

RESEARCH ARTICLE

Effects of ovalbumin glycoconjugates on alleviation of orally induced egg allergy in mice via dendritic-cell maturation and T-cell activation

Prithy Rupa¹, Soichiro Nakamura², Shigeru Katayama² and Yoshinori Mine¹

¹Department of Food Science, Ontario Agricultural College, University of Guelph, Guelph, Canada

²Food Biotechnology Laboratory, Graduate School of Agricultural Science, Shinshu University, Nagano, Japan

Scope: Glycation of allergens via Maillard reaction or chemical conjugation has been shown to influence susceptibility to food-induced allergies. It is hypothesized that mucosal immune response bias can be favorably altered by orally administering various forms of glycated ovalbumin (OVA).

Methods and results: Groups of Balb/c mice ($n = 10$) were orally sensitized to OVA and administered various forms of glycated OVA (glucose, mannose, glucomannan, galactomannan, and a mixture containing OVA and glucomannan). Outcomes post oral challenge were measured as clinical allergic signs, serum histamine, mouse mast cell protease 1 (MMCP-1), antibody activity, type-1/2 cytokines, percentage of T-regulatory cells (T-regs) and in vitro dendritic cell, and T-cell-related mechanisms. Clinical signs and specific IgE were decreased ($p \leq 0.05$), and T-reg cell percentage was increased in the mannose and glucomannan treated groups. The OVA-mannose group also had less histamine, MMCP-1, specific IgG, IL-4 and IL-17, and more IL-12p70 ($p \leq 0.05$). Other parameters measured did not differ significantly among groups. Also, OVA-glycated mannose reduced maturation and uptake by dendritic cells. Less activation of T cells and type-2 cytokine response in DC–T-cell cocultures were observed with OVA-glycated mannose stimulation.

Conclusion: This study validates, for the first time, the use of OVA-glycated mannose and glucomannan for potential beneficial dietary interventions for allergy.

Keywords:

D-mannose / Food allergy / Glucomannan / Glycation / Immune modulation

Received: January 22, 2013

Revised: July 19, 2013

Accepted: July 20, 2013

1 Introduction

Food allergy, defined as an adverse immune response (IR) to food proteins, affects 6% of children and 3–4% of adults [1]. Egg ranks among the “top 8” most allergenic foods, with some children gaining tolerance by the age of 5 [2]. However, allergy persists in others and allergic reactions to eggs in those patients are frequently severe [3, 4]. Oral tolerance studies suggest that tolerance occurs due to deletion of effector T cells and activation of regulatory T cells (T-regs) [5].

Antigen-specific hypoallergenic interventions to induce tolerance and/or prevent detrimental allergic IR are currently promising and various strategies have been proposed.

Conjugation with reducing sugars via the Maillard reaction under controlled dry heating conditions seems to be one such promising and safe strategy [6]. The effects of Maillard reaction on allergenicity of shellfish allergens [7], peanut allergens [8], β -lactoglobulin [9], and soy proteins [10] have been reported earlier. The effect may be carbohydrate (CHO) and/or allergen dependent [11]. However, controversial data exist from these studies. Binding of Ara h 1 and Ara h 2 to serum IgE in peanut allergic patients increased upon heating via Maillard reaction [8]. However, in other cases sugar conjugation has been proposed as a way to change allergen structures, reduce allergenicity, and enhance functional properties [10]. Research conducted by Nakamura et al. [12] recently demonstrated glycated buckwheat Fage-1 conjugated with glucomannan modulated and suppressed buckwheat allergy in mice. It has also been shown that

Correspondence: Professor Yoshinori Mine, Department of Food Science, Ontario Agricultural College, University of Guelph, Guelph, Ontario, N1G2W1, Canada

E-mail: ymine@uoguelph.ca

Fax: +1-519-824-6631

Abbreviations: CHO, carbohydrate; CT, cholera toxin; IR, immune response; MR, mannose receptor; OVA, ovalbumin

glucose-conjugated ovalbumin (OVA) did not trigger the maturation of bone marrow-derived murine myeloid dendritic cells (DCs) but enhanced activation of allergen-specific CD4⁺ T cells [13]. In a similar study, it was demonstrated that glycosylated OVA was more allergenic than native OVA [14].

It has been reported earlier that a CHO moiety attached to ovomucoid had a crucial role in pathogenesis of allergy [15]; however others [16] have reported that the CHO did not have a role. We confirmed earlier that CHO had an inhibiting effect on antigen binding [17] and an N-linked glycan in the third domain of ovomucoid was associated with suppression of allergic response in mice [18]. Also, with the recent developments in strategies for modulating glycosylation *in vivo*, it is now feasible to directly examine the global influence of glycans on self-tolerance and allergy [19]. It has been reported that OVA (structurally related to other allergens) can engage the mannose receptor (MR) of DCs and mediate the internalization of environmental allergenic glycoproteins [20].

We studied the various mannose-related glycosylated forms of OVA (monosaccharides and polysaccharides) and role of these forms in tolerance induction in a Balb/c mouse model. It is hypothesized that oral administration of various glycosylated OVA forms will subsequently reduce susceptibility and decrease severity of egg-induced allergy in mice. Glycosylated forms of OVA such as OVA-mannose (OVAMan-a monosaccharide), glucomannan and galactomannan (OVAGluMan, OVAGalMan-polysaccharides), and a mixture containing glucomannan and OVA (mix) were used for treatment modalities. Glycosylated OVA conjugated to glucose (OVAGlu) was used as a positive control based on studies done earlier [13,14]. Outcomes were measured as clinical allergic signs, serum histamine, mouse mast cell protease enzyme activity (MMCP), specific antibody-related isotype activity (IgG, IgE, IgG1, IgG2, IgA), cytokines (IL-4, IFN- γ , IL-12, IL-10, TGF- β , IL-17), and T-reg. Also, related mechanisms in context of DC maturation and uptake, and antigen recognition and presentation in cocultures with T cells were examined *in vitro*.

2 Materials and methods

2.1 OVA-saccharide conjugate preparation

CHOs such as D-mannose, D-glucose (Sigma; Oakville, ON, Canada), glucomannan (Konjac; Sunnyvale, CA, USA), and galactomannan (Sunfiber, partially hydrolyzed guar gum, 20 kDa; Taiyo International, MN, USA) were used in the study. Maillard reaction as described by Usui et al. [21] was carried out using monosaccharides mannose (OVAMan) and glucose (OVAGlu) at 1:1 ratio (w/w; OVA:CHO). Briefly the samples were mixed at 1:1 ratio, and freeze-dried and incubated at 55°C for 72 h at a 65% relative humidity using saturated potassium iodide solution. As a control, native OVA was heated in a similar manner without CHO. Chemical conjugation method was used for OVA-polysaccharides

Table 1. Molar binding ratio of conjugates

Molar binding ratio of OVA:CHO conjugates	
OVA:Man	1:52.9 (mol/mol)
OVA:Glu	1:37.2 (mol/mol)
OVA:GlucoMannan	1:2.1 (mol/mol)
OVA:GalactoMannan	1:5.3 (mol/mol)

(OVAGluMan and OVAGalMan) since the yield with Maillard reaction was very low. Briefly, the Konjac glucomannan was first hydrolyzed using 0.25 N HCl for 1 h at room temperature as described by Suzuki et al. [22] to yield low molecular weight (15 kDa) glucomannan, and both polysaccharides were oxidized using sodium periodate and conjugated to OVA by reductive alkylation using sodium cyanoborohydride [23]. All samples were dialyzed with PBS to get rid of nonconjugated saccharides. A mixture containing OVA (1 mg) and glucomannan (3.3 mg) was used to treat one group since it was reported earlier that hydrolyzed glucomannan alone prevented IgE production both *in vivo* and *in vitro* [22].

2.2 GE (SDS-PAGE)

The SDS-PAGE was carried out to confirm the attachment and purity of saccharides in a 12% polyacrylamide separating gel with a stacking gel of 5%. Electrophoresis was performed using a Mini Protean 3 Apparatus (BioRad) at 120 mA for 45 min. Each sample was dissolved in Laemmli sample buffer (BioRad) in the presence of 5% 2-mercaptoethanol, heated at 97°C for 4 min, and subjected to electrophoresis. Gels were stained with CBB R-250 [24].

