

Oral immunotherapy with immunodominant T-cell epitope peptides alleviates allergic reactions in a Balb/c mouse model of egg allergy

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Abstract

Background: Allergen-specific T-cell epitopes are obvious targets for immunotherapeutic interventions in allergic disease. T-cell epitope peptides given orally may provide a practical way of inducing tolerance and preventing allergy.

Objective: This study investigates oral immunotherapy (OIT) with T-cell epitope peptides of the dominant egg-white allergen ovomucoid (Ovm) in a Balb/c mouse model of egg allergy.

Methods: Groups of mice were orally sensitized to Ovm and subsequently administered Ovm T-cell epitopes [single peptide 157–171 (SP) or multiple peptide (157–171)₃ (MP)], followed by oral challenge with Ovm. Outcomes post oral challenge were measured as clinical signs, serum histamine, antibody activity (IgG, IgE, IgG1, IgG2, IgA), cytokines (IL-4, IFN- γ , IL-12p70, IL-10, TGF- β , and IL-17), and T regulatory cells (Tregs).

Results: Clinical signs were less frequent in both SP and MP groups ($P \leq 0.05$). Specific IgE was less and IgA was more in both groups; however, SP-treated mice had less histamine and IgG1 and more IgG2-related antibodies indicating a bias toward the type-1 response ($P \leq 0.05$). Concentration of type-2 cytokine interleukin-4 (IL-4) was significantly less in both groups and IL-12p70 and IL-10 were more in SP-treated mice ($P \leq 0.001$). Interferon- γ , IL-17, and TGF- β did not differ significantly. There was significant increase in the percentage of CD4+FOXP3+ and CD4+CD25+ cells in the SP group, indicating the significant role of Tregs in immune regulation.

Conclusion: In summary, we demonstrated that OIT with SP and MP comprising the immunodominant regions of Ovm was safe and significantly reduced subsequent frequency of allergy to Ovm, and validated potential use of Ovm T-cell epitope as an immunoregulator.

Allergen-specific immunotherapy (SIT) using intact allergens has been used to treat allergies (1); however, this type of therapy has been associated with the risk of adverse side-effects. To avoid this risk, peptide-based immunotherapy (PIT) using dominant T-cell epitopes has been proposed as an alternative treatment strategy. Administration of dominant T-cell epitopes induces either anergy, T-cell deletion, or active cellular suppression mediated by regulatory T cells (Tregs) (2). Evidence of PIT by a single T-cell epitope conferred tolerance and suppressed allergy via linked epitope suppression/bystander suppression. (3).

There is growing body of evidence that Tregs potently suppress IgE production. Defects in Treg activity likely contributes to the development of food allergy. Skewing of allergen-specific effector T cells to Tregs appears to be a crucial event in successful allergen-SIT (4). Both naturally occurring CD4+CD25+ Tregs and inducible allergen-specific IL-10-secreting Tregs (Tr1) inhibit effector cells and play a central role in the maintenance of peripheral homeostasis (5). We have recently identified ovalbumin (OVA) B- and T-cell epitopes (6, 7) and validated PIT, demonstrating the therapeutic potential of subcutaneously injected OVA T-cell

epitopes acting via the regulatory molecules TGF- β and FOXP3, to reduce allergy in a mouse model of food allergy (8).

Oral immunotherapy (OIT) has been documented recently for the treatment of peanut, milk, and egg allergies, and the overall rate of success ranged between 70% and 80% (9–12); however, in most cases, adverse side-effects have been reported. Thus, the use of dominant T-cell epitopes given orally may provide a safe and effective strategy against food allergies.

Oral immunotherapy with T-cell epitopes has been described for pollen allergy in animal (13) and human (14) subjects. This has resulted in reduction in allergen-specific T-cell proliferation and IgE. Furthermore, oral administration of a T-cell epitope of Cryj 2 inhibited signs of allergic rhinitis and hyperreactivity (15) and had less proliferative responses of T cells, IgE antibodies and cytokine production in Cryj 2-sensitized mice (16). Also, recently five dominant CD4⁺ T-cell epitopes of Arah2 peptides were identified as novel candidates for T-cell-targeted immunotherapy of peanut allergy (17).

