

# Allergen specific immunotherapy with plasmid DNA encoding OVA-immunodominant T cell epitope fused to Tregitope in a murine model of allergy

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## ABSTRACT

**Background:** Peptide-based immunotherapy (PIT) was introduced as an attractive approach in allergen-specific immunotherapy (AIT). However, PIT clinical trials have shown variable results, and immune response to peptides is not precisely predictable. On the other hand, induction of antigen-specific tolerance may be augmented when allergens are combined with the regulatory T cell epitope (Tregitope). This study aimed to evaluate the therapeutic administration of a plasmid DNA encoding Tregitope and ovalbumin (OVA) immunodominant epitope in the murine model of allergy.

**Methods:** Following the induction of allergic rhinitis by ovalbumin, vaccinated group received three doses of recombinant plasmid containing Signal peptide-Tregitope-OVA T cell epitope. After the final OVA challenge, clinical symptoms, histopathological changes, OVA-specific IgE level, and cytokine secretion pattern of spleen cells were examined.

**Results:** Our data are showing that AIT with the recombinant DNA vaccine significantly suppressed airway inflammation; reduced eosinophilic infiltration in the nasal mucosa; decreased expression level of *IL-4* and *IL-17* in spleen cells, while *IFN-γ*, *IL-10*, and *TGF-β* expression were increased. Moreover, OVA-specific IgE levels were also decreased.

**Conclusion:** These results suggest that Tregitope-immunodominant T cell epitope fusion can act as a safe and effective approach in DNA-based allergen-specific immunotherapy.

## 1. Introduction

Allergen-specific immunotherapy (AIT) is the disease-altering treatment based on immune-tolerance induction, effectively reducing allergic symptoms [1]. AIT consists of sublingual (SLIT; sublingual immunotherapy) or subcutaneous (SCIT; subcutaneous immunotherapy) repeated allergen administration. The effectiveness of AIT has been shown in some allergic disorders such as insect stings, seasonal pollinosis, perennial pet, and mite allergies [2–4]. However, these conventional immunotherapies are associated with adverse side effects of local or systemic reactions, which in some cases can be life-

threatening [4,5]. In AIT, severe allergic reactions may occur due to the presence of B cell epitopes in the allergen extract. Besides, it may elicit a new IgE response in treated individuals against other components in the allergen extract [5,6].

Peptide-based immunotherapy (PIT) represents a new therapeutic strategy for allergic diseases. In PIT, allergen-derived immunodominant T-cell epitopes are used as an alternative therapeutic agent. As a result, B-cell epitopes' absence leads to overcoming the risk of IgE crosslinking and allergic reactions in conventional allergen immunotherapy methods [7–10]. PIT clinical trials in allergic diseases, predominantly for cat allergy, have shown variable results [11–14]. Moreover, immune

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responses to epitopes is not precisely predictable [15–18], possibly dependent on the MHC binding affinity. Therefore, there is a growing need to develop an effective, safe, and cost-effective immunotherapy method.

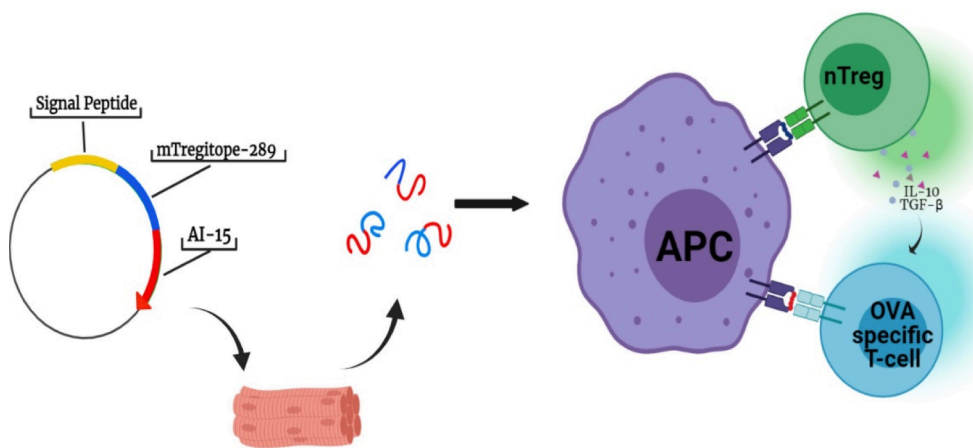
Regulatory T cell epitopes, termed Tregitopes, are highly conserved epitopes that have been identified in the IgG molecule by computational epitope mapping [19]. Tregitope peptides have the following characteristics: (1) Binding to several Major Histocompatibility Complexes class II (MHC II; HLA-DR); therefore, Tregitopes can be recognized broadly by a population. (2) Stimulating CD4 + CD25hiFoxP3 + regulatory T cell expansion and up-regulation of *TGF-β* and *IL-10*. (3) Suppression of effector T cell response in T cells' co-incubation with Tregitope and immunogenic antigens [19–21]. Tregitopes are presented by antigen-presenting cells (APCs) and then recognized in the context of MHC II by Tregitope specific natural regulatory T cells (nTreg). Activated Treg cells suppress APC by decrease in expression of the co-stimulatory molecules (i.e., CD80, CD86) and MHC II and upregulation of the inhibitory receptors such as Leukocyte Ig-like receptor B4 (LILRB4). Cytokines secreted by Treg (*TGF-β* and *IL-10*) and modulated APC leading to Suppression of T cell response and may convert these cells to adaptive regulatory T cells (aTreg), which are specific to antigen [19,20]. In other words, induction of antigen-specific tolerance may be augmented when allergens are combined with Tregitopes. Therefore, in diseases where there is an abnormal immune response against an antigen, such as autoimmune diseases and allergies, the binding of Tregitope to the antigen can serve as a promising therapeutic strategy.

