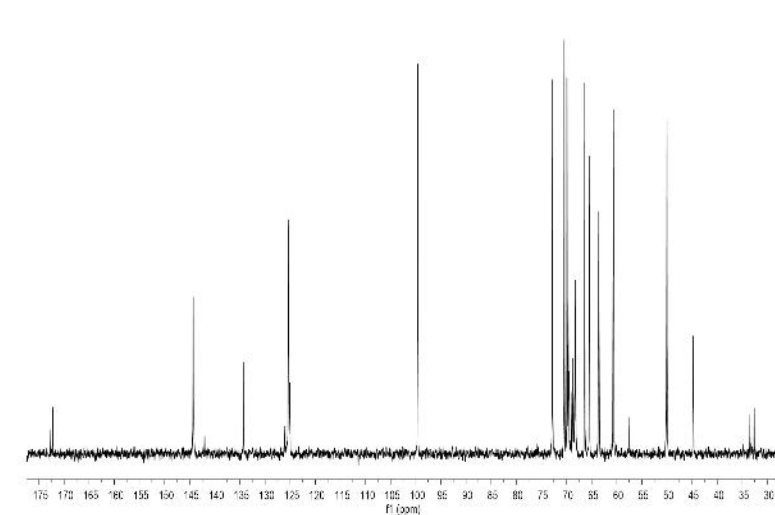
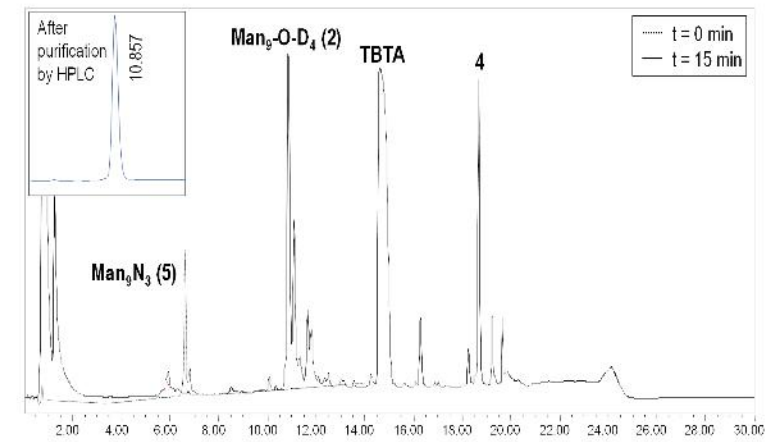
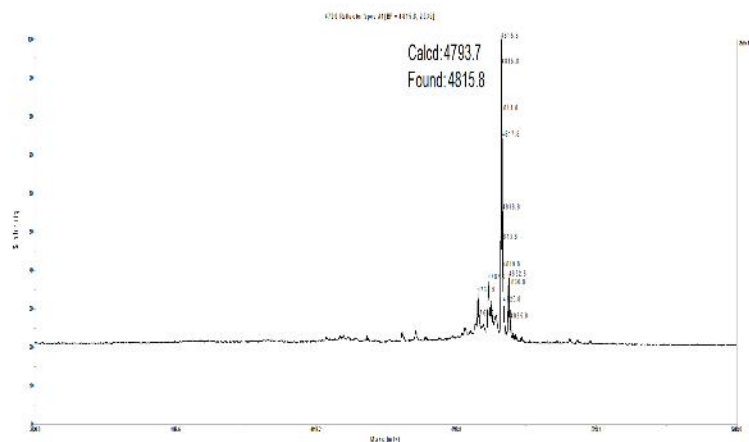


Figure S13

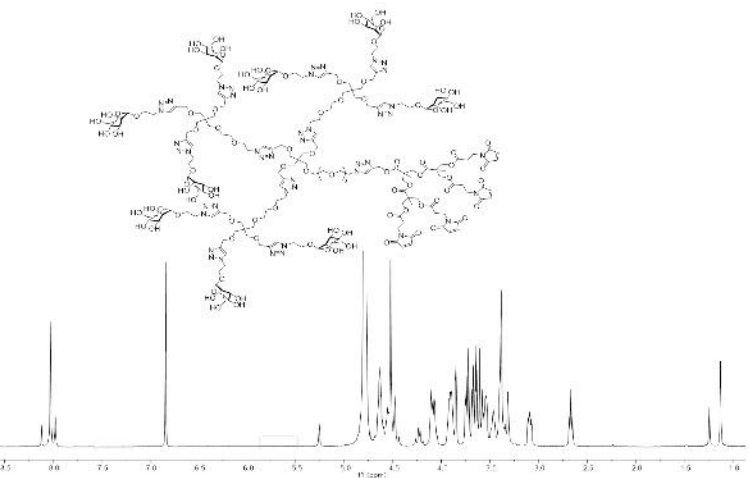
A) HPLC chromatogram



B) MALDI-ToF



C) $^1\text{H-NMR}$ (400 MHz, D_2O)