To the best of our knowledge OIT based on T-cell epitopes to treat food allergies have not been documented yet. Hence in the current study we sought to investigate a safe T-cell epitope-targeted OIT using the dominant T-cell epitope of the major egg-allergen ovomucoid (Ovm) for an immunotherapeutic approach to tolerance induction in a mouse model of food allergy. To circumvent the risk associated with systemic injections, an oral approach was attempted, and synthetic peptides comprising the immunodominant T-cell determinants of Ovm were administered orally to orally sensitized Balb/c mice. Outcomes were measured as clinical signs, serum histamine, antibody activity (IgG, IgE, IgG1, IgG2 and IgA), cytokines (IL-4, IFN- γ , IL-12, IL-10, TGF- β , and IL-17), and Tregs.

Materials and methods

Design of synthetic T-cell epitope peptides

Localization of T-cell epitope regions of Ovm (amino acids 100–114 and 157–171) was earlier documented for Balb/c mice (18). The immunodominant peptide 157–171 was chosen (18). Two synthetic peptides comprising 15 mer (157–171; single peptide, SP-DNKTYGNKSNFSNAV) and 51 mer [3 repeated units of SP linked by alanine residues (157–171)₃; multiple peptide, MP-DNKTYGNKSNFSNAVAADNKTYGNKSNFSNAVAADNKTYGNKSNFSNAV] were purchased (GL Biochem Ltd, Shanghai, China), with a purity of 99% as confirmed by mass spectrometry. Cysteine residues were replaced with serine as the peptide had 3.7- to 4.8-fold higher stimulation activity when replaced (18).

Experimental design

All animal-use protocols were approved by the University of Guelph Animal Care Committee under the guidelines of Canadian Council for Animal Care. A total of 40 female

Balb/c mice (6–8 weeks of age) were used in this study. All animals were housed in the campus animal facility under an egg-free diet (Teklad global diet) in a 12-h lighting cycle. The mice were orally sensitized twice per week for 4 weeks with 1 mg of Ovm (19) adjuvanted with 10 μ g of cholera toxin (CT; List Biologicals, Campbell, CA, USA) (8), as outlined in Fig. 1. The negative control group received CT alone ($n = 10$). Post sensitization, mice received SP and MP treatments (at similar concentration). All mice were orally challenged with 20 mg of Ovm, and clinical signs of allergy were monitored as described earlier (8). Blood and spleen were collected for the measurement of various biomarkers.

Assessment of clinical signs

The following clinical scores were assigned as described earlier (8): 0 = no signs; 1 = repeated scratching; 2 = puffiness around eyes and snout, diarrhea, pilar erecti, reduced activity with increased respiratory rate; 3 = wheezing, labored respiration, cyanosis; 4 = tremor and convulsions; and 5 = death. Total scores were obtained by adding scores for individual signs.

Serum histamine

Whole blood was collected and two serum samples were pooled in equal volumes within each group ($n = 5$ /group) according to the volume of blood required to conduct Enzyme-Linked Immunosorbent Assay (ELISA). Serum histamine concentrations were assayed using a commercial ELISA kit (Labor Diagnostika Nord, Nordhon, Germany).

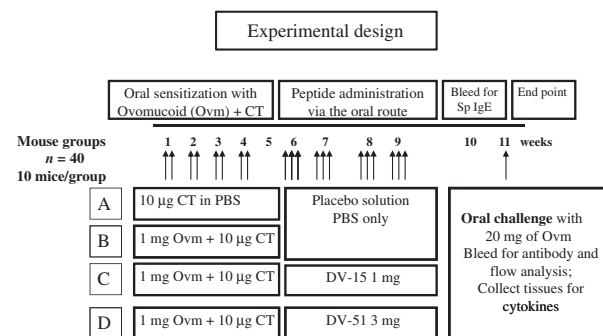


Figure 1 Outline of the experimental design. Mice were sensitized to 1 mg of ovomucoid (Ovm) and 10 μ g of cholera toxin for 4 weeks, twice per week followed by 4 weeks of oral treatments with peptides (single peptide, multiple peptide) given thrice a week. Negative control group received phosphate-buffered saline, and the positive control group received Ovm. Clinical signs of hypersensitivity were recorded immediately after oral challenge with 20 mg of Ovm, and 30 min later, all mice were killed for the collection of blood and spleen. Both blood and spleen were pooled within treatments after collection ($n = 5$).