In this study, we hypothesized that allergen-specific immunotherapy using Tregitope-allergen derived immunodominant T cell epitope DNA fusion would cause allergen-specific Treg generation. Thus, this study aimed to assess the therapeutic administration of a plasmid DNA encoding Tregitope-OVA immunodominant T cell epitope in OVA-induced allergic mice.

## 2. Materials and methods

### 2.1. Animals

Twenty four female BALB/c mice (age, 6–8 weeks) were obtained from Jundishapur Animal Care and Breeding Center (Ahvaz Jundishapur University of Medical Science (AJUMS) Ahvaz, Iran) and housed in standard cages under standard temperature ( $22 \pm 2^\circ\text{C}$ ), humidity (50–55%) and a 12 h light/dark cycle. Experimental protocols and animal handling were done in accordance with the institutional guidelines for animal research and approved by the AJUMS Animal Experiments Ethical Committee. (IR.AJUMS.REC.1399.001).



regulatory T cells (iTreg).

### 2.2. Plasmid construction

The recombinant vector containing OVA immunodominant T cell epitope (AI 15: AMVYLGAkdSTRtQI) that is previously introduced as a promising epitope for peptide-immunotherapy (22), Tregitope (Mouse Tregitope 289: EEQFNSTFRSVSELPIMHQ) (20), and an endoplasmic reticulum (ER)-targeting leader sequence (MKWVTFILLFLFSSAYS), which is providing a start codon and help to the chimeric peptide's secretion, was synthesized (Generay Biotech Co., China) (Fig. 1). The final sequence was codon-optimized for mouse expression and inserted in the eukaryotic expression vector pcDNA3.1 + by *Bam*HI and *Kpn*I digestion. The resulting recombinant vector, named TregOVAtope, and the empty plasmid were transformed to *Escherichia coli* Top 10F' using CaCl<sub>2</sub> and heat shock treatment. Then the amplified plasmid was isolated with Maxi Prep plasmid purification columns (Favorgen Biotech Corp, Taiwan). Cloning was confirmed by Sanger's DNA sequencing and enzymatic digestion with *Bam*HI/*Mlu*I (Fig. 2). Optimized DNA sequences:

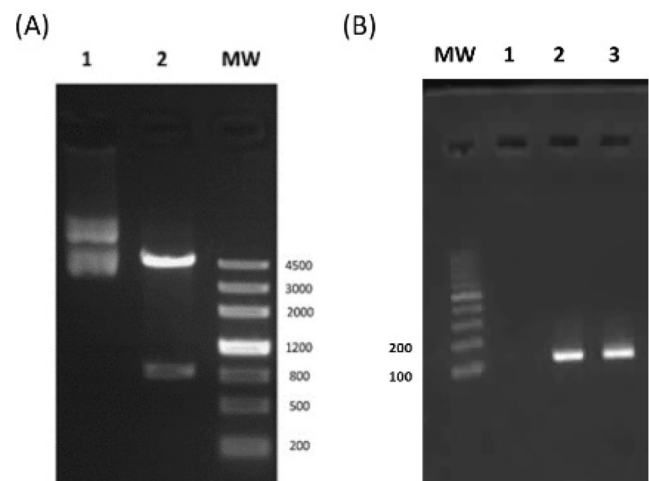


**Fig. 2.** (A) Agarose gel electrophoresis. Band 1- undigested pcDNA3.1+TregOVAtope plasmid. Band 2- pcDNA3.1+TregOVAtope plasmid digested with *Bam*HI and *Mlu*I restriction enzymes. MW- DNA molecular weight marker. (B) TregOVAtope mRNA (147 bp) expression was examined 16 days after last IM injection by RT-PCR. Mice were transfected with TregOVAtope plasmid (Band 2 and 3) or empty plasmids (Band 1). MW- DNA molecular weight marker.

