

A Dendritic Cells-Targeting Nano-Vaccine by Coupling Polylactic-Co-Glycolic Acid-Encapsulated Allergen with Mannan Induces Regulatory T Cells

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Keywords

Dendritic cells · Polylactic-co-glycolic acid · Treg cells · Allergen · Mannan · Immune tolerance · Allergen-specific immunotherapy

Abstract

Background: The efficacy of allergen-specific immunotherapy (AIT) is mainly depended on the tolerogenic immune responses elicited. Properly conjugated nano-vaccine has the advantages of both specific targeting and continuous and on-demand release of allergen. **Objectives:** The aim of this study is to investigate the effects of a dendritic cells (DCs)-targeting nano-vaccine for AIT. **Methods:** The nano-vaccine was produced by coupling polylactic-co-glycolic acid (PLGA)-encapsulated ovalbumin (OVA) with mannan. Allergen capture, human monocytes-derived DCs (hMoDCs) activation, and T cells responses were assessed by flow cytometry, confocal microscopy, quantitative real-time PCR, ELISA, and Cytometric Bead Array. Balb/c mice were immunized with the nano-vaccines, and the immune responses were analyzed. **Results:** OVA-PLGA nanoparticle (NP) displayed fa-

vorable safety profile. OVA-mannan-PLGA NP was captured more efficiently by hMoDCs than OVA-PLGA NP, which was mediated mainly through DC-specific intercellular adhesion molecule 3-grabbing nonintegrin. A tolerogenic phenotype of hMoDCs was induced by OVA-mannan-PLGA NP, but not OVA-PLGA NP, and increased number of regulatory T (Treg) cells was generated subsequently in vitro coculture. Immunization of Balb/c mice with OVA-mannan-PLGA NP resulted in lower serum level of OVA-specific immunoglobulins and less production of pro-inflammatory cytokines in splenocytes culture than the mice immunized with OVA-PLGA NP, PLGA NP, or OVA, while the number of splenic Treg cells was higher in OVA-mannan-PLGA group than in other groups. Moreover, preimmunization with OVA-mannan-PLGA NP significantly inhibited the Th2 immune response induced by OVA sensitization. **Conclusions:** The biocompatible PLGA-encapsulated OVA coupling with mannan has augmented ability for tolerance induction and could be developed as a novel vaccine for AIT.

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Introduction

Allergen-specific immunotherapy (AIT) has been employed for more than a century for the management of allergic diseases. Successful AIT holds the promise of not only alleviating the symptoms like allergic inflammation but more importantly, helping to remodel the disease process and to achieve long-lasting protection against later exposure to antigen. In reality, however, the efficacy of AIT is suboptimal, possibly due to the weak immunity being provoked, thus leading to the low compliance of AIT. Various methods have been proposed to improve the performance of AIT, including modification of allergen structure, new routes for allergen administration, and optimization of adjuvants [1, 2]. Recently, emerging interests have been directed to targeted delivery of allergen to dendritic cells (DCs), one pivotal type of antigen-presenting cells, which have been reported to play a key role in the immunoregulation involved in AIT. Previous studies demonstrate that DCs-targeting vaccines induce the production of Th1 cells or regulatory T (Treg) cells [3–6], which could balance the Th2 response witnessed in allergic diseases. Although similar strategies have been proposed and verified in terms of outcome, a rationally designed approach by targeting DCs to augment the action of AIT remains elusive.

For a given type of vehicle being delivered specifically to DCs, the choice of membrane targets plays a critical role. C-type lectin receptors (CLRs) are pattern recognition receptors that recognize carbohydrate structures in a Ca^{2+} -dependent fashion and mediate antigen uptake, inducing either immunogenic or tolerogenic immune responses [7, 8]. Recently, glycan-based targeting approaches have gained increasing attention [9]. Mannan is a polysaccharide composed of mannose residues existing widely in plants, fungi, and yeast and has been shown to enhance antigen presentation, DCs maturation, and immune responses [10, 11]. DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) is the main type of CLRs that recognize mannan [12]. In addition to the role as targeting moiety, recent findings reveal that mannan can also function as an adjuvant to prime the production of tolerogenic DCs through increasing their programmed cell death protein ligand 1 (PD-L1) expression [5]. These evidences together rationalize the selection of mannan for the DCs-targeting allergen delivery.

Nanotechnology has been widely explored in the field of targeted drug delivery in medicine during the past decade. Poly(lactic-co-glycolic acid) (PLGA) is one of the biocompatible materials approved by the Food and Drug

Administration of the USA, which can be used in human. Studies have shown that PLGA-encapsulated antigen could prime stronger cellular and humoral immune responses than soluble antigen [13], in the cases of both tumors and infectious diseases [14]. On this basis, we expect that the sustained while controlled release of allergens as rendered by PLGA vehicle could enhance the production of tolerogenic DCs, thereby leading to better outcome of AIT.

In the present study, we constructed ovalbumin (OVA)-loaded PLGA nanoparticles (NP) that were glycosylated with mannan (OVA-mannan-PLGA NP). Next, we quantified the uptake of OVA-mannan-PLGA NP by human monocytes-derived DCs (hMoDCs), followed by assessment of potential immune responses *in vitro* and *in vivo*, with OVA-PLGA NP as control for comparison. The results demonstrated that OVA-mannan-PLGA NP induced a tolerogenic phenotype of DCs as supported by the decreased expression of co-stimulatory molecules and pro-inflammatory cytokines, in addition to increased expression of interleukin (IL)-10 and PD-L1 by hMoDCs. OVA-mannan-PLGA NP also induced increased production of Treg cells *in vitro* and *in vivo*. Our study revealed the therapeutic potential of a novel nano-vaccine against allergy, and shed light on the future design of nano-strategies for AIT.

Materials and Methods

Preparation of NPs

Blank-PLGA NP and OVA-PLGA NP were prepared using a double emulsion solvent evaporation method. Briefly, 50 mg of 50:50 PLGA (RG503H, Sigma) was dissolved in 1 mL dichloromethane (DCM). A solution with OVA (Sigma) at a concentration of 40 mg/mL was prepared in deionized water. Using a microtip probe sonicator set at 35% amplitude (America Sonics VCX130), 200 μL of deionized water without antigen or the antigen solution containing 8 mg OVA was mixed with the PLGA/DCM solution for 40 s (20 s pulses) in an ice bath to form the W/O emulsion. This emulsion was then added to 10 mL of 2% (*wt/vol*) PVA (Mw 30–70 kDa, Sigma) in deionized water with probe sonicator set at 35% amplitude for 2 min (80 s pulses) to form the W/O/W emulsion. Then the mixture was added dropwise into 25 mL 0.3% (*wt/vol*) PVA and simultaneously stirred with a magnetic stirrer (CJJ78-1, Xicheng xinrui instrument factory) at 300 rpm for 4 h in an ice bath during which time the DCM solvent evaporated. Blank-PLGA NP or OVA-loaded PLGA NP was then collected via centrifugation (14,000 rpm, 30 min) at 4°C (Centrifuge 5810R, Eppendorf). OVA-mannan-PLGA NP was obtained by absorption. 10 mg OVA-PLGA NP were resuspended in PBS (pH 5.6), 10 mg mannan (Sigma) was added, and the mixture was placed on a magnetic stirrer at room temperature for 6–8 h and then concentrated by ultracentrifugation (12,000 g, 30 min). The collected NPs were washed

with deionized water for at least 3 times (12,000 g, 15 min), and the NPs were lyophilized (SCIENTZ-10N). The sample powder was stored at 4°C for use.