2.3 Determination of molar binding ratios

The CHO content of conjugates was determined by the phenol/sulfuric acid method [25], and protein content by the bicinchoninic acid method [26]. Conjugate binding ratios for binding of CHO to protein were calculated based on the concentration and molecular weight obtained using the following formula: (concentration of CHO/molecular weight of CHO) / (concentration of protein/molecular weight of the protein) [27]. In calculations, known molecular masses of CHO and OVA were used. Results are represented in Table 1.

2.4 Estimation of free amino acids

To quantify free amino acids in the conjugates, the trinitrobenzene sulphonic acid (ThermoScientific, Mississauga, ON, Canada) assay was used. Briefly, 20–200 μ g/mL of the proteins were dissolved in 0.1 M sodium bicarbonate buffer (pH 8.5). To 100 μ L of the protein solution, 50 μ L of diluted trinitrobenzene sulphonic acid solution was

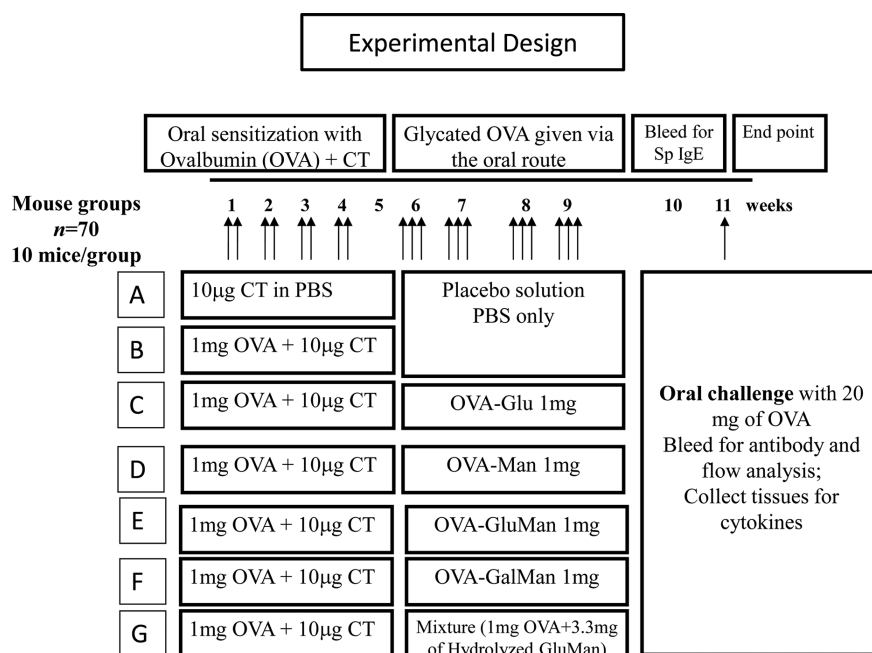


Figure 1. Outline of the experimental design. Mice were sensitized to 1 mg of OVA and 10 µg of CT for 4 weeks, twice per week, followed by 4 wk of oral treatments with glycated OVA forms (OVAGlu, OVAMan, OVAGluMan, OVAGalMan, and mixture) given thrice per week. Negative control group received PBS and the positive control group received OVA. Clinical signs of hypersensitivity were recorded immediately postoral challenge with 20 mg of OVA, and 30 min later all mice were killed for collection of blood and spleen. Both blood and spleen were pooled within treatments after collection (*n* = 5).

added and mixed well. The mixture was incubated at 37°C for 2 h. To the mixture, 0.5 mL of 10% SDS and 0.25 mL of 1 N HCl were added to stop and stabilize the reaction. Absorbance of the solution was measured at 335 nm. The concentration of primary amines was calculated in comparison to lysine standards.

2.5 Circular dichroism

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

2.6 Experimental design and animals

All animal use protocols were based on the guidelines of Canadian Council for Animal Care. Female Balb/c mice were purchased from Charles River Laboratories (Montreal, QC, Canada) and randomly divided into seven groups (*n* = 10/group). Figure 1 summarizes the treatment, sensitization, and sampling schedule. Briefly, mice (*n* = 60) were orally sensitized with 1 mg of OVA [28] adjuvanted with

10 µg cholera toxin (CT; List Biologicals, CA, USA) for 4 wk (twice/week), and orally treated with various glycated OVA forms (1 mg) for 4 wk (three times/week). In the end (week 11), mice were orally challenged with 20 mg of OVA and monitored for clinical allergic signs (0–30 min) by three independent observers in a blinded fashion [29]. Blood was collected for measurement of histamine, antibody activity, and flow cytometry. Spleen was collected for measuring cytokines.

2.7 Oral challenge and assessment of clinical signs

Clinical scores were assigned as described earlier [29] and total scores for each animal were obtained by adding scores for individual signs. In brief mice were challenged orally with 20 mg of OVA at the end point and observed for 30 min, and clinical scores were assigned in a blinded manner by three experienced observers. Scores were as follows: 0 = no signs; 1 = repeated scratching and/or rubbing around the snout and head; 2 = puffiness around eyes and snout, diarrhoea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3 = wheezing, labored respiration, cyanosis around the mouth and tail; 4 = tremor and convulsions; and 5 = death. Total scores for each animal were obtained by adding scores for individual signs.

2.8 Serum histamine and mouse mast cell assay (MMCP)

Serum histamine concentration was assayed using an ELISA kit (Labor Diagnostika Nord, Nordhon, Germany)

following the manufacturer's instructions. The mouse mast cell protease enzyme (MMCP) concentration was quantified by ELISA as per manufacturer's recommendation (eBiosciences, California, USA). Two blood samples were pooled within each group ($n = 5$) due to the limitations in the blood volume for conducting various biomarker assays.

2.9 ELISA

To measure anti-OVA-specific serum antibody activity, ELISA assays used standard methods of detection for IgG (H + L), IgG1, IgG2a, and IgE. OVA-specific murine IgG, IgE, IgG₁, and IgG₂ isotypes were detected as described previously [29], except for the coating antigen that was replaced by OVA (100 µg/mL). OD of test sample wells was measured at 405 nm when the positive control (from OVA-sensitized mice) OD reached 1.0. Mean values of OD were obtained from triplicates of test serum and from the controls. Individual serum means were expressed as percent of the positive control values for each plate: % of positive control activity = (mean test serum OD/mean positive control serum OD) × 100.

2.10 Cytokine analysis

Cytokine concentrations were measured in supernatants of OVA-stimulated (50 µg/well, 72 h) splenocytes as described previously [29]. In brief, individual spleens were collected aseptically at the end point post challenge from each mouse into ice-cold RPMI-1640 medium (Invitrogen, NY, USA). Two spleens were pooled within each group such that each group had five samples ($n = 5$). The medium contained sodium bicarbonate (1.5 g/L), glucose (4.5 g/L), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (50 U/mL), and streptomycin (50 µg/mL). Single-cell suspensions were prepared and 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 as unstimulated (control) or stimulated with 50 µg/well of OVA. Culture supernatants were collected after 72-h incubation at 37°C and stored in aliquots at –20°C. Concentration of type-1 and type-2 cytokines were measured in culture supernatant using murine capture ELISA cytokine kits following manufacturer's instructions (BD Pharmingen, Mississauga, Canada for IL-4 and IFN-γ; eBioscience for IL-10, IL-12, IL-17A, and TGF-β).

2.11 Determination of OVA-specific IgA in mouse fecal pellets

Mouse fecal pellets were freshly collected on a weekly basis at weeks 9, 10, and 11 (Fig. 1) from each mouse group cage and were processed as described earlier [29]. Concentrations of OVA-specific IgA were determined using an indirect

ELISA procedure. In brief, 1.0 g of fecal pellets were added to 7 mL of PBS buffer in a 15 mL conical tube and vortexed for 2×30 s. Samples were subsequently centrifuged at $1600 \times g$ for 15 min at 4°C to remove large fibrous particles, and supernatants were carefully collected in new tubes (2 mL) and centrifuged at $7200 \times g$ for 10 min at 4°C. The supernatant was collected for ELISA immediately or stored at –80°C for further analysis. Flat-bottom 96-well ELISA plates (Corning) were incubated with 100 µL/well of 100 µg/mL purified OVA in 100 mM of sodium bicarbonate buffer (pH 9.5) and incubated overnight at 4°C. Plates were subsequently washed four times with 200 µL/well of PBST, and blocked with 200 µL/well of 1% BSA in PBS and incubated for 2 h at 37°C. Additional four washes were performed (200 µL/well of PBST) and 100 µL/well of supernatant from fecal extracts was added onto the plate in triplicate wells for a 2-h incubation at 37°C. The plates were washed four times with PBST and incubated with 100 µL/well of biotinylated monoclonal antimouse IgA (1:500; BD Pharmingen) diluted in 1% BSA in PBST for 1 h at 37°C. The wells were further washed four times with 200 µL/well of PBST, and 100 µL/well of avidin–horse radish peroxidase conjugate (1:2000; BD Pharmingen) was applied onto the plate for 30-min incubation at 37°C. After a final four-wash cycle with PBST, OVA-specific IgA binding activity was detected by addition of 100 µL/well of 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich). The reaction was terminated after 30 min by addition of 50 µL/well of 2 N H₂SO₄, and the absorbance was determined at 450 nm using an ELISA microplate reader (BioRad).