D) $^{13}\text{C-NMR}$ (100 MHz, D_2O)

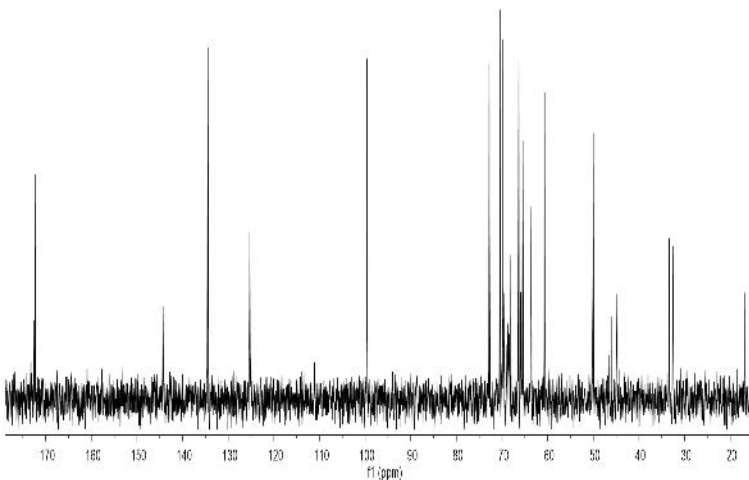
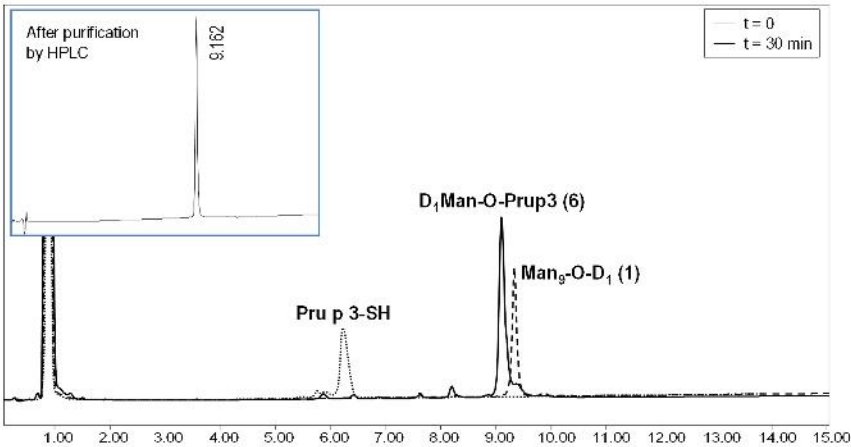


Figure S14

A) HPLC chromatogram



B) ESI-MS (deconvoluted)

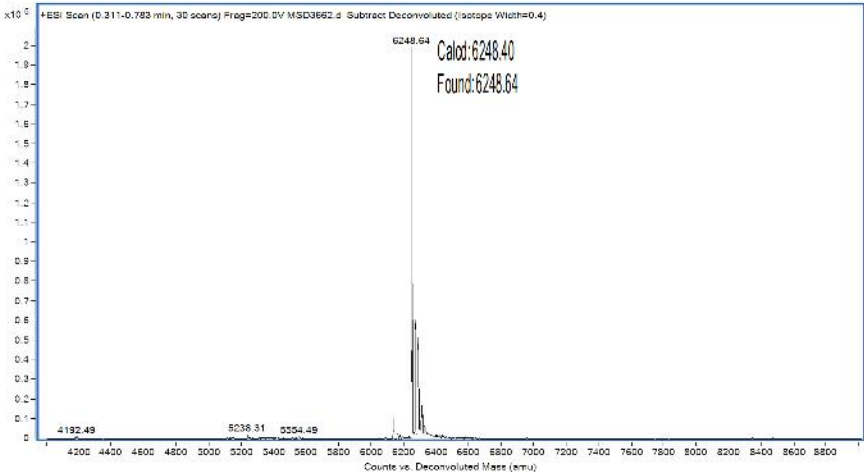
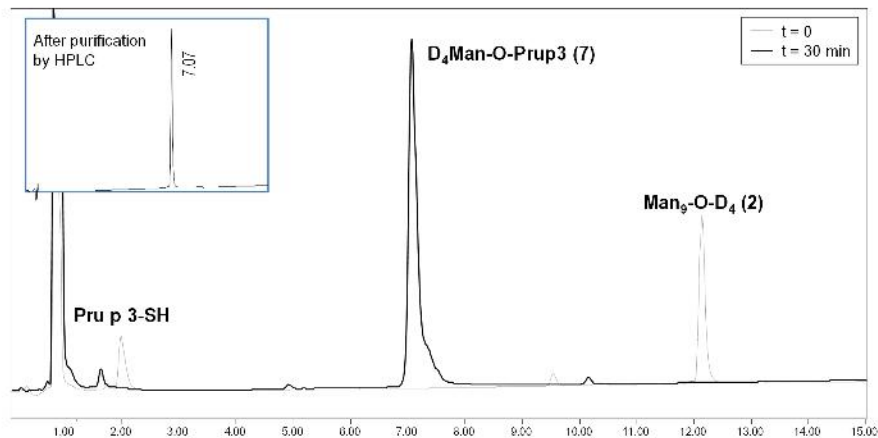


