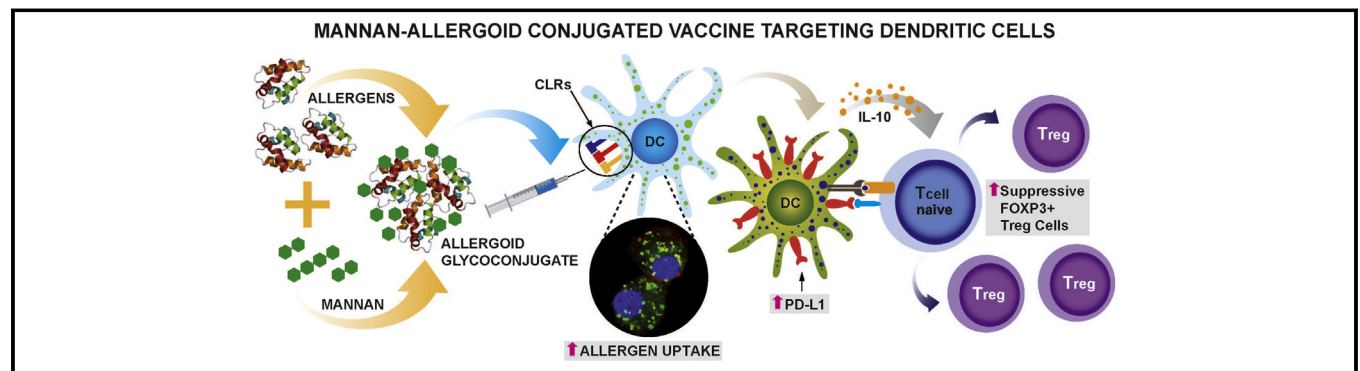


Novel vaccines targeting dendritic cells by coupling allergoids to nonoxidized mannan enhance allergen uptake and induce functional regulatory T cells through programmed death ligand 1

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GRAPHICAL ABSTRACT



Background: Allergen immunotherapy (AIT) is the only curative treatment for allergy. AIT faces pitfalls related to efficacy, security, duration, and patient compliance. Novel vaccines overcoming such inconveniences are in demand. **Objectives:** We sought to study the immunologic mechanisms of action for novel vaccines targeting dendritic cells (DCs) generated by coupling glutaraldehyde-polymerized grass pollen allergoids to nonoxidized mannan (PM) compared with glutaraldehyde-polymerized allergoids (P) or native grass pollen extracts (N).

Methods: Skin prick tests and basophil activation tests with N, P, or PM were performed in patients with grass pollen allergy. IgE-blocking experiments, flow cytometry, confocal microscopy, cocultures, suppression assays, real-time quantitative PCR, ELISAs, and ELISpot assays were performed to assess allergen capture by human DCs and T-cell responses. BALB/c mice were immunized with PM, N, or P. Antibody levels, cytokine production by splenocytes, and splenic forkhead box P3 (FOXP3)⁺ regulatory T (Treg) cells were quantified. Experiments with oxidized PM were also performed.

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Results: PM displays *in vivo* hypoallergenicity, induces potent blocking antibodies, and is captured by human DCs much more efficiently than N or P by mechanisms depending on mannose receptor- and dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin-mediated internalization. PM endorses human DCs to generate functional FOXP3⁺ Treg cells through programmed death ligand 1. Immunization of mice with PM induces a shift to nonallergic responses and increases the frequency of splenic FOXP3⁺ Treg cells. Mild oxidation impairs these effects in human subjects and mice, demonstrating the essential role of preserving the carbohydrate structure of mannan.

Conclusions: Allergoids conjugated to nonoxidized mannan represent suitable vaccines for AIT. Our findings might also be of the utmost relevance to development of therapeutic interventions in other immune tolerance-related diseases. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Allergen immunotherapy, vaccines, neoglycans, allergoids, mannan, immunomodulation, dendritic cells, regulatory T cells

Allergen immunotherapy (AIT) is currently the only curative treatment for allergic diseases.¹⁻⁴ AIT involves administration of high doses of the causative allergen to induce a state of permanent tolerance after treatment discontinuation.^{3,5} AIT induces a very rapid desensitization of mast cells and basophils, which impairs systemic anaphylaxis.^{5,6} Tolerance induction requires generation of allergen-specific regulatory T (Treg) cells, T_H1 cells, or both; allergen-specific regulatory B cells; decreases of allergen-specific IgE levels; and increases of blocking IgG and IgA levels.⁵ Although AIT is effective in many cases, it remains underused and is estimated to be used in less than 10% of patients with allergic rhinitis or asthma worldwide.¹ This is mainly due to several important drawbacks, including efficacy and safety concerns or the long duration of the treatment and low patient adherence. Alternative routes of vaccination and use of different hypoallergenic derivatives in combination with biological agents or novel adjuvants have been assayed to solve some of these problems; however, the progress in clinical trials is still moderate.⁷⁻⁹ Therefore there is an urgent need to develop more effective and safer vaccines.

Dendritic cells (DCs) are indispensable for the development of allergy or tolerance, and the rational design of vaccines targeting these cells represents a promising therapeutic alternative.¹⁰⁻¹² The induction of peripheral T-cell tolerance through generation of allergen-specific Treg cells is essential in the restoration of healthy immune responses to allergens after successful AIT,^{2,5,13,14} but the underlying immunologic mechanisms are not yet fully understood.¹⁵ Pathogen- or commensal-derived molecules and exogenous signals promote tolerogenic DCs that are able to induce functional Treg cells by using soluble and surface molecules, such as TGF- β , IL-10, indoleamine 2,3-dioxygenase (IDO), and programmed death ligand 1 (PD-L1).¹⁵⁻¹⁷

Myeloid C-type lectin receptors (CLRs) are pattern recognition receptors expressed by DCs that bind glycans in a Ca²⁺-dependent manner.¹⁸ CLR engagement not only mediates endocytosis but also cell signaling.^{19,20} Therefore targeting myeloid CLRs offers the additional advantage of shaping immune responses.^{21,22} Antibody-mediated CLR targeting has been successfully used to induce immunity or tolerance in patients with cancer or autoimmune diseases.²²⁻²⁴ Recently,

Abbreviations used

AIT:	Allergen immunotherapy
APC:	Allophycocyanin
BAT:	Basophil activation test
CFSE:	Carboxyfluorescein succinimidyl ester
CLR:	C-type lectin receptor
CT:	Cycle threshold
DC:	Dendritic cell
DC-SIGN:	Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
DMSO:	Dimethyl sulfoxide
EF1 α :	Elongation factor 1 α
FITC:	Fluorescein isothiocyanate
FOXP3:	Forkhead box P3
hmoDC:	Human monocyte-derived dendritic cell
ICOSL:	Inducible costimulator ligand
IDO:	Indoleamine 2,3-dioxygenase
mDC:	Myeloid dendritic cell
N:	Native grass pollen <i>Phleum pratense</i> allergens
NF- κ B:	Nuclear factor κ B
P:	Mannan-free glutaraldehyde-polymerized allergoids
PAO:	Phenylarsine oxide
pDC:	Plasmacytoid dendritic cell
PD-L1:	Programmed death ligand 1
PE:	Phycoerythrin
PM:	Glutaraldehyde-polymerized allergoids conjugated to nonoxidized mannan
PM OX:	Oxidized PM
SPT:	Skin prick test
Treg:	Regulatory T

glycan-based targeting approaches have also gained increasing attention.^{25,26} In this regard, mannan is a polysaccharide composed of mannose residues that can be found in plants, fungi, and yeast and has been shown to enhance antigen presentation, DC maturation, and immune responses.²⁷⁻²⁹ The mannose receptor (CD206), dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN; CD209), and Dectin-2 are the main CLRs expressed by DCs recognizing mannose-terminated sugars.^{29,30}

Glutaraldehyde-polymerized allergens are hypoallergenic allergoids that reduce undesired side effects during AIT.^{3,7} Moreover, allergoids alone or in combination with adjuvants have shown efficacy in clinical trials, but there is still some controversy in relation to their immunogenicity.³¹⁻³⁴ Here, for the first time, we show that allergoids coupled to nonoxidized mannan are novel hypoallergenic vaccines targeting DCs that enhance allergen uptake and promote healthy T-cell responses to allergens by generating functional forkhead box P3 (FOXP3)⁺ Treg cells through PD-L1 in human subjects. Mild oxidation impairs these features both in human subjects and mice. We uncover that preservation of the structural integrity of mannan is essential for the observed effects, which might well pave the way for the development of novel immunotherapeutic strategies not only for allergy but also for other immune tolerance-related diseases.

METHODS

Defatted grass pollen grains from *P pratense* (Iberpolen, Jaén, Spain) were extracted overnight with PBS (pH 7.2) and subjected to tangential flow

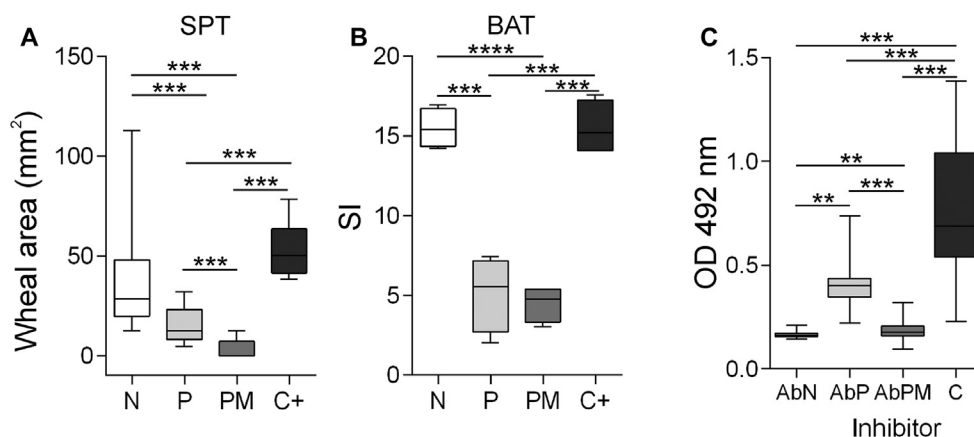


FIG 1. PM shows enhanced *in vivo* hypoallergenicity and induces potent blocking antibodies. **A**, SPT (n = 12). **B**, BAT (n = 4). **C**, Inhibition of serum IgE binding to N-coated plates by blocking antibodies induced by N (AbN), P (AbP), and PM (AbPM; n = 12). C, Negative control; SI, stimulation index; C+, positive control. Wilcoxon (Fig 1, A and C) or paired Student *t* (Fig 1, B) tests: ***P* < .01, ****P* < .001, and *****P* < .0001.

ultrafiltration (cutoff pore size, 100 kDa). The enriched native grass pollen *P pratense* allergens (N) contained in the filtrate were dialyzed with distilled water and lyophilized until use. Total protein content was measured by using the Bradford assay with serum albumin as the standard (Bio-Rad Laboratories, Hercules, Calif). The native mannan (nonoxidized) was purified from the yeast *Saccharomyces cerevisiae* (Lesaffre, Marcq-en-Baroeul, France), according to previously described protocols with slight modifications.^{35,36} N were polymerized and conjugated with mannan through the lysine residues from its mannoprotein, as previously described.^{37,38} Briefly, glutaraldehyde (25 mmol/L) was added to a solution containing a mixture of allergen and mannan (1:0.5 wt/wt) in PBS for 6 hours at 4°C in continuous stirring. The reaction was stopped with glycine (1.25 mol/L), followed by tangential flow filtration with distilled water (membrane cutoff, 100 kDa). Glutaraldehyde-polymerized allergoids conjugated to nonoxidized mannan (PM) were recovered in the concentrated retained fraction (>100 kDa). Stable allergen-mannan conjugates were confirmed by means of nuclear magnetic resonance, including 2 Dimensional-Diffusion Ordered Spectroscopy (2D-DOSY) and ¹H-¹³C Heteronuclear Single Quantum Correlation (HSQC) spectra.^{37,38} The same native allergen extract was subjected to the above protocol but without mannan to obtain mannan-free glutaraldehyde-polymerized allergoids (P). For some experiments, PM was treated with sodium periodate (5 mmol/L) at 4°C for 1 hour with continuous stirring in the dark. The reaction was stopped with ethylene glycol (1.8 mol/L), dialyzed against distilled water, and lyophilized to obtain oxidized PM (PM OX). PM oxidation was monitored and assessed by using 1-dimensional nuclear magnetic resonance spectra.³⁸

The study was approved by the Regional Ethics Committee of Comunidad Autónoma de Madrid (EudraCT: 2014-005471-88). Written informed consent was obtained from all subjects. The general clinical characteristics of the 12 patients with grass pollen allergy included in this study are summarized in Table E1 in this article's Online Repository at www.jacionline.org. Animal experiments were approved by the Ethics Committee of Hospital Clínico San Carlos and performed in accordance with the Spanish national and international/EU legislation regulated by D.C.86/609/CEE; RD 1201/2005.

