

Comparison of Glycated Ovalbumin–Monosaccharides in the Attenuation of Ovalbumin-Induced Allergic Response in a BALB/C Mouse Model

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Supporting Information

ABSTRACT: The aim of the present study was to compare various glycated ovalbumin (OVA)–monosaccharides, including OVA–mannose (Man), –glucose, –ribose, and –fructose, in the attenuation of OVA-induced allergic response in a BALB/C mouse model and the potential mechanisms of immunological modulation. The glycated OVA forms were prepared by Maillard reactions. OVA-Man significantly reduced the frequency of allergic signs. Mouse mast cell protease enzyme concentration was significantly reduced in the OVA-Man group (549.80 ± 84.67 ng/mL, $p < 0.05$). The OVA-Man group also had a lower histamine concentration (30.96 ± 1.12 ng/mL) as compared with the positive control OVA group (44.43 ± 0.71 ng/mL, $p < 0.05$). Both specific IgG and IgE were significantly reduced in the OVA-Man-treated group ($p < 0.05$). The OVA-Man group exhibited decreased concentrations of IL-4 (67.98 ± 3.11 pg/mL) and IL-17 (67.98 ± 3.11 pg/mL) and an increased concentration of IL-12 (336.70 ± 18.69 pg/mL, $p < 0.05$) compared with the positive control. Mannosylation played a vital role in allergen recognition, implicating deleterious downstream Th2 cell activation, cytokine secretion, and IgE production. This result indicates that different glycans target specific DC receptors, and differential DC processing, antigen presentation, and T cell response leads to altered variation in allergic response. OVA-Man exhibited minimal DC internalization, DC processing, MHC antigen presentation, and antigen-specific T cell activation, resulting in an attenuated allergic response and validating its efficacy as a potential immunotherapeutic candidate to treat egg allergy.

KEYWORDS: ovalbumin, glycation, dendritic cells, mannose receptor, MHC class II

INTRODUCTION

Food allergy is mainly induced by glycosylated proteins eliciting type 2 immune response. Dendritic cells (DCs) are the major antigen presenting cells, acting as a backbone for the immune system and playing a major role in re-elicitation and induction of type 2 mediated inflammation in allergic diseases.¹ DCs are able to recognize antigens at the site of entry through various pathogen associated molecular patterns, such as toll-like receptors, C-type lectin receptors, and others. Depending upon the stimulus encountered, DCs can alter naive T helper (Th) cells, differentiating them into functionally distinct subsets: Th1, Th2, or Th17 effector T cells or regulatory T cells (Tregs). A strategy involving targeting the antigens encountered by DCs in vivo has been proposed in the past.²

The mannose receptor (MR) is a C-type lectin expressed by DCs.³ MRs fall under the pattern recognition molecules that link innate and adaptive immunity and recognize a wide range of both endogenous and exogenous ligands.⁴ There is abundant expression of MRs on monocyte derived DCs, which may confirm that MRs act as antigen recognition tools. It was shown that MRs contribute to the generation of multiple HLA-restricted, antigen-specific T cell responses.⁵ Several other studies have implicated a role for MRs in the modulation of DC function and allergen recognition.^{6–8} MR was demonstrated to be the main receptor on DCs for a number of glycated allergens from diverse sources and was shown to play a key role in Th2 cell polarization.⁹ Recently carbohydrate moieties on allergens were shown to have a vital role in recognition by innate immune

cells, implicating downstream deleterious Th2 cell activation and IgE production.¹⁰

Ovalbumin (OVA), a major egg allergen, constitutes 54% of the total egg white protein. The OVA protein is made up of 385 amino acids and has a molecular mass of 45 kDa. We have recently demonstrated that mannosylated OVA attenuates clinical signs of allergy in mice via DC maturation and T cell activation.¹¹ We then proceeded to define the influence of mannosylation on other common allergens, such as egg white, peanut, and whey protein. It was demonstrated that only egg white had effects similar to mannosylated OVA on allergy reduction.¹²

The glycosylation patterns of allergens play important roles in the allergenicity of proteins, and differential glycosylation patterns may impact antigen uptake, processing, and presentation and the balance between type 1 and 2 immune responses.¹³ Differential receptor binding, either to MR or DC-specific intercellular-adhesion-molecule-3-grabbing nonintegrin (DC-SIGN), on DCs could target allergens to different locations, thereby altering immune responses. Alternative receptors for glycated antigens could be involved in the reduction of allergic responses. To have a better understanding of the roles of other monosaccharides, we examined several commonly encountered

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Experimental Design

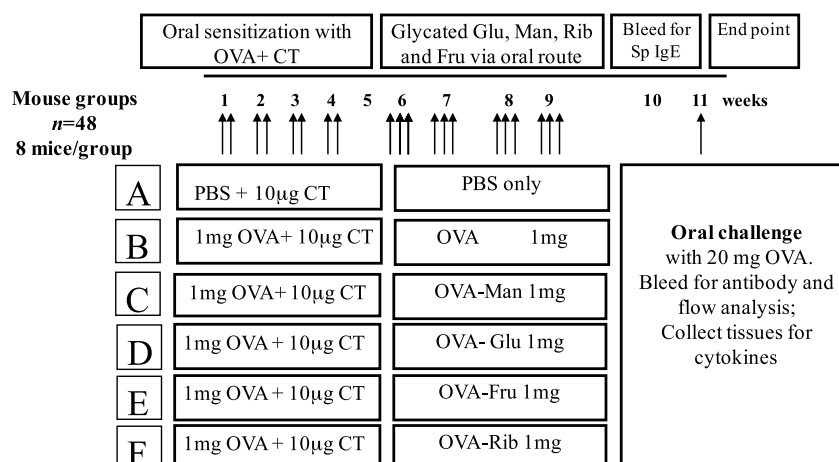


Figure 1. Experimental outline. Mice were sensitized to 1 mg of ovalbumin (OVA) and 10 μg of cholera toxin for 4 weeks, twice per week; this was followed by 4 weeks of oral treatments with glycosylated OVA forms (OVA-Glu, OVA-Man, OVA-Fru, and OVA-Rib), given thrice per week. The negative control group (CT) received phosphate-buffered saline, and the positive control group received OVA. Clinical signs of hypersensitivity were recorded after oral challenge with 20 mg of OVA, and 30 min later, all mice were euthanized for collection of blood and spleen. Both blood and spleen were pooled within treatments after collection ($n = 4$).

monosaccharides, such as glucose, fructose, and ribose, for their glycosylation effects on OVA-induced allergy. It was hypothesized that our prior observation of enhanced type 1 immune response and decreased allergic signs with mannose-ligated OVA would be duplicated, and other monosaccharide conjugates would have similar effects. This study provides the first comparison of various glycosylated OVA–monosaccharides to mannose-ligated OVA with emphasis on the pivotal role of mannose in attenuating allergy. We have demonstrated that glycan modification using mannose to OVA can strongly reduce uptake, lead to less induction of T cells, and lead to a more balanced immune response, thereby reducing allergy. Induction of a balanced immune response validates mannose-ligated OVA as a potential candidate molecule for immunomodulation of allergy.

MATERIALS AND METHODS

Chemicals. Monosaccharides D-mannose, D-glucose, D-fructose, and D-ribose were purchased from Sigma (Oakville, ON, Canada). OVA was isolated in the laboratory and confirmed for its purity (over 98%) using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, Figure S1). All other chemicals used in this work were purchased from Sigma (unless otherwise specified).

OVA–Monosaccharide Conjugation. Maillard reactions were carried out in 1:1 ratios (w/w, OVA/monosaccharide) as described earlier.¹¹ In brief, the monosaccharides were added to OVA (mixed in Milli Q water) and freeze-dried. The mixture was incubated at 55 °C for 3 days (72 h) at 65% relative humidity using saturated potassium iodide solution in a laboratory incubator. As a control, native OVA was heated in a similar manner without the monosaccharide. All samples were dialyzed using 30 kDa molecular cut off dialysis tubing (Fisher Scientific, Ottawa, Canada) with phosphate-buffered saline (PBS) for 3 days at 4 °C in order to get rid of the nonconjugated monosaccharides.