Generation of hMoDCs from Human Peripheral Blood

Density gradient centrifugation was used to isolate PBMCs from the peripheral blood of healthy donors. The study was approved by the Ethics Committee of the Institute of Dermatology in Chinese Academy of Medical Sciences, and written informed consent was obtained from the participants. Monocytes were sorted from PBMCs using positive selection with the help of human CD14 microbeads (MACS, Miltenyi Biotec) according to the instruction. Adherent monocytes were further cultured with recombinant human GM-CSF (100 ng/mL, R&D Systems) and IL-4 (20 ng/mL, R&D Systems) to obtain hMoDCs as described before. After 5 days of culture, immature hMoDCs were harvested, and the expression of specific surface markers was analyzed by flow cytometry (FCM) (online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000512872).

hMoDCs Treatment and Cytokine Quantification

The hMoDCs were cultured in 24-well plates at a density of 5×10^5 – 10^6 cells per well and allowed to adhere overnight. For NP uptake experiment, immature hMoDCs were incubated with OVA-mannan-PLGA or OVA-PLGA (containing 8 µg/mL OVA) labeled with Rhodamine123 (83702, Sigma-Aldrich, St. Louis, MO, CA, USA) at 37°C, and the cells were washed with cold PBS, 2 mmol/L EDTA, and 0.5% BSA twice before surface staining with antihuman DC-SIGN-FITC (Abcam) and analyzed using FCM and confocal microscopy. Anti-DC-SIGN-blocking antibody (5 µg/mL, MAB161, R&D) was used to test the specificity for DC-SIGN. For hMoDCs maturation assay, OVA, PLGA NP, OVA-PLGA NP, or OVA-mannan-PLGA NP were incubated with hMoDCs for 24 h. The concentration for OVA was 8 µg/mL, and OVA-PLGA NP and OVA-mannan-PLGA NP containing the same dose of OVA were used. The expression of co-stimulatory markers on hMoDCs was analyzed by FCM. Pro-inflammatory cytokines (IL-1β, IL-6, IL-12p70, IL-10, and tumor necrosis factor [TNF]-α) in the culture supernatant of hMoDCs at 24 h were collected and analyzed by Cytometric Beads Array kit (Cat. 551811, BD, USA) according to the protocol recommended. Expression of PD-L1, IL-10, IL-4, IL-6, and TNF-α in hMoDCs was analyzed by quantitative real-time PCR (qRT-PCR).

DC-T Cell Coculture

Naive CD4⁺ T cells were purified from PBMCs using the Naive CD4⁺ T Cell Isolation Kit (Miltenyi Biotec, Germany). hMoDCs were first incubated with the NPs for 24 h as described above, and then the NP-treated hMoDCs were cocultured with allogeneic T cells at a DCs/T-cell ratio of 1:10 in the presence of recombinant human IL-2 (R&D System, 20 ng/mL) for 7 days. The proliferation of T cells was analyzed by a CellTrace™ CFSE Cell Proliferation Kit (Thermo Fisher) (online suppl. Fig. 2). The cultured cells were collected for intracellular IL-4 (Catalog No.17-7049-81, eBioscience, San Diego, CA, USA) and CD4⁺CD25^{high}Foxp3⁺ Treg cells analysis by FCM.

FCM and Confocal Microscopy

For surface staining, 5×10^5 hMoDCs were incubated with antibodies in PBS containing 5% fetal bovine serum (FBS) and 0.1%

NaN₃ for 30 min on ice in the dark and washed twice with PBS containing 5% FBS. The antibodies used included FITC-conjugated anti-CD14, APC, or FITC-conjugated anti-DC-SIGN (Abcam), PE-conjugated anti-CD11c, APC-conjugated anti-CD80, FITC-conjugated anti-CD86, APC-conjugated anti-CD83, FITC-conjugated anti-HLA-DR antibodies (antibodies were purchased from Miltenyi Biotec, Germany, except indicated). For intracellular staining of T cells, 25 ng/mL PMA and 1 µg/mL Ionomycin were added in the culture supernatant of T cells 6–8 h before collecting cells for FCM, and 10 µg/µL BFA and 1.7 µg/mL Monensin were added 4 h before FCM. 5×10^5 cells were stained with FITC-conjugated anti-CD4 antibody and (or) APC-conjugated anti-CD25 antibody first, then cells were fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (eBioscience, Thermo Scientific, Waltham, MA, USA), then stained with APC-conjugated anti-IL-4 antibody (BD Biosciences) or PE-conjugated anti-Foxp3 antibody (BioLegend) or isotype control. The samples were run on FACSVerse (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). For confocal microscopy, the cell surface staining was the same as that of FCM, and the cells were then stained with DAPI (Roche Diagnostics, Chicago, IL, USA) and were imaged by confocal microscopy (Olympus FV1000).

Quantitative Real-Time RT-PCR

Total RNA was extracted from cultured hMoDCs using Trizol (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to standard protocol. Complementary DNA was prepared with PrimeScript RT Master Mix (Vazyme, China), and the following qRT-PCR was carried out using AceQ qPCR SYBR Green Master Mix (Vazyme) according to the manufacturer's instructions. Gene expression was determined using the ABI Prism 7300 detection system (Applied Biosystems, Foster City, CA, USA). Results were normalized to β-actin and evaluated using the ΔΔCT method to calculate the fold increase. Primers used for qPCR were obtained from Realgene (China), and sequences were given in online suppl. Table 1.

Mice and Treatment

Balb/c mice were purchased from the Vital River Co. (Import commission agent of Charles River Laboratories, Beijing, China). All the mice were kept on a 12-h Light/dark cycle under specific pathogen-free conditions. Mice were immunized by injection subcutaneously with OVA, PLGA NP, OVA-PLGA NP, or OVA-mannan-PLGA NP once a week for 8 weeks. The dose for OVA was 100 µg, and OVA-PLGA NP and OVA-mannan-PLGA NP containing the same dose of OVA were used. Blood was taken from the tail vein and subjected for OVA-specific IgE, IgG1, and IgG2a analysis by ELISA. Single-cell suspension was prepared from the spleen according to conventional protocols. The splenic CD4⁺CD25^{high}FOXP3⁺ Treg cells in the immunized mice were analyzed by FCM as described above. Splenocytes were cultured in RPMI-1640 containing 10% FBS and were stimulated with 100 µg/mL OVA, and the cytokines (IL-4, IFN-γ, and IL-10) production in 48-h culture supernatants were measured using ELISA kits (Abcam, UK). For intervention experiment, after 8 weeks' vaccination with OVA, PLGA NP, OVA-PLGA NP, or OVA-mannan-PLGA NP as described above, the mice were intraperitoneally sensitized with OVA (100 µg) mixed with Al (OH)₃ weekly for 3 weeks. Blood was taken from the tail vein and subjected for OVA-specific IgE,