All procedures are fully described in the Methods section in this article's Online Repository at www.jacionline.org.

RESULTS

Allergoids conjugated to nonoxidized mannan shows increased *in vivo* hypoallergenicity and induces potent blocking antibodies

We hypothesized that coupling mannan to allergoids would significantly enhance their allergen uptake by DCs and promote

tolerance while retaining hypoallergenicity. As a proof of concept, we generated novel grass pollen vaccines by coupling mannan from the cell wall of the yeast *S cerevisiae* to allergen-enriched *P pratense* pollen extracts in the presence of glutaraldehyde without prior sugar oxidation (see Fig E1 in this article's Online Repository at www.jacionline.org). This strategy generates stable neoglycans of polymerized allergens (allergoids) conjugated with nonoxidized mannan (ie, PM) with low *in vitro* IgE-binding reactivity.^{37,38} The *in vivo* hypoallergenic character of PM was assessed and compared with the same batch of native (N) and glutaraldehyde-polymerized (P) allergen-enriched *P pratense* pollen extracts by using skin prick tests (SPTs) in patients with well-defined grass pollen allergy. The capacity of PM to induce IgE cross-linking in skin mast cells was significantly lower than that of N and P (Fig 1, A). *Ex vivo* basophil activation tests (BATs) showed that PM and P display a similar reduced capacity to activate basophils from patients with grass pollen allergy than seen with N (Fig 1, B). Immunization of rabbits with PM induced potent allergen-specific blocking antibodies able to inhibit the IgE binding of sera from patients with grass pollen allergy to N more efficiently than those induced by P and with similar effectiveness than those induced by N (Fig 1, C).

Allergoids conjugated to nonoxidized mannan target CLRs and are captured more efficiently by human DCs than conventional allergoids and native allergens

Freshly prepared human monocyte-derived dendritic cells (hmoDCs) displayed an immature phenotype and expressed significant levels of the mannose receptor (CD206), DC-SIGN (CD209), and Dectin-2 (see Fig E2, A, in this article's Online Repository at www.jacionline.org). N, P, and PM were equally labeled with specific fluorophores when using sulfhydryl but not amino-reactive probes (see Fig E2, B). Kinetic uptake experiments monitored by means of flow cytometry with equally fluorescence-labeled N, P, and PM revealed that PM is captured by hmoDCs much more efficiently than P and N at all assay times (Fig 2, A). Representative dot plots and confocal microscopic images of the internalization of the different extracts after

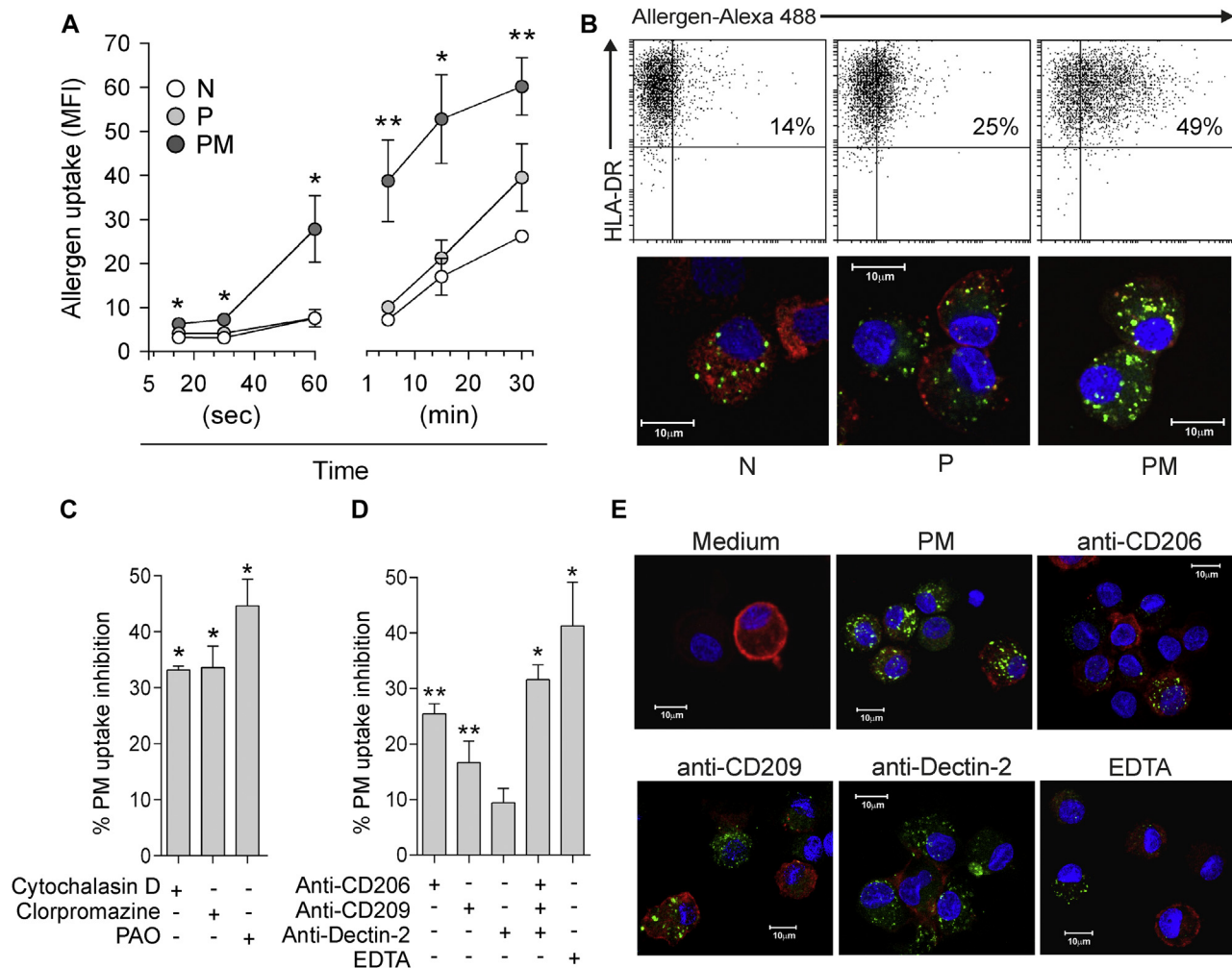


FIG 2. PM is captured much more efficiently by human DCs than N or P by targeting CLRs. **A**, Timing of fluorescence-labeled N, P, and PM allergen uptake by hmoDCs ($n = 5$). **B**, Representative flow cytometric plots and confocal images. **C** and **D**, Inhibition of the capture of fluorescence-labeled PM with the indicated compounds (Fig 2, C) or blocking antibodies or EDTA related to vehicle or isotype control (Fig 2, D; $n = 4$). **E**, Representative confocal images. Fluorescence-labeled allergens (green), HLA-DR (red), and 4'-6-diamidino-2-phenylindole dihydrochloride (blue). Values are means $\pm$ SEMs. Paired Student t test: * $P < .05$ and ** $P < .01$.

15 minutes of incubation are shown (Fig 2, B). We performed uptake inhibition experiments using cytochalasin D, chlorpromazine, or phenylarsine oxide (PAO). Around 45% of the PM uptake was inhibited by PAO, indicating that the process is highly dependent on receptor-mediated internalization mechanisms (Fig 2, C). To identify which CLRs were involved in taking up PM, we used specific blocking antibodies for mannose receptor (CD206), DC-SIGN (CD209), or Dectin-2. EDTA inhibited around 39% of the capture of PM, and blocking the 3 CLRs simultaneously reached an inhibition of around 32% (Fig 2, D). Mannose receptor (26%) and DC-SIGN (16%) were the main CLRs contributing to the PM uptake by hmoDCs (Fig 2, D and E).

Human DCs stimulated with PM increase PD-L1 expression and produce IL-6 and IL-10 through mechanisms depending on CLR-mediated activation of nuclear factor κ B

We treated hmoDCs with N, P, and PM and studied the expression pattern of different surface molecules and cytokine

production. hmoDCs stimulated with N, P, or PM expressed comparable high levels of HLA-DR and CD86 as expected for DCs with no expression of inducible costimulator ligand (ICOSL; Fig 3, A). CD83 expression was significantly higher in P- and PM-treated than N-treated hmoDCs, with lower levels induced by PM than P (Fig 3, A). PM treatment significantly increased expression of the inhibitory molecule PD-L1 compared with N and P both at the protein and mRNA levels (Fig 3, A, and see Fig E3 in this article's Online Repository at www.jacionline.org), suggesting that PM is able to induce a more tolerogenic phenotype on DCs. We also analyzed the expression pattern of the IDO, retinaldehyde dehydrogenase (RALDH), and suppressor of cytokine signaling (SOCS) genes at the mRNA level and did not find significant differences in expression of these molecules (see Fig E3). PM-treated hmoDCs produced significantly higher levels of IL-6 and IL-10 and lower IL-4 levels than N- or P-treated hmoDCs (Fig 3, B). We did not detect production of IL-12 in any case and detected very low levels of IL-23 only after N stimulation (Fig 3, B). Experiments with specific blocking antibodies showed that mannose receptor (CD206) and Dectin-2