Molar Binding Ratio. Conjugate binding ratios for binding of monosaccharides to OVA were calculated on the basis of the concentration and molecular weight using the following formula: (conc. of monosaccharide/mol wt of monosaccharide)/(conc. of OVA/mol wt of OVA). Monosaccharide binding to OVA was determined by the

previously described phenol/sulfuric acid colorimetric method^{11,12} at 490 nm with D-mannose as the standard, and protein concentration was measured using the standard bicinchoninic acid method. Results are represented in Table S1.

SDS-PAGE Gel. Conjugation of the products was confirmed by running the end products on a polyacrylamide SDS-PAGE gel. Each sample was mixed with 2× sample loading buffer and loaded onto a 12% polyacrylamide separating gel with a 5% stacking gel. Electrophoresis was performed at a constant current of 30 mA using a mini Protean Apparatus (BioRad Laboratories, Hercules, CA). Subsequently, the gels were stained with 0.05% (w/v) Coomassie Brilliant blue-R250 (Sigma) for 1 h after electrophoresis and then destained in distilled water for 1 h and photographed.

Quantification of Free Amino Acids. The trinitrobenzenesulfonic acid (Thermo Scientific, Mississauga, ON, Canada) assay was used to quantify the free amino acids in the glycoconjugates. In brief, 200 μg/mL OVA glycoproteins were dissolved in 0.1 M sodium bicarbonate buffer (pH 8.5). To 100 μL of the protein solution, 50 μL of diluted trinitrobenzenesulfonic acid solution was added and mixed well. The mixture was incubated at 37 °C for 2 h. To stop the reaction, 0.5 mL of 10% SDS and 0.25 mL of 1 N HCl were added. Absorbance was measured at 335 nm using a spectrophotometer (Shimadzu model 1201S, Kyoto, Japan). The concentration of primary amines was calculated by comparison to lysine standards. Results are given in Table S2.

Circular Dichroism. The secondary structures of the conjugates were determined by circular dichroism (CD) spectroscopy. The CD spectra of all OVA monosaccharide conjugates were recorded using a Jasco J 600 spectropolarimeter (Easton, MD). All spectra were baseline-corrected and presented as mean residue molar ellipticity (θ) mean residue weights (MRWs) at a given wavelength. Far ultraviolet CD spectra were recorded at 20 °C in a 2 mm quartz cuvette (Hellma, Mullheim, Baden, Germany) at a protein concentration of 0.2 mg/mL. All measurements were performed in 10 mM phosphate buffer, pH 7, and were scanned four times using wavelengths from 190 to 260 nm.

Experimental Design: Mice and Sensitization. Forty-eight female BALB/C mice (6–8 weeks, 18–20 g) were purchased from Charles River Laboratories (Montreal, QC, Canada). Mice were housed on a 12 h light–dark cycle and permitted ad libitum access to water and normal chow. A summary of animal experiment is shown in Figure 1. Mice were randomly divided into six groups ($n = 8$ mice per

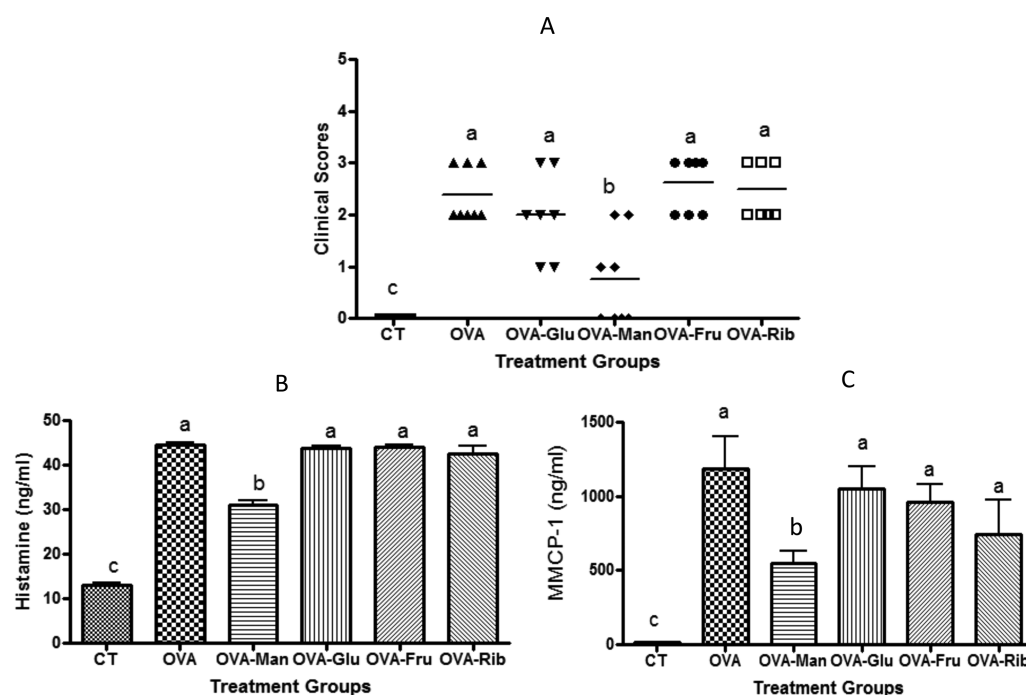


Figure 2. (A) Clinical Scores for individual mice. Total clinical scores for each mouse after OVA challenge were calculated. Average scores assigned by four independent observers in a blinded fashion are represented. Different letters indicate statistically significant differences. (B) Serum histamine concentration. Data for serum histamine concentration are represented as means $\pm$ standard deviations ($n = 4$ pooled sera). Different letters indicate statistically significant differences ($p < 0.05$) among groups of mice. (C) Mouse mast cell protease concentration (MMCP). Data for MMCP concentration are represented as means $\pm$ standard deviations.

group). All experimental protocols were run in compliance with the Canadian Council for Animal Care guidelines, and all animal use protocols were based on and approved by the University of Guelph Animal Care Committee (AUP 04R100). OVA (1 mg) was given by oral gavage (with each mouse receiving 10 μ g of cholera toxin (CT); List Biologicals, Campbell, CA) twice a week for 4 weeks, and various monosaccharide-conjugated forms of OVA (OVA-Glu, OVA-Man, OVA-Fru, and OVA-Rib) and the control OVA were administered orally thrice a week for 4 weeks, as described in Figure 1. On the day of experimentation, allergy was induced by administering 20 mg of OVA orally (week 11), and all mice were humanely euthanized for blood and tissue collection. Briefly, mice were challenged orally with 20 mg of OVM and observed for 30 min, and clinical scores were assigned in a blinded manner by four experienced observers. Scores were as follows: 0 indicated no signs; 1 indicated repeated scratching and rubbing around the snout and head; 2 indicated puffiness around eyes and snout, diarrhea, pilar erecti, and reduced activity or decreased activity with increased respiratory rate; 3 indicated wheezing, labored respiration, and cyanosis around the mouth and tail; 4 indicated tremors and convulsions; and 5 indicated death. Total scores for each animal were obtained by adding average scores for individual clinical signs.¹¹

Blood was collected for various biomarker assays (measurement of histamine levels and antibody activity, flow cytometry, etc.). All samples were stored at -20°C until further analysis. Spleen was collected for measuring cytokines. Outcome criteria were standardized by clinical scores of allergic signs and levels of serum antibody associated with IgG, IgE, and IgG1 (type 2 immune response) and with IgG2a and IgA (type 1 immune response). Type 2 (IL-4 and IL-10) and type 1 (IFN- γ and IL-12) cytokines and IL-17 were also quantified. Treatment effects were assessed by standardized induction of active immune response to OVA as an experimental food allergy. Expression of allergy and its immunological correlates were taken to indicate a type 2 immune response bias.

Measurement of Serum Histamine and Mouse Mast Cell Protease. Thirty minutes after induction of anaphylaxis, blood was taken for the analysis of histamine plasma concentration. Histamine

concentration was measured by ELISA according to the instructions of the manufacturer (Labor Diagnostika Nord, Nordhonn, Germany) using an enzyme immunoassay kit. The mouse mast cell protease enzyme (MMCP) serum concentration was assayed using a commercial ELISA kit as recommended by the manufacturer (eBioscience, San Diego, CA).