2.12 Flow cytometry

Blood was collected postchallenge from individual mouse and pooled ($n = 5$ /group). Flow cytometry staining protocol was carried out as recommended by the manufacturer (BD bioscience), and the sample was stained with 100 µL of FITC-conjugated antimouse CD25 antibody (558642; BD biosciences, California, USA) and phycoerythrin (PE)-conjugated antimouse-FOXP3 (clone FJKS-16; eBioscience), measured via the FacScan Flow Cytometer (BD), and analyzed using FCS Express 4 Plus software.

2.13 Splenic DC isolation, maturation, and uptake analysis

Spleens from three mice were used to isolate CD11c⁺ cells (Easysep CD11c⁺ kit; Stemcell Technologies, Vancouver, Canada) following the manufacturer's recommendations. More than 90% were positive for CD11c⁺ (data not shown). Monocytes thus obtained were cultured in complete RPMI-1640 medium containing 10% heat-inactivated fetal calf serum and incubated with 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF) and 10 ng/mL IL-4 (BD

Bioscience) for 6 days. The immature DCs were activated with 10 µg/mL CT for 48 h in the presence of various OVA glycyated forms (50 µg/mL). The DCs were stained for maturation against FITC-conjugated CD86, and MHC Class II (eBioscience) and PE-conjugated CD11c⁺ antibodies. Cells sorted from spleen DC preparations as CD11c⁺high and CD11c⁺medium were used to study uptake of FITC-conjugated OVA forms. The CD11c negative cells were excluded from the assay. Uptake of OVA forms by DCs was analyzed by labeling the proteins with 5 µg/mL of FITC (FITC kit; Sigma, Oakville, Canada) following the manufacturer's instructions. Antigen uptake was measured using 2.5 × 10⁵ cells/mL of DCs incubated for 3 h at 37°C with 5 µg/mL of labeled glycyated OVA forms or PBS (untreated). Uptake was terminated by washing the cells 3 × with ice-cold PBS containing 2% fetal bovine serum (FBS). Experiments were performed on ice to inhibit intracellular uptake as a control for surface binding of the antigens. Cells were resuspended in FACS buffer (PBS containing 2% bovine serum albumin and 0.1% sodium azide) and further stained with PE-conjugated antimouse CD11c⁺ antibody (eBioscience) and measured via BD FacScan.

2.14 Isolation of CD4⁺ T cells and assessment of cytokine production

Naive CD4⁺ cells were isolated using Easy Sep CD4⁺ kit (Stemcell Technologies) with a purity of 95% from splenocytes. The T cells (1 × 10⁵ cells/mL) were cocultured with myeloid DCs (2.5 × 10⁵ cells/mL) and stimulated with glycyated forms of OVA for 72 h. The concentrations of IL-4, IFN-γ (BD), IL-10, IL-12, IL-17, and IL-2 (eBioscience) in the supernatants were measured using ELISA.

2.15 Statistical analyses

All calculations were performed with GraphPad Prism software 4.0 (GraphPad Software Inc, La Jolla, Calif). One-way ANOVA with Tukey's Kramer posttest with 95% confidence interval ($p < 0.05$) was done. To compare the means of two groups, the Student's *t*-test was used for parametric data and the Mann–Whitney's test was used for nonparametric data.

3 Results

3.1 SDS-PAGE

SDS-PAGE pattern of native and various glycyated forms of OVA is represented in Fig. 2. The molecular weights of both OVAMan (Fig. 2; lane 1) and OVAGlu (Fig. 2; lane 2) were higher as compared to that of native OVA (molecular weight >45 kDa; (Fig. 2; lane 3). Nonglycyated OVA (45 kDa) band was not visible, confirming negligible amounts

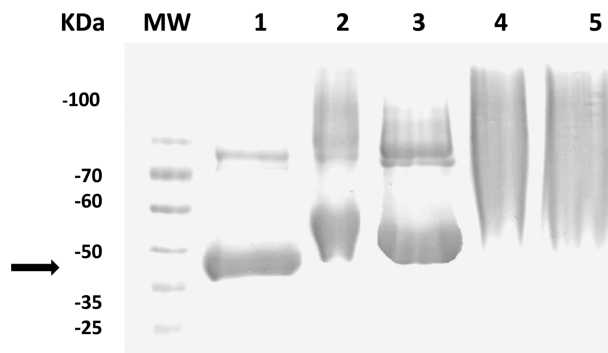


Figure 2. Electrophoretic profile (SDS-PAGE gel) of native and glycyated OVA. Lane 1 represents native OVA. Lane 2 represents OVA-Glucose; lane 3 represents OVA-Mannose; lane 4 represents OVA-Glucomannan; and lane 5 represents OVA-Galactomannan.

of intact OVA content in the glycyated fractions. Polysaccharides OVAGluMan (Fig. 2; lane 4) and OVAGalMan (Fig. 2; lane 5) had diffused aggregated bands, indicating significant modification of the protein caused by glycyation. The binding ratios of the glycyated conjugates are given in Table 1. Free amino acid estimation for various glycoconjugates is summarized in Table 2.

3.2 Suppression of allergic phenotype

Clinical signs and clinical scores were less severe and less frequent in the OVAMan and OVAGluMan treated groups (Fig. 3A; $p < 0.05$). Significant reduction of histamine concentration (Fig. 3B; $p < 0.05$) and MMCP (Fig. 3C; $p < 0.05$) were found only in the OVAMan treated group. All other groups did not differ significantly ($p > 0.05$).

3.3 Ig isotype-related antibody activity

Significant difference in OVA-specific IgE-related antibody activity (Fig. 3E; $p < 0.05$) was observed with OVAMan and OVAGluMan treated groups. OVAMan group also had less specific IgG (3D; $p < 0.05$). However, no significant difference

Table 2. Estimation of free amino acids using 2,4,6-trinitrobenzene sulphonic acid assay

OVA conjugates	Free amino acids (µmol/mL)
OVA	16.64
OVAGlu	1.08
OVAMan	0.76
OVAGluMan	0.74
OVAGalMan	0.56