Figure SI5

A) HPLC chromatogram



B) ESI-MS (deconvoluted)

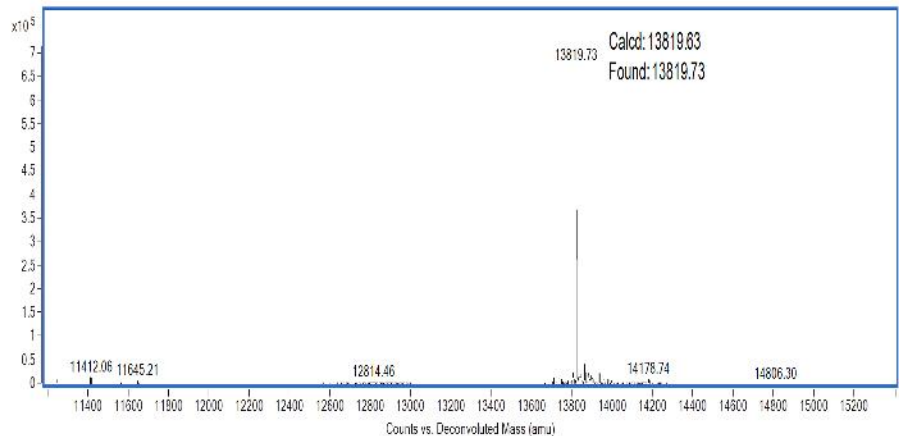
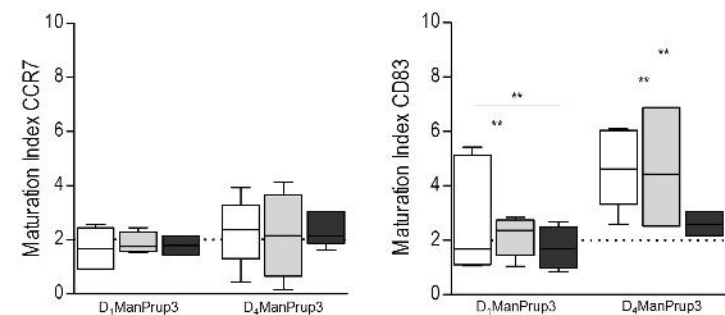
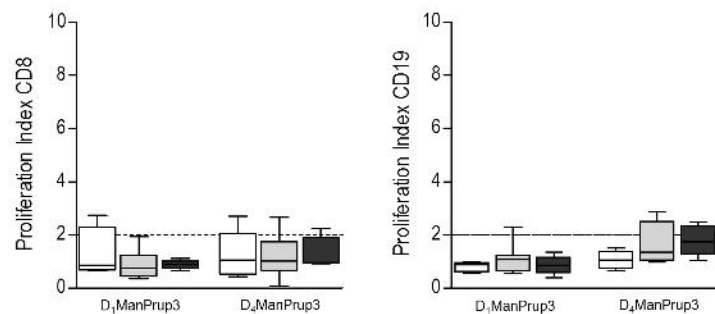
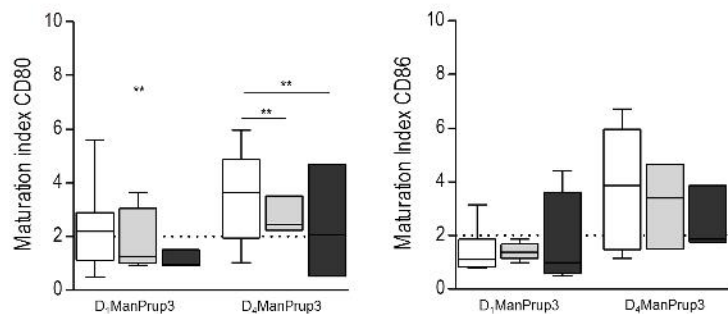
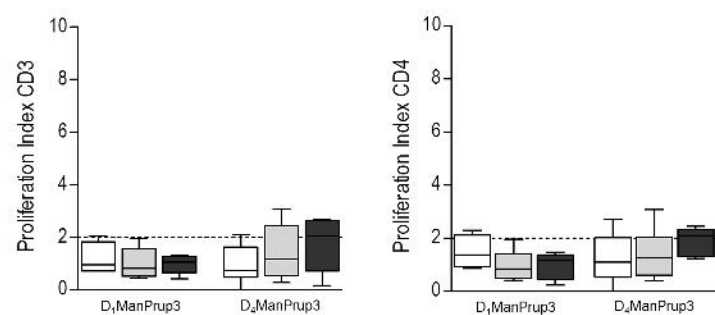


Figure S16

A



B



□ No anti-body

▒ anti-CD206

■ anti-CD209

Supporting information

Pru p 3-glycodendropeptides based on mannose promote changes in the immunological properties of human dendritic and T-cells in LTP-allergic patients.