Measurement of Allergen-Specific IgG, IgE, IgG1, and IgG2a Isotypes. OVA-specific immunoglobulin E (IgE), G (IgG), G1 (IgG1), and G2a (IgG2a) concentrations were measured using indirect sandwich-type ELISAs according to standard protocols, as described earlier.¹¹ Serum allergen-specific IgE, IgG1, and IgG2a subclasses were examined. Briefly, 96-well microtiter plates (Costar Corning Inc., Corning, NY) were coated with 100 μ g/mL OVA (in 100 μ L of 50 mM carbonate buffer, $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$, pH 9.6) for 24 h at 4°C . Rabbit anti-mouse-IgG conjugated to alkaline phosphatase was used for the detection of IgG, and rat monoclonal anti-mouse-IgE was used for the detection of IgE; these were followed by streptavidin–HRP conjugate. For IgG1 and IgG2a, rat monoclonal anti-mouse-IgG1 and -IgG2a were used, and these were also followed by streptavidin–HRP conjugate. Plates were washed three times with 200 μ L of 0.05% Tween phosphate-buffered saline (PBST 0.01 M, pH 7.4) per well, after being coated with 2% bovine serum albumin/PBS at 37°C for 1 h. Washing was repeated, serum was diluted (1:10, v/v, for IgE; 1:100, v/v, for IgG, IgG1, and IgG2a) in 0.05% PBST and added at 100 μ L per well, and the plates were incubated for 2 h at room temperature. Controls included wells without serum. Plates were washed again, and the bound antibodies were detected by adding the respective detection antibodies at room temperature for 1 h. Specific binding activity was detected by addition of 100 μ L of 3,3',5,5'-tetramethylbenzidine substrate (Sigma) per well. The reaction was terminated with 2 N H_2SO_4 after 30 min, and absorbance at 415 nm was measured by a microtiter reader (Bio-Rad, Model 550). Data are represented as percent of positive control (sera from mice that had high OVA-specific IgG activity), calculated as $(\text{OD of test serum})/(\text{OD of positive control} - \text{OD of negative control}) \times 100\%$.

Cytokine Analysis. Cytokine concentrations in supernatants of OVA-stimulated (50 μ g/well, 72 h) splenocytes were measured as

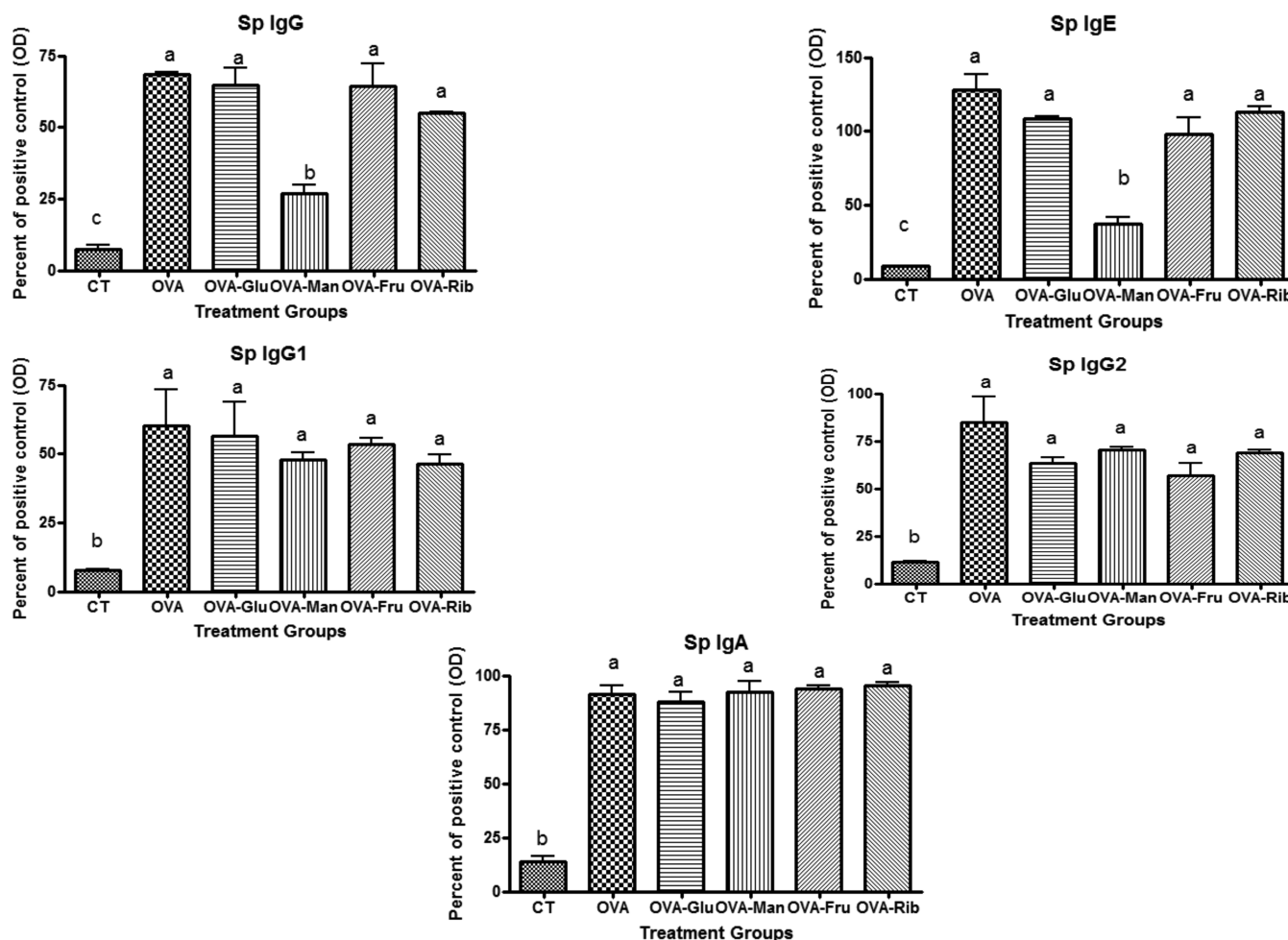


Figure 3. Antibody activity. Ovalbumin (OVA)-specific serum IgG (whole molecule)-, IgE-, IgG1-, IgG2a-, and IgA-related antibody activities were determined. Rabbit anti-mouse-IgG conjugated to alkaline phosphatase was used for the detection of IgG, and rat monoclonal anti-mouse-IgE was used for IgE; these were followed by streptavidin–HRP conjugate. Data are represented as percent of positive control (sera from mice that had high OVA-specific IgG) activity, calculated as (OD of test serum)/(OD of positive control – OD of negative control) $\times$ 100%. Rat monoclonal anti-mouse-IgG1 and -IgG2a were used followed by streptavidin–HRP conjugate for measuring IgG1 and IgG2a. For OVA-specific IgA, pooled fecal samples collected from weeks 9–11 were used. Biotinylated rat monoclonal anti-mouse-IgA antibody followed by avidin–HRP conjugate were used to determine IgA activity. Different letters indicate statistically significant differences.

described previously.¹¹ Briefly, individual spleens were collected aseptically at the end point postchallenge from each mouse. Two spleens were pooled within each group, such that each group had four samples ($n = 4$). Single cell suspensions were prepared, and splenocyte cell viability was assessed by trypan blue exclusion. Cells were cultured in 24 well plates in triplicate at a density of 2.5×10^6 cells/mL, unstimulated (control) or stimulated with 50 μ g/well OVA. Culture supernatants were collected after 72 h of incubation at 37 °C and stored in aliquots at –20 °C. Concentrations of cytokines were measured in culture supernatants using murine capture ELISA cytokine kits following the manufacturer's instructions (BD Pharmingen, Mississauga, ON, Canada, for IL-4 and IFN- γ ; eBioscience for IL-10, IL-12, IL-17A, and TGF- β).

Determination of OVA-Specific IgA. Mouse fecal pellets were freshly collected on a weekly basis at weeks 9, 10, and 11 (Figure 1) from the cages for each mouse group and were processed as described earlier.¹¹ Concentrations of OVA-specific IgA were determined using an indirect ELISA procedure as described before.¹¹

Flow Cytometry. Labeling was performed at 4 °C using standard techniques. Blood was collected postchallenge from individual mice and pooled ($n = 5$ mice/group). The flow cytometry staining protocol was carried out as recommended by the manufacturer (BD Biosciences, San Jose, CA), and the sample was stained with 100 μ L of FITC-conjugated anti-mouse-CD25 antibody (558642, BD Biosciences) and

PE-conjugated anti-mouse-FOXP3 antibody (clone FJKS-16, eBioscience) as described by the manufacturer. Analysis of labeled cells was performed using a FacScan Flow Cytometer (BD Biosciences). Live cells were gated on the basis of forward and 90° light scatter characteristics. At least 10 000 events were acquired from each sample and all data sets were analyzed using FCS Express 4 Plus software (BD Biosciences).