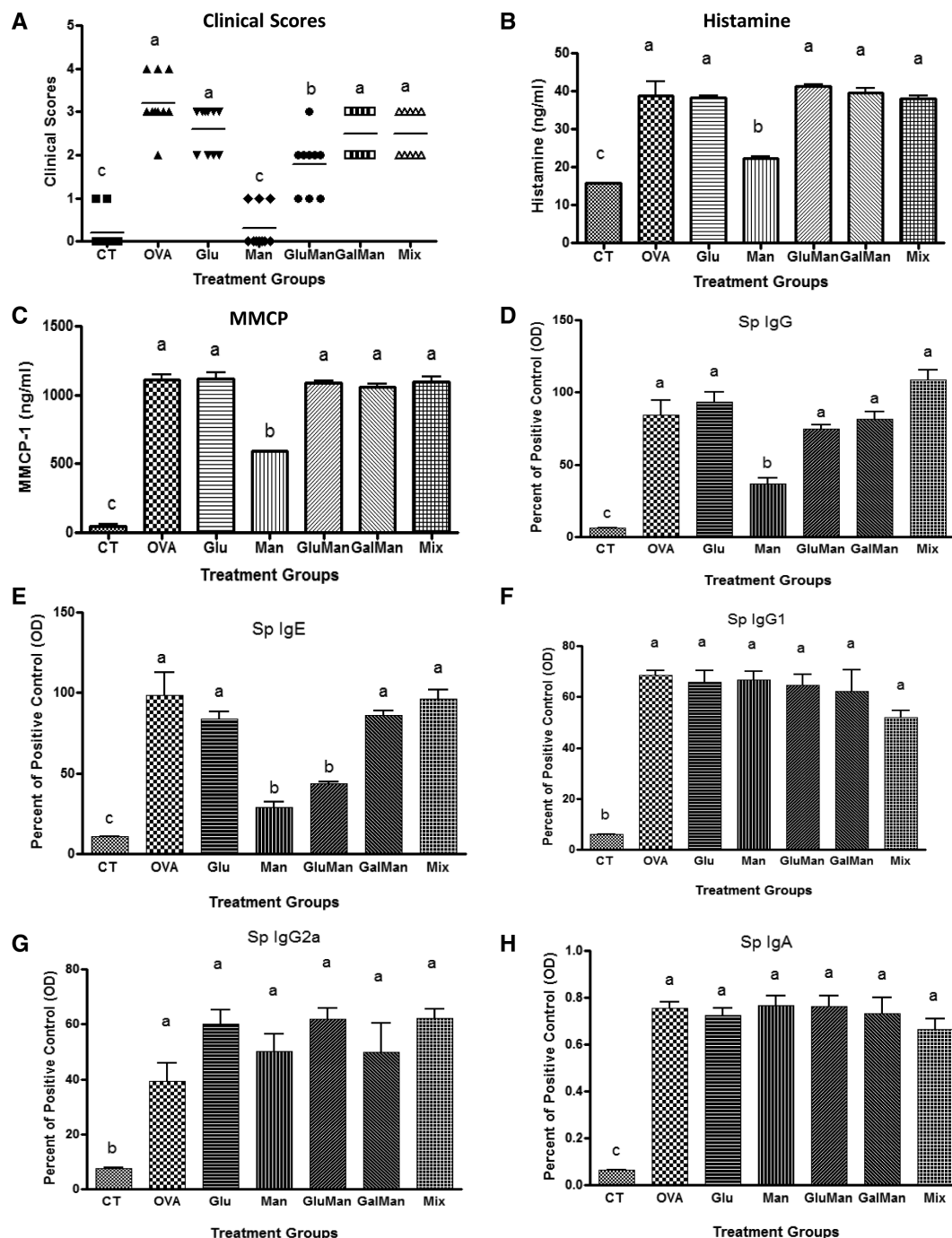


Figure 3. (A) Clinical scores for individual mice. Total clinical scores for each mice post-OVA challenge were calculated. Average scores assigned by three independent observers in a blinded fashion are represented. Different letters indicate statistically significant differences. (B) Serum histamine concentration. Data for serum histamine concentration are represented as mean $\pm$ SD ($n = 5$ pooled sera). Different letters indicate statistically significant differences ($p < 0.05$) between groups of mice. (C) Mouse mast cell protease concentration (MMCP). Data for MMCP concentration are represented as mean $\pm$ SD. (D and E) OVA-specific serum IgG (whole molecule) and IgE-related antibody activity. Rabbit antimouse IgG conjugated to alkaline phosphatase was used for detection of IgG and rat monoclonal antimouse IgE was used for IgE followed by streptavidin–horse radish peroxidase 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 serum–OD of negative control) $\times$ 100%. Different letters indicate statistically significant differences at $p < 0.05$. F and G represent OVA-specific serum IgG1-, IgG2-, and IgA- related antibody activity. Rat monoclonal antimouse IgG1 and IgG2a were used, followed by streptavidin–horse radish peroxidase conjugate. H represents OVA-specific IgA was detected from pooled fecal samples collected from weeks 9–11, using biotinylated rat monoclonal antimouse IgA antibody followed by avidin–horse radish peroxidase conjugate. Different letters indicate statistically significant differences.

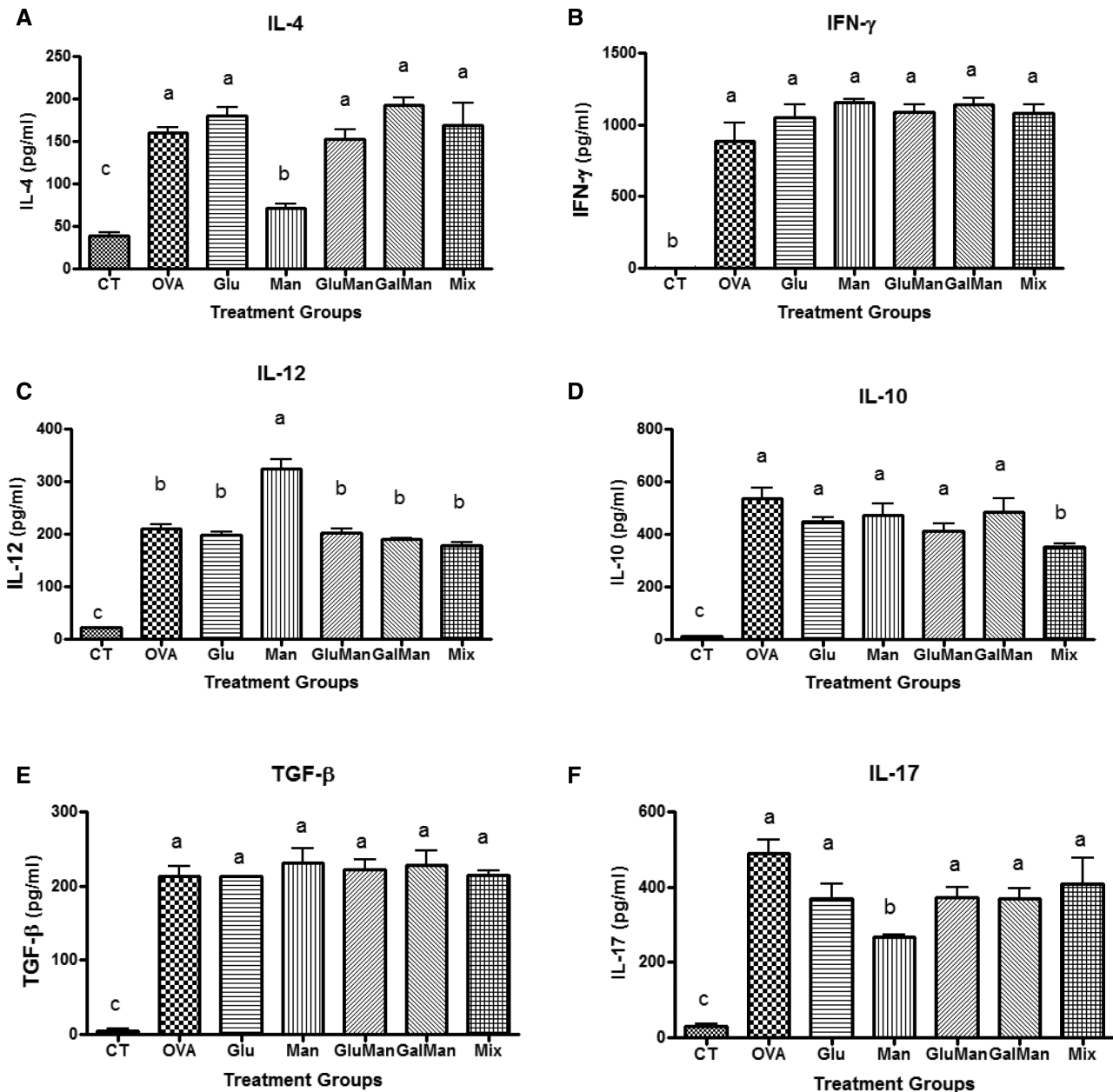


Figure 4. Cytokine concentration. Spleen was collected from each mice and pooled within groups ($n = 5$), and splenocytes were isolated and cultured at 2.5×10^6 cells/mL concentration of cells in triplicate wells as unstimulated (control) or stimulated with $50 \mu\text{g}/\text{well}$ of OVA for 72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

in IgG1, IgG2a, and specific IgA activity was detected between groups otherwise treated (Fig. 3F–H; $p > 0.05$).

3.4 Cytokine concentration

OVA-specific proliferation of cells was used to measure cytokine production from splenocytes. The OVAMan treated group had less IL-4 and IL-17, and more IL-12 cytokine as

compared to groups otherwise treated (Fig. 4, $p < 0.05$). Other cytokines such as IFN- γ , IL-10, and TGF- β did not differ significantly between groups ($p > 0.05$).