**Fig. 1.** The proposed mechanism of action of TregOVAtope plasmid DNA in Antigen-specific adaptive tolerance induction (ASATI). After translating the sequence, Sp-Tregitope-AI15 is directed to the endoplasmic reticulum (ER). In ER, the signal peptide is cleaved and Tregitope-AI15 (TregOVAtope) is secreted. Tregitope-AI15 is processed in APCs via the MHC class II pathway. Tregitope is presented to natural Treg (nTreg) and OVA T cell epitope (AI-15) presented to OVA-specific CD4<sup>+</sup> T cell. Activated nTreg cell suppress APC by decrease in expression of the co-stimulatory molecules (i.e., CD80, CD86) and MHC II and upregulation of the inhibitory receptors such as Leukocyte Ig-like receptor B4 (LILRB4). Cytokines secreted by Treg (*TGF-β* and *IL-10*) and modulated APC leading to Suppression of OVA-specific T cell response and may convert these cells to induced reg-

ATGAAGTGGGTGACCTTCATCAGCCTGCTGTTCTGTTTCAGCTCCG  
CCTACAGCGAGGAGCAGTTCAACAGCACCTTCCGGAGCGTGAGCGAG  
CTGCCATCATGCACCAGGCCATGGTGACCTGGGCGCCAAGGACTC-  
CACCCGGACCCAGATCTAA.

### 2.2.1. Detection of TregOVAtope mRNA expression

Muscle tissue was isolated at the injection site to confirm plasmid expression *in vivo*. Total RNA was isolated by RNA isolation kit (Yekta Tajhiz Azma, Iran). After RNA extraction, DNA contamination was removed from extracted RNA by DNase I (Sinaclon, Iran) and complementary DNA (cDNA) synthesis from total RNA (1 µg) was carried out using a first strand cDNA synthesis kit (Yekta Tajhiz Azma, Iran). The polymerase chain reaction to evaluate the expression of TregOVAtope mRNA was performed by RT-PCR. (Fig. 2).

### 2.3. Immunization

The experimental protocol for induction of allergic rhinitis was carried out as previously explained [22–24] with slight modification. Briefly; mice were sensitized with 0.1 ml of 25 µg ovalbumin (Sigma, USA) adsorbed on 2 mg alum, injected intraperitoneally three times, one week apart. Seven days later, mice were intranasally challenged (1% OVA solution in PBS), 10 µl in each nasal cavity, on days 21 to 25. The normal control group was received PBS instead of OVA. In order to evaluate the production of OVA-specific IgE and to confirm the induction of allergies, ocular blood was collected on day 25 of the mice (two mice in each group) and the level of OVA-specific IgE in their serum was examined by the enzyme-linked immunosorbent assay (ELISA) kit (East Biopharm, China). TregOVAtope vaccine group was injected intramuscularly with three doses of the plasmid (100 µg in 0.1 ml Phosphate-Buffered-Saline PBS) after the sensitization phase. The allergic placebo-treated group was received empty plasmid, and normal control and AR groups just received PBS (Fig. 3). Two weeks after the last injection of the TregOVAtope vaccine, the final challenge was performed on days 53 and 54. In other words, animals were challenged intranasally twice with OVA (before and after the TregOVAtope vaccine). After the final intranasal OVA-challenge mice were sacrificed for further analyses on day 55.

#### 2.3.1. Preparation of Alum adsorbed ovalbumin

1.89 g of aluminum potassium sulfate (ALK (SO<sub>4</sub>)<sub>12</sub>H<sub>2</sub>O) was dissolved in 10 ml of distilled water and adjusted to pH: 6.5 using NaOH. After incubation at room temperature, it was centrifuged and the supernatant was discarded. The resulting precipitate was dissolved in 5 ml of distilled water and each 10.5 µl of it contained 2 mg of Alum. Due to the instability of the solutions, the next steps were performed just before the injection. 10.5 µl of alum solution was mixed with 25 µg of ovalbumin and incubated for 20 min at room temperature to form Alum adsorbed ovalbumin. After centrifugation, the supernatant was discarded and the precipitate was dissolved in 100 µl of PBS and used for each intraperitoneal injection into a mouse.

### 2.4. Evaluation the allergic symptoms

After the last intranasal challenge, on day 55, mice were placed in monitoring cages. Immediately after the instillation of 20 µl OVA into bilateral nasal cavities, sneezing and nose scratching movements were counted for ten minutes.

### 2.5. Histopathologic analysis

Immediately postmortem, mice heads were removed and lower jaws were discarded. After separating the skin, soft tissues, and muscles, the upper jaw was immersed in 4% paraformaldehyde for 24 h. Following the fixation step, tissues were rinsed with tap water and then decalcified in 10% EDTA at 4 °C for ten days. After rehydration in 30% sucrose in PBS for three days, tissues were embedded in optimal cutting temperature compound (Tissue-Tek OCT, Miles, Elkhart, IN) at – 196 (liquid nitrogen). Tissues were stored at –70 °C. Five-micron thick cryostat sections were stained with Hematoxylin and Eosin (H&E). The number of infiltrated eosinophils in the nasal septum, nasal turbinate, and maxillary turbinate were counted in non-overlapping high-power-fields (×400) under a light microscope.