Dendritic Cell Isolation, Maturation, and Uptake Analysis.

Spleens from three naive mice were used to isolate cells by magnetic separation (Easysep CD11c+ Kit, Stemcell Technologies, Vancouver, Canada) following the manufacturer's recommendation. More than 90% were positive for CD11c+ (data not shown). The adherent cells were cultured for 7 days in complete RPMI-1640 medium containing 10% heat-inactivated fetal calf serum and incubated with 10 ng/mL GM-CSF and 10 ng/mL IL-4 (BD Biosciences). The immature DCs were activated with 10 μ g/mL CT for 48 h in the presence of various glycated OVA forms (50 μ g/mL). The phenotypes of the DCs were assessed by staining for maturation markers using FITC-conjugated CD86 and major histocompatibility complex (MHC) class II (eBioscience) and PE-conjugated CD11c+ antibodies. Antigen uptake was measured using 2.5×10^5 cells/mL of DCs incubated for 3 h at 37 °C with 5 μ g/mL labeled glycated OVA forms or PBS (untreated). Uptake was terminated by washing the cells three times with ice-cold PBS containing 2% FBS. Cells were suspended in FACS buffer

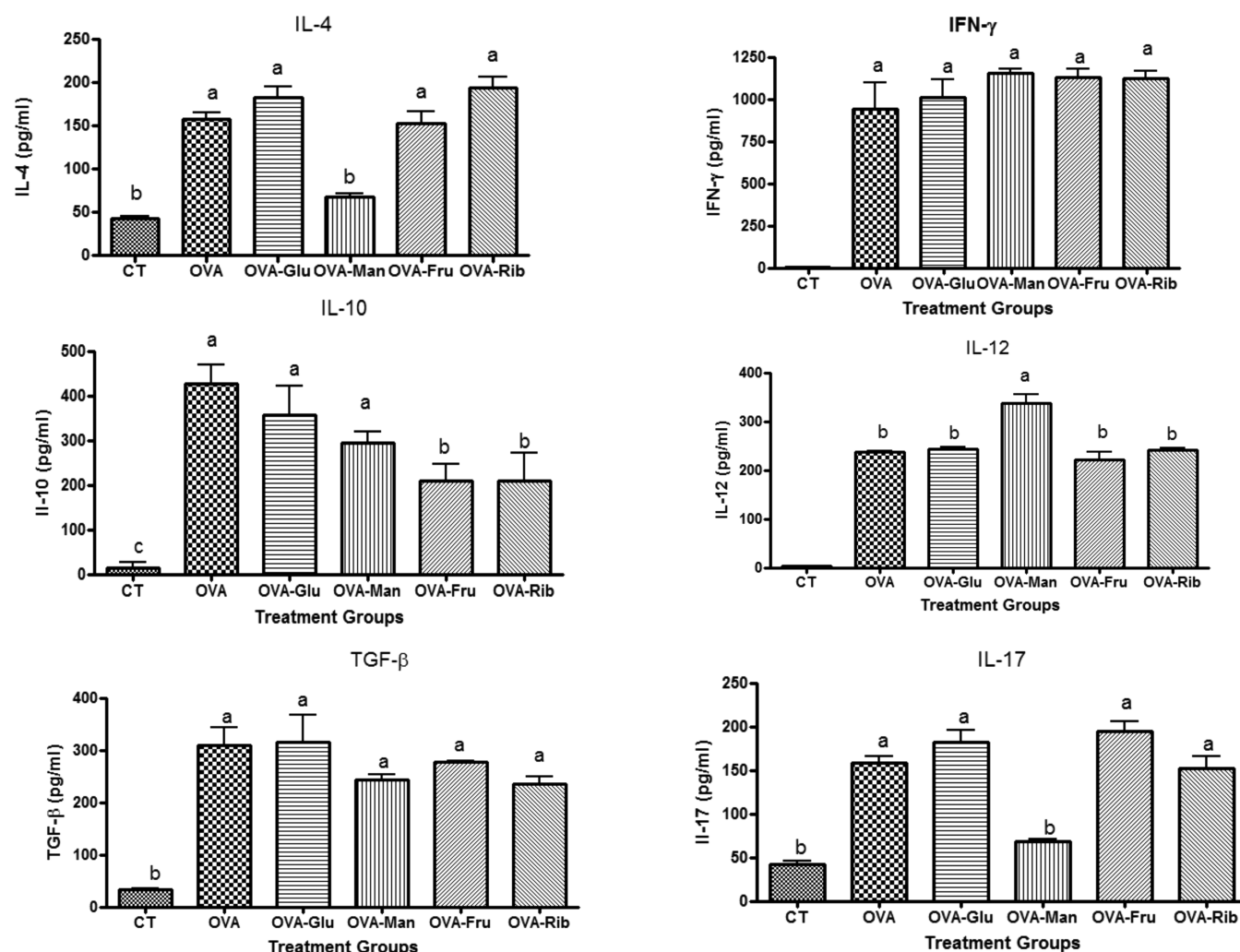


Figure 4. Cytokine concentration. Spleen was collected from each mouse and pooled within groups ($n = 5$), and splenocytes were isolated and cultured at 2.5×10^6 cells/mL in triplicate wells, unstimulated (control) or stimulated with $50 \mu\text{g}/\text{well}$ OVA for 72 h. The cytokine concentrations of cell culture supernatants for IL-4, IFN- γ , IL-10, IL-12, TGF- β , and IL-17 were determined by ELISA. Different letters indicate significant difference among groups for each cytokine. Significance was taken at $p < 0.05$.

(PBS containing 2% bovine serum albumin and 0.1% sodium azide) and further stained with PE-conjugated anti-mouse-CD11c+ antibody (eBioscience), and cells were washed and analyzed immediately by BD FacScan. Effects of OVA uptake was analyzed by preincubation with mannan (50 mg/mL, Sigma), antagonists of mannose-specific receptors, α -methyl-D-mannopyranoside (50 $\mu\text{g}/\text{mL}$, Sigma), and specific blocking antibodies against MR (2.5 $\mu\text{g}/\text{mL}$, BioRad) and DC-SIGN (eBioscience) to investigate if uptake of OVA was blocked by competitive binding to mannose receptor, on the basis of an earlier study.¹¹ Mannose alone was also used as a control to check effects of OVA and mannose uptake.

Isolation of CD4+ T Cells and Assessment of Cytokine Production. Naive CD4+ cells were isolated from splenocytes using an Easy Sep CD4+ Kit (Stemcell Technologies) with a purity of 95%. The T cells (1×10^5 cells/mL) were cocultured with mDCs (2.5×10^5 cells/mL) and stimulated with various glycosylated forms of OVA (5 $\mu\text{g}/\text{mL}$) for 72 h. The concentrations of IL-2, IL-4, IFN- γ (BD), IL-10, IL-12, and IL-17 (eBioscience) in the culture supernatants were measured using ELISAs.

Statistical Analyses. All calculations were performed with GraphPad Prism software 4.0 (GraphPad Software Inc., La Jolla, CA). Data from animal studies were analyzed using one-way ANOVA with Tukey–Kramer post-test. Statistical tests to compute p values were calculated for median $\pm$ SEM values. Results were considered as significant at p values ≤ 0.05 . To compare the means of two groups,

Student's t test was used for parametric data, and the Mann–Whitney test was used for nonparametric data.

RESULTS

SDS-PAGE. The SDS-PAGE profiles of native and various glycosylated monosaccharides of OVA are represented in Figure S1. The molecular weights of all OVA–glycoconjugates (OVA-Glu, OVA-Man, OVA-Fru, and OVA-Rib) were higher than that of native OVA (molecular weight > 45 kDa; Figure S1, lane 2). The nonglycosylated OVA (45 kDa) band was not visible, confirming negligible amounts of intact OVA content in the glycosylated fractions. The binding ratios of the glycosylated conjugates are given in Table S1. Free amino acid estimations for various glycoconjugates are summarized in Table S2.