3.5 Increase in regulatory T cells indicates immune regulation

The proportion of CD25⁺ Foxp3⁺ T-regs was increased in both the OVAMan and OVAGluMan treated groups (Fig. 5B,

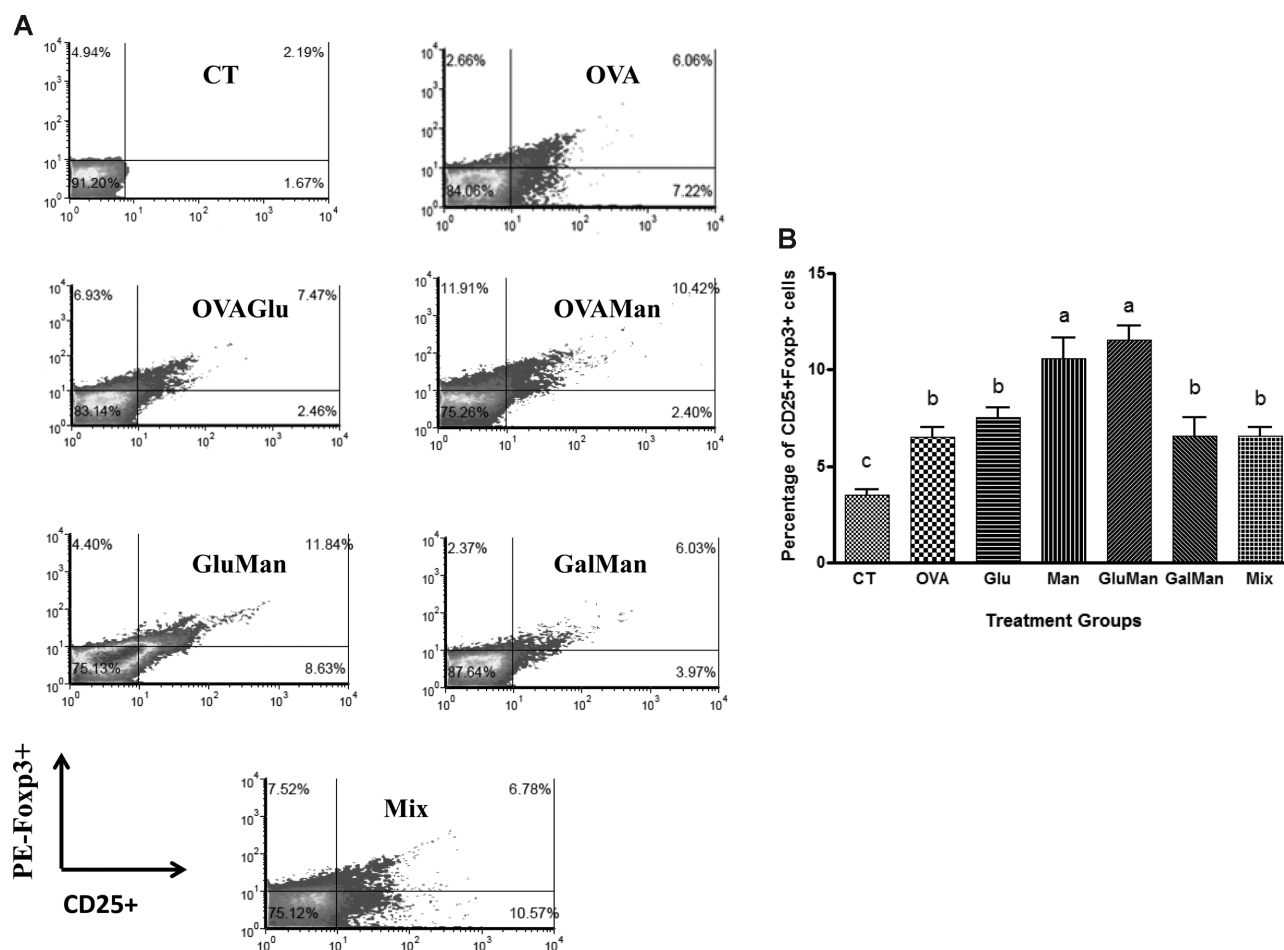


Figure 5. Flow cytometry. (A) A representative image of CD25⁺ Foxp3⁺ cells for each group is shown. (B) Percentage of CD25⁺ Foxp3⁺ cells was determined by FACS from whole blood of mice collected at the end point of the experiment. Different letters indicate statistically significant differences between groups.

$p < 0.05$). A representative image for each treatment group is shown in Fig. 5A. All other groups treated otherwise did not differ significantly ($p > 0.05$).

3.6 OVAMan impedes maturation and inhibits uptake as measured with splenic DCs

DC maturation phenotype as measured by costimulatory markers CD86 and MHC Class II revealed that OVAMan treated group had significantly less expression of both the costimulatory markers (Fig. 6A and B, $p < 0.05$) indicating altered expression of these surface molecules. The antigen uptake of splenic DCs revealed less uptake in both the CD11c^{high} and CD11c^{medium} sorted cells with OVAMan (Fig. 7A, $p < 0.05$). There was no significant difference observed between other treated groups ($p > 0.05$). Only basal level of uptake of FITC-OVA was observed in CT treated group. Results shown are representative images of two identical uptake experiments conducted.

3.7 Cytokine production in cocultures of T cells and DCs reveals a balanced profile by OVAMan

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 OVAMan group, indicating less CD4⁺ T-cell activation. Also, cocultures revealed less IL-4 (Fig. 7B, $p < 0.05$) and more IL-12 (Fig. 7B, $p < 0.05$) in the OVAMan group. Interestingly no significant difference among groups was observed with IFN- γ , IL-10, and IL-17 ($p > 0.05$).

3.8 CD secondary structure reflects functional role in suppression of allergic response

The CD profile of OVAMan was different than that of the native OVA (Fig. 8A); in particular the structure of the α -helix side chain residues of native OVA was altered from that of OVAMan. The n - π^* transition at 222 nm of OVAMan was decreased compared to the native analogue. The wavelength