ELISA

Standard methods were used to measure IgG (H+L)-, IgG₁-, IgG₂- and IgE-related antibody activity. Ovm-specific murine IgG and IgE antibodies were detected as described previously (8). Antibodies associated with IgG₁ and IgG₂ were detected using biotinylated monoclonal rat antibodies (BD Biosciences, Mississauga, ON, Canada) and streptavidin–HRP conjugates (BD Biosciences) (20). Optical densities (OD) were quantified (iMark; BioRad, Mississauga, ON, Canada), and the reaction was stopped when OD of the positive control reached 1.0. Results were expressed as percentage of the positive control OD (sample OD/[positive control OD – negative control OD]) × 100.

Cytokine analysis

Individual spleens were collected aseptically from each mouse into ice-cold RPMI-1640 medium (Invitrogen, Grand Island, NY, USA), and two spleens were pooled within each group ($n = 5$ /group). Single-cell suspensions were prepared as described previously (20) and stimulated with 50 µg/well of Ovm. Culture supernatants were collected after 72 h. Cell proliferation was measured by WST-1 assay (Roche, Laval,

QC, Canada). Cytokine concentration was measured using ELISA kits for IL-4 and IFN- γ (BD Biosciences) and for IL-10, IL-12, IL-17A, and TGF- β (eBioscience, Mississauga, ON, Canada) as outlined by the manufacturers. Data were expressed as sample means for test replicates.

Determination of Ovm-specific IgA

Fecal pellets were freshly collected on weeks 9, 10, and 11 (posttreatments) from each cage ($n = 2$ /group for each week; 6 samples/group) and were processed as described earlier (8). Concentrations of Ovm-specific IgA were determined using an indirect ELISA (8).

Flow cytometry

Blood was collected in 1.5-ml heparinized tubes from individual mouse, and two samples were pooled ($n = 5$ /group). Using whole blood, extracellular staining with FITC-conjugated anti-mouse-CD4 and PE-conjugated anti-mouse-CD25 (BD Biosciences) and intracellular staining with anti-mouse-FOXP3 (eBioscience) were performed as described by the manufacturer (BD Biosciences). Cells were acquired using FACSCalibur™ (BD Biosciences) and analyzed using FCS