Clinical Signs. The in vivo effects of the glycosylated OVA forms were tested in a murine model of food allergy on the basis of the management of clinical allergic signs in sensitized mice. Figure 2A illustrates the clinical signs observed in different groups of mice. After challenge, animals in the positive control group developed statistically significant clinical scores (2.38 ± 0.18) of food allergy compared with those in the negative control group (CT). There were no significant effects from OVA-Glu (2.00 ± 0.27), OVA-Fru (2.62 ± 0.18), and

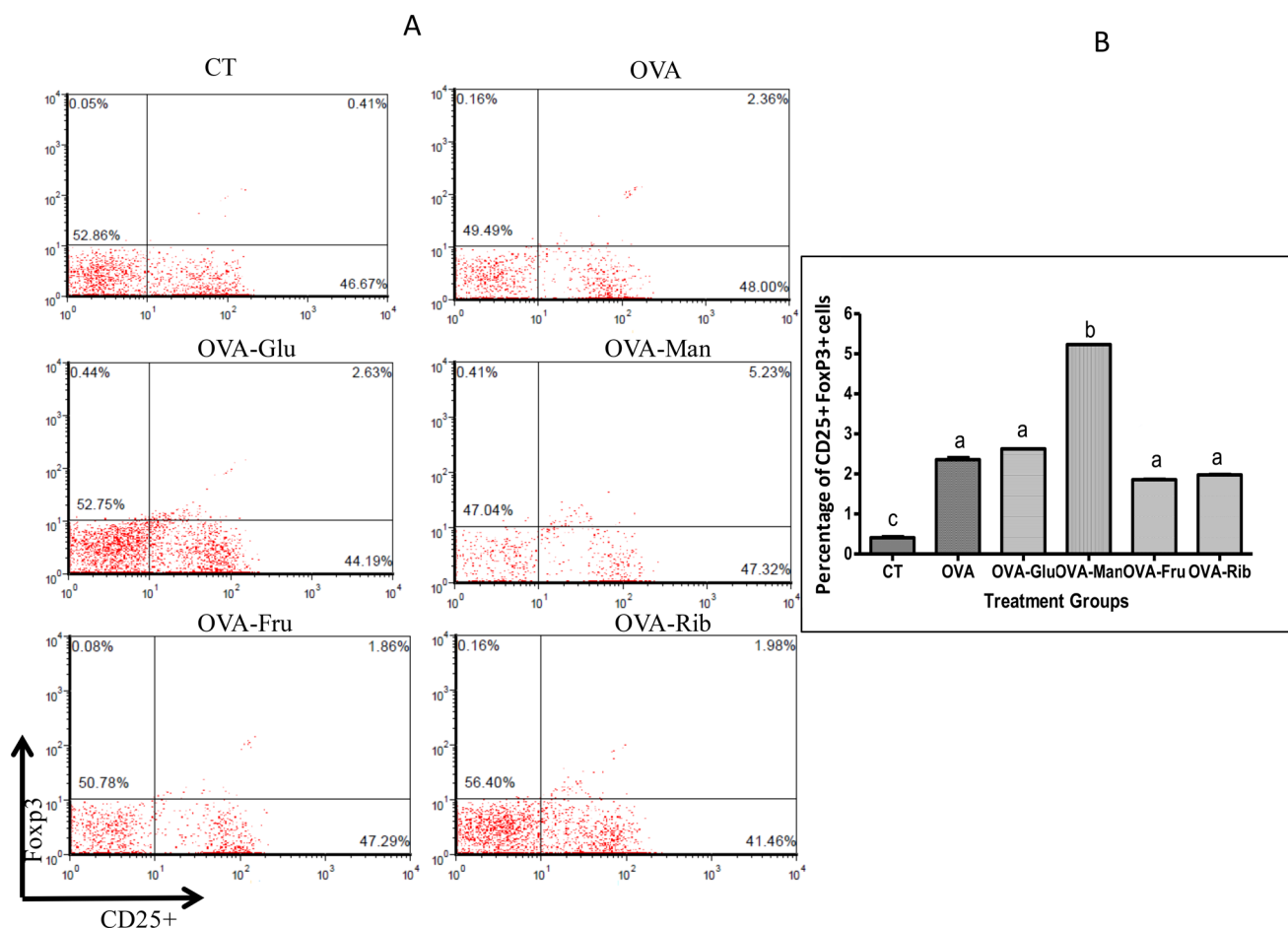


Figure 5. Flow cytometry. (A) Representative images of CD25⁺ Foxp3⁺ cells for each group. (B) Percentages of CD25⁺ Foxp3⁺ cells determined by FACS from whole blood of mice collected at the end point of the experiment. Different letters indicate statistically significant differences among groups.

OVA-Rib (2.50 ± 0.19) treatment on the frequency of clinical signs of allergy ($p > 0.05$). Treatment with mannosylated OVA (OVA-Man) significantly reduced the frequency of allergic signs (0.75 ± 0.31 , $p < 0.05$).

Histamine and MMCP Concentration. MMCP-1 concentration was evaluated as a measure for mast cell activation and degranulation. MMCP-1 concentration increased significantly in the positive control group (1183.00 ± 217.80 ng/mL) when compared with in the negative control group (CT, 17.60 ± 1.19 ng/mL). MMCP-1 concentration was significantly reduced in the OVA-Man group (549.80 ± 84.67 ng/mL, Figure 2C, $p < 0.05$). The OVA-Man group also had a lower histamine concentration (30.96 ± 1.12 ng/mL) as compared with that in the positive control OVA group (44.43 ± 0.71 ng/mL, Figure 2B, $p < 0.05$). Other groups did not differ significantly ($p > 0.05$).

OVA-Specific Antibody Isotypes. The results show that the allergic mice differed from the control mice. OVA-specific IgE ($128.30 \pm 10.11\%$), IgG1 ($60.29 \pm 13.06\%$), IgG2a ($84.60 \pm 13.92\%$), and IgA ($91.36 \pm 4.09\%$) antibody activities increased significantly in the positive control group (Figure 3), when compared with in the negative control group. Administration of OVA-Man had a significant impact on the concentrations of immunoglobulin isotypes. Both specific IgG and IgE were significantly reduced in the OVA-Man-treated group ($p < 0.05$). Other isotypes did not differ significantly ($p > 0.05$). This and the previous data suggest that OVA-Man likely causes a

reduction in allergic signs via its effects on two pathways commonly involved in the allergic cascade, namely, mast cell activation and antibody-mediated immunity.

Cytokine Concentration. To further identify potential mechanisms via which OVA-Man exerted beneficial effects, ex vivo cytokine profiles of antigen-stimulated lymphocytes isolated from spleen were evaluated. OVA-specific proliferation of cells was used to measure cytokine production from splenocytes. As shown in Figure 4, cells from the negative control CT group secreted low levels of cytokines. Cells from the positive control OVA group secreted increased levels of IL-4 (158.30 ± 7.11 pg/mL), IFN- γ (941.80 ± 15.37 pg/mL), IL-10 (426.60 ± 43.28 pg/mL), IL-12 (366.30 ± 44.80 pg/mL), IL-17 (158.30 ± 7.11 pg/mL), and TGF- β (308.60 ± 35.70 pg/mL) compared with those of the negative control group. Cells from mice given OVA-Man exhibited decreased concentrations of IL-4 (67.98 ± 3.11 pg/mL) and IL-17 (67.98 ± 3.11 pg/mL) and an increased concentration of IL-12 (620.50 ± 41.97 pg/mL, Figure 4, $p < 0.05$) compared with those of the positive control. There were no differences in IFN- γ , IL-10, and TGF- β expression ($p > 0.05$). IL-10 was decreased in the OVA-Fru and OVA-Rib groups ($p < 0.05$).