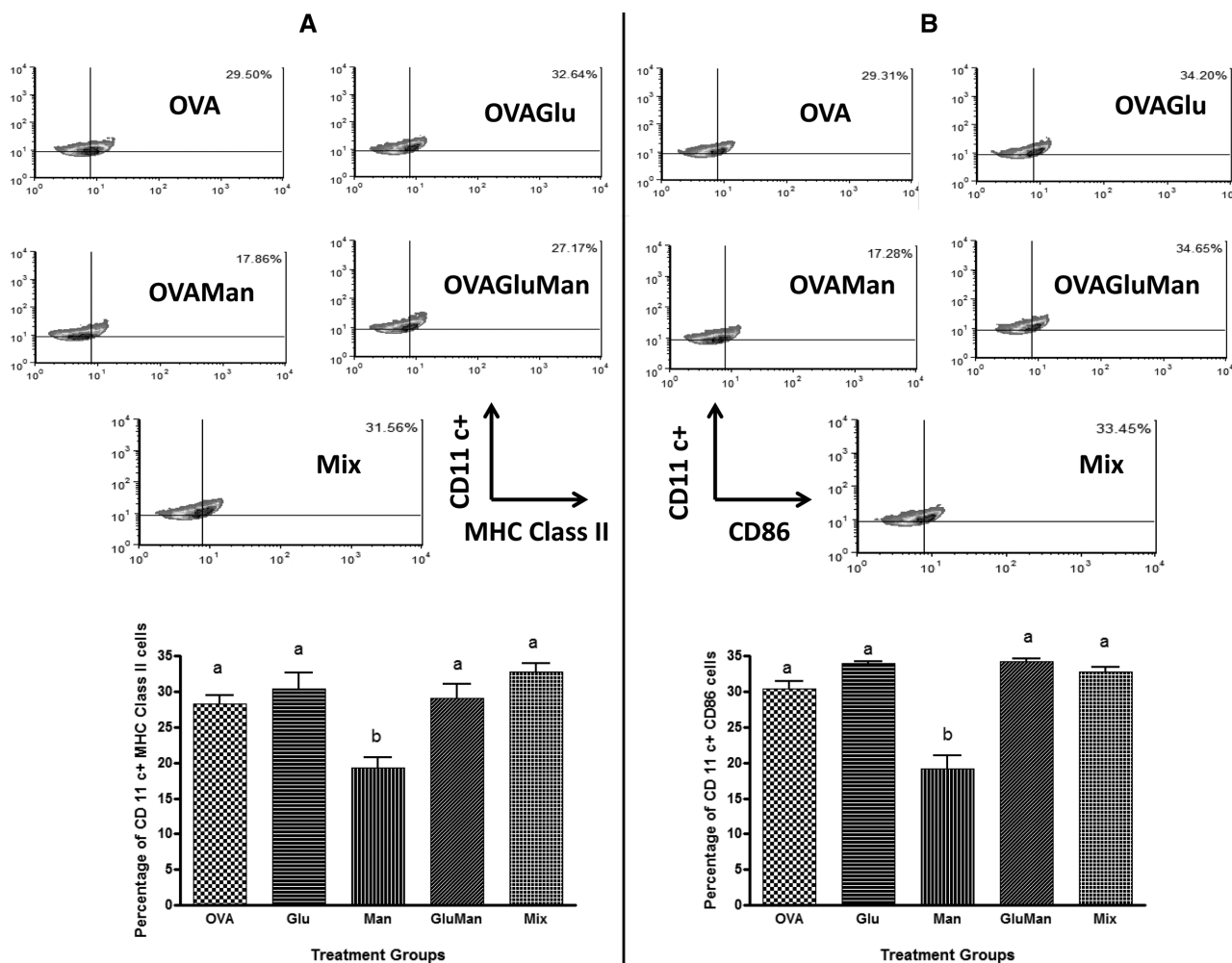


Figure 6. Flow cytometric analysis of DC phenotype. DCs cultured as described in Methods and assessed by levels of expression of CD11c⁺ and costimulatory molecules major histocompatibility complex (MHC) class II and CD86, stimulated with CT for maturation and various glycosylated forms of OVA. The data shown are representative FACS images showing expression levels of MHC Class II and CD86 maturation for various groups. Different letters indicate statistically significant differences ($p \leq 0.05$) between groups in the graph.

minima at 207 and 222 nm, and the maximum at 191 nm, both typical characteristics of α -helical configuration, were reduced in OVAMan (Fig. 8A). This indicates that OVA-Man may have a slightly less compact structure than the native form. The structure of OVAGlu did not differ as compared to native OVA (Fig. 8B).

4 Discussion

There is a growing body of evidence that glycation structures influence resistance or susceptibility to food allergies. This research tested the hypothesis that glycosylated OVA forms may alter allergic IR in a Balb/c mouse model of egg allergy. Response to various treatments was assessed by allergic clinical signs; histamine and mast cell protease concentration; serum anti-OVA antibody as IgG, IgG1, IgG2, and IgE; as

well as cytokine production; percentage of T-reg cells; and in vitro response using DCs and T cells. Interestingly, in this study, both OVAMan and OVAGluMan were effective in reducing allergic clinical signs. Strikingly, OVAMan was found to exert various effects and differed in most of the IR phenotype parameters and other correlates of clinical signs measured. This alteration in IR was type-1 biased and reflected by significant differences in histamine and mast cell protease concentration, isotype-specific antibody-related activity, type-1/2 cytokine profiles, and proportion of T-reg cells. Furthermore, in vitro DC uptake, maturation, T-cell activation, and type-2 response were reduced by OVAMan. OVAGluMan treatment also had IR altering activity, reflected by reduced clinical signs and specific IgE, and increased proportion of T-reg cells. Other groups did not have an effect on change in IR bias or reduction of clinical signs. The observation that the mixture group did not elicit an effect was in contrast to what

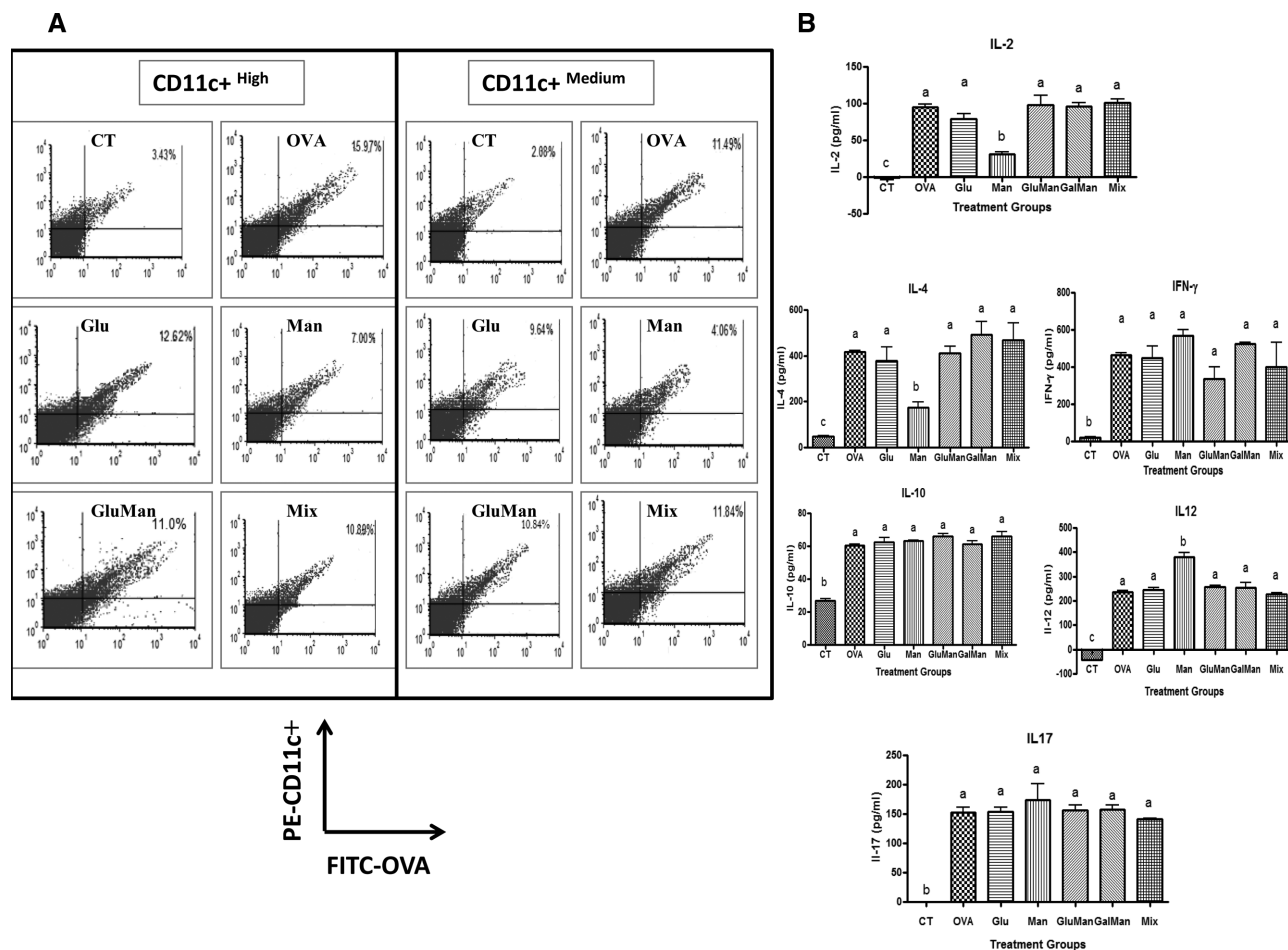


Figure 7. (A) The uptake of OVA glycosylated forms by CD11c⁺high and CD11c⁺medium sorted DCs. Wild-type DCs were incubated with 5.0 µg/mL of FITC-conjugated native OVA or glycosylated OVA forms for 3 h. The uptake of labelled OVAs by the DCs was analyzed by means of flow cytometry with PE-conjugated CD11c⁺ antibody. The data are representative of two separate experiments. (B) Purified CD4⁺ T cells were co cultured with myeloid DCs and stimulated with native OVA, or various glycosylated OVA forms for 72 h. The concentration of IL-2, IL-4, IFN-γ, IL-10, IL-12, IL-17 in the culture supernatant was measured by means of ELISA. The data are representative of two separate experiments ($p < 0.05$).

Suzuki et al. had observed earlier [22] that hydrolyzed konjac glucomannan alone suppressed the production of plasma IgE and prevented germline class-switching and IgE production. While mechanistic studies were not conducted here, it seems likely that the allergic response was driven by the presence of OVA content in the mixture and therefore may have prevented the attenuation of allergic signs.