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MATERIALS AND METHODS

Chemical synthesis and characterization of GDPs with the corresponding synthetic intermediates.

Here we describe the syntheses of the Prup3-GDPs with mannose and different linkers. We have previously prepared GDPs with different peptide epitopes of Pru p 3 using a similar approach [52]. The sequence of the Pru p 3 peptide was ASSNGIRNVNNLARTPDRQAC. It was based on the 26–46 region of Pru p 3, with some modifications: the Cys residues in positions 27–28 were replaced with Ser to avoid cross-linking problems, and with a C-terminal non-native Cys residue to allow conjugation to the mono- and tetravalent systems. The new synthetic strategy described here confers an important advantage as it uses glycodendritic platforms that can be functionalized using easily a thiol-ene reaction by a terminal cysteine present at one end of the peptide epitope. The glycodendritic platform contains a glycodendron moiety at one site and either 1 or 4 copies of a maleimide group at the other site. The glyco-maleimide dendrons can be used as common intermediates to be conjugated with any type of peptidic epitopes, highlighting the potential versatility of this approach. Prup3-GDPs, D₁ManPrup3 and D₄ManPrup3, were prepared in good yields of high purity as demonstrated by HPLC and mass spectrometry. The details of the synthesis of these structures have been previously published [27]. However, D₁Man_O-Prup3 or D₄Man_O-Prup3 with ether linkers, are described below (Figure SI1). Besides, dendropeptides (DPs) without mannoses (D₁Prup3 or D₄Prup3) have been prepared as structure controls.

General protocol for glyco-maleimide dendrons synthesis 1-2.

To a solution of the maleimido derivative 3 [53] or the tetravalent dendron 4 [52] (7.65 μmol) in DMSO (83 μL), another solution of CuBr (5.10 μmol) and TBTA (10.20 μmol) in the same solvent (166 μL) was added. Subsequently, a solution of glycodendron 5 [54] (5.10 μmol) in sodium phosphate buffer (16 mM, pH 7.4, 249 μL) was added to the former solution. After shaking for aprox. 30 min at room temperature (the progression of the reaction was monitored

by analytical RP-HPLC), the reaction mixture was directly purified by semipreparative RP-HPLC to give target compounds **1-2** (Figures SI2-SI3).

Glyco-maleimide dendron Man₉-O-D1 (1).

Following the general procedure, using compound **3** as starting material, and purification by semipreparative RP-HPLC (column C₈ and the elution with a linear gradient from 15 to 25% of solvent B into A over 30 min, at 3 mL/min flow rate (A: H₂O (0.05% TFA), B: ACN (0.1% TFA)), compound **1** (8.1 mg, 2.04 μmol, 40%) was obtained as a white solid. Characterization data are shown in Figure SI2.

¹H-NMR (400 MHz, D₂O) 8.05 (s, 1H, H_{triazole}), 8.02 (s, 9H, H_{triazole}), 7.97 (s, 3H, H_{triazole}), 6.75 (s, 2H, H_{maleimide}), 5.15 (s, 2H, OCOCH₂C_{triazole}), 4.79 (under D₂O, 9H, H-1), 4.70-4.54 (m, 26H, OCH₂CH₂N, O_{linker}CH₂CH₂N), 4.52 (s, 18H, OCH₂C_{triazole}), 4.47 (s, 6H, OCH₂C_{triazole}), 4.08 (m, 9H, OCHHCH₂N), 3.96-3.87 (m, 17H, OCHHCH₂N, O_{linker}CH₂CH₂N), 3.85 (m, 9H, H-2), 3.77-3.70 (m, 11H, H-6a, CH₂CH₂N_{maleimide}), 3.69-3.51 (m, 43H, H-3, H-5, H-6b, CH₂O), 3.46 (m, 8H, CH₂O), 3.38 (m, 24H, CCH₂O), 3.34 (br s, 2H, CCH₂OCH₂CH₂O), 3.31 (br s, 6H, CCH₂OCH₂CH₂O), 3.08 (m, 9H, H-4), 2.67 (t, 2H, J_{H,H} = 6.6, CH₂CH₂N_{maleimide});