Secondary Structure. The CD profile of OVA-Man had a reduced and compact structure as compared with that of the native OVA (Figure S2); in particular, the structure of the α -helix side chain residues of OVA-Man was altered from that of native OVA. The $n-\pi^*$ transition at 222 nm of OVA-Man

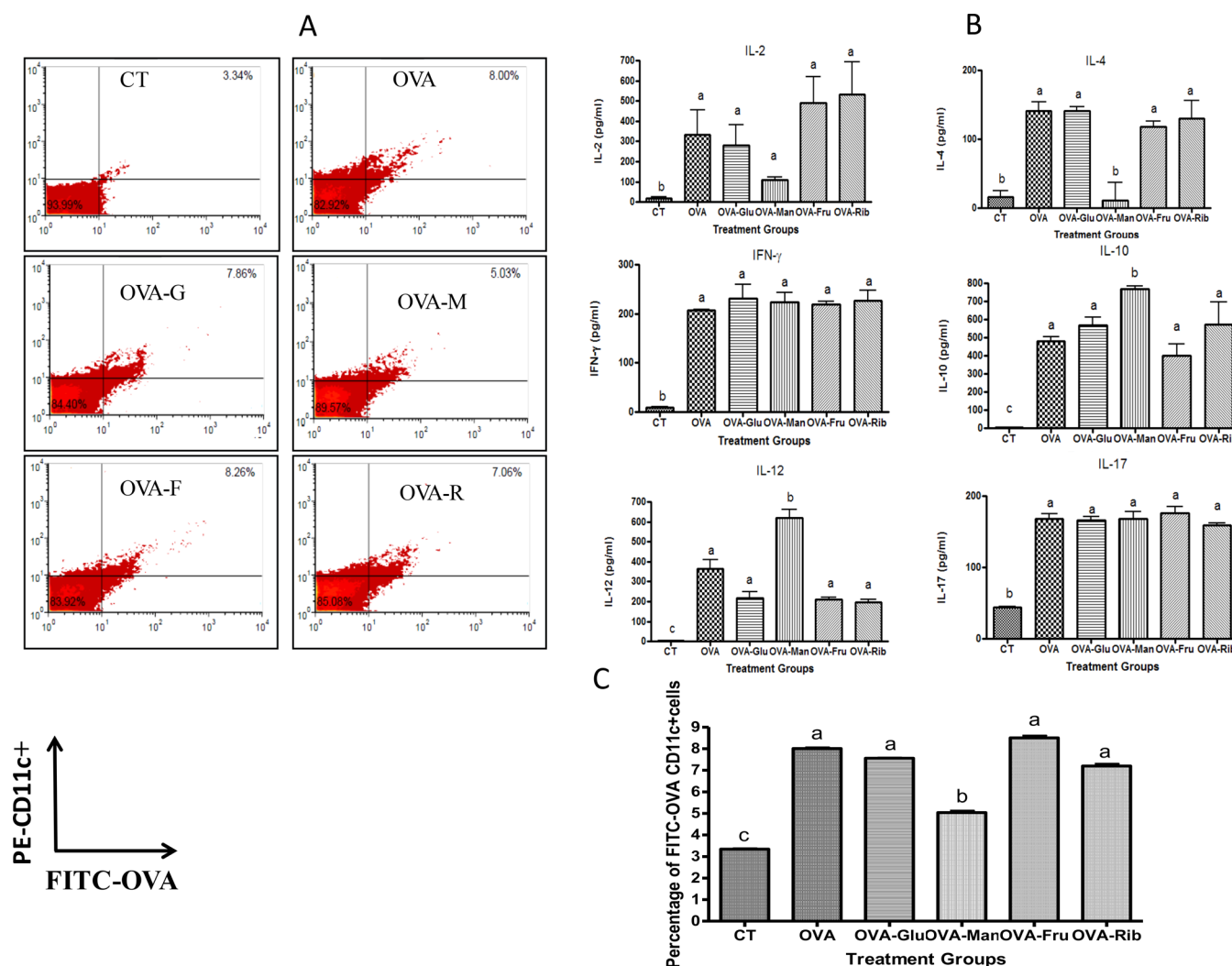


Figure 6. (A) Uptake of OVA glycosylated forms by CD11c⁺ sorted DCs. Wild-type DCs were incubated with 5.0 μ g/mL FITC-conjugated native OVA or glycosylated OVA forms for 3 h. The uptake of labeled OVAs by the DCs was analyzed by means of flow cytometry with PE-conjugated CD11c⁺ antibody. (B) Concentrations of IL-2, IL-4, IFN- γ , IL-10, IL-12, and IL-17 in the culture supernatant measured by means of ELISA. Purified CD4⁺ T cells were cocultured with DCs and stimulated with native OVA or various glycosylated OVA forms for 72 h. The data are representative of two separate experiments ($p < 0.05$). (C) Percentage of FITC OVA CD11c⁺ uptake of cells determined by FACS. Different letters indicate statistically significant differences among groups.

was decreased compared with that of the native analogue. The wavelength minima at 207 and 222 nm and the maximum at 191 nm, both typical characteristics of α -helical configuration, were reduced in OVA-Man. The structures of other OVA glycoconjugates did not differ as compared with native OVA.

Regulatory T Cell Proportion. The percentage of CD25⁺ Foxp3⁺ T regulatory cells was significantly increased in the OVA-Man group (Figure 5, $p < 0.05$). Representative images for each treatment group are shown in Figure 5. All other groups treated otherwise did not differ significantly ($p > 0.05$).

DC–T Cell Coculture Experiment. The antigen uptake of splenic DCs revealed less uptake in CD11c⁺ sorted cells in the OVA-Man group (Figure 6, $p < 0.05$). There were no significant differences observed among the other treated groups ($p > 0.05$). Only a basal level of uptake of FITC–OVA was observed in the CT group. Also, there was a significant difference ($p < 0.05$) in the uptake of OVA with addition of mannan and mannan-specific receptor antagonist (α -methyl-D-mannopyranoside, Figure 7). Blocking with mannan or α -methyl-D-mannopyranoside decreased the antigen uptake of DCs

(Figure 7) as compared with that in the native OVA group; however, addition of mannose to OVA (OVA + mannose) did not result in any effects. Furthermore, preincubation with anti-MR antibody blocked antigen uptake, but that was not the case with anti-DC-SIGN. The results shown are representative images of two identical uptake experiments conducted. Surface marker expression on DCs was assessed using flow cytometry. Of the cells analyzed for DC maturation markers, both CD86 and MHC class II markers were low, with the OVA-Man group (Figure 8, $p < 0.05$) indicating altered expression of these surface molecules. Other groups did not differ significantly ($p > 0.05$). IL-4 and IL-2 cytokine expression was low in the OVA-Man-treated group as compared with that in the OVA-positive control group (Figure 6, $p < 0.05$). The IL-2 concentration, as a marker of T cell activation, in cocultures containing T cells and DCs revealed less production ($p < 0.05$) in the OVA-Man group, indicating less CD4⁺ T cell activation. In addition, cocultures revealed that both IL-12 and IL-10 were found to be increased in the OVA-Man group ($p < 0.05$). Cytokine levels remained unaltered in other treatment groups.