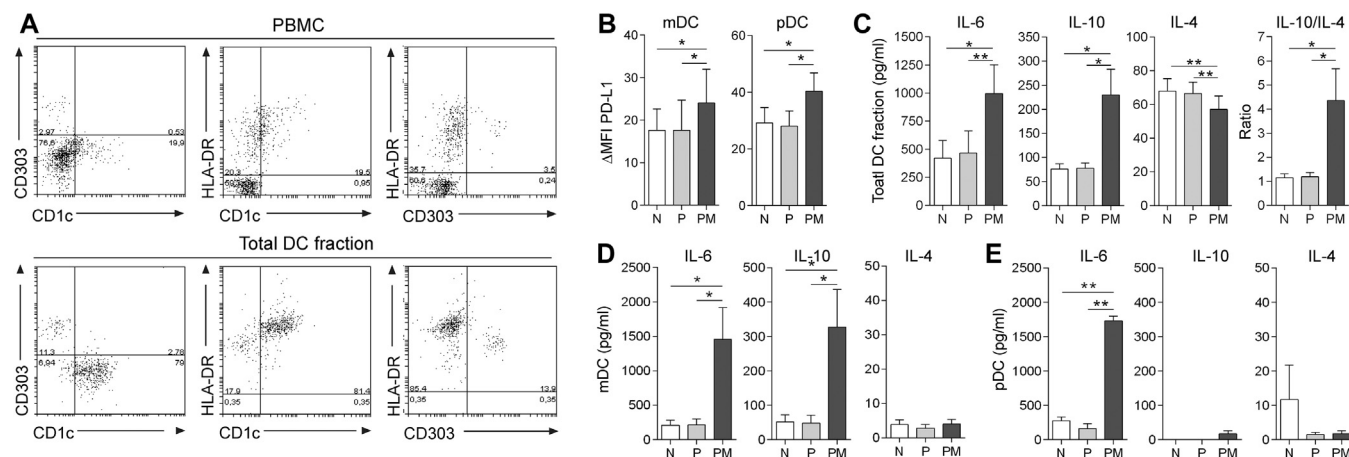
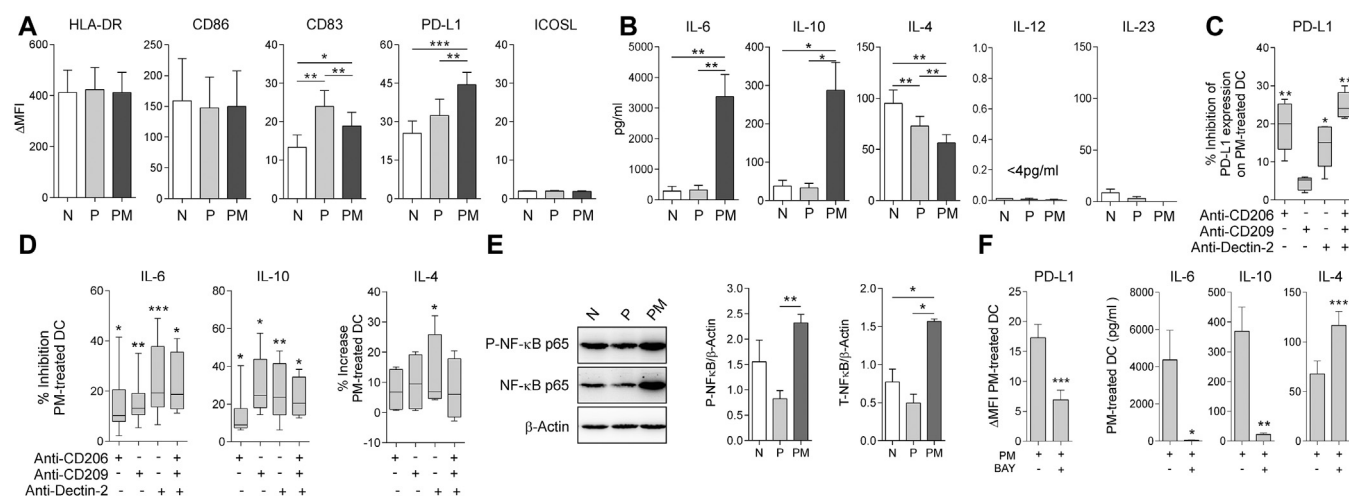


FIG 4. PM immunomodulates the phenotype and function of human mDCs and pDCs. **A**, Dot plots of different DC subsets in PBMCs and the total DC fraction. **B**, Δ MFI (after isotype control subtraction) of PD-L1 on HLA-DR⁺CD1c⁺CD303⁻ mDCs and HLA-DR⁺CD1c⁻CD303⁺ pDCs from the total DC fraction stimulated with N, P, or PM. **C-E**, Cytokines after stimulation of total blood DCs (Fig 4, **C**), enriched mDCs (Fig 4, **D**), or enriched pDCs (Fig 4, **E**) with N, P, and PM for 18 hours (mean $\pm$ SEM, $n = 4$). Paired Student t test: * $P < .05$ and ** $P < .01$.

are involved in PM-induced PD-L1 expression (Fig 3, **C**), whereas CD206, DC-SIGN, and Dectin-2 contributed at different extents to the observed effects at the cytokine level (Fig 3, **D**). Purified mannan also produced significant IL-6 and IL-10 levels (data not shown), suggesting that the capacity of PM to induce the production of these cytokines by DCs is likely due to the incorporation of mannan to the allergoid. The activation of nuclear factor κ B (NF- κ B) was significantly higher after stimulation with PM than N or P (Fig 3, **E**), and the pharmacologic inhibition of this transcription factor significantly reduced PM-induced PD-L1 expression, abolished IL-6 and IL-10 production, and increased IL-4 levels (Fig 3, **F**).

From peripheral blood, we isolated a human DC fraction containing myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs; Fig 4, **A**). Both mDCs and pDCs activated with PM also showed higher expression PD-L1 than DCs activated with N or P (Fig 4, **B**). The total human blood DC fraction activated with PM also produced significantly higher IL-6 and IL-10 levels and lower IL-4 levels than seen after activated with N or P (Fig 4, **C**). Interestingly, the IL-10/IL-4 ratio was also significantly higher when human DCs were activated with PM than with N or P (Fig 4, **C**), thus confirming the results obtained for hmoDCs. PM-activated mDCs produced significantly higher levels of IL-6 and IL-10

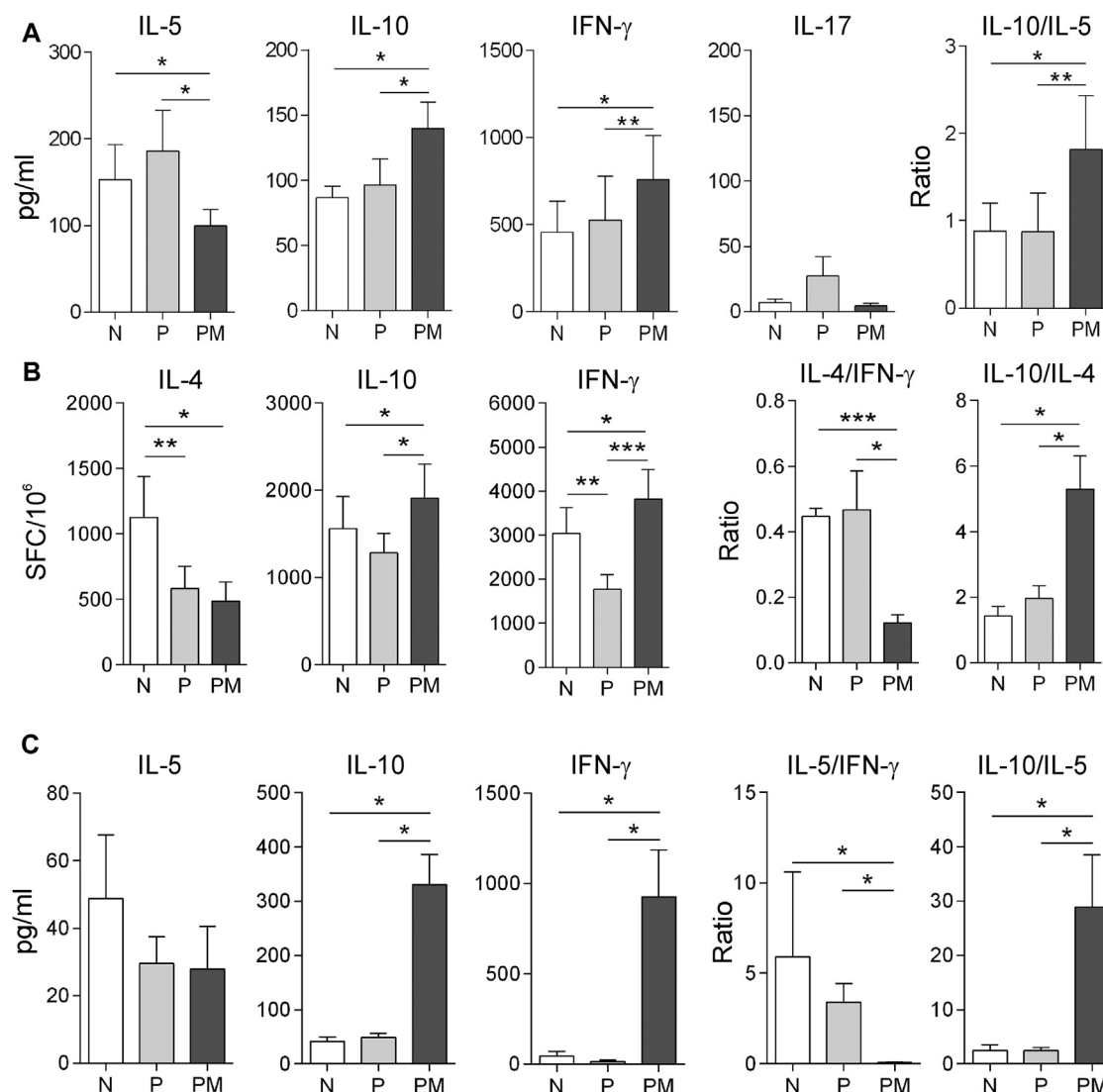


FIG 5. PM promotes the generation of healthy responses to allergens in human subjects. **A**, Cytokines produced by allogeneic naive CD4⁺ T cells primed by N-, P-, or PM-activated hmoDCs after 5 days (n = 6). **B**, ELISpot analysis of allergen-specific cytokine-producing T cells after *in vitro* expansion of naive and memory T cells primed with autologous N-, P-, or PM-activated DCs (n = 14). SFC, Spot-forming cells. **C**, Cytokines produced by PBMCs from allergic donors (n = 6) after activation with N, P, or PM for 5 days. Values are means $\pm$ SEMs. Paired Student *t* test: **P* < .05, ***P* < .01, and ****P* < .001.

than N- or P-activated mDCs, without significant production of IL-4 detected in any case (Fig 4, D). PM-activated pDCs also produced significantly higher levels of IL-6 than N- or P-activated pDCs, but they did not produce substantial levels of IL-10 or IL-4 (Fig 4, E).

Allergoids conjugated to nonoxidized mannan promote the generation of functional FOXP3⁺ Treg cells through PD-L1 in human subjects

To study the capacity of human DCs activated with PM to polarize T-cell responses, we performed coculture experiments. N-, P-, and PM-treated hmoDCs induced proliferation of allogeneic naive CD4⁺ T cells in a similar manner (see Fig E4 in this article's Online Repository at www.jacionline.org). PM-treated hmoDCs generated T cells producing significantly

higher IFN- γ and IL-10 levels and lower IL-5 levels than T cells primed by N- or P-treated hmoDCs (Fig 5, A). There was no significant production of IL-17 in any case. The IL-10/IL-5 ratio was significantly higher when T cells were generated by hmoDCs activated with PM than with N or P (Fig 5, A). These data, together with IFN- γ levels, suggest that PM-activated hmoDCs promote the generation of T_H1 cells and a T_H2/Treg cell shift in favor of Treg cells (Fig 5, A). Supporting these results, the same T-cell profile was observed by using ELISpot analysis when naive and memory T cells were primed by N-, P-, or PM-treated hmoDCs with *ex vivo* immunization protocols in an antigen-specific manner (Fig 5, B). Interestingly, and in line with these data, the IL-5/IFN- γ ratio was significantly lower when PBMCs from allergic patients were stimulated with PM than with N or P (Fig 5, C). In contrast, the IL-10/IL-5 ratio was increased after PM stimulation (Fig 5, C).