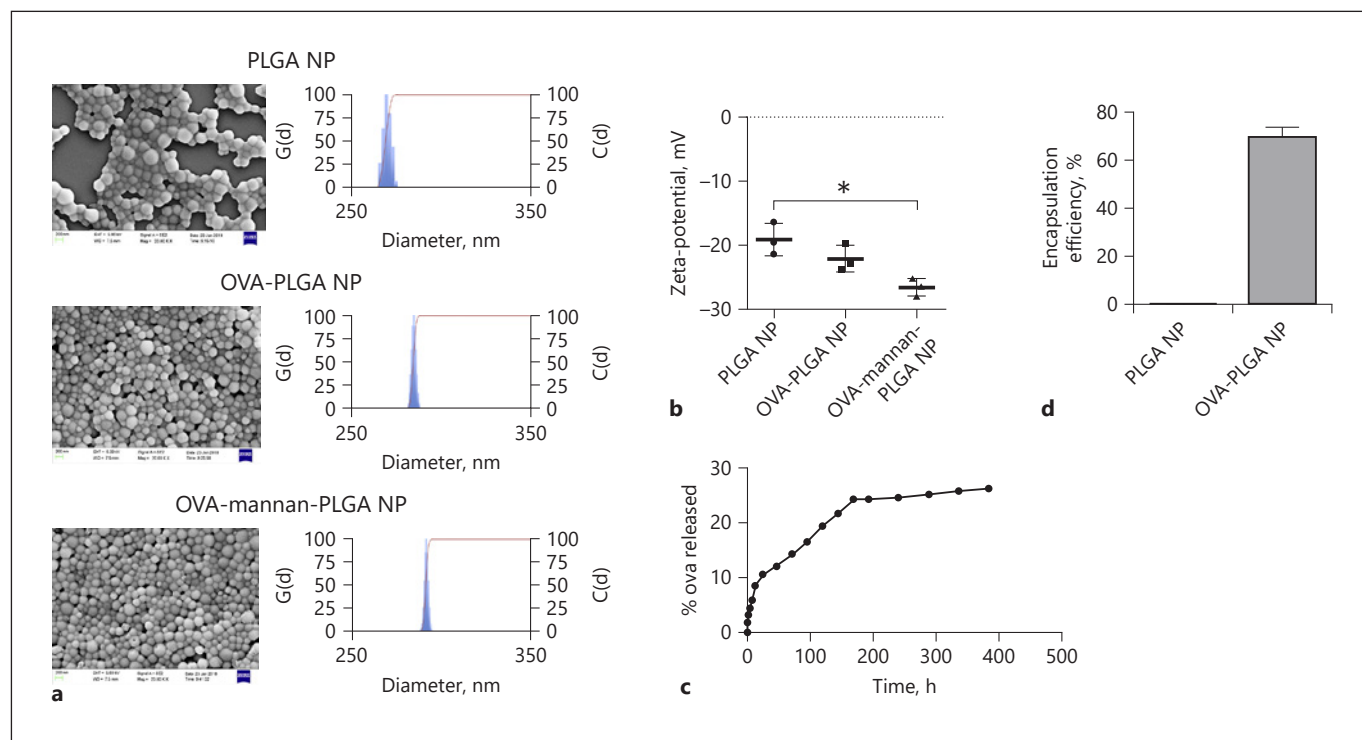


Fig. 1. Characterization of PLGA NP, OVA-PLGA NP, and OVA-mannan-PLGA NP. **a** Images of scanning electron microscopy and particle size distribution. **b** Zeta potential. Data are shown as mean $\pm$ SD. **c** Release curve of OVA from OVA-PLGA NP. **d** Encapsulation efficiency of OVA-PLGA NP. Data are representatives of at least 3 independent experiments. PLGA, polylactic-co-glycolic acid; OVA, ovalbumin; NP, nanoparticle.

IgG1, and IgG2a analysis by ELISA. The sensitized mice were challenged intraperitoneally with 5 mg of OVA 2 weeks post the last intraperitoneal sensitization. Changes in the animals' core body temperature were monitored for the ensuing 60 min.

ELISA for Serum OVA-Specific Immunoglobulin

For detection of OVA-specific IgE, a capture ELISA was applied. Briefly, the microplates were coated with purified rat-anti-mouse IgE (2 μ g/mL; BD Pharmingen), then blocked with 10% FBS, followed by incubation with diluted sera for 2 h at room temperature. Biotin-labeled OVA (Invitrogen) was added afterward, and then the microplates were incubated with avidin-HRP. OVA-specific IgE (2C6, Bio-rad) was used for the standard curve. For the detection of OVA-specific IgG1 or IgG2a, the microplates were coated with OVA (10 μ g/mL) (Sigma-Aldrich), and after incubation with diluted serum, rat-anti-mouse IgG1, or IgG2a (BD Pharmingen) were added.

Statistical Analysis

Statistical comparisons among different groups were analyzed with ANOVA using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Quantitative data were presented as mean $\pm$ SD. p value of <0.05 was considered statistically significant.

Data Availability

The authors declare that all other relevant data supporting the findings of the study are available in this article and its online suppl. files or from the corresponding author upon request.

Results

Characterization of NPs

We first characterized the morphology of PLGA NP, OVA-PLGA NP, and OVA-mannan-PLGA NP using scanning electron microscopy. The result showed that most of the NPs adopted a quasi-spherical structure with little to no aggregation, and the mean diameter of PLGA NP, OVA-PLGA NP, and OVA-mannan-PLGA NP was 267.3 ± 1.1 , 272 ± 15.7 , and 288.5 ± 8.2 nm, respectively (diagram showing the size distribution was in Fig. 1a). Surface charge of PLGA NP, OVA-PLGA NP, and OVA-mannan-PLGA NP was assessed, and the mean zeta potential was around -20 mV for all 3 types of NPs (Fig. 1b). Additionally, the loading efficiency of OVA was around 65% (Fig. 1c). We then quantitatively measured the cumulative release profile of OVA by BCA assay, and as shown in Figure 1d, the OVA as payload was liberated continuously from PLGA in medium bearing a pH around 7.0 at 37°C , in which the physiological condition was mimicked.

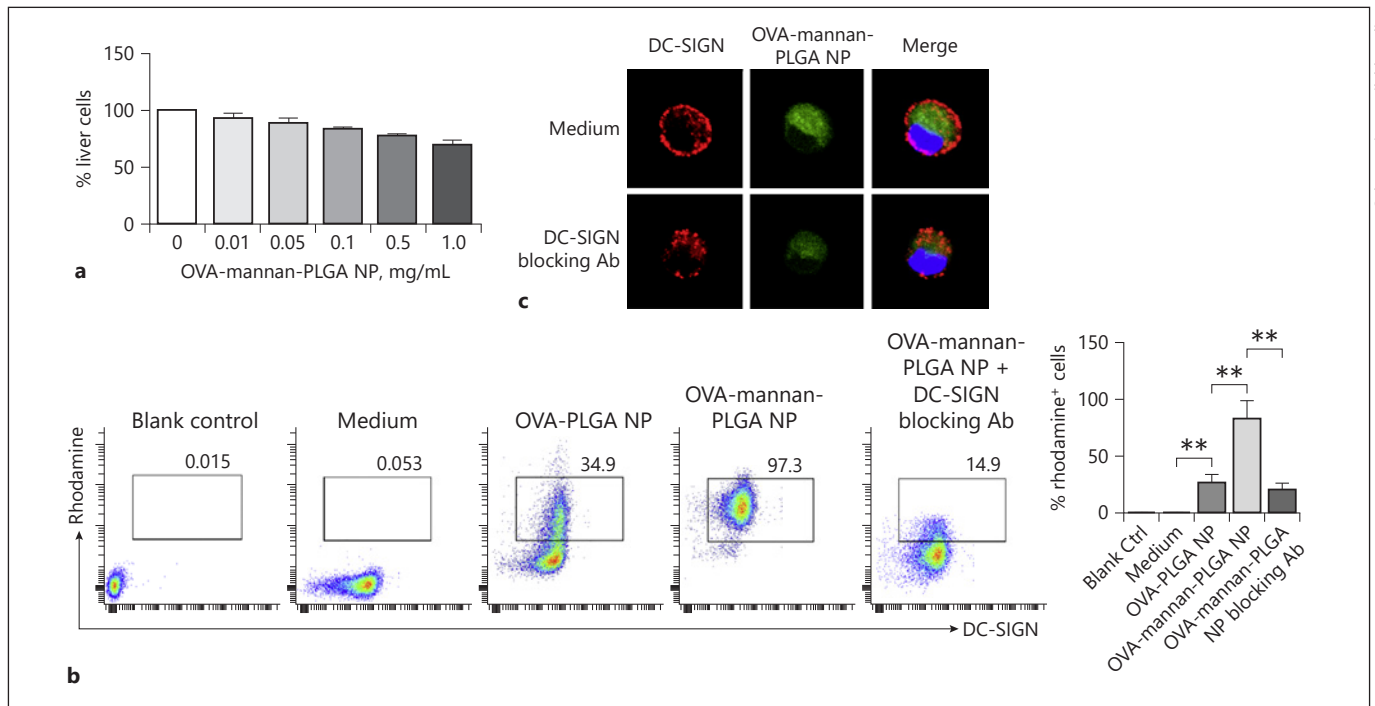


Fig. 2. Enhanced uptake of OVA-mannan-PLGA NP by hMoDCs. **a** Viability of hMoDCs when cocultured with OVA-mannan-PLGA NP. Data are shown as mean $\pm$ SD. **b, c** hMoDCs were incubated with Rhodamine123-labeled OVA-PLGA NP or OVA-mannan-PLGA NP and stained with anti-DC-SIGN antibody. Results of FCM, the statistic data (**b**), and confocal microscopy (**c**)

were shown. Anti-DC-SIGN antibody was used as the blocking antibody. Data are representatives of at least 3 independent experiments. PLGA, polylactic-co-glycolic acid; OVA, ovalbumin; hMoDCs, human monocytes-derived dendritic cells; NP, nanoparticle.