Research conducted earlier has resulted in contradictory reports. It was reported that glucose glycosylated OVA led to increased allergic IR [13, 14], but on the other hand oral administration of buckwheat allergen Fage-1 conjugated to glucomannan reduced allergic signs in Balb/c allergic mice [12]. Antigenicity of cedar major allergen Cry j 1 was found to be greatly diminished with Cry j 1-conjugated galactomannan [30]. Both Cry j 1-galactomannan and Cry j 1-mannose were effectively transported into the gut and colocalized with

DCs in the gut using histological analysis. Furthermore, a recent article described, thermal processing of OVA induced activation of different T-cell subpopulations, shift towards type-1 response, and ultimately reduced its allergenicity [31]. Our results confirm and extend this finding in that the data clearly indicated that both OVAMan and OVAGluMan were able to suppress allergic IR. Also, in the present study, we found that glucose glycosylated OVA did not have an effect on reduction of allergic response, supporting earlier evidence [13, 14]. It is also of interest to note that OVA-conjugated glucomannan did have an effect in this study, similar to what was observed earlier [12, 30].

It was of interest to note that the concentration of the type-1-associated cytokine IL-12 and the type-2-associated IL-4 and IL-17 cytokines measured in the OVAMan treated group were more able to regulate cytokine production toward a balanced

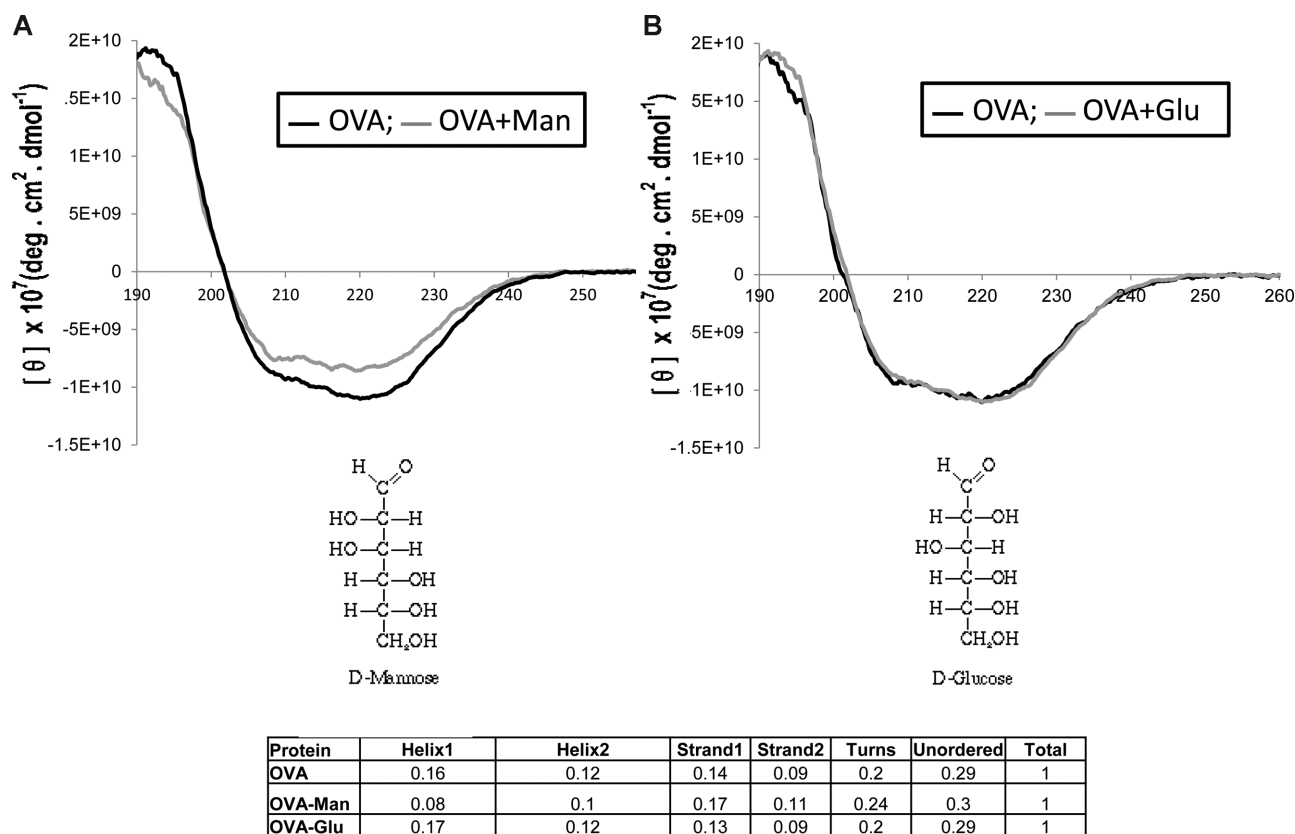


Figure 8. Circular dichroism spectrum of OVA monosaccharide conjugates. The far UV spectra were measured by scanning four times from 190 to 250 nm. The proteins (0.2 mg/mL) were dissolved in 10 mM phosphate buffer, pH 7, and blank containing phosphate buffer was measured and subtracted from the spectra. The dark line denotes the native OVA spectrum and the faded line denotes the conjugated spectrum.

response, than those otherwise treated. This explains a well-maintained homeostasis and balanced immune regulation mediated by OVAMan. Involvement of T-regs was also of interest in this study since induction of allergen-specific T-regs is a critical event that leads to the development of healthy IR [32]. Therefore, we measured the proportion of circulating CD25⁺ FoxP3⁺ cells in mice from each treatment group. In this study increase in T-regs in both the OVAMan and OVAGluMan treated groups was observed. This observation complements our other findings indicating that the increase in T-regs in this case may have been controlled via the modulation of IL-17 response and increase in type-1 response (IL-12). However, since the regulatory cytokines (IL-10 and TGF-β) did not differ among groups, it may be postulated that this mechanism of suppression is independent of cytokines such as IL-10 and TGF-β. Also with regards to OVAGluMan group, although increase in T-reg proportions was observed this did not complement other parameters such as the cytokines measured. Thus, the complexity of actual regulatory interactions involved in the type-1:type-2 paradigms still remains unclear and needs to be elucidated further.

In an attempt to have a better understanding of the role of the various glycated CHO forms in OVA allergen recognition

by the innate immune system, we examined the influence of these CHO forms on allergen recognition by DCs. DCs express innate pattern recognition receptors such as toll-like receptors that respond to their ligands by producing high amounts of IL-12, which promotes a type-1 response [33]. It has been reported earlier that DCs recognize OVA via interactions with MR and thus MR is known to play a key role in DC-mediated antigen recognition and internalization of OVA [20]. Furthermore, it has been shown that DCs are able to incorporate OVA via the MR pathway as well as via macropinocytosis as described earlier [34]. Interestingly in this study, using in vitro type-2 CT-stimulated DC-activation model, we found less maturation and less uptake with OVA-Man. This confirmed that OVAMan altered the phenotype of DCs via changes in surface molecule expression. It was also observed that the uptake of OVA and its glycated forms were completely inhibited by adding MR inhibitor mannan at 200 μg/mL (data not shown). This confirms mannan as a biological ligand for DCs.

In vitro coculture experiments with T cells and DCs revealed less type-2 (IL-4) and more type-1 (IL-12) cytokine production, and decreased T-cell activation as evidenced by reduced IL-2 production in the OVAMan group. This

observation supported the in vivo finding since the DCs not only affected in vitro polarization of T cells, but also the in vivo function of the obtained polarized T cells as observed with the cytokine data. The only exception to this was the IL-17 production that differed between the two data sets (in vivo and in vitro), and the reason for this remains to be elucidated. T-cell response to DCs is of pivotal importance in the induction of immunity with regards to antigen presentation and mediating effector IR. DCs orchestrate innate inflammatory responses and adaptive immunity through activation of T cells. The resulting phenotype induced by OVAMan was marked by an overall reduction in antigen presentation capability as evidenced by less maturation and uptake of DCs, dampened lymphocyte-stimulating capacity, and reduced cytokine responsiveness in cocultures. The secondary structure further revealed that alpha-helix was reduced in OVAMan and this may have led to suppression of allergic response, indicating a structure–function relationship. However, the CD spectra of polysaccharides were not measured in this study due to technical difficulties and thus mechanism related to suppression in allergic response with OVA-GluMan needs to be further explored. Also, in a recent study it was reported that changes in OVA secondary structure caused by thermal processing induced a shift toward a type-1 response and ultimately reduced allergenicity in a mouse model of food allergy [31] similar to what was observed here, which validates further that structure does play a role in reducing allergy.