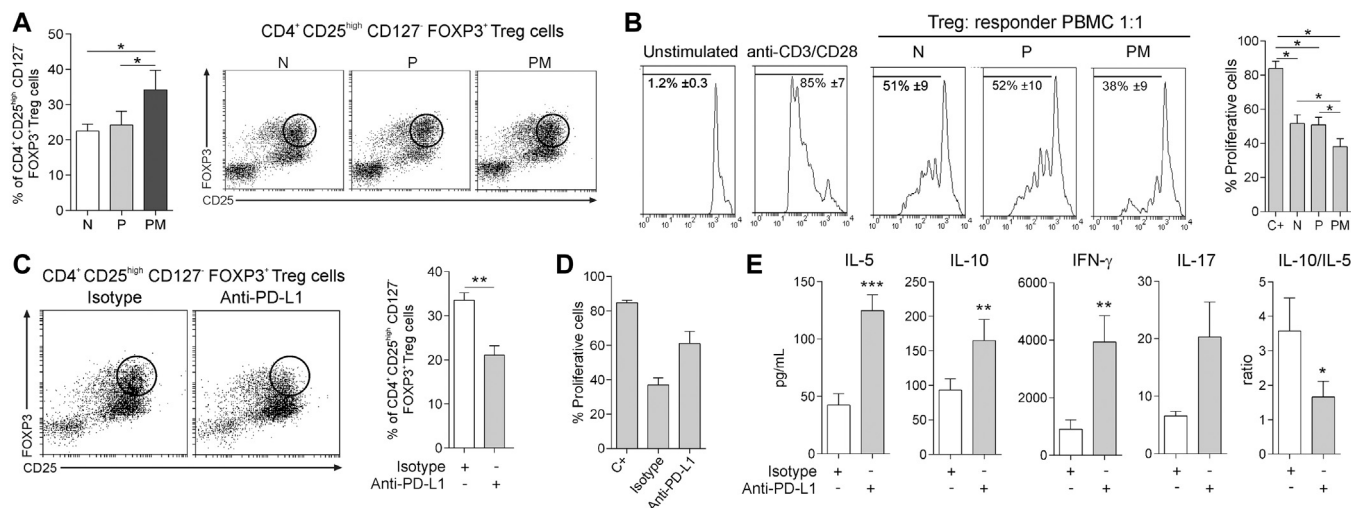


FIG 6. PM promotes the generation of functional FOXP3⁺ Treg cells through PD-L1 in human subjects. **A**, Percentage of induced CD4⁺CD25^{high}CD127⁻FOXP3⁺ Treg cells by allogeneic N-, P-, and PM-treated hmoDCs after 5 days (lymph gate, n = 4). **B**, Proliferation of CFSE-labeled PBMCs gated on CD4⁺ cells after 5 days of coculture with autologous purified FOXP3⁺ Treg cells induced by allogeneic N-, P-, or PM-activated hmoDCs (ratio 1:1). The percentage of proliferating responder PBMCs stimulated with plate-bound anti-CD3 and soluble anti-CD28 for each condition at a 1:1 ratio is shown (n = 3). **C**, CD4⁺CD25^{high}CD127⁻FOXP3⁺ Treg cells generated by allogeneic PM-treated hmoDCs in the presence of isotype control or PD-L1 blocking antibodies (n = 5). **D**, Suppressive capacity of Treg cells induced by PM-treated hmoDCs when inhibiting PD-L1. Percentage of proliferating responder PBMCs stimulated with plate-bound anti-CD3 and anti-CD28 for each condition (1:1 ratio). One of 4 representative examples is shown. **E**, Cytokines produced by allogeneic naive CD4⁺ T cells cocultured with PM-treated hmoDCs for 5 days in the presence of isotype control or PD-L1 blocking antibodies added at the beginning of coculture (n = 9). Values are means $\pm$ SEMs. Paired Student t test: **P* < .05, ***P* < .01, and ****P* < .001.

To further investigate the capacity of PM-treated human DCs to induce Treg cells, we performed allogeneic coculture experiments and analyzed the generation of FOXP3⁺ Treg cells. Human monocyte-derived DCs treated with PM induced significantly higher numbers of CD4⁺CD25^{high}CD127⁻FOXP3⁺ Treg cells than N- or P-treated hmoDCs (Fig 6, A). The FOXP3⁺ Treg cells generated by PM-treated hmoDCs inhibited the proliferation of autologous PBMCs in a dose-dependent manner (see Fig E5 in this article's Online Repository at www.jacionline.org) and showed a slight but significantly more potent suppression capacity than those generated by N- or P-treated hmoDCs when assayed at a Treg cell/PBMC ratio of 1:1 (Fig 6, B). The generation of functional FOXP3⁺ Treg cells by PM-treated hmoDCs was significantly reduced when blocking PD-L1 (Fig 6, C), and the remaining FOXP3⁺ Treg cells showed reduced capacity to inhibit proliferative responses, as demonstrated by using conventional suppression assays (Fig 6, D). PD-L1 blocking also significantly enhanced cytokine production of the T cells generated by PM-activated hmoDCs and reduced IL-10/IL-5 ratio (Fig 6, E).

Oxidation of mannan impairs the capture of PM by human DCs and tolerogenic properties both in human subjects and mice

To assess the role of keeping the structural integrity of mannan after conjugation to allergoids in the observed effects, we subjected fluorescence-labeled PM to mild oxidation (PM OX) without affecting the intensity of fluorescence emission (data not shown) and compared the ability of hmoDCs to internalize PM

and PM OX by using flow cytometry and confocal microscopy. The oxidation process destroyed the mannan structure as monitored by 1-H nuclear magnetic resonance (NMR) studies (see Fig E6 in this article's Online Repository at www.jacionline.org). Oxidation of PM significantly reduced the capacity of hmoDCs to internalize the glycoconjugate after 30 and 60 minutes of incubation (Fig 7, A). Representative dot plots and confocal images are displayed (Fig 7, B). Similarly, oxidation significantly inhibited PD-L1 expression induced by PM in hmoDCs (Fig 7, C). PM OX-activated hmoDCs produced significantly lower IL-6 and IL-10 levels and higher IL-4 levels than PM-activated hmoDCs (Fig 7, D). As shown in Fig 7, the IL-10/IL-4 ratio was also significantly reduced after PM oxidation. The percentage of generated CD4⁺CD25^{high}CD127⁻FOXP3⁺ Treg cells in allogeneic cocultures was significantly reduced when hmoDCs were activated with PM OX compared with PM (Fig 7, E).

To assess the *in vivo* relevance of these findings, we immunized subcutaneously BALB/c mice with N, P, PM, or PM OX and analyzed the induction of CD4⁺CD25^{high}FOXP3⁺ Treg cells in the spleen. The percentage of CD4⁺CD25^{high}FOXP3⁺ Treg cells (gated as shown in Fig E7 in this article's Online Repository at www.jacionline.org) was significantly higher in splenocytes from mice immunized with PM than with N or P, and oxidation of PM significantly abolished this increment (Fig 7, F). The ratio of serum IgG_{2a}/IgE levels specific for native grass pollen extract was significantly higher in mice immunized with PM than in those immunized with N or P, and oxidation of PM also abolished this increment (Fig 7, G). In the same way the IL-10/IL-4 ratio after *in vitro* stimulation with native grass pollen extract was significantly higher in

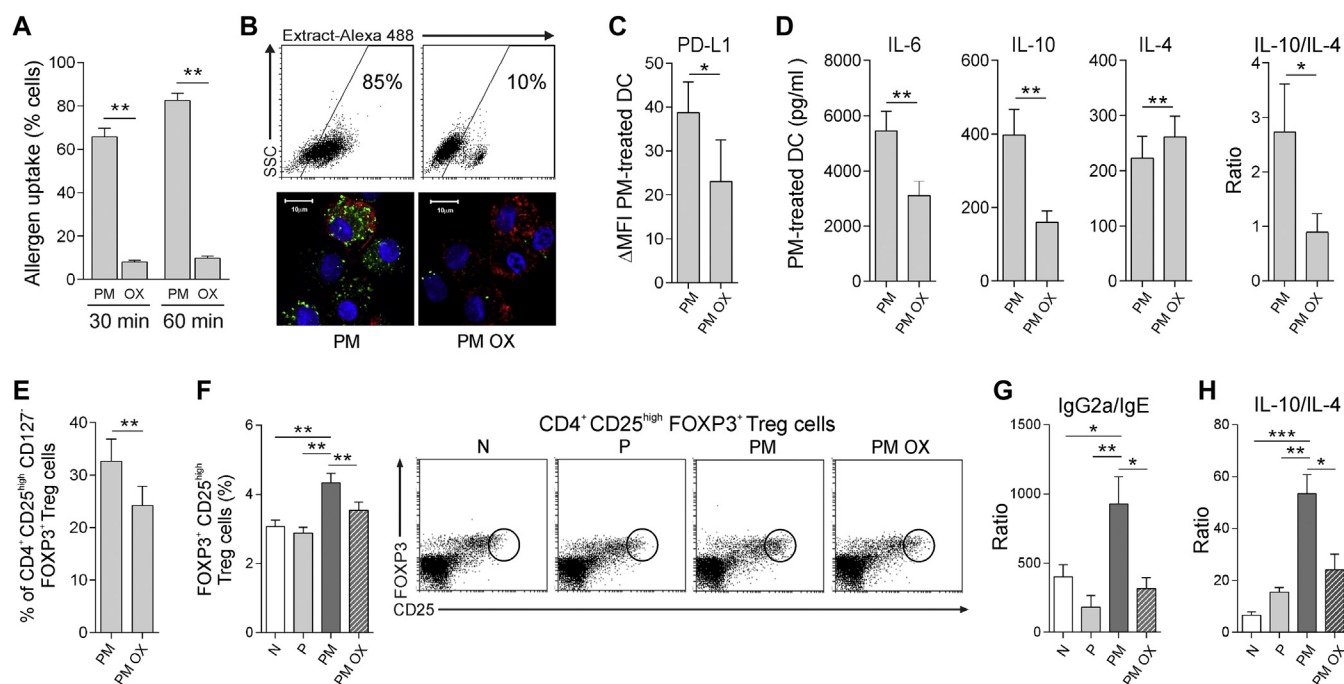


FIG 7. Capacity of PM to promote healthy immune responses to allergens depends on keeping the structure of nonoxidized mannan in the generated conjugate. **A**, Uptake of fluorescence-labeled PM or PM previously subjected to oxidation (PM OX) after 30 or 60 minutes of incubation with hmoDCs. **B**, Dot plots and confocal images after 60 minutes. PM/PM OX (green), HLA-DR (red), and 4'-6-diamidino-2-phenylindole dihydrochloride (blue) are shown. **C**, PD-L1 expression on hmoDCs treated with PM or PM OX for 18 hours. Δ MFI after isotype control subtraction is shown. **D**, Cytokines produced by hmoDCs stimulated with PM or PM OX for 18 hours. **E**, Percentage of CD4⁺CD25^{high}CD127⁻FOXP3⁺ Treg cells generated by allogeneic PM- or PM OX-treated hmoDCs after 5 days. **F**, Percentage of CD4⁺CD25^{high}FOXP3⁺ Treg cells in spleens of mice immunized with N, P, PM, or PM OX. Representative dot plots are shown. **G**, Ratio of serum grass pollen-specific IgG2a/IgE from mice immunized with N, P, PM, and PM OX. **H**, IL-10/IL-4 ratio produced by splenocytes from mice immunized with N, P, PM, or PM OX after *in vitro* stimulation with N. Values are means $\pm$ SEMs of 3 (Fig 7, A) or 5 (Fig 7, C-E). For Fig 7, F-H, n = 5 individual mice per condition of 1 of 3 independent experiments. The paired Student *t* test was used for Fig 7, A-E, and the unpaired Student *t* test was used for Fig 7, F-H: **P* < .05, ***P* < .01, and ****P* < .001.

splenocytes from mice immunized with PM than P or N, and this increment was impaired when PM was subjected to oxidation (Fig 7, H).