Enhanced Capture of OVA-Mannan-PLGA NP by hMoDCs via DC-SIGN

The possible toxicity of OVA-mannan-PLGA NP on hMoDCs was first evaluated. After incubation of hMoDCs with the NP for 24 h, the cell viability was around 70% even with the high concentration of OVA-mannan-PLGA NP (1 mg/mL) as shown in Figure 2a, indicating that OVA-mannan-PLGA NP was safe and did not have apparent toxicity. We then analyzed the uptake of OVA-PLGA NP and OVA-mannan-PLGA NP by hMoDCs using laser scanning confocal microscopy and FCM. Enhanced uptake of OVA-mannan-PLGA NP by hMoDCs was revealed compared to OVA-PLGA NP (Fig. 2b, c), which met our expectation that the presence of targeting moiety would benefit endocytosis. When DC-SIGN-blocking antibody was used, a nearly 60% reduction in the binding of OVA-mannan-PLGA NP by hMoDCs was observed (Fig. 2b), indicating that DC-SIGN was involved in the uptake of OVA-mannan-PLGA NP by hMoDCs.

Tolerogenic Phenotype of hMoDCs Induced by OVA-Mannan-PLGA NP

The immunoregulatory function of the NPs was then analyzed. The hMoDCs incubated with OVA-mannan-PLGA NP expressed decreased surface levels of CD86, CD83, and HLA-DR compared with those treated with OVA-PLGA NP, PLGA NP, and OVA (Fig. 3a; online suppl. Fig. 3). And increased production of IL-10 and TNF- α and decreased production of IL-6 were observed in the hMoDCs treated with OVA-mannan-PLGA NP compared with those treated with OVA or OVA-PLGA NP, as revealed by the results of ELISA or qRT-PCR (Fig. 3b, c). OVA-mannan-PLGA NP also induced increased expression of PD-L1 in hMoDCs (Fig. 3a, c). These data indicated that OVA-mannan-PLGA NP was able to induce tolerogenic hMoDCs.

Increased Generation of Foxp3⁺ Treg Cells by hMoDCs Treated with OVA-Mannan-PLGA NP

We next analyzed the polarization of allogenic naive CD4⁺ T cells cocultured with NP-treated hMoDCs. After

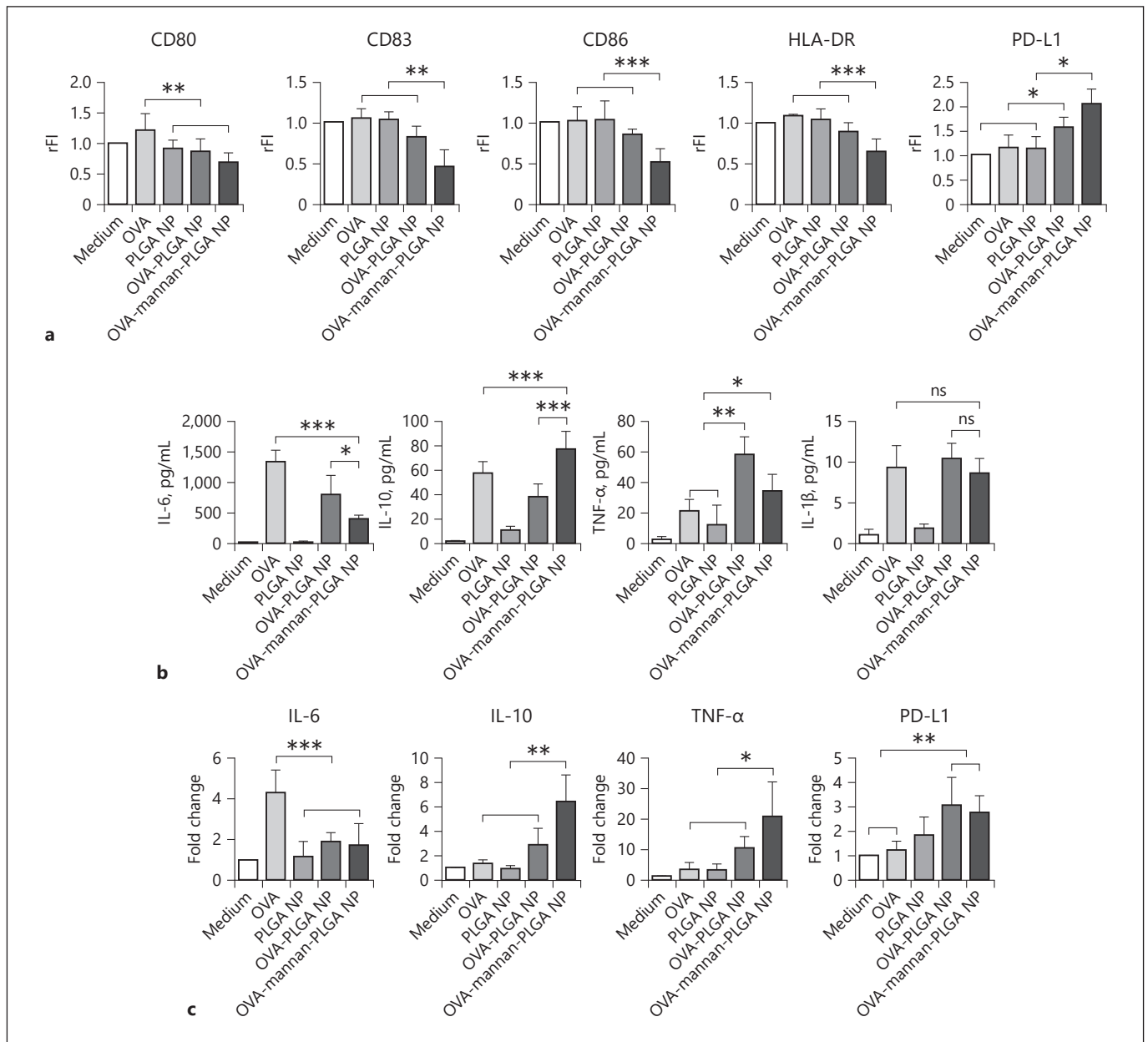


Fig. 3. Tolerogenic phenotype of hMoDCs induced by OVA-mannan-PLGA NP. Different NPs were incubated with hMoDCs in vitro for 24 h, and the expression of co-stimulatory molecules and PD-L1 (relative mean fluorescence intensity, rFI) were shown in (a), and production of various cytokines at the protein level and mRNA level was shown in (b, c), respectively. Data are shown as

mean $\pm$ SD and are representatives of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. PLGA, polylactic-co-glycolic acid; OVA, ovalbumin; hMoDCs, human monocytes-derived dendritic cells; PD-L1, programmed cell death protein ligand 1; NP, nanoparticle.

6 days of culture, there was no difference in the numbers of Th1, Th17, or Th22 cells among different groups (data not shown); while the number of IL-4-producing T cells was significantly higher in the group of OVA-treated hMoDCs than that of other groups (Fig. 4a, c). Increased

number of CD4⁺CD25^{high}FOXP3⁺ Treg cells was found in the group of hMoDCs treated with OVA-PLGA NP or OVA-mannan-PLGA NP compared with the groups of hMoDCs treated with OVA or medium control (Fig. 4b, d), and the OVA-mannan-PLGA NP group showed the