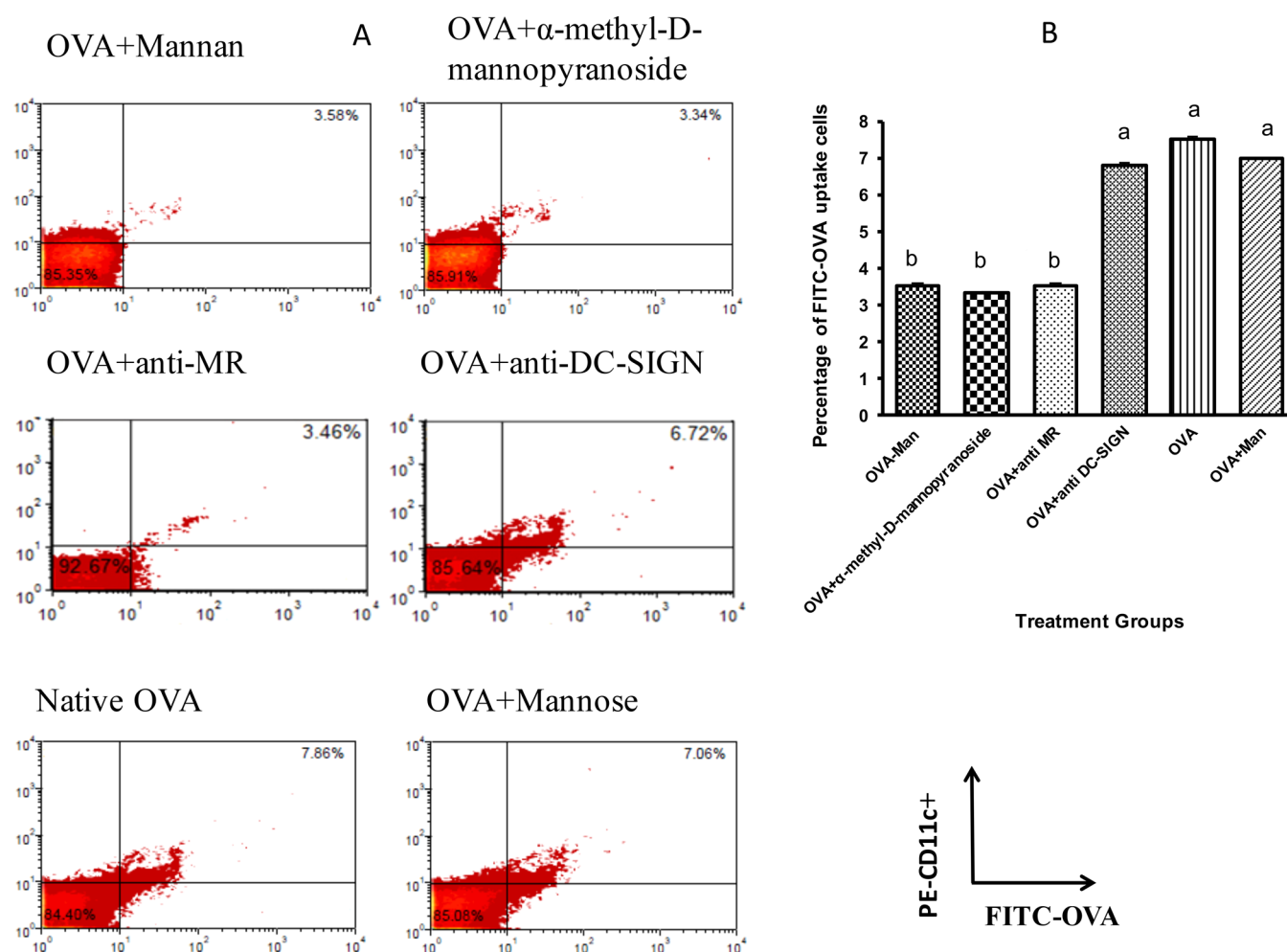


Figure 7. Uptake experiment after blocking with mannan, α -methyl-D-mannopyranoside, and anti-DC-SIGN or anti-MR antibodies. (A) Uptake of OVA glycosylated forms by CD11c+ sorted DCs. Mannose with OVA was also used as a control to check uptake. Wild-type DCs were further incubated with 5.0 μ g/mL FITC-conjugated native OVA for 3 h. The uptake of labeled OVAs by the DCs was analyzed by means of flow cytometry with PE-conjugated CD11c+ antibody. (B) Percentage of FITC-OVA CD11c+ uptake of cells determined by FACS. Different letters indicate statistically significant differences among groups.

Interestingly no significant differences among groups were observed with IFN- γ and IL-17 ($p > 0.05$, Figure 6).

DISCUSSION

DCs express several MRs on their surface, which play important roles in antigen recognition, uptake, and presentation and, in particular, priming of specific T cell responses.¹⁴ Glycosylated antigens, like mannosylated BSA, have been shown to enhance MHC class II restricted T cell responses via the mannose receptor when compared with nonmannosylated BSA.¹⁵ An earlier study has demonstrated that yeast-produced mannosylated ovalbumin, as compared with the nonmannosylated OVA fraction, is more potent at stimulating Ag-specific T cell responses in an MR-dependent manner.¹⁶ We have recently shown that mannosylated OVA attenuates clinical signs of allergy in mice and in vitro.¹¹ We have reconfirmed the effects of OVA-Man here in abrogation of allergic response and have shown that other monosaccharides conjugated to OVA, such as glucose, ribose, and fructose, have no role in allergy reduction. The desired outcome of inducing tolerance needs to target the delivery of the antigen to the DC irrespective of the choice of antigen conjugation.

We aimed to target the use of high affinity mannose receptor ligands because it was shown earlier that mannosylated

dendrimers were able to target DCs and induce strong CD4 and CD8 immune responses.¹⁷ It was also shown that glycan modification of antigens can greatly increase CD8 and CD4 T cell responses.¹⁸ Al-Ghoul et al. found that recombinant hypermannosylated Der p 1 was taken up more readily by DCs than natural Der p 1.¹⁰ Also, lower uptake of glycosylated allergens by MR-deficient DCs was observed earlier.⁹ This is in contrast to what was observed in the present study and earlier studies.^{11,12} There seems to be diversity with recognition, and uptake was totally dependent on the nature of the glycosylated protein initiating the immune response. The mode of mannose conjugation to a given allergen structure may be important as the differential immune response result. This could likely be one of the reasons why other monosaccharides conjugated to OVA in this study did not alleviate signs of allergy. It is also possible that because mannosylated OVA and egg white structurally differ from Der p 1, the recognition and inhibition of uptake differed accordingly. It is of interest to note that binding and uptake were inhibited in the presence of free mannan, suggesting that the uptake was MR dependent. Mannan blocking had similar effects to mannosylated OVA exhibiting less uptake by DCs. Also, it was earlier demonstrated that blocking the mannose receptor with mannan strongly reduced

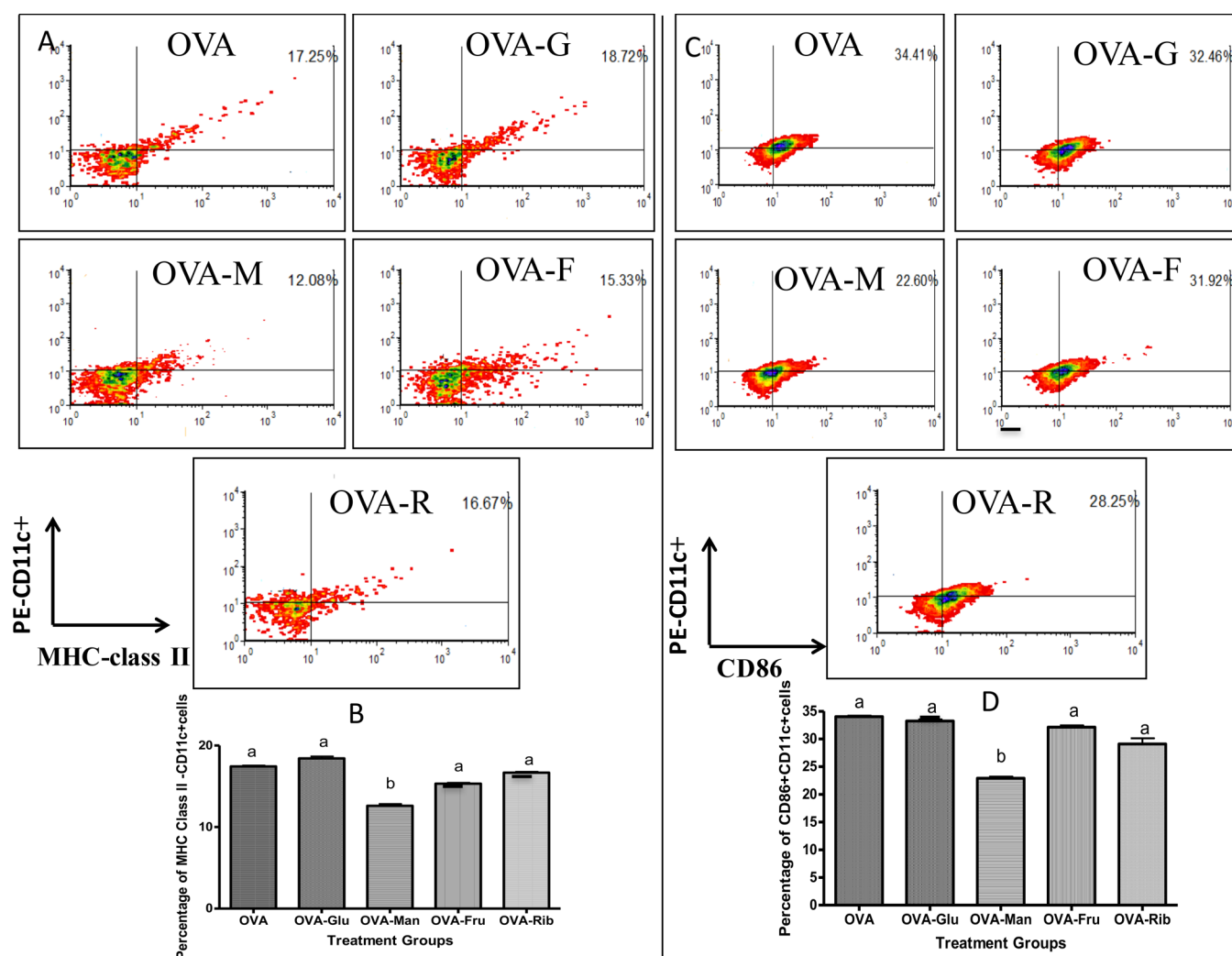


Figure 8. Flow cytometric analysis of DC phenotype. (A,C) Representative FACS images showing expression levels of (A) MHC class II and (C) CD86. (B,D) Expression levels CD11c⁺ and costimulatory molecules (B) MHC class II and (D) CD86 in DCs cultured as described in the Materials and Methods and stimulated with the CT conditions for maturation and various glycosylated forms of OVA. Different letters indicate statistically significant differences ($p < 0.05$) among groups in the graph.