DISCUSSION

In this study we have provided evidence that allergoids coupled to native mannan (nonoxidized) represent novel suitable preparations to formulate improved vaccines for AIT. PM displays *in vivo* hypoallergenicity, induces potent blocking antibodies in rabbits, is captured by human DCs much more efficiently than N or P, and endorses cells with the capacity to generate functional FOXP3⁺ Treg cells through PD-L1. In mice immunization with PM also increases the frequency of splenic FOXP3⁺ Treg cells and induces a shift to nonallergic responses. Mild oxidation impairs these effects in both human subjects and mice, indicating that preservation of the carbohydrate structure of mannan during conjugation is essential for the reported features. This study shed light into the basic structural and immunologic mechanisms that place allergoids conjugated to nonoxidize mannan as suitable vaccines for AIT.

AIT represents the only treatment for allergy with the capacity to modify the course of the disease^{1,2}; however, it still faces several drawbacks in terms of efficacy, security, duration, and

patient compliance, and therefore novel vaccines that overcome such inconveniences are in demand.^{7,9} Mannan from *S cerevisiae* can be conjugated with allergens under nonoxidizing conditions by using glutaraldehyde, which results in very stable mannan-allergoid complexes, as detected by using nuclear magnetic resonance.^{37,38} Here we have performed a detailed immunologic study of PM in comparison with N and P to validate the suitability of this conjugate as novel vaccines for AIT before clinical translation.

Immunization of rabbits with PM induced potent blocking antibodies to the nominal native allergen, which is a key feature for successful AIT.³⁹⁻⁴¹ PM is captured by human DCs much more rapidly and efficiently than N or P through mechanisms mainly depending on mannose receptor- and DC-SIGN-mediated endocytosis. This enhanced allergen uptake could help increase the effective dose of the vaccine without increasing its allergen concentration, which, together with the higher *in vivo* hypoallergenicity showed by PM, might well contribute to the design of efficacious, shorter, and safer immunotherapy protocols.^{9,32,42,43}

Human DCs activated with PM produce high levels of IL-10 and significantly increase the expression of PD-L1, which are molecules involved in the generation of different types of functional Treg cells.^{5,14,44-46} Our data indicate that both mDCs

and pDCs contribute to the production of IL-6, but only mDCs produce IL-10 after PM stimulation. The expression of PD-L1 on DCs has been demonstrated to be linked to their capacity to induce functional FOXP3⁺ Treg cells both in mice and human subjects.⁴⁵⁻⁴⁸ In mice PD-L1 promotes functional FOXP3⁺ Treg cells during tolerance induction to allergens associated with changes in lung microbiota of neonates.⁴⁵ In human subjects *Staphylococcus aureus* converts neonatal conventional CD4⁺ T cells into FOXP3⁺ Treg cells through the PD-1/PD-L1 axis.⁴⁶ The bacterial components directly involved in such processes were not investigated in any of these studies. Herein we show that PM is able to significantly increase the expression of PD-L1 on human DCs and promote the generation of functional FOXP3⁺ Treg cells only when the structural integrity of the native mannan (ie, nonoxidized) is retained. PM-induced PD-L1 expression on DCs required signaling through mannose receptor and DC-SIGN, leading to NF- κ B activation. Blocking of PD-L1 impairs the generation of functional FOXP3⁺ Treg cells, enhances the production of T-cell effector cytokines, and significantly decreases the IL-10/IL-5 ratio. It has been previously shown that PD-L1 ligation impedes the T-cell receptor signaling pathway, which can lead to differentiation into FOXP3⁺ Treg cells.⁴⁷⁻⁵⁰ In line with our results, compelling experimental evidence has also demonstrated that under certain circumstances, fully mature DCs might also promote the generation of Treg cells.⁵¹⁻⁵³ Interestingly, we show that mice immunized with PM also display significantly larger numbers of splenic FOXP3⁺ Treg cells and higher IL-10/IL-4 ratios than those immunized with N or P. The ratio of serum allergen-specific IgG_{2a}/IgE levels was significantly higher in PM-immunized mice than in N- or P-immunized mice, indicating that PM promotes *in vivo* healthy immune responses to allergens.

Importantly, our data uncover that keeping the integrity of the mannan structure is essential for the reported immunologic properties of PM, which also might be of great relevance when designing glycan-based targeting strategies for other different therapeutic interventions.^{18,25,29} Oxidation leads to the structural disruption of mannan, which might condition functional properties.⁵⁴ For example, immunization with MUC1 coupled to oxidized mannan or to oxidized and subsequently reduced mannan promoted differential immune responses in mice. Only mice immunized with oxidized mannan-MUC1 induced potent tumor-specific CD8⁺ T-cell responses.^{55,56} Recently, targeting antigens to Mannose receptor by coupling them to oxidized mannan has been shown to suppress pathogenic autoimmune responses through expansion of anergic antigen-specific T_H1 and T_H17 cells without generating Treg cells.⁵⁷ In this experimental model PD-L1 expression was downregulated on DCs by oxidized mannan.⁵⁷ Hypoallergenic neoglycoconjugates created by coupling allergens to oxidized mannan were reported to enhance immunogenicity, but their tolerance induction capacity was not assessed.⁵⁸ In contrast to these approaches, our method of conjugation avoids initial sugar oxidation and retains the integrity of the mannan structure in PM, which seems to be essential for the observed immunogenicity and tolerogenic capacity. In this study we used grass pollen allergens as a proof of concept, but the same allergen uptake and immunomodulatory properties have been observed when coupling nonoxidized mannan to other relevant allergenic sources, such as house dust mites or different tree pollens (data not shown), according to this innovative method of conjugation. Interestingly, BSA

coupled to individual mannose molecules without prior sugar oxidation was shown to induce oral tolerance in a food allergy mouse model by targeting lamina propria DCs through SIGNR1, the mouse homologue for human DC-SIGN.⁴⁴ These DCs generated type 1 IL-10-producing but not FOXP3⁺ Treg cells. These data support that the structural integrity of mannoses might be a requisite to generate Treg cells. The differences observed in this type of induced Treg cell might be justified not only by the different experimental system but also because we coupled the whole mannan polysaccharide to allergoids instead of single mannose residues to purified BSA, which results in very different molecular entities. Recognition by different CLR is highly dependent on the carbohydrate branching, spacing, and multivalency.^{18,25} Our data show that PM can simultaneously target mannose receptor, DC-SIGN, and Dectin-2, which contribute to different extents to each of the reported functional outcomes after global integration of the induced signaling pathways. The strength and duration of signaling and induced functional response not only depend on the type of targeted CLR but also on specific features of the ligand, such as low or high affinity, soluble or particulated, and particle size.¹⁸⁻²⁰

In summary, for the first time, we have demonstrated that allergoids coupled to mannan through a method that avoids sugar oxidation might well represent novel hypoallergenic vaccines for AIT, which enhance allergen uptake and promote the generation of functional FOXP3⁺ Treg cells through PD-L1 in human subjects. These findings might be of the utmost importance for the development of alternative therapeutic interventions for other immune tolerance-related diseases.

Clinical implications: Glutaraldehyde-polymerized allergoids coupled to native nonoxidized mannan represent novel suitable preparations to formulate vaccines that might well help in the design of efficacious, shorter, and safer AIT protocols.

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METHODS

Material, media, and reagents

The medium used for cell cultures was RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated FBS (Gibco, Grand Island, NY), antibiotics (penicillin and streptomycin; Life Technologies, Grand Island, NY), MEM Vitamin Solution, L-glutamine, nonessential amino acids, sodium pyruvate (Life Technologies, Grand Island, NY), and Normocin (InvivoGen, San Diego, Calif). Flow cytometric antibodies were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany; human anti-HLA-DR–fluorescein isothiocyanate [FITC], anti-HLA-DR–allophycocyanin [APC], anti-CD83–APC, anti-CD86–phycoerythrin [PE], anti-CD303–PE, anti-CD1c–FITC, anti-CD127–PE, anti-CD4–APC, anti-CD25–peridinin chlorophyll protein complex [PerCP], anti-CD206–APC, anti-CD209–PE [FcR Blocking]); BioLegend (San Diego, Calif; human anti-FOXP3–Alexa Fluor 488, mouse anti-FOXP3–Alexa Fluor 488, mouse anti-CD4–PerCP, and mouse anti-CD25–PE), and BD Pharmingen (San Jose, Calif; human anti-PD-L1–FITC and human anti-ICOSL–PE). Blocking antibodies were anti-CD206 (clone 15-2, BioLegend), anti-CD209 (clone 120507; R&D Systems, Minneapolis, Minn), anti–Dectin-2 (clone 545943, R&D Systems), and anti-PD-L1 (clone 29E.2A3, BioLegend) and corresponding isotype controls. None of the reagents contained detectable amounts of LPS, as determined by using the Limulus assay (Cambrex Bio Science, Walkersville, Md).

SPTs and BATs

SPTs were performed with N, P, or PM in 12 patients with well-defined grass pollen allergy in accordance to standard procedures.^{E1} Each patient was simultaneously tested by duplicates with the 3 different preparations (N, P, and PM) by using the Prick-Film technology (Inmunotek, Madrid, Spain) by the same operator. Histamine dihydrochloride and physiologic saline solutions were used as positive and negative controls, respectively. Results of SPTs were automatically processed and quantified by using planimetry with the proper software (Prick-Film) and expressed as median wheal size in square millimeters. A wheal area of less than 7 mm² was considered negative. BATs were performed with N, P, and PM extracts by using heparinized whole blood from 4 patients with well-defined grass pollen allergy and the BasoTest (ORPEGEN Pharma, Baden-Württemberg, Germany). Anti-human IgE antibodies (BD Pharmingen) and physiologic saline solutions were used as positive and negative controls, respectively. Stimulation indexes were calculated as the ratio between the percentage of activated basophils with the stimulus and the negative control. The study was approved by the Regional Ethics Committee of Comunidad Autónoma de Madrid (EudraCT:2014-005471-88). Written informed consent was obtained from all subjects.

ELISA inhibition of the binding of serum IgE from allergic patients to N extract by blocking antibodies induced after immunization of rabbits with N, P, or PM

New Zealand rabbits were immunized subcutaneously with 0.5 mg of N, P, or PM mixed with complete Freund adjuvant. The rabbits were given 3 booster doses with incomplete Freund adjuvant at 14-day intervals. After 2 weeks from the last immunization, rabbits were bled to collect whole blood from the carotid artery. The blocking capacity of the serum antibodies generated by means of immunization of rabbits with N, P, and PM was analyzed by using ELISA inhibition assays, as previously described,^{E2} with minor modifications. Microtiter plates (Corning Inc, Corning, NY) were coated with 100 µL/well of N (5 µg/mL) overnight at 4°C. Plates were washed 3 times with PBS and 0.1% vol/vol Tween-20 and blocked for 1 hour with PBS, 0.1% vol/vol Tween-20, and 3% wt/vol dehydrated milk. Then plates were incubated with rabbit serum immunized against N, P, and PM extracts diluted 1:50 in PBS, 0.1% vol/vol Tween-20, and 3% wt/vol dehydrated milk. After 1 hour of incubation, plates were washed 4 times with PBS and 0.1% vol/vol Tween-20 and incubated for 2 hours with individual serum from 10 patients with well-defined grass pollen allergy diluted 1:10 in PBS, 0.1% vol/vol Tween-20, and 3% wt/vol dehydrated milk. After 4 washes, bound

IgE antibodies were detected by incubating with mouse anti-human IgE antibodies (1:5000 diluted), followed by horseradish peroxidase–coupled goat anti-mouse IgG (diluted 1:2500; Dako, Glostrup, Denmark). The peroxidase reaction was developed by using fresh enzyme substrate (0.03% H₂O₂ and 0.63 mg/mL *o*-phenylenediamine in 0.1 mol/L sodium citrate, pH 5.0), and the reaction was stopped with 3 N H₂SO₄. OD was measured at 492 nm in an ELISA reader. Each OD value was the mean of 2 determinations after blank subtraction.

