

## Nicotine, as a novel tolerogenic adjuvant, enhances the efficacy of immunotherapy in a mouse model of allergic asthma

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### Abstract

An increasing trend in the incidence of allergic diseases including asthma and related morbidity and mortality is observed worldwide during the last decades. Allergen-specific immunotherapy is suggested for the treatment of some allergic diseases; nevertheless, there is always a menace of uncommon, but life-threatening reactions due to increasing the administration of allergen extract doses. Hence, improving its efficacy may reduce the required doses as well as the risk of such reactions. The current study aimed at examining the effects of nicotine (NIC), as a tolerogenic adjuvant, on the improvement of immunotherapy efficacy in a mouse model of allergic asthma. BALB/c mice were sensitized using alum and ovalbumin (OVA) on the days 0 and 7. Mice received OVA either alone or together with NIC (1 or 10 mg/kg) on the days 21, 23, and 25. Then, the mice were challenged with OVA 5% using a nebulizer on the days 35, 38, and 41 and sacrificed the next day. Co-administration of OVA and NIC decreased the inflammation of the lung tissue, eosinophils count in the bronchoalveolar lavage (BAL) fluid, the serum level of OVA-specific immunoglobulin E, as well as interleukin (IL)-4 production, while increasing the population of antigen-specific regulatory T-cells (Treg cells) and transforming growth factor- $\beta$ /IL-4 (TGF- $\beta$ /IL-4) ratio compared to the OVA and control groups in a dose-dependent manner. Collectively, the findings suggest that administration of NIC plus the allergen increased immunotherapy efficacy through decreasing allergic inflammation and allergic responses intensity, and increasing Treg cells population.

**Keywords:** Allergy; Asthma; Cholinergic anti-inflammatory response; Nicotine; immunotherapy.

### INTRODUCTION

Despite the improvements in understanding the mechanisms of allergic diseases and advances in their treatment, the incidence rate of allergic diseases including asthma has increased during the last decades. Asthma is a global health problem and it is estimated that about 300 million patients living with asthma worldwide (1). Both pharmacotherapy and immunotherapy methods are utilized to treat allergic asthma. However, immunotherapy is the only therapeutic method that can alter the nature of the immune responses against the allergen.

Allergen-specific immunotherapy (SIT) is suggested as a treatment of choice for allergic diseases, in which, gradually increasing doses of the allergen are frequently administered to moderate the symptoms (2). Although pharmacotherapy is useful in the relief of allergic diseases symptoms, SIT is the only therapeutic method modifying the nature of immune system responses to the allergen (3). Subcutaneous immunotherapy (SCIT) is the best route of administration for the treatment of allergy.

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A classical SIT consists of repeated subcutaneous administration of the responsible allergen. However, despite its efficacy, there are drawbacks on using of SCIT because of local, even rare, and potentially systemic allergic reactions producing, especially due to the high-dose administration of the allergen, and it is time-consuming (4). Due to these disadvantages, only a small percent of patients with allergy consent to receive SCIT (5). Hence, safer or more effective SCIT is needed using adjuvants, alternate administration routes, and better allergens (6).

Some materials such as toll-like receptors agonists (7), bacterial toxins (8), and active form of vitamin D (calcitriol) (9) are used as adjuvants to increase the efficacy of immunotherapy. However, most of them ameliorate allergic responses by augmenting T-helper (Th) 1 immune response. Despite investigations and introduction of some new adjuvants, due to their costs and safety concerns, aluminum hydroxide (alum) is the commonest adjuvant used in the SCIT introduced empirically by vaccines a century ago (4,10). Alum deviates the immune responses toward Th 2 (11) that are in contrary to the known therapeutic mechanisms of SIT. Furthermore, alum accumulates in tissue in SIT, which is a safety concern (4,12). Hence, it is wise to look for the new effective adjuvants with the minimal side effects and costs.