¹³C-NMR (100 MHz, D₂O) 172.7 (CO), 172.3 (CO_{maleimide}), 144.3 (C_{triazole}), 144.2 (C_{triazole}), 142.0 (C_{triazole}), 134.3 (CH_{maleimide}), 126.1 (CH_{triazole}), 125.3 (CH_{triazole}), 125.1 (CH_{triazole}), 99.5 (C-1), 72.8 (C-4, CH₂O), 70.4 (C-3, CH₂O), 69.9 (C-2), 69.6 (CH₂O), 69.5 (CH₂O), 68.9 (CCH₂OCH₂CH₂O), 68.7 (CH₂O), 68.6 (CH₂O), 68.4 (O_{linker}CH₂CH₂N), 68.2 (CCH₂O), 66.4 (C-5), 65.4 (OCH₂CH₂N, O_{linker}CH₂CH₂N), 63.6 (OCH₂C_{triazole}), 63.5 (OCH₂C_{triazole}), 60.6 (C-6), 57.4 (OCOCH₂C_{triazole}), 50.0 (OCH₂CH₂N, O_{linker}CH₂CH₂N), 44.8 (CCH₂O), 33.5 (CH₂CH₂N_{maleimide}), 32.6 (CH₂CH₂N_{maleimide});

MALDI-ToF *m/z* calcd. for C₁₅₈H₂₅₂N₄₀O₈₀: 3989.7; found: 4012.3 [M+Na]⁺; HPLC t_R = 6.76 min (Column: analytical C₈; A: H₂O (0.05 % TFA), B: Acetonitrile (0.1 % TFA), 15 to 25% linear gradient of B into A over 15 min, F = 1 mL/min).

Glyco-maleimide dendron Man₉-O-D₄ (2).

Following the general procedure, using compound 4 as starting material, and purification by semipreparative RP-HPLC (column C₁₈ and the elution with a linear gradient from 12 to 50% of solvent B into A over 30 min, at 3 mL/min flow rate (A: H₂O (0.05% TFA), B: ACN (0.1% TFA)), compound 2 (9.1 mg, 1.89 μ mol, 37%) was obtained as a white solid. Characterization data are shown in Figure SI3.

¹H-NMR (400 MHz, D₂O) 8.12 (s, 1H, H_{triazole}), 8.03 (s, 9H, H_{triazole}), 7.98 (s, 3H, H_{triazole}), 6.84 (s, 8H, H_{maleimide}), 5.26 (s, 2H, OCOCH₂C_{triazole}), 4.79 (under D₂O, 9H, H-1), 4.68-4.54 (m, 26H, OCH₂CH₂N, O_{linker}CH₂CH₂N), 4.52 (s, 18H, OCH₂C_{triazole}), 4.48 (s, 6H, OCH₂C_{triazole}), 4.23 (AB system, 4H, C_aCH₂O), 4.15-4.02 (m, 17H, OCHHCH₂N, C_bCH₂O), 3.96-3.87 (m, 17H, OCHHCH₂N, O_{linker}CH₂CH₂N), 3.85 (m, 9H, H-2), 3.78-3.70 (m, 17H, H-6a, CH₂CH₂N_{maleimide}), 3.70-3.51 (m, 43H, H-3, H-5, H-6b, CH₂O), 3.46 (m, 8H, CH₂O), 3.38 (m, 24H, CCH₂O), 3.35 (br s, 2H, CCH₂OCH₂CH₂O), 3.31 (br s, 6H, CCH₂OCH₂CH₂O), 3.09 (m, 9H, H-2), 2.67 (t, 8H, J_{H,H} = 6.6, CH₂CH₂N_{maleimide}), 1.24 (s, 3H, (CH₃)_a), 1.13 (s, 6H, (CH₃)_b);

¹³C-NMR (100 MHz, D₂O) 172.5 (CO_a, CO_b, CO), 172.3 (CO_{maleimide}), 144.3 (C_{triazole}), 134.4 (CH_{maleimide}), 125.3 (CH_{triazole}), 125.2 (CH_{triazole}), 125.1 (CH_{triazole}), 99.5 (C-1), 72.8 (C-4, CH₂O), 70.4 (C-3, CH₂O), 69.9 (C-2), 69.6 (CH₂O), 69.5 (CH₂O), 68.9 (CCH₂OCH₂CH₂O), 68.7 (CH₂O), 68.6 (CH₂O), 68.5 (O_{linker}CH₂CH₂N), 68.3 (CCH₂O), 66.4 (C-5), 65.9 (OC_aH₂, OC_bH₂), 65.4 (OCH₂CH₂N, O_{linker}CH₂CH₂N), 63.7 (OCH₂C_{triazole}), 63.6 (OCH₂C_{triazole}), 60.6 (C-6), 57.9 (OCOCH₂C_{triazole}), 50.0 (OCH₂CH₂N, O_{linker}CH₂CH₂N), 46.5 (C_{q,a}), 46.1 (C_{q,b}), 44.8 (CCH₂O), 33.4 (CH₂CH₂N_{maleimide}), 32.5 (CH₂CH₂N_{maleimide}), 16.9 (C_bH₃), 16.8 (C_aH₃);

MALDI-ToF *m/z* calcd. for C₁₉₄H₂₉₁N₄₃O₉₈: 4793.7; found: 4815.8 [M+Na]⁺; HPLC t_R = 10.86 min (Column: analytical C₁₈; A: H₂O (0.05 % TFA), B: Acetonitrile (0.1 % TFA), 12 to 50% linear gradient of B into A over 15 min, F = 1 mL/min).