Flow cytometry and confocal microscopy

Labeling of cell-surface markers was performed at room temperature for 15 minutes in PBS, 2 mmol/L EDTA, and 0.5% BSA. Cells were then washed with PBS, 2 mmol/L EDTA, and 0.5% BSA and resuspended in PBS and 0.1% paraformaldehyde. For analysis of FOXP3 expression in human T cells primed with DCs, cells were first subjected to surface staining with anti-human CD4–PerCP, CD127–PE, and CD25–APC (Miltenyi Biotec). After fixation and permeabilization, cells were stained with anti-human FOXP3–Alexa Fluor 488 (BioLegend), according to the manufacturer's recommendations. The same protocol described above was carried out for the phenotypic characterization of CD4⁺CD25^{high}FOXP3⁺ Treg cells in freshly isolated mouse splenocytes using anti-mouse CD4–PerCP and CD25–PE antibodies for surface staining and anti-mouse FOXP3–Alexa Fluor 488 (BioLegend) for intranuclear staining. The corresponding isotype controls were included in all stainings. Flow cytometric analysis was performed with a FACSCalibur cytometer (Becton Dickinson) and further by using Weasel v2.5 software. For confocal microscopy, stained cells were subjected to cytospin (600g for 8 minutes), fixed with ice-cold methanol for 5 minutes, and stained with ProLong Gold–DAPI (Dako) 24 hours before analysis. Images were acquired and analyzed by using the confocal microscope Leica SP-2 AOBs (Leica, Wetzlar, Germany).

Differentiation of hmoDCs and isolation of total DC fraction from human PBMCs

PBMCs from healthy donors were isolated by using Ficoll-Paque Plus (GE Healthcare, Fairfield, Conn) density gradient centrifugation from heparinized blood. Monocytes were isolated from PBMCs with anti-human CD14 microbeads (Miltenyi Biotec) by using positive magnetic selection in autoMACS (Miltenyi Biotec), according to the manufacturer's recommendations. Purified monocytes were cultured in RPMI with recombinant human GM-CSF and IL-4 (100 ng/mL each; PeproTech, Rocky Hill, NJ). After 6 days of culture, immature hmoDCs were harvested, and the expression of specific surface markers was analyzed by means of flow cytometry for phenotype determination. The total DC fraction was obtained from PBMCs with the Blood Dendritic Cell Isolation Kit II (Miltenyi Biotec), according to the manufacturer's protocol. The presence and content of mDC and pDC subsets within the obtained total DC fraction was analyzed by using flow cytometry with anti-human CD1c–FITC, CD303–PE, CD19–PEyC7, and HLA-DR–APC (Miltenyi Biotec). An enriched fraction of mDCs and pDCs was obtained from peripheral blood by using the CD1c (BDCA-1)⁺ and CD304 (BDCA-4/Neuropilin-1) DC isolation kits, respectively (Miltenyi Biotec), as previously described with minor modifications.^{E3} In all cases cell viability was routinely checked by using trypan blue cell exclusion in the light microscope and/or YO-PRO/propidium iodide staining and flow cytometric analysis. N, P, or PM did not show toxicity on DCs at the concentrations used, and significant differences in cell viability were not detected for all the assayed conditions.

Fluorescent labeling of N, P, and PM

N, P, and PM allergen extracts were labeled with Alexa Fluor 488 dye conjugated with a thiol-reactive group (Alexa Fluor 488 C₅ maleimide, Life Technologies) or with an amine-reactive group (Alexa Fluor 488 succinimidyl ester, Life Technologies), according to the manufacturer's instructions. For the thiol-reactive probe labeling, extracts dissolved in PBS (5 mg/mL) were incubated for 15 minutes with 25 mmol/L Tris(2-carboxyethyl) phosphine (TCEP) in a nitrogen atmosphere. One hundred micrograms of Alexa Fluor 488 C₅ maleimide dissolved in dimethyl sulfoxide (DMSO; 2 mg/mL) was

added to the extracts. After 2 hours of incubation with constant stirring in the dark, the reaction was stopped by adding 20 μ L of β -mercaptoethanol and maintaining stirring for 15 minutes. Uncoupled free Alexa Fluor 488 was removed by means of gel filtration with a PD-10 column with PBS. The fluorescence-labeled allergens were concentrated with Nanosep 3K Omega (Pall Corporation, Port Washington, NY), and the degree of labeling was checked with the FLUOstar OPTIMA fluorescence reader (BMG Labtech, Ortenberg, Germany). Labeling of allergens with Alexa Fluor 488 succinimidyl ester probes according to the manufacturer's indications did not render final products with equal degree of fluorescence because of the lack of free lysine residues during polymerization with glutaraldehyde in P and PM.

Allergen uptake by hmoDCs and inhibition assays

Immature hmoDCs were cultured with 50 μ g/mL N, P, PM, and PM OX equally labeled with Alexa Fluor 488 C₅ maleimide (Invitrogen, Carlsbad, Calif) at different times at 37°C. Cells were washed with cold PBS, 2 mmol/L EDTA, and 0.5% BSA 2 times before surface staining with anti-human HLA-DR-FITC and analyzed by using flow cytometry and confocal microscopy. For inhibition assays, cells were preincubated with cytochalasin D (5 μ mol/L; Sigma, St Louis, Mo), chlorpromazine (7.5 μ g/mL, Sigma), PAO (2 μ mol/L, Sigma), EDTA (2 mmol/L, Sigma), DMSO as a vehicle control, anti-human CD206 mAb (10 μ g/mL, BioLegend), anti-human CD209 mAb (20 μ g/mL, R&D Systems), or an isotype control for 30 minutes at 37°C or with anti-human Dectin-2 (20 μ g/mL, R&D Systems) or a corresponding isotype control for 2 hours. Afterward, labeled or unlabeled N, P, and PM were added to the cells and incubated for 30 minutes (for uptake assays) or 18 hours (for cytokine quantification). For the NF- κ B inhibition assays, cells were incubated with BAY 117082 (5 μ mol/L, Sigma) or DMSO as a vehicle control for 30 minutes before PM stimulation. For all these assays, titration of dose and time responses was optimized previously.

Cytokine quantification

Cytokine quantification in cell-free supernatants was performed by means of ELISA with specific commercial kits, according to the manufacturer's protocols for IL-4, IL-5, IL-6, IL-10, IFN- γ (BD PharMingen), and IL-17 (R&D Systems). The ELISA reader (Bio-Rad Laboratories, Hercules, Calif) was used for OD measurement.

Coculture experiments and proliferation assays

Naive CD4⁺ T cells were purified from PBMCs by using the Naive CD4⁺ T Cell Isolation Kit (Miltenyi Biotec). The obtained negative fraction was further purified with anti-human CD14 and anti-human CD45RO coupled to microbeads (Miltenyi Biotec) by means of negative selection to reach greater than 99% purity. N-, P-, and PM-treated hmoDCs were cocultured with purified allogeneic naive CD4⁺ T cells at a 1:5 ratio for 6 days, as previously described.^{E3} As controls, naive CD4⁺ T cells supplemented with IL-2 (100 U/mL, PeproTech) were cultured alone in complete RPMI. Purified naive CD4⁺ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (ThermoFisher Scientific, Madrid, Spain) for 5 minutes at 4°C and washed twice with medium. After 6 days of coculture with N-, P-, or PM-stimulated hmoDCs, cells were harvested and stained with anti-human CD4-PerCP, and proliferation was determined based on CFSE dilution using flow cytometry. PD-L1 inhibition assays were performed by adding anti-human PD-L1 (10 μ g/mL) to cocultures. After 6 days, cells were harvested for further assays and cytokine quantification.

CFSE-labeled PBMCs from allergic donors were cultured with N, P, or PM (50 μ g/mL) for 5 days. Proliferative responses and cytokine levels in cell-free culture supernatants were determined by means of flow cytometry and ELISA, respectively.

Treg cell suppression assay

CD4⁺CD25^{high}CD127⁻FOXP3⁺ Treg cells induced by allogeneic N-, P-, or PM-stimulated hmoDCs were enriched with anti-human CD25 microbeads (Miltenyi Biotec), according to the manufacturer's instructions. The unlabeled enriched fraction of CD4⁺CD25^{high}CD127⁻FOXP3⁺ Treg cells was mixed with CFSE-labeled autologous PBMCs (responder cells) at different ratios

and stimulated with plate-bound anti-human CD3 antibody (1 μ g/mL, clone OKT3; eBioscience, San Diego, Calif) and soluble anti-human CD28 (1 μ g/mL, clone CD28.6; eBioscience) for 5 days. For control purposes, CFSE-labeled PBMCs were cultured alone with or without stimulation. Proliferation of CD4⁺ T cells was determined by using CFSE dilution with flow cytometry.

ELISpot identification of allergen-specific IFN- γ -, IL-10-, and IL-4-producing T cells after expansion by mean of *ex vivo* immunization with N-, P-, or PM-activated autologous hmoDCs

Immature hmoDCs were stimulated with 100 μ g/mL of N, P, or PM extracts for 8 hours and subsequently matured for 18 hours with 1 μ g/mL peptidoglycan. Matured hmoDCs were restimulated for an additional 6 hours with N, P, or PM and irradiated (3000 rad). These cells were cocultured with autologous monocyte-depleted PBMCs in the presence of IL-7 (20 U/mL) and supplemented at day 5 with IL-2 (10 U/mL). After 7 days, the same protocol was carried out in the same way to complete a total of 3 *ex vivo* immunizations with autologous N-, P-, or PM-treated hmoDCs. Seven days after the last immunization, expanded allergen-specific T cells producing IFN- γ , IL-10, and IL-4 were identified by using the ELISpot assay. Briefly, 96-well high-affinity plates (Millipore, Temecula, Calif) were coated with 10 μ g/mL of capture mAb against IFN- γ , IL-10, or IL-4 (Millipore) at 4°C for 18 hours. A total of 25,000 expanded allergen-specific T cells were added to each well and stimulated with 25,000 autologous PBMCs, which were previously incubated with 100 μ g/mL of the allergen, for 18 hours in a 5% CO₂ atmosphere at 37°C and processed as previously described.^{E4} Briefly, biotinylated detection antibody was added and incubated for 2 hours at room temperature. Plates were washed, incubated with alkaline phosphatase streptavidin for 1 hour in the dark at room temperature, and developed with SIGMAFAST BCIP/NBT (Sigma). The reaction was stopped by rinsing the plates with PBS-Tween (0.05%) and washed with distilled water. Each well was then examined for positive dots. The number of dots in each well was counted automatically with an ELISpot reader system (Immunospot Reader System). The obtained spots represent the number of cells that secreted the cytokine of interest (spot-forming cells). Each testing condition is assayed separately and compared with the negative control counts (spot-forming cells without antigenic stimulus). More than 50 spot-forming cells per well and a value at least 3 times greater than the background was considered a positive result.