The role of immunoglobulin E (IgE) is widely investigated in IgE-mediated allergic diseases. IgE antibody, which is specific to the allergen, binds to its particular receptor on the surface of the cell and crosslinking of IgE by the allergen results in degranulation of the immune cells such as mast cells. Therefore, reducing IgE production in allergic diseases is a desired goal in allergy treatment (13,14).

Increasing evidence indicates the important role of transforming growth factor- $\beta$ /IL-4 (TGF- $\beta$ ) and interleukin (IL)-10 in the production of regulatory T-cells (Treg cells) in allergic diseases. They are imperative cells in maintaining the self-tolerance and suppressing activated immune cells involved in the allergic inflammation. Treg cells are important critical cells in both

self-tolerance and suppression of harmful immune responses. Studies indicate decreased expression of Foxp3 and the number of Treg cells in patients with allergic diseases compared to the healthy controls (15). Hence, regarding the decreased population of Treg cells in patients with allergy, increasing the number of allergen-specific Treg cells may be an appropriate goal in SIT process. However, immunotherapy increases Treg cells number, but it occurs in an almost long run (16).

The cholinergic anti-inflammatory pathway is an example of interaction between the central nervous system and the immune system. The pathway uses vagus nerve to trigger an anti-inflammatory procedure in order to suppress potentially damaging inflammation (17,18). Two kinds of receptors are responsible for stimulating the pathway; nicotinic and muscarinic receptors (19). Nicotine (NIC), which is a non-selective nicotinic receptor agonist, demonstrates anti-inflammatory properties and applies its effects by binding to nicotinic receptors. Research indicated that NIC effects are mainly mediated by binding to alpha 7 subunits of nicotinic receptors (20). Anti-inflammatory effects of NIC and also its therapeutic potential in the treatment of various diseases such as alzheimer, parkinson, ulcerative colitis, and inflammatory bowel disease are studied extensively (21); however, to the best of the authors' knowledge the current study used it as a tolerogenic adjuvant in combination with allergen immunotherapy for the first time.

Animal models are valuable tools to investigate the effects of a substance or the mechanisms of diseases. Among these models, mouse models are commonly used by the researchers. Mouse models of allergic asthma share multiple clinical features of the disease with those in humans. For instance, similar to the human beings, mice are able to develop a Th2 immune response against an allergen, produce antigen-specific IgE, and also develop an airway inflammation because of the allergic responses. Hence, a mouse model of allergic asthma was used in the current study to investigate

the effects of the co-administration of NIC and ovalbumin (OVA) on the immunotherapy efficacy (22).

The current study aimed at examining the hypothesis that the administration of NIC (as an adjuvant) together with the allergen induce an anti-inflammatory microenvironment in which the involved cells shift the immune response onto the tolerance against the allergen in a mouse model of allergic asthma. Since a high dose of OVA efficiently suppresses the allergic inflammation, the present study used a suboptimal dose of OVA for immunotherapy.

## MATERIALS AND METHODS

### Animals

A total of ninety five healthy male BALB/c mice, aged 6-8 weeks, weighing 20-25 g were used to conduct all the experiments. Since 6-8 week old mice have a mature immune system and produce better immune responses, this age range was selected. Also, in order to prevent any possible impacts of hormonal changes during menstrual cycle of the female mice on the treatment results, only male mice were enrolled. The mice were purchased from Razi Vaccine and Serum Research Institute (Karaj, I.R. Iran) and kept in a specific, pathogen free environment in polystyrene cages with free access to water and standard food. All mice were adapted to the laboratory environment for at least one week before the experiments. All the experiments performed in accordance with the Animal Care and Use Protocol of Urmia University of Medical Sciences, Urmia, Iran (Ethics Committee approval No. IR.umsu.rec.1393.208).