General protocol for the preparation of GDPs 6-7.

To a solution of glyco-maleimide dendron 1-2 (0.75 μ mol, 1 eq.) in sodium phosphate buffer (50 mm, pH 7.4, 1134 μ L), another solution of peptide Pru p 3 SH [19] (1.1 eq. for each maleimide) in H₂O (567 μ L) was added. After shaking for aprox. 30 min at r.t. (the progression

of the reaction was monitored by analytical RP-HPLC), the reaction mixture was lyophilized, and then purified by semipreparative RP-HPLC to give target compounds **6-7** (Figures SI4-SI5).

GDP D₁Man₀Prup3 (6).

Following the general procedure, using glycodendron 1 as starting material, and purification by semipreparative RP-HPLC (column C₁₈ and elution with a linear gradient from 12 to 30% of solvent B into A over 30 min, at 3 mL/min flow rate (A: H₂O (0.05% TFA), B: ACN (0.1% TFA)), compound **6** (3.8 mg, 0.61 μmol, 82%) was obtained as a white solid. Characterization data are shown in Figure SI4.

ESI-MS (deconvoluted) *m/z* calcd. for C₂₄₇H₄₀₆N₇₆O₁₁₁S: 6248.40; found: 6248.64 [M]⁺, 6271.23 [M+Na]⁺, 6288.45 [M+K]⁺; HPLC *t_R* = 9.16 min (Column: analytical C₁₈; A: H₂O (0.05 % TFA), B: Acetonitrile (0.1 % TFA), 12 to 30% linear gradient of B into A over 15 min, F = 1 mL/min).

GDP D₄Man₀Prup3 (7).

Following the general procedure, using glycodendron 2 as starting material, and purification by semipreparative RP-HPLC (column C₁₈ and elution with a linear gradient from 15 to 40% of solvent B into A over 30 min, at 3 mL/min flow rate (A: H₂O (0.05% TFA), B: ACN (0.1% TFA)), compound **7** (6.8 mg, 0.49 μmol, 65%) was obtained as a white solid. Characterization data are shown in Figure SI5.

ESI-MS (deconvoluted) *m/z* calcd. for C₅₅₀H₉₀₇N₁₈₇O₂₂₂S₄: 13819.63; found: 13819.73 [M]⁺; HPLC *t_R* = 7.07 min (Column: analytical C₁₈; A: H₂O (0.05 % TFA), B: Acetonitrile (0.1 % TFA), 15 to 40% linear gradient of B into A over 15 min, F = 1 mL/min).

Sample collection and storing.

Peripheral blood was obtained from all subjects by venopunction. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density gradient (Rafer S.L, Zaragoza, Spain) and stored in liquid nitrogen in RPMI 1640 medium supplemented with 2mM of L-glutamine (BioWhittaker, Pittsburgh, PA), gentamicin (5mg/mL) and streptomycin (50ng/mL) (both from

Normon, Madrid, Spain), 40% of fetal bovine serum (BioWhittaker) and DMSO (10%) (Sigma, St. Louis, MO). Samples were biobanked according to standard procedures of the HRUM-IBIMA Biobank, Andalusian Public Health System Biobank.

Generation of monocyte-derived DCs (moDCs).

Monocytes were purified from PBMCs by positive selection using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD14⁺ fraction was differentiated to become moDCs using 200ng/mL of recombinant human granulocyte-macrophage colony-stimulating factor and 100ng/mL of recombinant human IL-4 (both from R&D Systems Inc, Minneapolis, MN) for 5 days at 5% CO₂ and 37°C [55, 56]. The CD14⁺ fraction was frozen in 10% DMSO for performing further lymphocyte proliferation tests.

DC maturation studies.