### 2.6. Measurement of OVA-Specific IgE

Blood samples were collected by heart puncture, and sera were isolated for evaluation of ovalbumin-specific immunoglobulin E (OVA sIgE). According to the manufacturer's instruction, the serum levels of OVA sIgE were evaluated by the enzyme-linked immunosorbent assay (ELISA) kit (East Biopharm, China).

### 2.7. Quantitative RT-PCR (qRT-PCR)

One day after the last local challenge with ovalbumin, mice were killed. After lysis of splenic red blood cells, total RNA was isolated from the spleen cells by RNA isolation kit (Yekta Tajhiz Azma, Iran). Complementary DNA (cDNA) synthesis from total RNA (1 µg) was carried out using a first strand cDNA synthesis kit (Yekta Tajhiz Azma, Iran). The polymerase chain reactions to evaluate the relative expression level of mRNA for *TGF-β*, *IL-10*, *IL-4*, *IFN-γ*, and *IL-17* were performed with ABI Step-One™ (Applied Biosystems) Real-Time PCR machine. Polymerase chain reactions were performed with the following oligonucleotide primer pairs: *TGF-β*-forward: 5'- ACCGCAACAACGCCATCTATGAG –3', *TGF-β*-reverse: 5'- GCGTATCAGTGGGGTTCAGCAG –3', *IL-10*-forward: 5'-CTGGACAACATACTGCTAACCGACTC-3', *IL-10*-reverse: 5'TCTTCAC CTGCTCCACTGCCTTG –3', *IL-4*-forward: 5'- TCACAGGAGAAGGGA CGCCATG –3', *IL-4*-reverse: 5'- TGGACTTGGACTCATTCATGGTGC –3', *IFN-γ*-forward: 5'- GCCTAGCTCTGAGACAATGAACG –3', *IFN-γ*-reverse: 5'- GCCAGTTCCTCCAGATATCCAAG –3', *IL-17*-forward: 5'- CTACAG TGAAGGCAGCAGCGATC –5', *IL-17*-reverse: 5'- CTTTCCCTCCGCATT-GACACAG –5', *GAPDH*-forward: 5'- TCCCGTAGACAAAATGGTGAAGG –3', *GAPDH*-reverse: 5'- ATGTTAGTGGGGTCTCGCTCCTG –3'. All

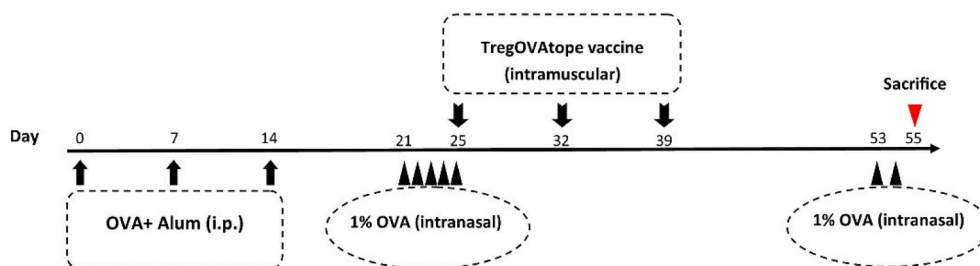


**Fig 3.** The experimental protocol. Allergic mice were injected with OVA and Alum adjuvant intraperitoneally three times, one week apart, followed by two intranasal OVA challenges. Mice in TregOVAtope group were injected intramuscularly with the recombinant DNA vaccine (100 µg in 100 µl PBS) on days 25, 32, and 39. Allergic placebo-treated and control groups were received empty plasmid and PBS, respectively.

target gene were normalized to a house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and relative expression were calculated using the  $2^{-\Delta\Delta CT}$  method.

## 2.8. Statistical analysis

All data analysis were performed using SPSS software version 22 (SPSS Inc, Chicago, IL, USA). The comparison of variables between IBM SPSS Statistics 22. The comparison of variables between groups was carried out by analysis of variance (ANOVA) with the Tukey's post hoc test. Kruskal–Wallis rank sum tests followed by pairwise comparisons were performed for non-parametric values. P-values < 0.05 was considered statistically significant.

## 3. Results

### 3.1. Nasal symptoms in OVA-induced allergic rhinitis

Mice in allergic rhinitis groups showed a significant ( $P < 0.0001$ ) increase in nasal rubbing and sneezing frequency compared to the control group (healthy animals). Interestingly, in mice that received the DNA vaccine, the incidence of sneezing and nose rubbing movements was significantly decreased ( $P < 0.0001$ ) compared to the AR group (Fig. 4).

### 3.2. Histopathological changes in the nasal mucosa

In the allergic and placebo-treated group, hyperplasia, vasodilatation, and increased eosinophil influx were observed in the nasal submucosa. The number of eosinophils in the allergic and placebo-treated group was significantly ( $P < 0.0001$ ) higher than the health group (control). Interestingly, immunotherapy with the TregOVAtope remarkably decreased eosinophil infiltration compared to the allergic group. Neutrophil and monocyte infiltration was not observed obviously in each group (Fig. 5).