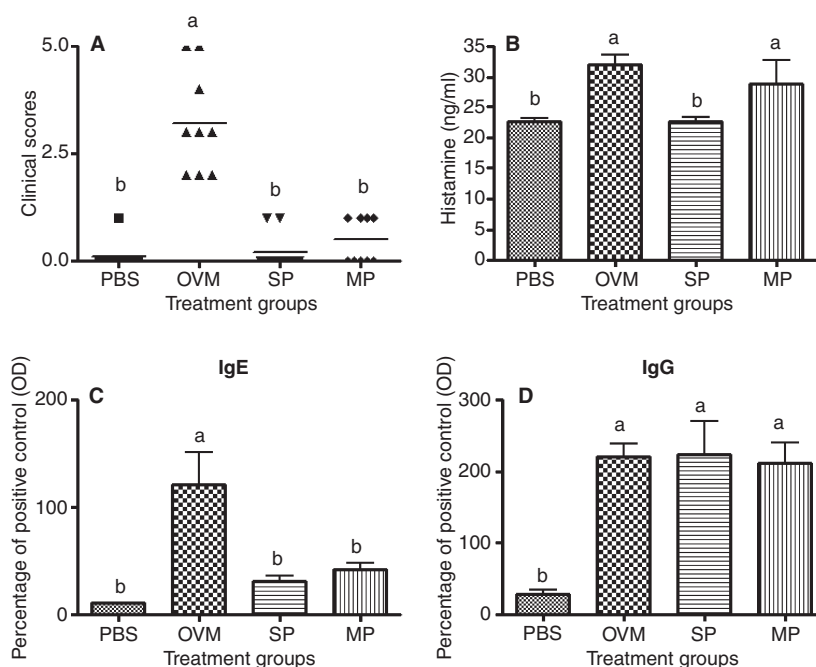


Figure 2 (A) Clinical scores for individual mice. Total clinical scores for each mice post ovomucoid challenge on week 11 were calculated. Average scores assigned by three independent observers in a blinded fashion are represented. Treatment groups differed significantly ($P \leq 0.05$; GraphPad Instat) in frequency of mice expressing allergic signs. (B) Serum histamine concentration. Data for serum histamine concentration are represented as mean $\pm$ standard deviation ($n = 5$ pooled sera). Different letters indicate statistically significant differences ($P < 0.05$) between groups of mice. (C, D) Ovomucoid (Ovm)-specific serum IgE- and IgG (whole molecule)-

related antibody activity. Ovm-specific serum antibody activity was measured by ELISA in triplicates for each sample. 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 followed by streptavidin–HRP conjugate. Data are represented as percentage of positive control (sera from mice that had high Ovm-specific IgG) activity, calculated as [optical density (OD) of test serum]/(OD of positive control serum-OD of negative control) $\times 100\%$. Significance was taken at $P \leq 0.05$. Different letters indicate statistically significant differences.

Express 4 Plus Research Edition software (Denovo software, Los Angeles, CA, USA).

Circular dichroism

The secondary structure of Ovm protein and SP and MP peptides was estimated using a Jasco J 600 spectropolarimeter (Easton, MD, USA) as described earlier (21). The 3D structure for domain III of Ovm and Ovm sequence were retrieved from Protein Data Bank.

Statistical analysis

All calculations were carried out with GraphPad Prism software 4.0 (GraphPad Software Inc., San Diego, CA, USA). Results were analyzed using one-way ANOVA test with Tukey's *post hoc* multiple comparison test. To compare the means of two groups, the Student's *t*-test for parametric data and Mann-Whitney's test for nonparametric data were used.

Results

Reduction in clinical signs

Clinical scores pooled by treatment indicate a significant difference in frequency of mice showing clinical signs in the control group as compared to the SP and MP groups (Fig. 2A; $P < 0.0001$; chi-squared test). There was no significant

difference in frequency between the SP and MP groups ($P > 0.05$). Also, a significant reduction in histamine concentration in the SP-treated group was observed (Fig. 2B; $P < 0.05$). No difference was observed between the control (Ovm) and MP mice.

Reduced Ovm-specific IgE detected in treated groups

There was significant difference in IgE-related antibody activity detected in the treated groups (SP and MP) before (data not shown) and after challenge (Fig. 2C; $P < 0.05$). No activity was detected in CT mice.

Immunoglobulin isotype-related bias reflects treatment effects

There was no significant difference in IgG-related antibody activity detected between groups (Fig. 2D; $P > 0.05$). However, the treatment of mice with SP induced less IgG₁-related antibody activity and more IgG_{2a}-related antibody activity ($P < 0.05$) (Fig. 3A,B). The MP group did not differ significantly ($P > 0.05$).

Specific IgA response indicates mucosal immune regulation by peptide treatment

Administration of SP and MP both made it possible to induce a significant increase in fecal-specific IgA (Fig. 3C; $P < 0.05$).