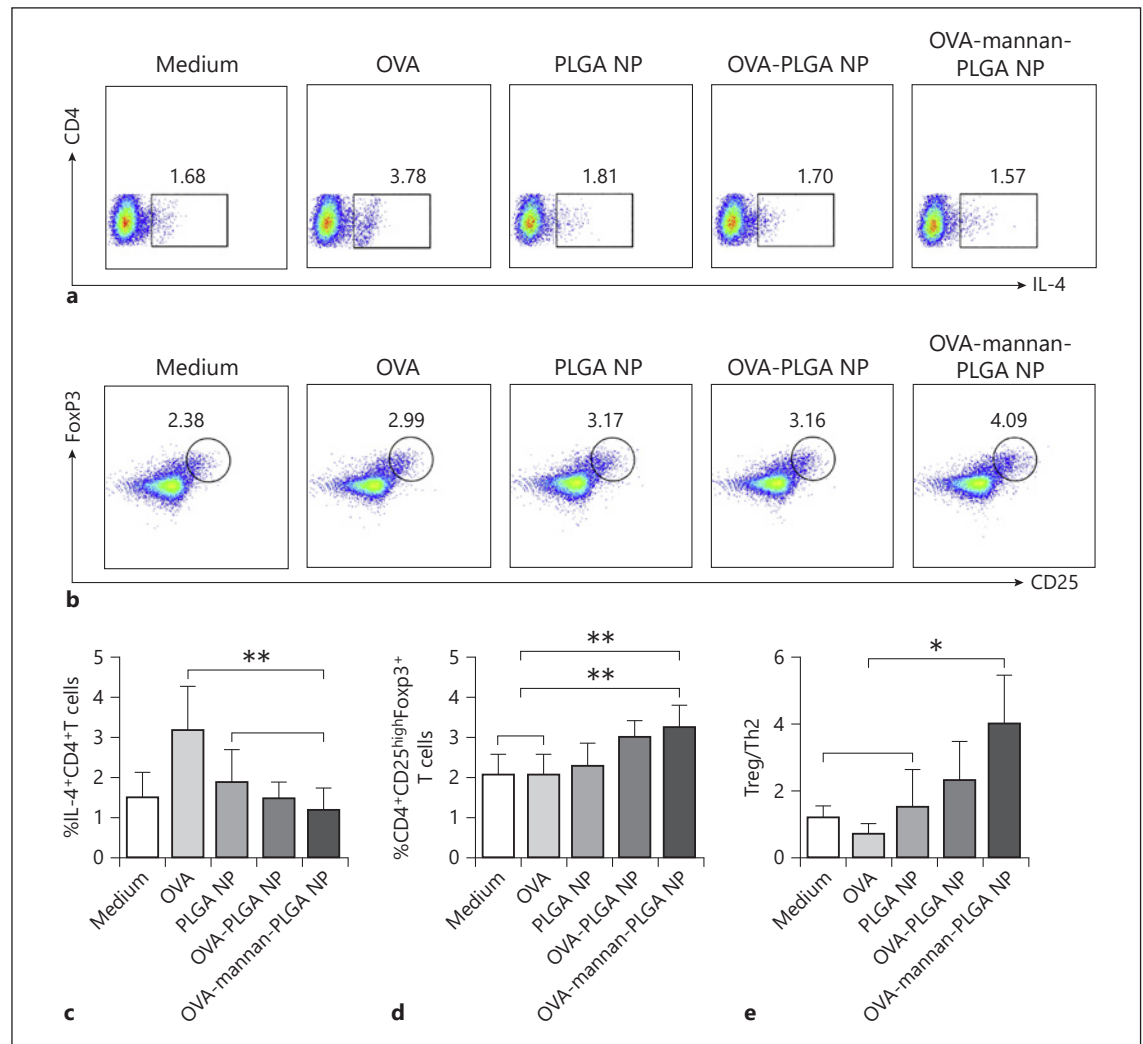


Fig. 4. Increased generation of Foxp3⁺ Treg cells induced by OVA-mannan-PLGA NP. CD4⁺ naive T cells purified from PBMC were cocultured with the hMoDCs that had been stimulated with various NPs and subjected to intracellular staining and FCM. The results of FCM were shown in (a, b), and the statistical results were

shown in (c, d). The ratio of Treg/Th2 was shown in (e). Data are representatives at least 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. PLGA, polylactic-co-glycolic acid; OVA, ovalbumin; hMoDCs, human monocytes-derived dendritic cells; Treg, regulatory T; NP, nanoparticle.

highest number of Treg cells. The ratio of Treg/Th2 cells in the OVA-mannan-PLGA NP group was also higher than in other groups. These data suggested that the hMoDCs treated with OVA-loaded PLGA NP not only inhibited the polarization of Th2 cells but also promoted the differentiation of Treg cells, and OVA-mannan-PLGA NP-treated hMoDCs had stronger tolerogenic effects.

Tolerogenic Immune Responses in Mice Induced by OVA-Mannan-PLGA NP Immunization

Balb/c mice were immunized subcutaneously with the NPs to assess their immunoregulatory effects in vivo. In-

creased number of splenic CD4⁺CD25^{high}FOXP3⁺ Treg cells was revealed in the mice immunized with OVA-mannan-PLGA NP compared with those in the mice immunized with OVA-PLGA NP, PLGA-NP, or OVA (Fig. 5a, b). The mice immunized with OVA-mannan-PLGA NP did not show increased serum levels of OVA-specific IgE, IgG1, or IgG2a as the mice immunized with OVA, and there was also no increase in the serum antibodies levels in the mice immunized with OVA-PLGA NP or PLGA-NP (Fig. 5c). The splenocytes from the immunized mice were then cultured in vitro with OVA, and the supernatant was analyzed for the production of IFN- γ ,