### 3.3. Serum level of OVA-specific IgE

To further evaluate the effect of the recombinant DNA vaccine on humoral immune response, the levels of OVA sIgE were assessed by ELISA (Sensitivity: 2.56 ng/ml). As shown in Fig. 6, the DNA vaccine administration containing Tregitope linked allergen derived immunodominant peptide significantly decreased OVA-specific IgE level in the serum of OVA-sensitized mice compared to untreated allergic mice group and allergic placebo-treated group which received empty plasmid ( $P < 0.0001$ ). Moreover serum levels of OVA-specific IgE increased in the allergic and placebo-treated mice on day 25. Due to this increase in specific antibodies and clinical manifestations, it can be concluded that the development of an allergic mouse model has been successfully

performed.

### 3.4. Promotion of a regulatory T cell biased response upon administration of TregOVAtope vaccine

To evaluate the tolerogenic effect of TregOVAtope vaccine in allergic rhinitis, we assessed the levels of *IL-4*, *IL-17*, *IFN- $\gamma$* , *IL-10*, and *TGF- $\beta$*  in spleen cells in mouse AR model. As shown in Fig. 6, the *IL-17* and *IL-4* expression level were increased significantly in AR and allergic placebo-treated group ( $P < 0.0001$ ). An increase in the relative expression of *IL-10* was observed in the AR group compared to the normal group ( $P < 0.05$ ), but this increase was not significant compared with the allergic placebo-treated group. Interestingly, increased *IL-17* and *IL-4* were remarkably reduced by TregOVAtope vaccine ( $P < 0.0001$  and  $P < 0.001$ , respectively). Also, decreased level of *IFN- $\gamma$*  in the allergic group was significantly increased in the TregOVAtope group ( $P < 0.0001$ ). Moreover, *TGF- $\beta$*  and *IL-10*, Treg-associated cytokines, were significantly elevated in the TregOVAtope group compared with the AR group ( $P < 0.0001$  and  $P < 0.01$ , respectively) (Fig. 7).

## 4. Discussion

Conventional allergen-specific immunotherapies, which are based on whole allergen administration in treating allergic diseases, involve the risk of anaphylactic reactions. Peptide immunotherapy has been identified as a promising alternative to other forms of allergen immunotherapies [25]. However, the results of PIT clinical trials vary considerably. Moreover, the immune response to epitopes is not precisely predictable [15–18], possibly dependent on the MHC binding affinity. As a result, atopic individuals with different MHC alleles may elicit different T helper-biased responses to the same epitope. Therefore, the development of an effective and safe immunotherapy method is needed. The aim of this study was to assess a novel immunotherapy approach in the immediate hypersensitivity treatment.

In IgE-mediated allergic disease, Th2-type cytokines play a major role in the disease pathogenesis [26]. The imbalance of cellular responses in T helper subsets is an important factor in the type I hypersensitivity development. Previous studies have shown that Tregitope when administered with an antigen, is able to induce specific tolerance against the antigen [6,27–29]. The immunosuppressive properties of Tregitope have been demonstrated in diseases including allergies to cockroaches [6], type 1 diabetes [30] and allergic asthma [29]. Tregitopes induce anti-inflammatory cytokines such as *TGF- $\beta$*  and *IL-10* secretion and reduce the co-stimulatory molecules on the surface of the APC cell, leading to a shift towards the Treg response [30]. In this study, increased expression of *TGF- $\beta$*  and *IL-10* could indicate the activation of regulatory T cells. Due to the decrease in *IL-4*, which is an essential cytokine for IgE production [31], and the increase in *TGF- $\beta$*  and *IL-10* cytokines, as well as the proposed mechanism of action for Tregitope, it

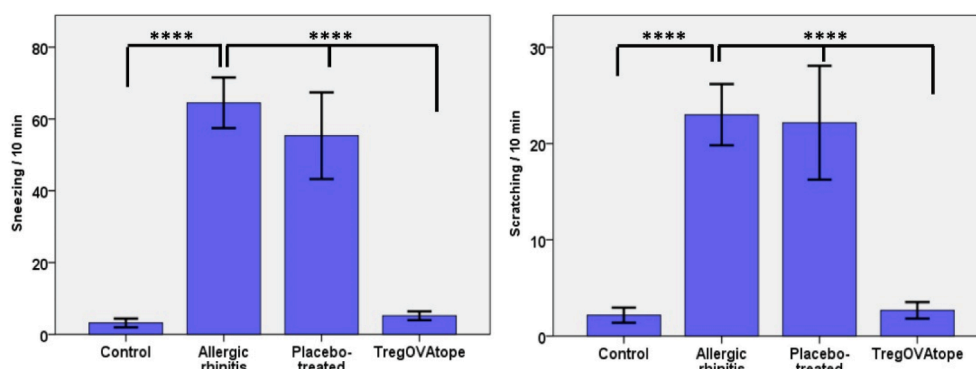


Fig. 4. Effect of TregOVAtope DNA vaccine on nasal symptoms. The data are shown as the mean  $\pm$  SEM, \*\*\*\* $P < 0.0001$ ,  $n = 6$  per group.