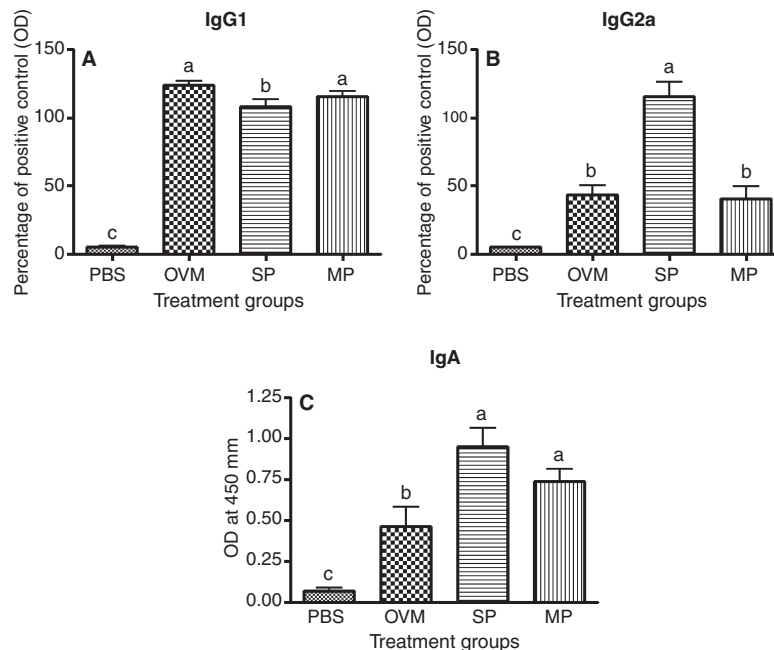


Figure 3 Ovomuroid (Ovm)-specific serum IgG1-, IgG2-, and IgA-related antibody activity. (A, B) Ovm-specific IgG1 and IgG2a activity was measured by ELISA in triplicates for each sample. Rat monoclonal anti-mouse IgG1 and IgG2a were used followed by streptavidin-HRP conjugate. (C) Specific IgA was detected from pooled fecal samples collected from weeks 9–11 using biotinylated

rat monoclonal anti-mouse IgA antibody followed by avidin-HRP conjugate. Data are represented as percentage of positive control (sera from mice that had high Ovm-specific IgG) activity, calculated as [optical density (OD) of test serum]/(OD of positive control serum-OD of negative control) $\times 100\%$. Significance was taken at $P \leq 0.05$. Different letters indicate statistically significant differences.

Cytokine response

The IL-4 cytokine was reduced in both SP and MP groups (Fig. 4A; $P < 0.05$). It is also noteworthy that reduced synthesis of IL-4 in the treated group did not increase the production of IFN- γ (Fig. 4B; $P > 0.05$); however, both IL-12 and IL-10 production was increased significantly in the SP-treated group (Fig. 4C,D; $P < 0.001$). Cytokines TGF- β and IL-17A did not differ significantly (Fig. 4E,F; $P > 0.05$). Treatments did not affect cell proliferation (data not shown).

Increase in Tregs indicates immune regulation

The percentage of CD4+CD25+- and CD4+FOXP3+-expressing cells as determined by FACS analysis increased dramatically in the SP-treated group (Fig. 5A,B; $P < 0.05$). Such increase was not observed in other groups ($P > 0.05$). A representative image of the FITC-stained CD4+ and PE-

stained FOXP3 cells for all treatment groups is shown in Fig. 5C–F. Red color indicates gated lymphocytes.

Secondary structure reflects functional role in suppression of allergic response

The CD profiles of both the SP and MP peptides were different from that of the native Ovm (Fig. 6A); in particular, the structure of the α -helix side chain residues of native Ovm was different from that of the peptides. The wavelength minima at 207 and 222 nm and the maximum at 191 nm, both typical characteristics of α -helical configuration, were absent in both peptides. The SP peptide had a 100% randomly coiled, disordered structure and a negative sign at around 195 nm corresponding to n - π^* transition (Fig. 6B). The structure of MP was more ordered as compared to SP and less ordered as compared to native Ovm (Fig. 6C). It was also shown from the 3D structure of Ovm domain III that the SP was more disordered in nature (Fig. 6D).