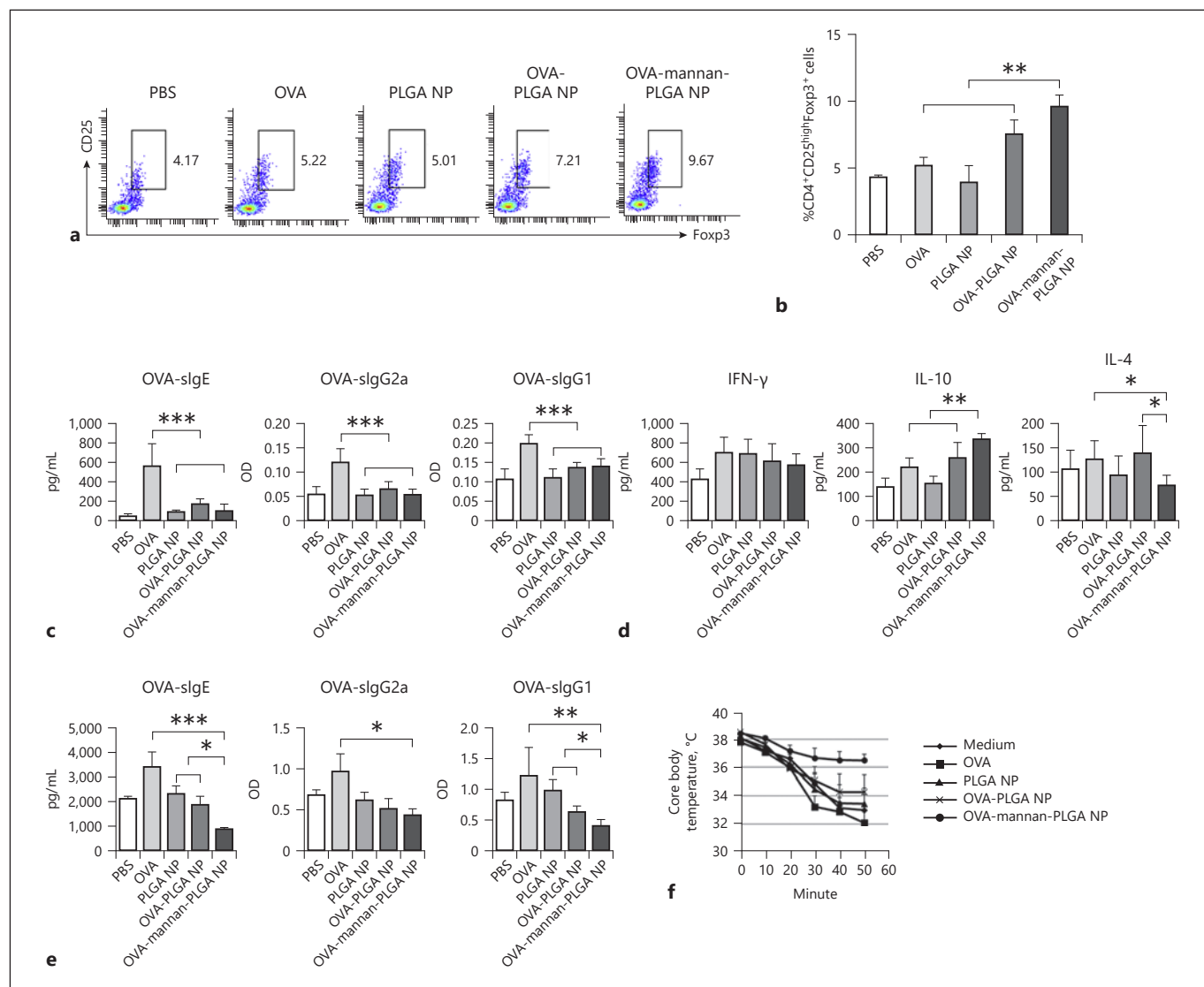


Fig. 5. Humoral and cellular immune responses in the mice immunized with OVA-mannan-PLGA NP. **a–d** Balb/c mice were immunized subcutaneously with different NPs weekly for 8 weeks. The numbers of CD4⁺CD25^{high}Foxp3⁺ Treg cells in the spleens were analyzed by FCM (**a**, **b**); the serum levels of OVA-specific antibodies were analyzed by ELISA (**c**); the production of cytokines in vitro culture of splenocytes from the immunized mice was analyzed by ELISA (**d**). **e–f** Balb/c mice were immunized with the NPs weekly for 8 weeks as above and were then intraperitoneally

sensitized with OVA plus Al(OH)₃ weekly for 3 weeks, and the serum levels of OVA-specific IgE, IgG1, and IgG2a were analyzed by ELISA (**e**). The sensitized mice were challenged intraperitoneally with OVA, and changes in the animals' core body temperature were monitored (**f**). Data are shown as mean $\pm$ SD and are representatives at least 3 independent experiments. $n = 5$ per group. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. PLGA, polylactic-co-glycolic acid; OVA, ovalbumin; Treg, regulatory T; NP, nanoparticle.

IL-10, and IL-4. The result showed that the level of IL-10 was significantly higher, while IL-4 was significantly lower, in the supernatant of splenocytes from the mice immunized with OVA-mannan-PLGA NP than those from the mice immunized with OVA-PLGA NP, PLGA-NP, or OVA (Fig. 5d). Moreover, effects of the NPs immunization on subsequent OVA sensitization were analyzed.

The mice were immunized with the NPs for 8 weeks and then were intraperitoneally sensitized with OVA weekly for 3 weeks. The serum levels of OVA-specific IgE, IgG1, and IgG2a in the mice pre-immunized with OVA-mannan-PLGA NP group were significantly lower than those from the mice pre-immunized with other NPs and OVA (Fig. 5e), indicating that immunization with OVA-man-

nan-PLGA NP significantly inhibited the subsequent OVA sensitization, which was further confirmed by the challenge experiment through intraperitoneal injection with OVA (Fig. 5f).

Discussion

The population suffering from allergic diseases has been increasing dramatically worldwide during the past several decades, and AIT represents the only curative therapy for allergy; however, the overall efficacy for AIT is not high, which is mainly due to the weak immune responses being provoked. Induction of Treg is one of the major mechanisms for AIT, and generation of tolerogenic DCs via allergen modification [15–18] is a promising strategy for immune regulation of allergy. In the present study, we constructed OVA-loaded PLGA NP coupling with native mannan and aimed to target DCs by binding DC-SIGN to induce tolerogenic responses. Our results showed that OVA-mannan-PLGA NP was taken up more efficiently by hMoDCs via DC-SIGN, and a tolerogenic phenotype of hMoDCs was induced subsequently. Increased production of Tregs was found in both in vitro coculture with hMoDCs treated with OVA-mannan-PLGA NP and in vivo mouse model immunized with OVA-mannan-PLGA NP. The well-designed NP in our study demonstrated enhanced ability for tolerance induction, which could be developed as a novel vaccine for AIT.

Various approaches have been pursued to improve the efficacy of AIT, which include utilization of adjuvants, conjugation with monoclonal antibody, construction of hypoallergenic variants, etc. [19] Encapsulation of allergens into NP is one of the most important strategies to improve the efficacy of AIT [20]. NP can serve as a carrier or adjuvant for allergen, protect allergen from degradation, and facilitate the delivery of the allergen to target cells [21]. NP-coupled allergen can achieve similar efficacy with lower doses of allergen compared with conventional allergen [22]. Moreover, NP promote the uptake of allergen by antigen-presenting cells, avoiding the activation of basophils or mast cells induced by the crosslinking of FcεRI by IgE-allergen, and therefore decrease the risk of undesirable side effects [23]. Among the numerous nanomaterials, PLGA has obtained the Food and Drug Administration approval for human usage. PLGA not only protects allergen from degradation but also demonstrates nonspecific degradation behavior upon hydrolysis or enzymatic degradation, permitting the sustained release of allergen, which would improve the efficacy of AIT

[24, 25]. In the present study, we used PLGA as a carrier for OVA, and found that OVA was released from the carrier gradually, and the peak of the release was on day 15. To increase the targeting efficiency, OVA-PLGA NP was conjugated with mannan, a specific ligand of CLRs expressing on DCs, and enhanced uptake of the mannan-conjugated PLGA-OVA NP by hMoDCs was observed, compared to OVA-PLGA NP. The PLGA NP and conjugation with mannan have been utilized in infectious diseases and autoimmune diseases, and our study set up a platform for the treatment of allergy by combining these 2 strategies for the first time.

DCs play a crucial role in linking the innate and adaptive immunity, and hence, DCs-targeting has been attracting great interests for improvement of the effectiveness of vaccines against pathogens or allergens [5, 26]. DCs not only induce immunogenic responses but also maintain immune homeostasis [27]. DCs can display a semi-mature phenotype and exert tolerogenic function in the absence of co-stimulatory signals, inducing the production of CD4⁺CD25⁺ Foxp3⁺ Treg cells [28]. Tolerogenic DCs can also produce IL-10 to expand allergen-specific type 1 Treg (Tr1) cells [29, 30], and PD-L1 expression on DCs induces various types of functional Treg cells [31, 32]. Studies have proved that IL-10-producing DCs induce allergen-specific Treg cells that suppress the proliferation of Th2 cells and the inflammatory cytokine production by Th2 cells from house dust mite-allergic patients [33–35]. In our study, tolerogenic properties of OVA-mannan-PLGA NP were demonstrated, which may due to the mannan-CLRs mediated targeting. Previous studies have shown that oral administration with mannose-rich allergen induces immune tolerance through SIGNR1 expressing on DCs of the oral lamina propria, resulting in IL-10 production and Treg cells differentiation [36]. It is also reported that allergoids coupled to mannan enhance allergen uptake and promote the generation of functional FOXP3⁺ Treg cells through PD-L1 in human subjects [5]. In our study, OVA-mannan-PLGA NP, but not OVA-PLGA or PLGA NP, induced tolerogenic hMoDCs with semi-mature phenotype manifested by increased expression of IL-10 and PD-L1, indicates that mannan mediates the tolerogenic effects.

Collectively, our data strongly suggest that OVA-mannan-PLGA NP well represent next-generation vaccines that could enhance the allergen uptake by DCs and induce immune tolerance to allergens and would be useful in subcutaneous immunotherapy for both food and airborne allergen. It meets several desirable requirements for an ideal allergy vaccine: stable protein-based antigen,

stable and sustained allergen release, induction of tolerogenic DCs and Tregs, and inhibition of Th2 response. Targeting of DCs by PLGA-encapsulated allergen coupled to native mannan represents novel suitable preparations to formulate vaccines that might promote the efficacy of AIT.

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Statement of Ethics

The study was approved by the Ethics Committee of the Institute of Dermatology in Chinese Academy of Medical Sciences (2015-KY-009), and written informed consent was obtained from the participants (or their parents or guardians). The animal ex-

periments were performed in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals of the Institute of Dermatology in Chinese Academy of Medical Sciences, which conform to internationally accepted standards.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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Author Contributions

H. Wen, L. Qu, Y. Zhang, B. Xu, S. Ling, X. Liu, and Y. Luo performed the experiments; X. Yao, D. Huo, and W. Li designed the experiments and wrote the manuscript; and X. Yao designed the experiments and coordinated the research.