To assess changes in the DC maturation state after stimulation, 10⁵ moDCs /mL were incubated in 96-well plates (Thermo-Fisher AS, Roskilde, Denmark) with D₁Prup3, D₄Prup3 (both dendrimers without mannoses), GDPs with mannoses, and ester linkers (D₁ManPrup3 or D₄ManPrup3) and GDPs with mannose and ether linkers (D₁Man_{-O}-Prup3 or D₄Man_{-O}-Prup3) at 10nM which were shown to be effective in preliminary experiments. Moreover, 25µg/mL natural Pru p 3 [43] (Bial Laboratory, Zamudio, Spain) and 1mg/mL lipopolysaccharide (Sigma) were included as positive controls, and complete medium as negative control, for 48 hours at 37°C in 5% CO₂. After this, DCs were harvested and their maturation status assessed by analysing the expression of CCR7, CD80, CD83, CD86 molecules (BD Pharmingen and Biolegend, San Diego, CA). To evaluate changes in MR and DC-SIGN expression, DCs were incubated with CD206 and CD209 antibodies (Abs) respectively (BD Biosciences). Cells were acquired in a FACSCanto II Cytometer (BD Biosciences, Milpitas, CA) and data analysed using FlowJo® software (Tree Star, Inc., Ashland, OR). Results were expressed as maturation index (MI) as has been described [10, 43] and the expression of CD206 and CD209 on moDCs was represented as mean fluorescence intensity (MFI).

Specific proliferative response of different cell subpopulations.

The specific proliferation of different lymphocyte subpopulations was evaluated using, as APCs, autologous moDCs pre-stimulated with 10 nM of different Prup3-GDPs for 48h. Proliferation was determined using a 5,6-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) dilution assay. A total of 1.5×10^6 /mL pre-labelled CD14⁺ cells were cultured with different Prup3-GDPs-pre-stimulated-DCs (10:1 ratio) at a final volume of 250 μ L of complete medium, in 96-well plates for 6 days at 37°C and 5% CO₂. Ten μ g/mL phytohemagglutinin (Sigma) was used as positive proliferative control and unstimulated DCs as negative proliferative control. The proliferative responses were assessed by flow cytometry, analysing CFSE_{low} expression in the different cell subsets as lymphocytes T (CD3⁺T, CD4⁺T and CD8⁺T) and lymphocytes B (CD19⁺B). Results were expressed as proliferation index (PI). The PI was calculated for each cell subset as $([\% \text{ CFSE}^{\text{low}} \text{ stimulated PBMCs+DC}] - [\% \text{ CFSE}^{\text{low}} \text{ stimulated PBMCs}]) / (\% \text{ CFSE}^{\text{low}} \text{ unstimulated PBMCs+DCs})$ [10, 43, 57].

Cytokine determination.

To determine cytokine production from Pru p 3 or the different GDPs-specific T lymphocytes, the supernatants from the lymphocyte culture were collected after 6 days of culture, and cytokine production (IL-4, IL-10, IL-13 and IFN- γ) was determined with a human ProcartaPlex Multiplex Immunoassays kit (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's indications and detected in a Bio-Plex 200 (Bio-Rad, Hercules, CA). Assay sensitivity was defined as the concentration of analyte corresponding to the mean fluorescent intensity (MFI) of the background plus two standard deviations (SD) of the background MFI. The corresponding values to lower limit of quantitation (LLOQ) for each analyte were, for IFN γ (LLOQ= 18.42 pg/mL), IL-10 (LLOQ= 2.21 pg/mL), IL-4 (LLOQ= 13.09 pg/mL) and IL-13 (LLOQ= 2.84 pg/mL). Data were analysed using Bio-Plex® Data Analysis Software (Bio-Rad, Hercules, CA).

Basophil Activation Test.

Heparinized blood was incubated with 20 μ L of stimulation buffer, 10 μ L/mL IL-3 and 1 μ L of monoclonal antibody (mAb) CCR3 (BioLegend INC, San Diego, USA) for 10 min at 37°C.

Then, 100 μ L of different Prup3-GDPs and native allergen, were added at tenfold concentrations (0.1-0.00001 μ M) 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, NJ, USA) as a positive control. After 5 min on ice, to stop the degranulation process, samples were incubated with mAb anti-CD203c and CD63 (BioLegend) for 15-20 min at 4°C. Finally, lysing solution (BD Biosciences) was used for red cell disruption. Cells were analysed in a FACSCalibur flow cytometer (BD Biosciences). Results were presented as percentage of cells expressing CCR3⁺CD203⁺CD63⁺.

Inhibition assays.

To determine that CLRs are involved in the interaction of the different molecules with DCs, the DCs were pre-incubated with 10 μ g/mL of anti-human CD206 mAb (anti-CD206) (Biolegend, San Diego, CA) and anti-human CD209 mAb (anti-CD209) (Beckman Coulter, Brea, CA) for 1h at 37°C. Afterwards, different GDPs were added to the cells for 48 hours at 37°C in 5% CO₂. The GDPs-treated DCs without specific blocking antibodies were considered as controls. Results were expressed as MI.

In addition, we measured changes in the specific proliferative response of different cell subpopulations by blocking the CLRs on moDCs. The autologous moDCs were pre-incubated with anti-CD206 or anti-CD209 as previously described. Then, the CD14⁺ cells were included in the culture (10:1 ratio) at a final volume of 250 μ L of complete medium, in 96-well plates and incubated for 6 days at 37°C and 5% CO₂. The proliferative response was expressed as PI.