Immunoblot analysis

Freshly isolated hmoDCs were treated with N, P, and PM extracts at 50 μ g/mL. After 15 minutes at 37°C, cells were harvested and lysed with PBS and 0.1% Triton X-100 supplemented with protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride and 1 μ g/mL aprotinin and leupeptin; Roche, Mannheim, Germany) for 30 minutes at 4°C with vortexing every 10 minutes. Lysates were centrifuged for 15 minutes at 10,000g and 4°C, and supernatants were collected for protein quantification with Micro BCA Kit (Pierce, Rockford, Ill). Thirty micrograms of total protein from cell lysates was separated by means of SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was incubated with the indicated antibodies used as follows: phospho-NF- κ B p65, total-NF- κ B p65 (1:1000; Cell Signaling, Danvers, Mass), and β -actin (1:1000, Sigma) as primary antibodies and goat anti-rabbit (1/3000, Bio-Rad Laboratories) or goat anti-mouse (1:2500, Pierce) conjugated with horseradish peroxidase as a secondary antibody. Reactive bands were visualized with the ECL chemiluminescence system (Bio-Rad Laboratories). The OD of the reactive bands was quantified with Fujifilm multigauge software, and values were expressed relative to the β -actin loading control.

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR

RNA was isolated from harvested cells by using an RNeasy Mini Kit (Qiagen, Hilden, Germany). According to the manufacturer's instructions.

cDNA was generated with a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). Real-time quantitative PCR was performed on cDNA by using FastStart Universal SYBR Green Master (Rox; Roche). The sequences of the used pair primers were as follows: PD-L1 (forward, AAGATGAGGATA TTTGCTGTCTTTATATTC; reverse, GTCCTTGGAACCGTGACAGT), IDO (forward, AGAAGTGGGCTTTGCTCTGC; reverse, TGGCAAGACCT TACGGACATCTC), RALDH1 (forward, CTGCCGGGAAAAGCAATCT; reverse, AAATTCAACAGCATTGTCCAAGTC), RALDH2 (forward, AG GGCAGTTCTTGCAACCAT; reverse, GCGTAATATCGAAAGGTTTGA TGA), SOCS1 (forward, CCCTGGTTGTTGTAGCAGCTT; reverse, CAA CCCCTGGTTTGTGCAA) SOCS3 (forward, CCTCAGCATCTCTGTCCG AAGA; reverse, GCATCGTACTGGTCCAGGAAGT), and elongation factor 1 α (EF1 α ; forward, CTGAACCATCCAAT; reverse, GCCGTGTGGCAATC-CAAT). Samples were run on a real-time PCR system (ABI Prism 7900 HT; Applied Biosystems, Foster City, Calif). Data were normalized to EF1 α and displayed as $2^{-\Delta CT}$ values multiplied by 10^4 , with Δ cycle threshold (ΔCT) defined as the difference between the CT value for the gene of interest and EF1 α .

Immunization of mice with N, P, PM, or PM OX; quantification of induced serum allergen-specific IgG_{2a} and IgE levels; and cytokine profiles of splenocytes stimulated *in vitro* with N

BALB/c female mice (6 weeks old) were immunized 3 times with 20 μ g (200 μ L at 100 μ g/mL) of N, P, PM, or PM OX by means of subcutaneous injection every 15 days and killed 7 days after the last immunization. Serum specific IgE and IgG_{2a} reactivities to N induced with each treatment were determined by means of ELISA. Spleens were used to prepare single-cell suspensions according to conventional protocols. The presence of splenic

CD4⁺CD25^{high}FOXP3⁺ Treg cells in the different immunized mice was quantified, as described above. Splenocytes were stimulated *in vitro* with N, and cytokine (IL-4 and IL-10) production was measured by using Multiplex Cytometric kits (eBioscience) in 48-hour culture supernatants. Animal experiments were approved by the Ethics Committee of Hospital Clínico San Carlos and performed in accordance with the Spanish national and international/European Union legislation regulated by D.C.86/609/CEE; RD 1201/2005.

Statistical analyses

In all experiments data represent means $\pm$ SEMs of the indicated parameters. Statistical differences were determined by using the paired or unpaired Student *t* test or the Wilcoxon test, as indicated in each figure by using Prism software 6.0 (GraphPad Software, La Jolla, Calif). Significance is indicated in each figure.

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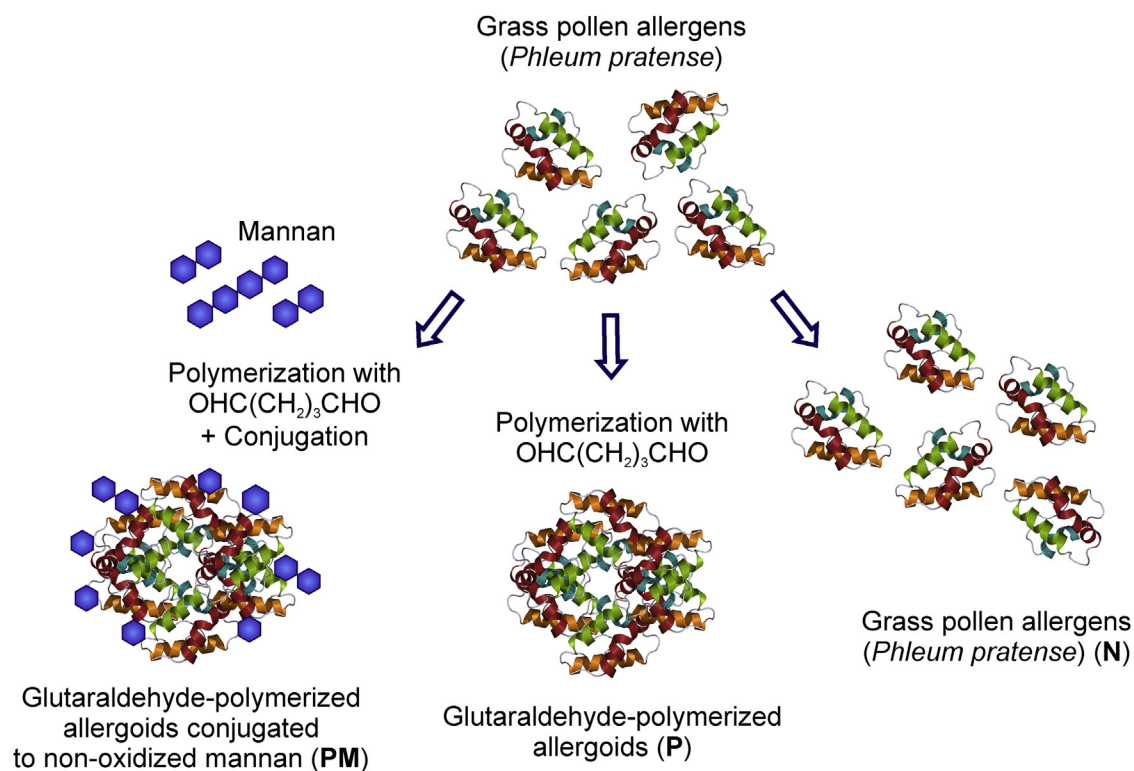


FIG E1. Chemical strategy to generate allergoids conjugated to nonoxidized mannan. Taking advantage of the presence of lysine residues in the purified mannan preparation, native grass pollen *P. pratense* allergen extract was polymerized and conjugated with mannan in a single step with glutaraldehyde to generate glutaraldehyde-polymerized allergoids conjugated to nonoxidized mannan (PM). Part of the same native allergen extract was also subjected to polymerization without mannan to obtain a mannan-free glutaraldehyde-polymerized allergoids (P) or used without polymerization as native allergen extract (N).

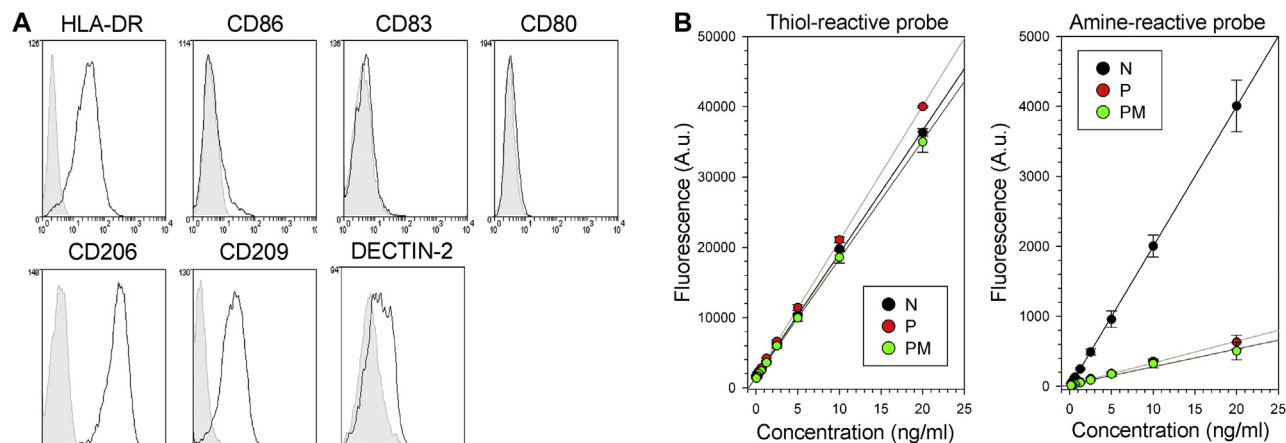


FIG E2. Phenotypic characterization of hmoDCs and comparison of different fluorescent probes for extract labeling. **A**, Representative histograms for the phenotype of immature hmoDCs. Freshly differentiated hmoDCs were analyzed by using flow cytometry for HLA-DR, CD86, CD83, CD80, CD206, CD209, and Dectin-2 expression (*open histograms*). *Shaded histograms* represent the reactivity of fluorochrome-matched isotype controls. **B**, Fluorescence intensity of different concentrations of N, P, and PM extracts labeled with thiol-reactive Alexa Fluor 488 (*left*) or amine-reactive Alexa Fluor 488 (*right*).

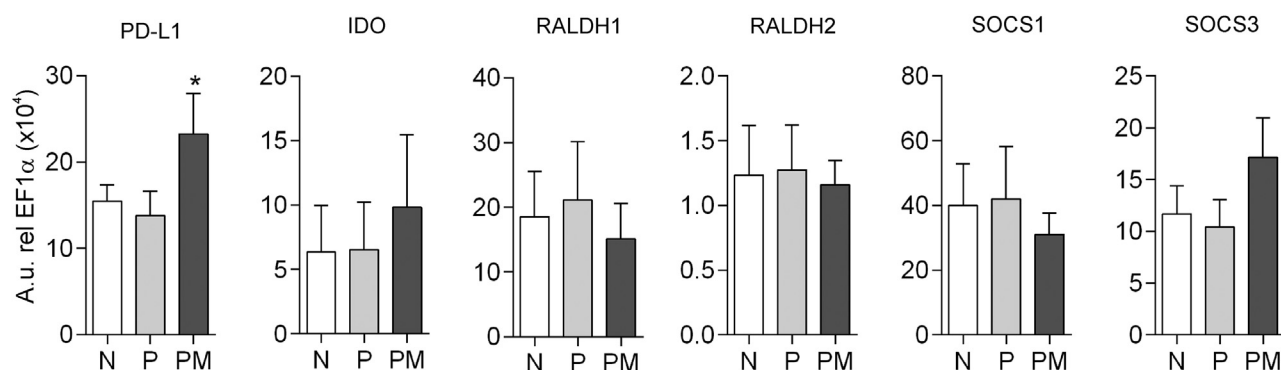


FIG E3. PD-L1 mRNA expression is increased in PM-treated hmoDCs. mRNA expression levels of PD-L1, IDO, RALDH1, RALDH2, SOCS1, and SOCS3 genes in hmoDCs treated with N, P, or PM. Arbitrary units (A.u.) are $2^{-\Delta CT}$ values multiplied by 10^4 , with ΔCT defined as the difference between the CT value for the gene of interest and EF1 α . Data are means $\pm$ SEMs (n = 4). Statistical significance was determined with the Student *t* test: **P* < .05.