### Materials

OVA (Grade V, Sigma, St. Louis, Missouri, USA, Cat number: A5503, LOT number: #SLBK7542V); nicotine (Santa Cruz Biotechnology, Dallas, Texas, USA, Cat number: SC-203161, LOT number: #10115); bovine serum albumin (BSA, Sigma, St. Louis, Missouri, USA, Cat number: A9418, LOT number: #129H14205); RPMI 1640 (Gibco, Loughborough, UK, LOT number: #1597562); fetal bovine serum (FBS, Gibco, Loughborough, UK, LOT number: #42F6350K); goat anti-mouse IgE HRP conjugated (Abnova, Neihu District, Taipei City, Taiwan, Cat number: PAB29743, LOT number: #12); mouse regulatory T cell staining kit (eBioscience, San Diego, California, USA, Cat. number: 88-8115, LOT number: #126196017); mouse IL-4 ELISA kit (eBioscience, San Diego, California, USA, Cat. number: BMS613, LOT number: #91744021); mouse TGF-Beta ELISA kit (eBioscience, San Diego, California, USA, Cat. number: BMS613, LOT number: #126196017).

### Sensitization, treatment, and immunotherapy protocol

To sensitize the mice against the allergen, each animal received 100  $\mu$ L of alum and 10  $\mu$ g of OVA intraperitoneally, on the days 0 and 7. Five groups were considered to examine the hypothesis. According to Table 1, each group received its specific treatment subcutaneously on the days 21, 23, and 25. Mice challenged with OVA aerosols (5% dissolved in sterile saline) three times on the days 35, 38, and 41 using a nebulizer for 20 min (Ultrasonic Nebulizer, Omron NE-U17, Japan). Twenty four h following the final challenge, on the day 42, five mice per group were sacrificed to perform the further experiments (23,24).

**Table 1.** The description of the groups and the treatments received.

Groups	Treatments
Control	Sterile saline(100 $\mu$ L)
OVA	(OVA 10 $\mu$ g/mL) + sterile saline (100 $\mu$ L)
OVA-NIC10	NIC (10 mg/kg) + OVA (100 $\mu$ g) + sterile saline (100 $\mu$ L)
OVA-NIC1	NIC (1 mg/kg) + OVA (100 $\mu$ g) + sterile saline (100 $\mu$ L)
Normal	Healthy and non-allergic mice which did not receive any treatment

OVA, Ovalbumin; NIC, nicotine.

***Evaluation of inflammation in the lung tissue***

A deep anesthesia was achieved using a ketamine and xylazine (80 and 10 mg/kg, respectively) mixture (25). Blood samples were taken from the heart to measure OVA-specific IgE titers. A tracheal cannula was used to puncture the trachea and then 1 mL of 10% formalin was inserted slowly into the lung via the cannula. Thereafter, the lung was removed and fixed in 50 mL formalin. The lung tissue samples were stained using the hematoxylin and eosin (H&E) method. A pathologist who was blind to the groups evaluated and analyzed the degree and intensity of inflammation. The current study assessed and graded the lung inflammation using a semi-quantitative scoring system as described previously; the highest degree of inflammation and aggregation of the inflammatory cells around most of the bronchioles and vessels was considered as +5, and the lowest degree of inflammation with a slight number of inflammatory foci was considered as +1 (26).

***Analysis of the bronchoalveolar lavage fluid***

After anesthetizing the mice on the day 42, their tracheas were punctured with a cannula. The airways were lavaged two times using a 0.9 mL cold PBS (~4 °C, pH 7.5) containing 5% BSA, and then kept on ice. The BAL fluid of each mouse was centrifuged at 2000 rpm (4 °C for 5 min) and then, the sediment was resuspended in 100 µL PBS; then the suspension was smeared on a glass slide and dried at room temperature. Next, the slides were floated in a methanol dish for 5 min and after the evaporation of methanol; they were stained by the Wright-Giemsa staining technique. To count eosinophils number, 20 arbitrary fields per slide were considered, in which at least 200 cells were counted (27,28).