Statistical analysis.

The Friedman test was used to find significant differences due to the effects of different Prup3-GDPs between subjects from the same group. In the case that the Friedman test indicated the existence of significant differences between treatments, we used the Wilcoxon signed rank test to compare between pairs of related samples, resulting in seven post-hoc tests (D₁Prup3 vs. D₁ManPrup3, D₁ManPrup3 vs. D₁Man₀.Prup3, D₄Prup3 vs. D₄ManPrup3, D₄ManPrup3 vs. D₄Man₀.Prup3, D₁Prup3 vs. D₄Prup3, D₁ManPrup3 vs. D₄ManPrup3, and D₁Man₀.Prup3 vs. D₄Man₀.Prup3). Bonferroni correction was used to reduce the threshold for significance to

0.0071. A similar procedure was used for the CLR analysis, in which each of the 7 treatments were compared to immature DCs, resulting in 7 post-hoc tests, and thus a Bonferroni value of 0.0071. Friedman and later post-hoc Wilcoxon signed rank tests with Bonferroni correction were also used in the analyses of the effects of blocking on the interaction of Prup3-GDPs with CLRs, reducing the threshold for significance to 0.025, as there were four groups and two comparisons (without blocking vs. with for anti-CD206 and anti-CD209).

We also compared the effects of the same treatment between the different groups of subjects (LTP-allergic patients vs. tolerant controls) to examine the effect of Pru p 3 and the Prup3-GDPs with ester linkers, using the Kruskal-Wallis test. This showed the existence of significant differences between groups, therefore we applied Mann-Whitney U test to compare between allergic patients and tolerant controls groups receiving the same treatment. This showed three post-hoc tests reducing the threshold for significance to 0.016. In addition, to examine the effect of the different Prup3-GDPs between LTP-allergic patients vs. tolerant control, the statistical analysis was same, using the Kruskal-Wallis test followed of Mann-Whitney U test to show the existence of significant differences between groups. This resulted in six post-hoc tests, as each of the six treatments were compared between allergic patients and tolerant controls, therefore Bonferroni correction was used to reduce the threshold for significance to 0.0083.

FIGURE LEGENDS

Figure SI1. Synthetic scheme for the preparation of GDPs 6 and 7.

Figure SI2. A) HPLC monitoring of the click chemistry reaction for obtaining compound **1** and the peak corresponding to the same compound after HPLC purification (left). **B)** MALDI-ToF for the compound **1**: MW calculated 3989.7 (M⁺) Dalton; Found 4012.3 Dalton (M+Na). **C)** ¹H-NMR spectrum (400 MHz, D₂O) for the compound **1**. **D)** ¹³C-NMR spectrum (100 MHz, D₂O) for the compound **1**. MALDI-ToF *m/z* calcd. for C₁₅₈H₂₅₂N₄₀O₈₀: 3989.7; found: 4012.3 [M+Na]⁺; HPLC *t_R* = 6.76 min (Column: analytical C₈; A: H₂O (0.05 % TFA), B: Acetonitrile (0.1 % TFA), 15 to 25% linear gradient of B into A over 15 min, F = 1 mL/min).

Figure SI3. A) HPLC monitoring of the click chemistry reaction for the obtaining the compound **2** and the peak corresponding to the same compound after HPLC purification (left). **B)** MALDI-ToF for the compound **2**: MW calculated 4793.7 (M+) Dalton; Found 4815.8 Dalton (M+Na). **C)** ¹H-NMR spectrum (400 MHz, D₂O) for the compound **2**. **D)** ¹³C-NMR spectrum (100 MHz, D₂O) for the compound **2**.

Figure SI4. A) HPLC monitoring of the thiol-ene reaction for the obtaining the compound **6** and the peak corresponding to the same compound after HPLC purification (left). **B)** ESI-MS (deconvoluted) for the compound **6**: MW calculated 6248.40 (M+) Dalton; Found 6248.64 Dalton (M+).

Figure SI5. A) HPLC monitoring of the thiol-ene reaction for the obtaining the compound **7** and the peak corresponding to the same compound after HPLC purification (left). **B)** ESI-MS (deconvoluted) for the compound **7**: MW calculated 13819.63 (M+) Dalton; Found 13819.73 Dalton (M+).

Figure SI6. Box and whisker plots represent median and interquartile range of **A)** MI for D_nManPrup3-stimulated DCs and **B)** PI of lymphocyte subpopulations after being incubated with D_nManPrup3-stimulated DCs, with or without blocking antibodies in tolerant controls (n=14). Wilcoxon test was used for pairwise comparisons in samples of the same group, showing significant differences ** as (p < 0.025, Bonferroni correction).