References

- Pfaar O, Lou H, Zhang Y, Klimek L, Zhang L. Recent developments and highlights in allergen immunotherapy. *Allergy*. 2018;73(12):2274–89.
- Komlosi ZI, Kovacs N, Sokolowska M, van de Veen W, Akdis M, Akdis CA. Highlights of novel vaccination strategies in allergen immunotherapy. *Immunol Allergy Clin North Am*. 2020;40(1):15–24.
- Soria I, López-Relaño J, Viñuela M, Tudela JL, Angelina A, Benito-Villalvilla C, et al. Oral myeloid cells uptake allergoids coupled to mannan driving Th1/Treg responses upon sublingual delivery in mice. *Allergy*. 2018;73(4):875–84.
- Yu S, Han B, Liu S, Wang H, Zhuang W, Huang Y, et al. Derp1-modified dendritic cells attenuate allergic inflammation by regulating the development of Th1/Th2 cells and regulatory T cells in a murine model of allergic rhinitis. *Mol Immunol*. 2017;90:172–81.
- Sirvent S, Soria I, Cirauqui C, Cases B, Manzano AI, Díez-Rivero CM, et al. 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. *J Allergy Clin Immunol*. 2016;138(2):558–e11.
- Daneshmandi S, Nourizadeh M, Pourpak Z, Pourfathollah AA. Eliciting Th1 immune response using casein- (Alpha S1) loaded dendritic cells. *Iran J Allergy Asthma Immunol*. 2017;16(2):159–68.
- Lepenes B, Lee J, Sonkaria S. Targeting C-type lectin receptors with multivalent carbohydrate ligands. *Adv Drug Deliv Rev*. 2013;65(9):1271–81.
- Unger WW, van Kooyk Y. “Dressed for success” C-type lectin receptors for the delivery of glyco-vaccines to dendritic cells. *Curr Opin Immunol*. 2011;23(1):131–7.
- van Kooyk Y, Unger WW, Fehres CM, Kalay H, García-Vallejo JJ. Glycan-based DC-SIGN targeting vaccines to enhance antigen cross-presentation. *Mol Immunol*. 2013;55(2):143–5.
- Tada H, Nemoto E, Shimauchi H, Watanabe T, Mikami T, Matsumoto T, et al. Saccharomyces cerevisiae- and Candida albicans-derived mannan induced production of tumor necrosis factor alpha by human monocytes in a CD14- and Toll-like receptor 4-dependent manner. *Microbiol Immunol*. 2002;46(7):503–12.
- Apostolopoulos V, Thalhhammer T, Tzakos AG, Stojanovska L. Targeting antigens to dendritic cell receptors for vaccine development. *J Drug Deliv*. 2013;2013:869718.
- Salazar F, Sewell HF, Shakib F, Ghaemmaghami AM. The role of lectins in allergic sensitization and allergic disease. *J Allergy Clin Immunol*. 2013;132(1):27–36.
- De Souza RJ, Esparza I, Ferrer M, Sanz ML, Irache JM, Gamazo C. Nanoparticulate adjuvants and delivery systems for allergen immunotherapy. *J Biomed Biotechnol*. 2012;2012:474605.
- Mittal A, Raber AS, Schaefer UF, Weissmann S, Ebensen T, Schulze K, et al. Non-invasive delivery of nanoparticles to hair follicles: a perspective for transcutaneous immunization. *Vaccine*. 2013;31(34):3442–51.
- Starchenka S, Heath MD, Lineberry A, Higenbottam T, Skinner MA. Transcriptome analysis and safety profile of the early-phase clinical response to an adjuvanted grass allergoid immunotherapy. *World Allergy Organ J*. 2019;12(11):100087.
- Ellis AK, Frankish CW, O’Hehir RE, Armstrong K, Steacy L, Larché M, et al. Treatment with grass allergen peptides improves symptoms of grass pollen-induced allergic rhinoconjunctivitis. *J Allergy Clin Immunol*. 2017;140(2):486–96.
- Benito-Villalvilla C, Soria I, Subiza JL, Palomares O. Novel vaccines targeting dendritic cells by coupling allergoids to mannan. *Allergy J Int*. 2018;27(8):256–62.

- 18 Samadi N, Klems M, Heiden D, Bauer R, Kitzmüller C, Weidmann E, et al. Nitrated food proteins induce a regulatory immune response associated with allergy prevention after oral exposure in a Balb/c mouse food allergy model. *Allergy*. 2020 Feb;75(2):412–22.
- 19 Gunawardana NC, Durham SR. New approaches to allergen immunotherapy. *Ann Allergy Asthma Immunol*. 2018;121(3):293–305.
- 20 Pohlitz H, Bellinghausen I, Frey H, Saloga J. Recent advances in the use of nanoparticles for allergen-specific immunotherapy. *Allergy*. 2017;72(10):1461–74.
- 21 Di Felice G, Barletta B, Bonura A, Butteroni C, Corinti S, Colombo P. Nanoparticles adjuvants in allergology: new challenges and pitfalls. *Curr Pharm Des*. 2015;21(29):4229–39.
- 22 Scholl I, Weissenböck A, Forster-Waldl E, Untersmayr E, Walter F, Willheim M, et al. Allergen-loaded biodegradable poly(D,L-lactic-co-glycolic) acid nanoparticles down-regulate an ongoing Th2 response in the BALB/c mouse model. *Clin Exp Allergy*. 2004;34(2):315–21.
- 23 Hayen SM, Kostadinova AI, Garssen J, Otten HG, Willemsen LE. Novel immunotherapy approaches to food allergy. *Curr Opin Allergy Clin Immunol*. 2014;14(6):549–56.
- 24 Pohlitz H, Frey H, Saloga J. Could allergen-specific immunotherapy benefit from the use of nanocarriers? *Nanomedicine*. 2016;11(11):1329–31.
- 25 Chereddy KK, Vandermeulen G, Preat V. PLGA based drug delivery systems: promising carriers for wound healing activity. *Wound Repair Regen*. 2016;24(2):223–36.
- 26 Appay V, Douek DC, Price DA. CD8+ T cell efficacy in vaccination and disease. *Nat Med*. 2008;14(6):623–8.
- 27 Steinman RM, Banchereau J. Taking dendritic cells into medicine. *Nature*. 2007;449(7161):419–26.
- 28 Raich-Regue D, Glancy M, Thomson AW. Regulatory dendritic cell therapy: from rodents to clinical application. *Immunol Lett*. 2014;161(2):216–21.
- 29 Gentile DA, Schreiber R, Howe-Adams J, Trecki J, Patel A, Angelini B, et al. Diminished dendritic cell interleukin 10 production in atopic children. *Ann Allergy Asthma Immunol*. 2004;92(5):538–44.
- 30 Escobar A, Aguirre A, Guzmán MA, González R, Catalán D, Acuña-Castillo C, et al. Tolerogenic dendritic cells derived from donors with natural rubber latex allergy modulate allergen-specific T-cell responses and IgE production. *PLoS One*. 2014;9(1):e85930.
- 31 Gollwitzer ES, Saglani S, Trompette A, Yadava K, Sherburn R, McCoy KD, et al. Lung microbiota promotes tolerance to allergens in neonates via PD-L1. *Nat Med*. 2014;20(6):642–7.
- 32 Rabe H, Nordstrom I, Andersson K, Lundell AC, Rudin A. Staphylococcus aureus convert neonatal conventional CD4(+) T cells into FOXP3(+) CD25(+) CD127(low) T cells via the PD-1/PD-L1 axis[J]. *Immunology*. 2014;141(3):467–81.
- 33 Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol*. 2003;111(6):1255–61.
- 34 Mahnke K, Ring S, Bedke T, Karakhanova S, Enk A. Interaction of regulatory T cells with antigen-presenting cells in health and disease. *Chem Immunol Allergy*. 2008;94:29–39.
- 35 Pacciani V, Gregori S, Chini L, Corrente S, Chianca M, Moschese V, et al. Induction of anergic allergen-specific suppressor T cells using tolerogenic dendritic cells derived from children with allergies to house dust mites. *J Allergy Clin Immunol*. 2010;125(3):727–36.
- 36 Zhou Y, Kawasaki H, Hsu SC, Lee RT, Yao X, Plunkett B, et al. Oral tolerance to food-induced systemic anaphylaxis mediated by the C-type lectin SIGNR1. *Nat Med*. 2010;16(10):1128–33.