***Measurement of transforming growth factor- $\beta$  and interleukin-4 cytokines***

The spleen was aseptically removed and cell suspensions were provided in RPMI 1640 culture medium. Ammonium chloride buffer (0.9%) was used to lyse red blood cells

osmotically. Then, the cells were washed twice with RPMI 1640 (29) and finally the cells were resuspended in RPMI 1640 enriched by FBS (10% v/v). Trypan blue (0.4%, w/v) was used to determine the viability of the splenocytes. The cells were cultured in V-bottom microplates ( $1 \times 10^5$ /well) using RPMI 1640 containing FBS (10% v/v), 100 mg/mL streptomycin, and 100 IU/mL penicillin. The V-bottom microplates were used to culture the splenocytes in order to avoid and minimize the cell loss while discarding the supernatant. Based on the authors' experience, the cells do not attach well to the flat bottom microplates during the centrifugation and many of them are lost with the discarded supernatant. To stimulate the cells, OVA 40 µg/mL was added into each well and the plates were incubated at 37 °C in 5% carbon dioxide (CO<sub>2</sub>) for 72 h. After the incubation, the plates centrifuged and the supernatant was collected and kept at -70 °C. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to assess the concentrations of cytokines in the supernatants (30,31).

***Assessment of the serum levels of ovalbumin-specific immunoglobulin E***

To measure the titers of OVA-specific IgE in different groups, the blood samples of the mice were centrifuged at 2000 rpm (4 °C for 10 min) and the sera were collected and kept at -80 °C. The current study used a checkerboard assay to determine the optimum dilution of the sera and the optimum dose of OVA should be used in the ELISA assay. To perform the test, a 0.5 µg OVA was dissolved in 1 mL carbonate-bicarbonate buffer (pH 9.5, 0.1 M) and then, 200 µL of the dilution was added to each wells and incubated overnight at 4 °C in a humid chamber. Afterward, the plate was washed three times with PBS (PH 7.5) containing 0.05% Tween<sup>®</sup> 20 (PBST) and the free spaces were blocked with 250 µL of blocking reagent (5% BSA) and incubated at 37 °C for 2 h. Then, 200 µL of diluted sera was added to each well and incubated

at 37 °C for 2 h. The plate was washed again with PBST and then 200 µL of goat anti-mouse IgE conjugated with horseradish peroxidase was added to each well and then incubated at room temperature for 1 h. To dilute the antibody, a 100 µL Tween® 20 and 100 mg BSA were dissolved in 100 mL PBS and the antibody was diluted 2000 times. After completion of the incubation, the plate was washed three times with PBST and then, 200 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well and incubated for 30 min in the dark. Then, 50 µL of the stop solution (0.16 M sulfuric acid) was added and the absorbency was read at 450 nm wavelengths (30,32). Since no specific standards for OVA were available, the present study used the optical densities to compare the IgE levels between the groups.

#### ***Assessment of Treg cells population***

Seven days after the last challenge (the day 42), the mice were sacrificed and their spleens were aseptically removed. The spleens were homogenized gently using 1 mL cold RPMI 1640 media. Cold ammonium chloride (0.9%) was added to the tube containing homogenized spleen in order to lyse the red blood cells and the tube was incubated at room temperature for 5 min. Then, the cells were washed twice with RPMI 1640 and after the final washing, the tube was centrifuged and the sediment was re-suspended in RPMI 1640 containing 10% FBS. The viability of the splenocytes was determined using trypan blue (0.4%, w/v) and it was estimated above 98%. Then, a suspension of  $1 \times 10^7$  cell/mL was prepared using RPMI 1640 and 1 mL of the suspension was cultured in 24-well micro-plates. Then, 1 mL of RPMI 1640 containing 100 mg/mL streptomycin, 100 IU/mL penicillin, 10% FBS, and 200 µg/mL OVA was added to each well and the plates were incubated at 37 °C for 72 h. Then, the cells were transferred into the polystyrene tubes and centrifuged for 5 min (at 4 °C, 2000 rpm). A flow cytometry staining buffer was utilized to resuspend the