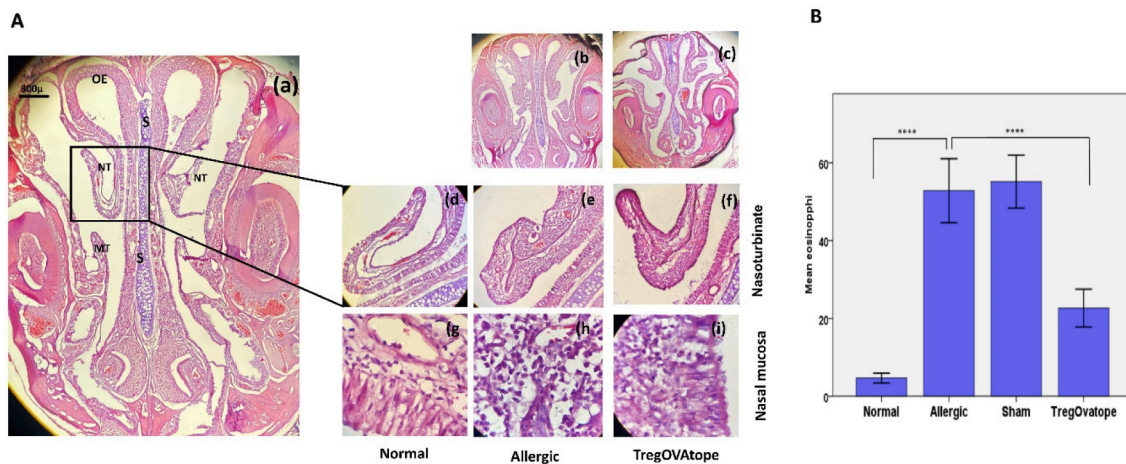


**Fig. 5.** (A) Histopathological changes in the nasal mucosa. (a) The nasal cavity of the mouse. OE, olfactory epithelium; NT, nasal turbinate; MT, maxillary turbinate; S, nasal septum. Evaluation of Histopathological changes in olfactory epithelium (b, c, d and e original magnification  $\times 200$ ), nasal turbinate (f, g, h and i, original magnification  $\times 200$ ) and inflammatory cell infiltration in nasal mucosa (j, k, l and m, original magnification  $\times 1000$ ) in different groups by hematoxylin & Eosin. (B) Comparison of eosinophil cell infiltration in the nasal mucosa. The number of infiltrated eosinophil in the AR mice group increased significantly ( $P < 0.001$ ) compared to health animal (Normal group) that indicates the successful induction of the allergic model and eosinophil infiltration in the vaccinated group were remarkably reduced ( $P < 0.001$ ). The data are shown as the mean  $\pm$  SEM. (\*\*\*\* $P < 0.0001$ ).

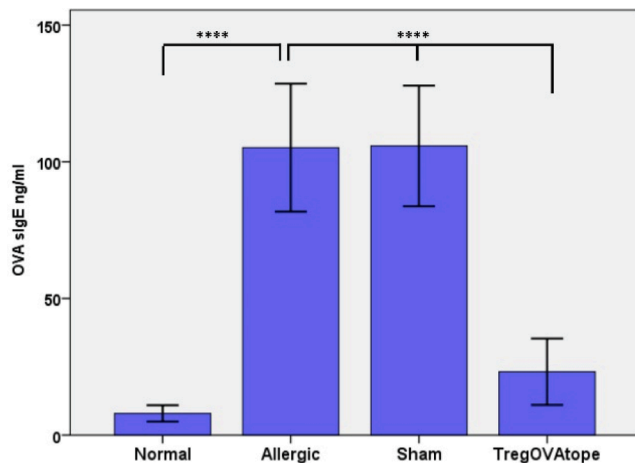


**Fig. 6.** Levels of OVA-specific antibody. Levels of ovalbumin (OVA)-specific IgE in serum after the last intranasal challenge. The data are shown as the mean  $\pm$  SEM. (\*\*\*\* $P$  value  $< 0.0001$ ).

is possible that specific anti-ovalbumin tolerance has been established. However, the study by Yang et al. demonstrated peptide immunotherapy with AI-15, an ovalbumin-derived T cell epitope, decreased *IL-4* and OVA sIgE levels and increased *IFN- $\gamma$*  [25] but the systemic anaphylactic score did not improve; besides, *TGF- $\beta$*  and *IL-10* expression levels decreased. It appears in their study, PIT by AI-15 resulted in a shift towards TH1 response. Interestingly, in the present study, due to tolerogenic effects of Tregitope on the immune response to AI-15, the levels of *TGF- $\beta$*  and *IL-10* increased, and the clinical symptoms improved so that no significant difference was observed between the healthy group and the TregOVAtope vaccine group. In addition to decreased *IL-4* expression level, *IFN- $\gamma$*  was also increased. Also, due to the increased expression of *TGF- $\beta$*  and *IL-10*, it can be concluded that the improvement in allergic status is due to the shift to regulatory T cell response. Although *TGF- $\beta$*  increased, *IL-17* expression levels also decreased in the TregOVAtope group, which may indicate a lack of secondary pro-inflammatory signal for Th-17 differentiation [32]. In a recent study [29], Tregitopes improved allergic airways disease and induced specific Tregs in the murine model, which is in line with the results obtained in