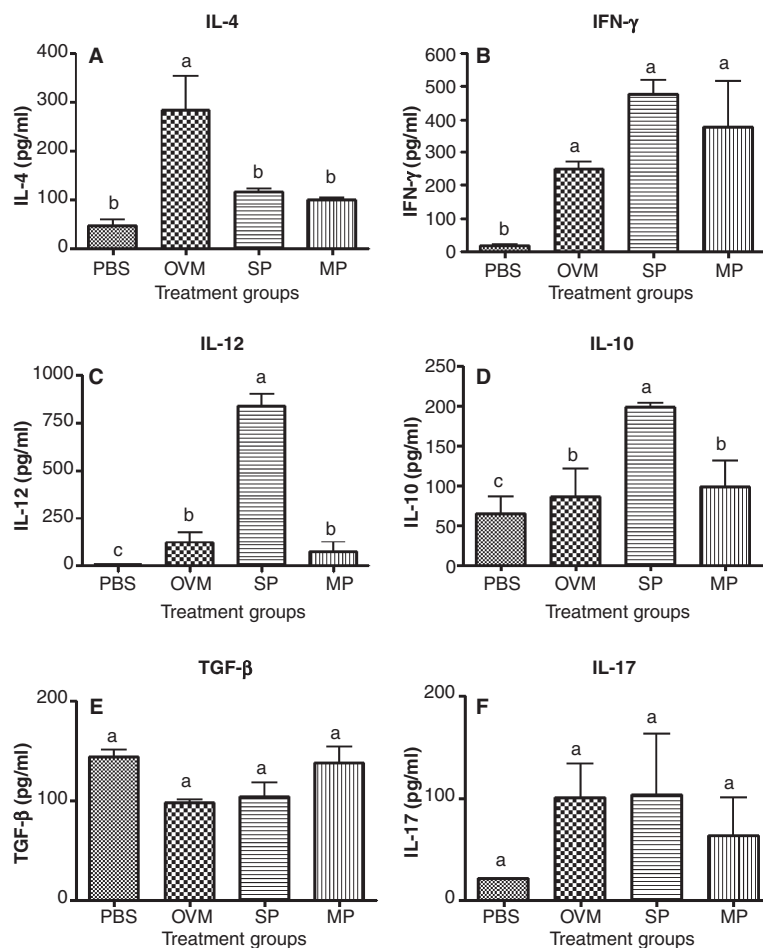


Figure 4 Cytokine concentration. Spleen was collected from each mice and pooled within groups ($n = 5$), and splenocytes were isolated and cultured at 2.5×10^6 cells/ml concentration of cells in triplicate wells as unstimulated (control) or stimulated with 50 μ g/well of

Ovm for 72 h. The cytokine concentration of cell culture supernatants for IL-4, IFN- γ , IL-12, IL-10, TGF- β , and IL-17 was determined by ELISA. Different letters indicate significant difference between groups for each cytokine. Significance was taken at $P \leq 0.05$.

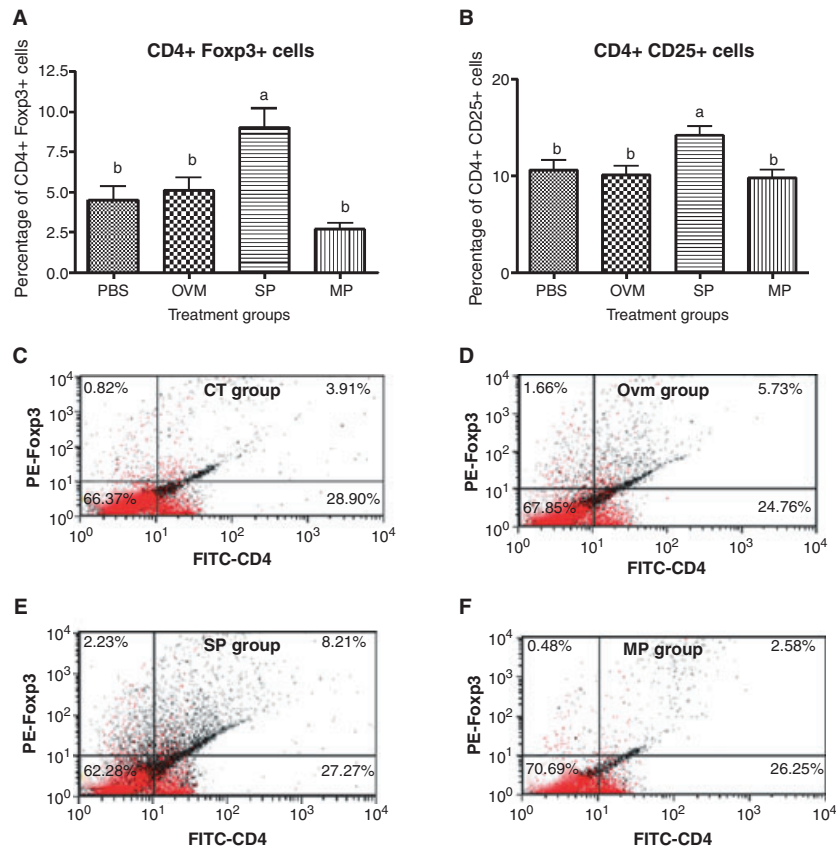


Figure 5 Flow cytometry. Percentage of CD4+Foxp3+ and CD4+CD25+ cells were determined by FACS from whole blood of mice collected at the end point of the experiment. There was a significant increase in CD4+Foxp3+ and CD4+CD25+ cells in the single peptide-treated group (* $P < 0.05$; ANOVA, GraphPad) (A, B). The