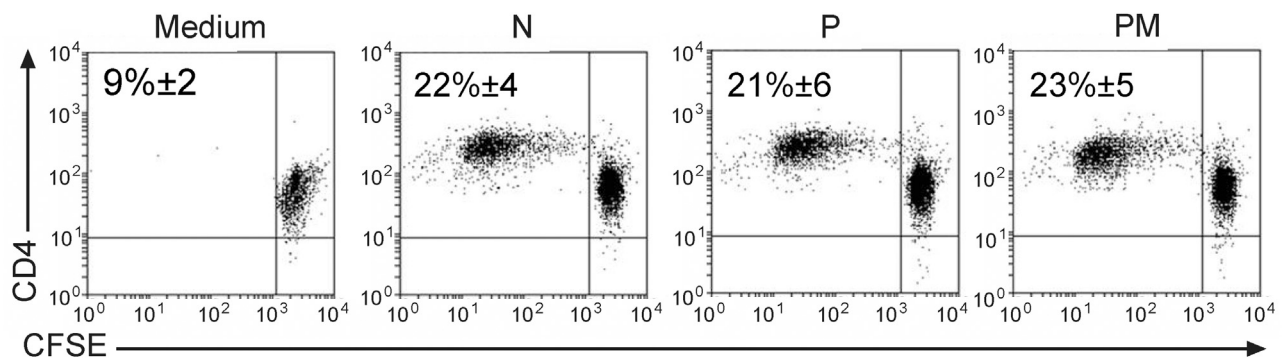


FIG E4. Representative dot plot of proliferating CFSE-labeled naive CD4⁺ T cells after 5 days of coculture with N-, P-, or PM-activated allogeneic hmoDCs. The frequency of proliferating cells is displayed (n = 6 independent experiments).

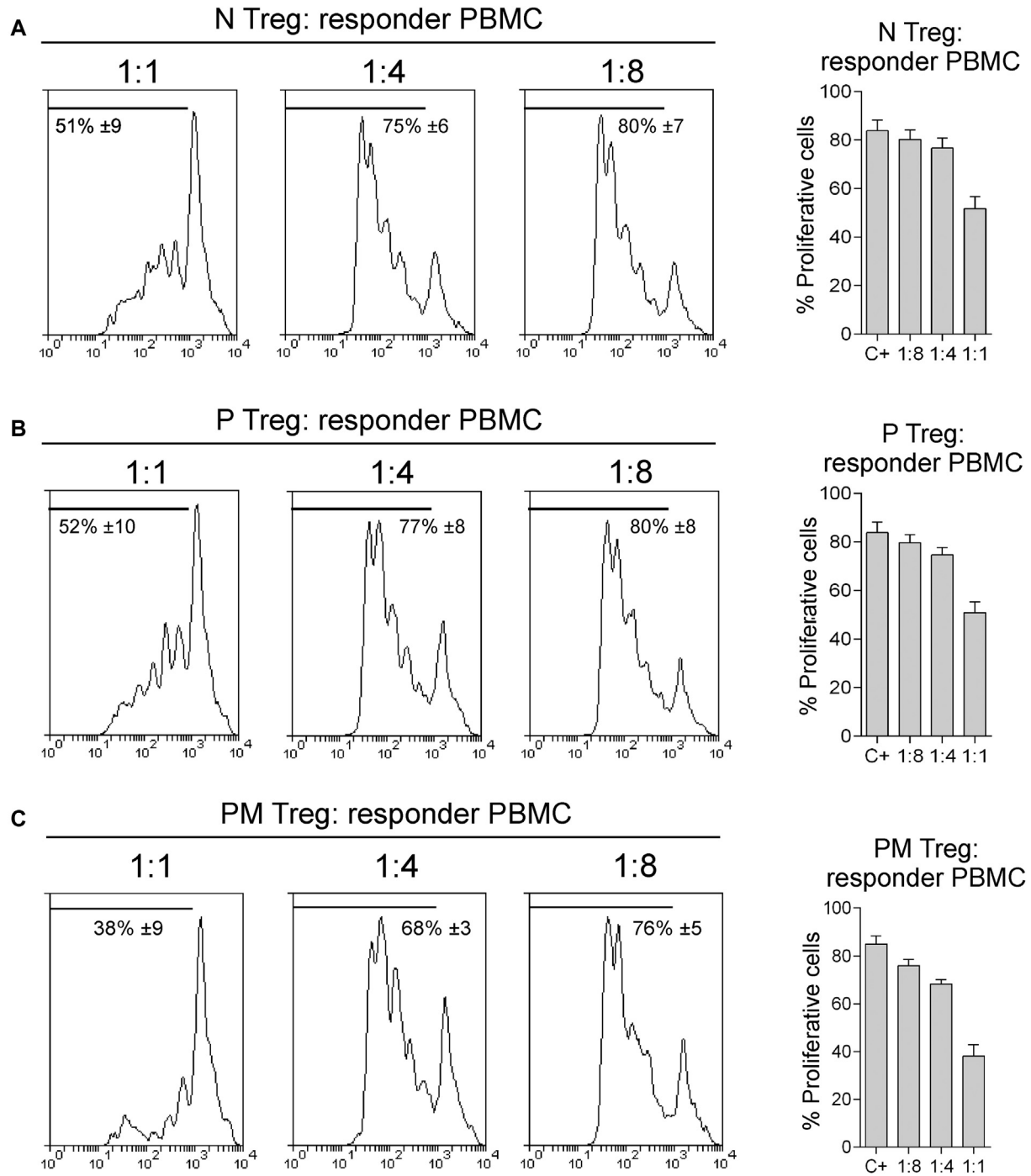


FIG E5. Suppression capacity of FOXP3⁺ Treg cells induced by allogeneic N-, P-, and PM-treated hmoDCs. Proliferation of CFSE-labeled PBMCs gated on CD4⁺ cells after 5 days of coculture with autologous purified CD4⁺CD25^{high}CD127⁺FOXP3⁺ Treg cells induced by N-treated (**A**), P-treated (**B**), and PM-treated (**C**) allogeneic hmoDCs is shown. Histograms show the percentage of proliferating responder PBMCs stimulated with plate-bound anti-CD3 and soluble anti-CD28 for each assayed condition at different Treg cell/responder PBMC ratios. The graph shows data from 3 independent experiments.

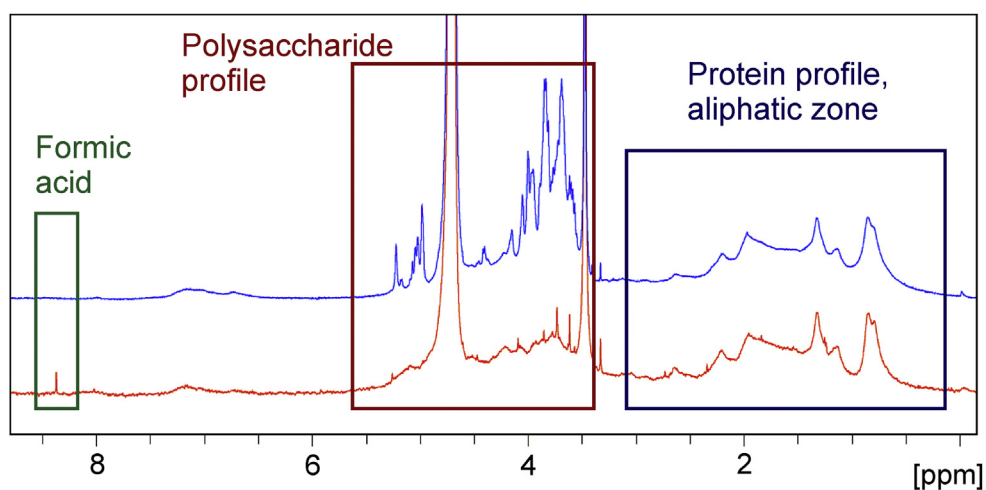


FIG E6. One-dimensional proton nuclear magnetic resonance spectrum of samples before and after oxidation (*blue and red traces*, respectively). The aliphatic part of the protein profile (from 0-3 ppm in the spectrum) is maintained after oxidation, whereas the carbohydrate-anomeric zone (from 3.5-5.5 ppm) is significantly perturbed. The signature of formic acid coming from the oxidation of terminal mannose residues is also observable (peak at 8.4 ppm).

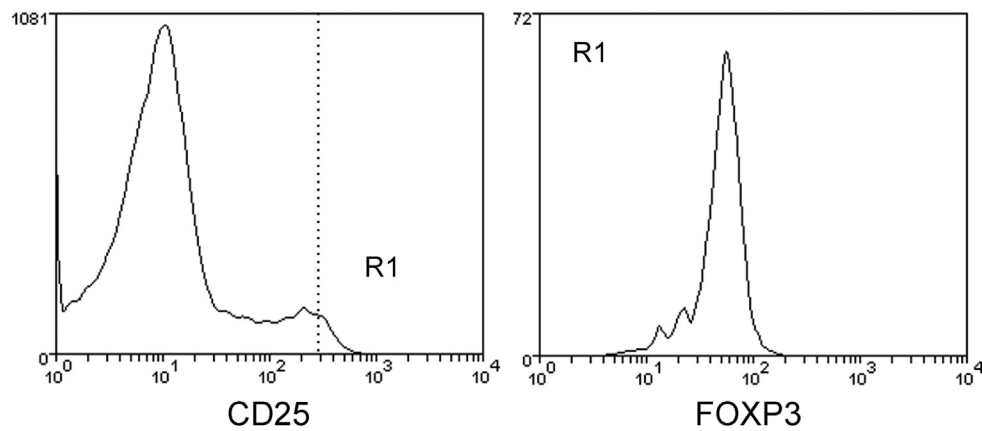


FIG E7. Gating strategy used to identify and compare the CD25^{high}FOXP3 Treg cell population in spleens from mice immunized with N, P, or PM. A representative histogram for CD25 and FOXP3 from mice immunized with PM is shown. In the CD25 histogram a shoulder for the gated population of cells with CD25^{high} expression is clearly visible. All these cells also express high FOXP3 levels.

TABLE E1. Clinical features of the patients with grass pollen allergy included in the study

Patient no.	Age (y)	Sex	Allergic to	Symptoms	SPT response (mm ²) with <i>N P pratense</i>	Other pollen allergies
1	70	Male	Grasses	RC	40.69	<i>Olea</i> species
2	29	Female	Grasses	RC-A	12.56	<i>Olea</i> species, <i>Cupressus</i> species
3	27	Male	Grasses	RC-A	18.09	<i>Platanus</i> species
4	78	Female	Grasses	RC	24.62	<i>Olea</i> species
5	54	Female	Grasses	RC	32.15	<i>Olea</i> species
6	63	Female	Grasses	RC-A	40.69	<i>Cupressus</i> species
7	25	Female	Grasses	RC	24.62	
8	33	Female	Grasses	RC-A	24.62	<i>Olea</i> species, <i>Cupressus</i> species
9	24	Male	Grasses	RC	113.04	<i>Olea</i> species, <i>Cupressus</i> species
10	11	Male	Grasses	RC	72.35	<i>Olea</i> species
11	46	Male	Grasses	RC	18.09	
12	35	Female	Grasses	RC	50.24	

A, Asthma; RC, rhinoconjunctivitis.