the present study. Although the immunomodulatory effects of Tregitopes and AL-15 peptide have been studied separately [25,29], but their mechanisms of action are different. In peptide immunotherapy with the OVA-immunodominant T cell epitope (AL-15), the level of *IFN- $\gamma$*  increased, while the level of the anti-inflammatory cytokines (*TGF- $\beta$*  and *IL-10*) decreased [25]. These results indicate a shift towards TH1 response. However, in the treatment with Tregitope, the level of *IFN- $\gamma$*  decreased and the protective effects of this treatment are due to the activity of regulatory T cells [29]. In the present study, in which a combination of these two peptides (AL-15\_Tregitope) was used as a therapeutic agent, the cytokine secretion pattern showed a mixed result. The level of *IFN- $\gamma$*  increased, which is similar to the results of the peptide immunotherapy with AI-15, but in the treatment with Tregitope, this cytokine showed an increased expression. On the other hand, the levels of anti-inflammatory cytokines (*TGF- $\beta$*  and *IL-10*) were increased in the present study, which is similar to the result of the treatment with Tregitope and different from the peptide immunotherapy with AL-15. The cytokine secretion pattern in immunotherapy with the combination of AL-15\_Tregitope in comparison with the peptide immunotherapy with AI-15 and treatment with Tregitope indicates that the protective effects of this approach are probably due to both the Tregitope and AI-15 sequences. Also, the combination of AL-15 and Tregitope has been much more effective in reducing anaphylactic score and disease recovery, which may indicate its synergistic effect and more effective performance. The main limitation of this study is the lack of comparison of the OVA-peptide (AI-15) construct with the dual construct encoding Tregitope\_AI-15. Therefore, it cannot be concluded that the addition of Tregitope to the immunodominant T cell epitope of ovalbumin is clinically beneficial. One of the limitations of peptide immunotherapy is the diversity of the immune response to the immunodominant T cell epitope, which is probably due to the diversity of MHC alleles in different individuals. One of the main reasons for addition of Tregitope to the immunodominant T cell epitope of ovalbumin in this study is the possible ability of Tregitope to unify the immune response to the antigenic peptide and induce the response of regulatory T cells. Therefore, in addition to evaluation of immunotherapy with OVA-peptide (AI-15) alone and its comparison with dual construct encoding Tregitope\_AI-15, it is suggested that Tregitope's ability to unify the immune response to allergen-derived peptides, which elicit different responses in different individuals, be investigated in future studies. It is also suggested that the effects of the plasmid DNA encoding Tregitope be examined.