multiple peptide group did not differ significantly. A representative image of CD4+Foxp3+ cells for each group is shown in (C–F). Red color indicates gated lymphocytes, and percentage of cells for each quadrant is given for the lymphocyte population. Different letters indicate statistically significant differences between groups.

Discussion

Current clinical trials on immunotherapy of food allergy rely on intact allergens for efficacy; however, there is an increased risk of adverse side-effects owing to the presence of intact B-cell epitopes. This can be overcome by the use of synthetic short peptides containing T-cell epitopes that have reduced the ability to cross-link allergen-specific IgE. Recently, we have shown that PIT with multiple T-cell determinants administered subcutaneously promoted suppressive effects in a mice model of OVA allergy (8). It is well known that SIT works at the T-cell level (22). Here, we demonstrate for the first time the use of T-cell determinants to alleviate clinical signs of food allergy validating OIT. It was found that both SP and MP significantly reduced allergy in Balb/c mice; however, changes with most of the clinical surrogate markers were associated with the SP group. Related mechanistic studies of SP-treated mice indicated reduction in clinical scores, histamine, IgE and IL-4 and increase in IgG_{2a}, IgA, IL-10, IL-12 cytokines, and CD4+CD25+ and CD4+FOXP3+ regulatory T cells. Paradoxically, the MP group did not differ

in other correlates except for specific IgE, IgA, and IL-4 production; however, the group suppressed clinical signs of allergy.

Specific IgG was equivalently induced in all mice regardless of the treatment group. There was less IgE-related antibody activity in both the treated groups and less IgG₁ in SP-treated mice (3A). It was earlier reported that measurement of Ovm-specific IgE was clinically useful as a surrogate marker in the diagnosis of egg allergy in humans (23). Consistent with this observation, we were able to confirm IgE as a clinical surrogate marker. The authors do acknowledge that measuring specific IgE alone as a correlate of allergy is not sufficient. Other regulatory functions were also induced by SP treatment such as increase in blocking antibody isotype (IgG_{2a}), IgA, and Tregs. The isotype switch bias to IgG_{2a} may confer protection against allergic signs. Given that mouse IgG_{2a} is a type-1 isotype, these results are consistent with previous reports in which antiallergic treatment of mice increased the expression of type-1 IgG antibody isotype (24).

Interleukin-4 and secretion of IgE and IgG₁ was found to be significantly reduced in both the treated groups; however,

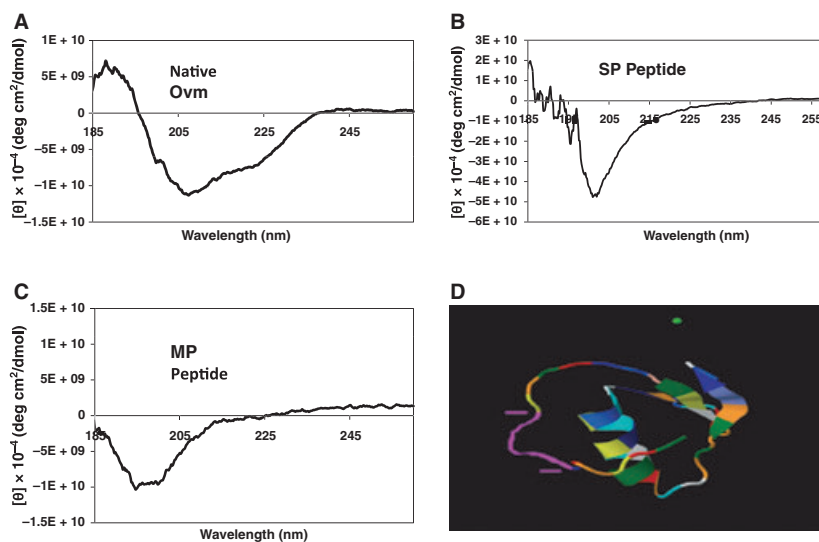


Figure 6 Circular dichroism (CD). Secondary structures of native Ovm, single peptide (SP) and multiple peptide (MP) peptides were obtained by measuring the far-UV spectra. The SP peptide had a more disrupted, randomly coiled structure as represented by CD

and three-dimensional structure of Ovm domain III (right panel – indicated in pink). The structure of the MP peptide was less rigid than that of the native Ovm, but more ordered as compared to the SP peptide.