Supplementary table 1: Sequences of qRT-PCR primers

Gene name	Primer sequences
β -actin	F: TCTGGCACCACACCTTCTA R: AGGCATACAGGGACAGCAC
IL-4	F: ATGGGTCTCACCTCCCAACT R: GATGTCTGTTACGGTCAACTCG
IL-10	F: GACTTTAAGGGTTACCTGGGTTG R: TCACATGCGCCTTGATGTCTG
PD-L1	F: TGGCATTGCTGAACGCATTT R: TGCAGCCAGGTCTAATTGTTTT
IL-6	F: ACTCACCTCTTCAGAACGAATTG R: CCATCTTTGGAAGGTTCAAGTTG
TNF-a	CCTCTCTCTAATCAGCCCTCTG GAGGACCTGGGAGTAGATGAG

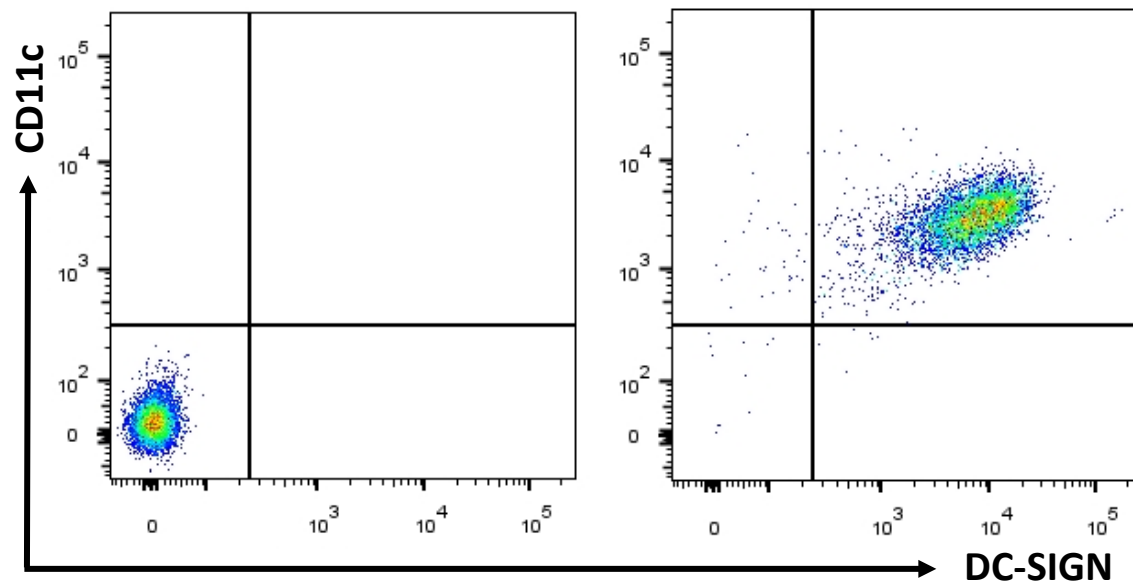
SUPPLEMENTARY FIGURE LEGENDS:

Supplementary Fig. 1: Analysis of hMoDCs by flow cytometry. CD14⁺ monocytes were purified from PBMC by magnetic beads, and were stimulated with recombinant human GM-CSF (100 ng/mL) and IL-4 (20 ng/mL) for 5 days, and the expression of specific surface markers was analysed by flow cytometry. CD14⁺ cells were gated and shown on CD11c and DC-SIGN plots.

Supplementary Fig. 2: Proliferation of T cells in co-culture with hMoDCs. Naïve T cells were stained with CFSE, and co-cultured with NPs-stimulated hMoDCs for 5 days. The cells were then labeled with anti-CD4 antibody and subjected for flow cytometry.

Supplementary Fig. 3: Tolerogenic phenotype of hMoDCs induced by OVA-mannan-PLGA NP. Different NPs were incubated with hMoDCs *in vitro* for 24 hours, and the expression of co-stimulatory molecules (histogram) were shown.

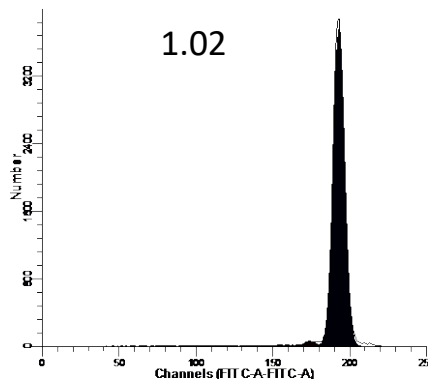
Supplementary Fig. 1



Supplementary Fig. 2

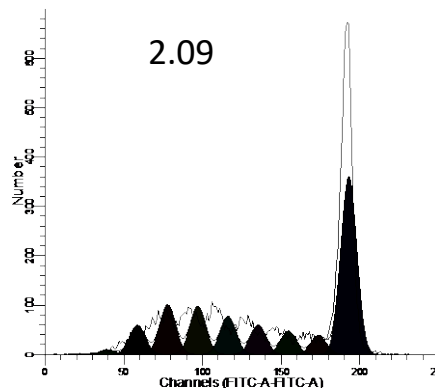
BLANK

1.02



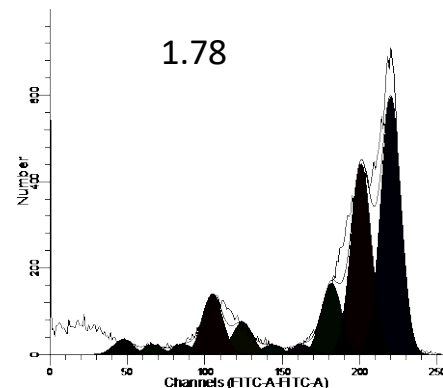
OVA

2.09



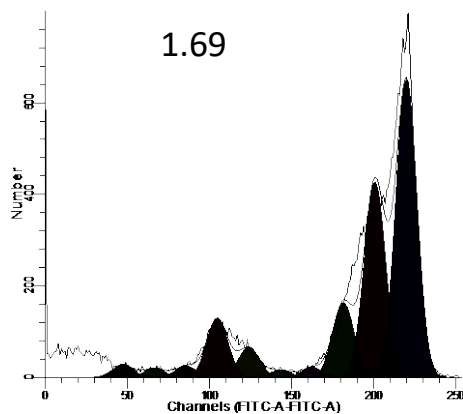
PLGA NP

1.78



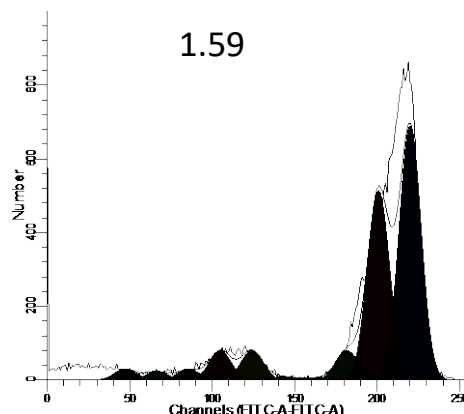
OVA-PLGA NP

1.69



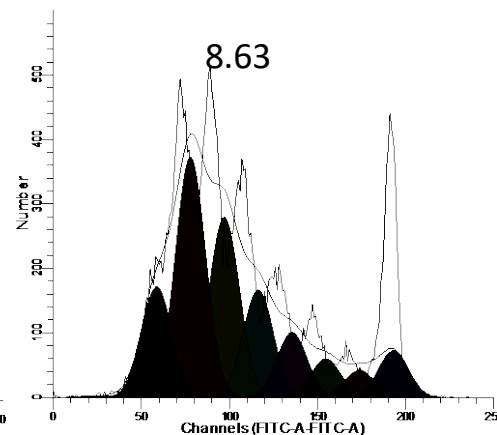
OVA-mannan-PLGA NP

1.59



PHA

8.63



Supplementary Fig. 3