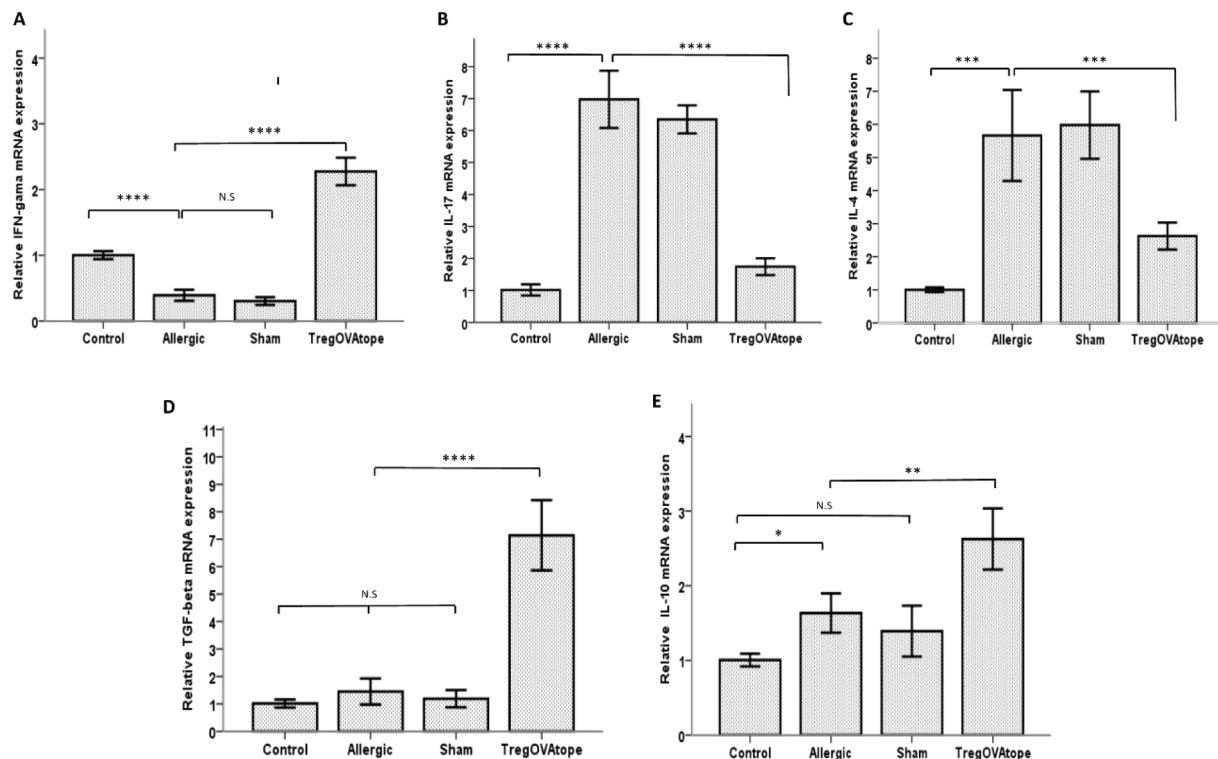


**Fig. 7.** Modulation of gene expression patterns in TregOVAtope-treated mice. The mRNA relative expression of *IFN-γ*, *IL-17*, *IL-4*, *TGF-β*, and *IL-10* (A to E respectively) were calculated by RT-PCR. Values are shown as mean ± SEM. (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; and \*\*\*\* $P < 0.0001$ ).  $n = 6$  per group.

In addition to examining the cellular immune response, the effect of TregOVAtope vaccine on the humoral immune response were also assessed. In this study, analysis of sera obtained from mice treated with TregOVAtope showed that OVA sIgE levels were significantly reduced. Similar results were observed to reduce specific IgE levels in PIT by AI-15 [25]. Paradoxically, IgE levels were not reduced in cockroach allergy immunotherapy [6], in which the allergen and Tregitope were delivered by liposomes, which may be due to differences in the delivery system.

Recently, plasmid DNA and mRNA vaccines against diseases such as type I hypersensitivity are being investigated [33]. In previous DNA-based immunotherapy in the treatment of allergic diseases, efforts have been made to induce TH-1 polarized response [34]. Among the adjuvants used to stimulate the TH-1 response is the amino acyl tRNA synthetase-interacting multifunctional protein 1 (AIMP1), which induces the expression of *IL-12* in dendritic and macrophage cells and thus leads to TH-1 activation [35,36]. Mycobacteria-derived antigen 85B (Ag85B) has also been introduced as an activator adjuvant of TH-1 cells [37]. The anti-allergic properties of plasmid DNA encoding Ag85B have been investigated [38]. Other strategies to increase the TH-1 polarized response in DNA-based immunotherapy include binding cytokines such as *GM-CSF*, *IFN-γ*, *IL-1β* to the allergen [39]. Although induction of the TH-1 response and inhibition of IgE production in immunization with DNA vaccine have been demonstrated, some studies have shown a mixed TH-1/TH-2 response [40], which is a concern for DNA-based immunotherapy in the treatment of allergies. Compared to the use of crude extracts in allergy immunotherapy, gene-based methods have advantages such as purity, stability, inexpensiveness, and a tendency to produce Th-1 and regulatory T cell response but involve the risk of anaphylactic reaction due to allergen release and neosensitization in the prophylactic approach [41]. However, in some study native allergen release was avoided by targeting allergen to degradation in subcellular compartments [42]; in our study, due to the absence of B cell epitopes of allergen [7,9–11], there are no concerns about native allergen release in the designed DNA vaccine. Although the immune system responds strongly to DNA-vaccines in rodents, the immunogenicity of DNA-vaccines in

humans is low [43]. In the present study, the aim of immunization by plasmid DNA encoding Tregitope and allergens was to induce a regulatory T cell response. For vague reasons, a strong immune response against DNA-vaccines is not developed in humans, but the ability of DNA-vaccines with the aim of inducing tolerance has not been studied. DNA-based immunotherapy with the aim of inducing tolerance and regulatory T cell response seems to be a more promising method in the treatment of human allergic diseases compared to inducing TH-1 cell response.

In summary, our data demonstrated that the plasmid DNA encoding the fusion of Tregitope and allergen-derived immunodominant T cell epitope significantly suppressed airway inflammation. TregOVAtope vaccine alleviates nasal symptoms; reduced eosinophilic infiltration in nasal tissues; decreased *IL-4* and *IL-17* expression in the spleen cells, and the levels of OVA-specific IgE were also decreased, but *IL-10* and *TGF-β* expression increased. In conclusion, these results suggest that DNA-based allergen-specific immunotherapy might be improved by combining Tregitope and immunodominant T cell epitope of allergen.

#### CRedit authorship contribution statement

**Ali Farhadi Biregani:** Conceptualization, Writing - original draft, Methodology. **Ali Khodadadi:** Software, Validation, Investigation. **Abbas Doosti:** Methodology, Investigation. **Ali Asadirad:** Formal analysis, Methodology. **Mohammad Ghasemi Dehcheshmeh:** Methodology. **Ata A. Ghadiri:** Writing – review & editing, Project administration, Supervision, Funding acquisition.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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