uptake of both FITC–OVA and FITC-conjugated advance glycation end products of OVA.¹⁹ This confirms MR binding engagement in blocking mannosylated OVA to a similar degree to blocking with mannan with regards to uptake of the allergen. These findings were unanticipated because DCs are thought to have strong binding affinity for high-mannose structures. An earlier report showed that immunization with mannosylated OVA peptide resulted in immunological unresponsiveness and induced poor T cell effector functions.²⁰

In the present study, DCs seemed to have taken up mannosylated OVA and utilized the peptide epitopes for antigen presentation, eliciting a preferential type 1 immune response. We observed that mannosylated OVA induced a reduced T cell response, as confirmed by our proliferation data, but simultaneously elicited a suppressive mechanism via tolerance induction, possibly because of higher bioavailability, thereby reducing allergy. It was shown earlier that mannosylated OVA induced enhanced antigen-specific CD4⁺ T cell proliferation, leading to enhanced immunogenicity as compared with that from nonmannosylated OVA, and uptake was primarily due to mannose-specific C-type lectin receptors (MRs).²¹ Blocking MRs by mannan, α -methyl-D-mannopyranoside, or anti-MR

antibody clearly demonstrated that MR is a major player for OVA uptake by DCs, and thereby, OVA-Man was shown to decrease its uptake and the maturation of DCs. Lam et al.²¹ studied the effects of differential N-linked and O-linked mannosylation on dendritic cells using various forms of recombinant glycosylated OVA. They reported that O-mannosylation of antigens is critical for optimal DC-SIGN binding, whereas either N- or O-mannosylation of antigen appears to act as a ligand for MR. In this study, OVA-Man was prepared by a Maillard reaction, as described earlier, and Man reacted with the amino groups of Lys or Arg via a Schiff base,¹¹ which differs from the OVA previously reported by Lam et al.²¹ The current report is also the first study that clearly demonstrates that Man-glycosylated OVA prepared by Maillard reactions can bind via ligands to MRs in DCs. Maillard reactions commonly occur in food processing and affect the flavors and textures of food products; therefore, it is also of great interest to understand the effects of Maillard reactions with regard to immune responses in the gut, including allergic reactions.

Multiple mechanisms may act synergistically in the inhibition of allergy development. It was observed that clinical signs of allergy, histamine and MMCP concentrations, were reduced

in the OVA-Man group. Also, OVA-specific IgG and IgE were reduced. However, the isotypes IgG1 and IgG2a did not differ. It is possible that there may be other IgG subisotypes that may have contributed to the induction of tolerance but were not tested here. It is of interest to note that although the Treg cell percentage increased in the OVA-Man group, the cytokines IL-10 and TGF- β were not enhanced. This is suggestive that there may be other mechanisms involved with the induction of tolerance, such as role of Th17 cells. The IL-17 cytokine was reduced in the OVA-Man group in our study (Figure 4). A pro-inflammatory role for IL-17 in autoimmune disorders has been favored, although there is an evidence that IL-17 has a dual role as a negative regulator.²² In conclusion, this work concludes that mannosylation of OVA attenuates allergy both in vitro and in vivo and expresses a tolerant immunosuppressive phenotype, as evidenced by various biomarkers of allergy, and may therefore be a suitable target for immune modulating therapy for OVA-induced allergy.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b02132.

Molar binding ratios for the conjugated OVA forms, measurement of free amino acids in the conjugated fractions, electrophoretic profile, and circular dichroism spectra of ovalbumin monosaccharide conjugates (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

DCs, dendritic cells; Tregs, regulatory T cells; Th, T helper cells; MR, mannose receptor; OVA, ovalbumin; DC-SIGN, dendritic-cell-specific intercellular-adhesion-molecule-3-grabbing nonintegrin; CD, circular dichroism; CT, cholera toxin; MMCP, mouse mast cell protease enzyme; MHC, histocompatibility complex; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline

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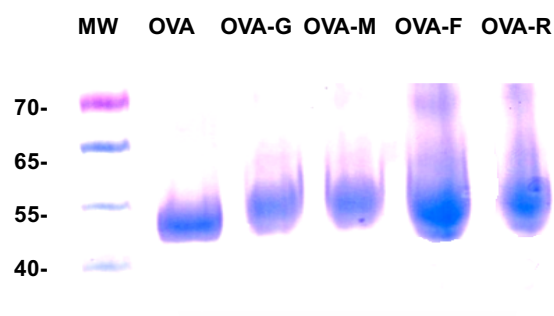
Suppl Table S1. The molar binding ratios for the conjugated OVA forms.

Conjugates	Molar binding ratio (mol/mol)
OVA:Glu	1: 35
OVA-Man	1: 55
OVA-Fru	1:60
OVA-Rib	1: 66

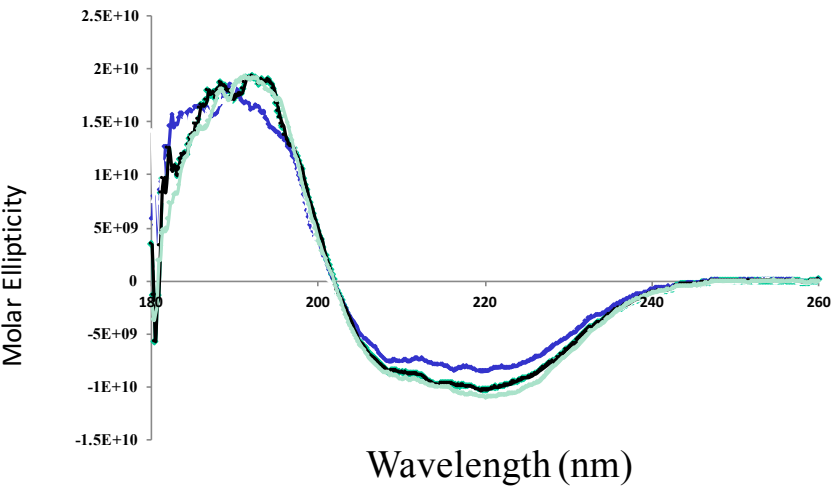
Suppl Table S2. The measurement of free amino acids in the conjugated fractions.

OVA Conjugates	Free amino acids (μmol/mL)
OVA	17.98
OVA-Glu	0.85
OVA-Man	0.74
OVA-Fru	0.55
OVA-Rib	0.56

Suppl Fig. S1. Electrophoretic profile (SDS-PAGE gel). Lane 1 represents native OVA. Lane 2 represents OVA-Glucose; lane-3 represents OVA-Mannose; lane-4 represents OVA-Fructose and lane-5 represents OVA-Ribose.



Suppl Fig. S2. Circular dichroism spectrum of ovalbumin monosaccharide conjugates. The far-UV spectra were measured by scanning four times from 190 nm to 250 nm. The proteins (0.2 mg/ ml) were dissolved in 10 mM phosphate buffer, pH 7 and blank containing phosphate buffer was measured and subtracted from the spectra. The dark line denotes the native ovalbumin spectrum and the blue line denotes the OVA-Man conjugated spectrum. Table indicates the secondary structure components as measured by CD spectrum



Protein	Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
OVA	0.16	0.12	0.14	0.09	0.2	0.29	1
OVA-Man	0.08	0.1	0.17	0.11	0.24	0.3	1
OVA-Glu	0.17	0.12	0.13	0.09	0.2	0.29	1
OVA-Fru	0.16	0.11	0.15	0.08	0.2	0.3	1
OVA-Rib	0.16	0.12	0.13	0.1	0.2	0.29	1

Suppl Fig. 2