Overall, these data suggest different mechanisms of the structurally different glycosylated forms of OVA, leading to the altered allergic IR via DC functions; however, detailed molecular mechanisms on what causes the mannose form to attenuate allergic response remains to be elucidated. This study provides the first investigation that both glycosylated OVAMan and OVAGluMan have the ability to alter allergic IR to OVA-induced egg allergy. Also regulation of DC differentiation and T-cell activation were found to be important mechanisms for controlling the initiation of IR with regards to OVAMan modulation. Overall, OVAMan and OVAGluMan both could potentially act as valid immune-regulating molecules to attenuate food allergies.

The authors would like to acknowledge funding support to Y. Mine from NSERC Discovery grant. The authors would like to thank all the staff at the Central Animal facility, University of Guelph and lab colleagues for their skillful technical assistance in help with the animal study. Y. M. designed and supervised the study, P. R. performed the experiment, and all authors interpreted the data and wrote the paper.

The authors have declared no conflict of interest.

5 References

- [1] Sicherer, S. H., Sampson, H. A., Food allergy. *J. Allergy Clin. Immunol* 2006, 117, S470–S475.
- [2] Wood, R. A., The natural history of food allergy. *Pediatrics* 2003, 111, 1631–1637.
- [3] Savage, J. H., Matsui, E. C., Skripak, J. M., Wood, R. A., The natural history of egg allergy. *J. Allergy Clin. Immunol.* 2007, 120, 1413–1417.
- [4] Escudero, C., Quirce, S., Fernández-Nieto, M., Miguel, J. et al., Egg white proteins as inhalant allergens associated with baker's asthma. *Allergy* 2003, 58, 616–620.
- [5] Mayer, L., Shao, L., Therapeutic potential of oral tolerance. *Nat. Rev. Immunol.* 2004, 4, 407–419.
- [6] Matsuda, T., Ishiguro, H., Ohkubo, I., Sasaki, M. et al., Carbohydrate binding specificities of monoclonal antibodies raised against lactose-protein Maillard adducts. *J. Biochem.* 1992, 111, 383–387.
- [7] Nakamura, A., Watanabe, K., Ojima, T., Ahn, D. H. et al., Effect of maillard reaction on allergenicity of scallop tropomyosin. *J. Agric. Food. Chem.* 2005, 53, 7559–7564.
- [8] Gruber, P., Becker, W. M., Hofmann, T., Influence of the maillard reaction on the allergenicity of rAra h 2, a recombinant major allergen from peanut (*Arachis hypogaea*), its major epitopes, and peanut agglutinin. *J. Agric. Food. Chem.* 2005, 53, 2289–2296.
- [9] Hattori, M., Miyakawa, S., Ohama, Y., Kawamura, H. et al., Reduced immunogenicity of beta-lactoglobulin by conjugation with acidic oligosaccharides. *J. Agric. Food. Chem.* 2004, 52, 4546–4553.
- [10] Wilson, S., Blaschek, K., de Mejia, E., Allergic proteins in soybean: processing and reduction of P34allergenicity. *Nutr. Rev.* 2005, 63, 47–58.
- [11] Taheri-Kafrani, A., Gaudin, J. C., Rabesona, H., Nioi, C. et al., Effects of heating and glycation of beta-lactoglobulin on its recognition by IgE of sera from cow milk allergy patients. *J. Agric. Food. Chem.* 2009, 57, 4974–4982.
- [12] Suzuki, Y., Kassai, M., Hirose, T., Katayama, S. et al., Modulation of immunoresponse in BALB/c mice by oral administration of Fag e 1-glucomannan conjugate. *J. Agric. Food. Chem.* 2009, 57, 9787–9792.
- [13] Ilchmann, A., Burgdorf, S., Scheurer, S., Waibler, Z. et al., Glycation of a food allergen by the Maillard reaction enhances its T-cell immunogenicity: role of macrophage scavenger receptor class A type I and II. *J. Allergy. Clin. Immunol.* 2010, 125, 175–183.
- [14] Hilmenyuk, T., Bellinghausen, I., Heydenreich, B., Ilchmann, A. et al., Effects of glycation of the model food allergen ovalbumin on antigen uptake and presentation by human dendritic cells. *Immunology* 2010, 129, 437–445.
- [15] Matsuda, T., Nakamura, R., Nakashima, I., Hasegawa, Y. et al., Human IgE antibody to the carbohydrate-containing third domain of chicken ovomucoid. *Biochem. Biophys. Res. Commun.* 1985, 129, 505–510.
- [16] Besler, M., Steinhart, H., Paschke, A., Allergenicity of hen's egg-white proteins: IgE binding of native and deglycosylated ovomucoid. *Food. Agric. Immunol.* 1997, 9, 277–288.
- [17] Zhang, J. W., Mine, Y., Characterization of IgE and IgG epitopes on ovomucoid using egg-white-allergic patient's sera. *Biochem. Biophys. Res. Commun.* 1998, 253, 124–127.

- [18] Rupa, P., Nakamura, S., Mine, Y., Genetically glycosylated ovomucoid third domain can modulate Immunoglobulin E antibody production and cytokine response in Balb/c mice. *Clin. Exp. Allergy* 2007, 37, 918–928.
- [19] Purcell, A. W., van Driel, I. R., Gleeson, P. A., Impact of glycans on T-cell tolerance to glycosylated self-antigens. *Immunol. Cell. Biol.* 2008, 86, 574–579.
- [20] Li, J., Jiang, H., Wen, W., Zheng, J. et al., The dendritic cell mannose receptor mediates allergen internalization and maturation involving notch 1 signalling. *Clin. Exp. Immunol.* 2010, 162, 251–261.
- [21] Usui, M., Saito, A., Taniguchi, N., Nishijima, N. et al., Reduction of antigenicity of Cry j 1, major allergen of Japanese cedar pollen, by the attachment of polysaccharides. *Biosci. Biotechnol. Biochem.* 2003, 67, 2425–2430.
- [22] Suzuki, H., Oomizu, S., Yanase, Y., Onishi, N. et al., Hydrolyzed Konjac glucomannan suppresses IgE production in mice B cells. *Int. Arch. Allergy Immunol.* 2010, 152, 122–130.
- [23] Guo, Z., Jennings, H., Protein-polysaccharide conjugation. *Methods Mol. Med.* 2001, 66, 49–54.
- [24] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227, 680–685.
- [25] Dubois, M., Gillies, K. A., Hamilton, J. K., Rebers, P. A. et al., Calorimetric method for determination of sugars and related substances. *Anal. Chem.* 1956, 28, 350–356.
- [26] Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K. et al., Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 1985, 150, 76–85.
- [27] Kanska, U., Budzynska, R., Nevozhay, D., Boratynski, J., Preparation of mannan-protein conjugates using high-temperature glycation. *Biotechnol. Appl. Biochem.* 2008, 49, 57–64.
- [28] Mine, Y., Yang, M., Epitope characterization of ovalbumin in Balb/c mice using different entry routes. *Biochem. Biophys. Acta.* 2007, 1774, 200–212.
- [29] Rupa, P., Mine, Y., Oral immunotherapy with immunodominant T-cell epitope peptides alleviates allergic reactions in a Balb/c mouse model of egg allergy. *Allergy* 2012, 67, 74–82.
- [30] Aoki, R., Saito, A., Azakami, H., Kato, A., Effects of various saccharides on the masking of epitope sites and uptake in the gut of cedar allergen cry j 1–saccharide conjugates by a naturally occurring Maillard reaction. *J. Agric. Food. Chem.* 2010, 58, 7986–7990.
- [31] Golias, J., Schwarzer, M., Wallner, M., Kverka, M. et al., Heat-induced structural changes affect OVA-antigen processing and reduce allergic response in mouse model of food allergy. *PLoS One* 2012, 7, e37156.
- [32] Akdis, C.A., Akdis, M., Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *J. Allergy Clin. Immunol.* 2009, 123, 735–746.
- [33] Bellinghausen, I., Modification of the innate immune function of dendritic cells by allergen-specific immunotherapy. *Clin. Exp. Allergy* 2010, 40, 12–14.
- [34] Burgdorf, S., Lukacs-Kornek, V., Kurts, C., The mannose receptor mediates uptake of soluble but not of cell-associated antigen for cross-presentation. *J. Immunol.* 2006, 176, 6770–6776.