IFN- γ did not differ between groups. This is consistent with an earlier finding that described reduction in IL-4 and no difference in IFN- γ concentrations in pigs pretreated with *Lactococcus lactis*, exhibiting less frequency to clinical signs of Ovm allergy (25). Significant increase in IL-12 production was observed in the SP group. A previous study claimed that DCs from IL-12-deficient mice failed to induce type-1 responses, suggesting a critical role of IL-12 in DC-induced type-1 response (26). Hence, it may be postulated that the bias to a type-1 response in the SP group is linked to the synthesis of IL-12 via DCs. Also, increased IL-10 production was observed in the SP-treated group. This is consistent with an earlier report that described role of IL-10 and modification of allergen-specific B-cell response following PIT in which increase in IL-10 was essential to peripheral tolerance of allergens (27). Thus, increase in IL-10 in SP group can be mechanistically attributed to induction of adaptive, allergen-specific Tregs. However, no significant difference was observed in TGF- β between groups. This is noteworthy, as TGF- β is also a regulatory cytokine, although it has been earlier reported that allergic suppression can possibly occur independently of TGF- β (28, 29), which may be the mechanism associated here. There was no significant difference in the amounts of IL-17A between groups. More recently, it has been reported that IL-17E – and not IL-17A – is associated with allergic sensitization (30). Hence, measuring IL-17E as a clinical surrogate marker for allergy would have been more useful.

There is a growing body of evidence that suggests that interactions between type-1 and -2 immune elements are not solely antagonistic, but may in fact modulate the IR in a much more complex manner involving other regulatory mediators (31). Two phases of oral tolerance can be distinguished:

clonal deletion and active suppression of the immune response by Tregs (32). Regulatory CD4+CD25+FOXP3+ cells are generally immune suppressive and important for tolerance and maintenance of immune homeostasis. It was earlier demonstrated by Gri et al. (33) that Tregs are able to inhibit mast cell degranulation via IL-10 production. In the present study, there was an increase in the percentage of CD4+FOXP3+ cells and CD4+CD25+ cells in the SP group. Based on this observation, it may be postulated that Tregs in the SP group may have influenced the mast cells and thus alleviated allergic signs via the production of IL-12 and IL-10. In this case it remains to be elucidated whether these Tregs are naturally occurring or antigen induced. Also, the role of Tregs showing multiple effects in controlling both allergen-specific immune and allergic inflammation has been discussed earlier (34). This may be attributed to increased production of specific IgA as shown in the SP-treated group, in which a decrease in IgE is associated with an isotype switch and induction of IgA.

Relationship between the allergic inhibitory specificity of the peptides (SP and MP) and the conformational flexibility of location on domain III of Ovm revealed that suppression of allergic response in this study may be due to the disordered structure of the peptide. It has earlier been reported that presentation of antigenic peptides by cells resulted in the generation of T cells with regulatory/immunosuppressive function (35). Also, the nature of structure of the peptide may be related to the communication of the peptides with DCs and may have induced a shift of immune polarization by recruitment of type-1 cells, and this may have led to the suppression of allergic response. At present, the nature of this polarizing stimulus remains speculative. Hence, further *in vitro* characterization of the structure–function relationship of

these peptides may provide valuable insights into the suppression mechanism involved. Several mechanistical evidences derived from this study suggest that OIT of T-cell epitope peptide modifies numerous surrogate clinical markers of allergy and validates a safe and practical approach to cure food allergies. These results could have important implications for therapeutic use of T-cell determinants in the treatment for allergies.

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Author contributions

YM designed and planned the study and revised the manuscript critically. PR executed the study, acquired the data, and drafted the manuscript.

Conflict of interest

No conflict of interest declared.

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