cells pellet and then a suspension containing  $1 \times 10^6$  cell/mL was prepared. The viability of the cells was determined using trypan blue (0.4%, w/v) that was above 70%. A commercially available mouse Treg staining kit was used to stain the cells and the assay was conducted according to the manufacturer's instructions. Finally, the samples were analyzed by flow cytometry device (Sysmex-Partec, GmbH, Germany) and FlowJo software (Version 7.6) was used to analyze the results. The cells with Foxp3 and CD4<sup>+</sup> markers were considered as Treg cells (33) and the percentages compared between the groups (34).

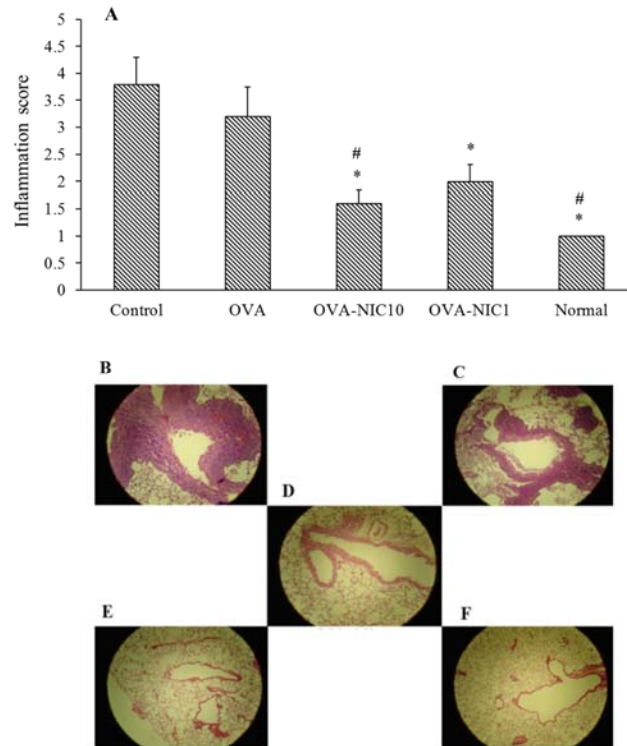
#### ***Statistical analysis***

For statistical analysis and to determine the differences between the groups, a one-way analysis of variance (ANOVA) followed by Tukey post hoc test was used.  $P \leq 0.05$  was considered statistically significant. Values were expressed as mean  $\pm$  standard error of mean (SEM).

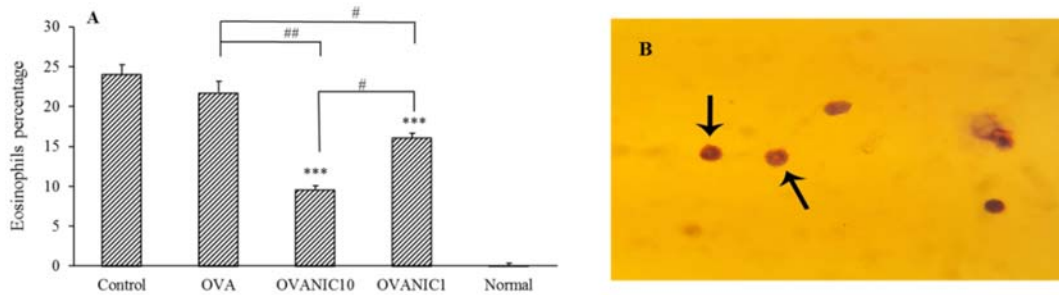
## **RESULTS**

#### ***Evaluation of inflammation in the lung tissue***

A pathologist in a single-blind manner evaluated the immigration of inflammatory cells and their accumulation around the bronchioles and vessels. As demonstrated in Fig. 1A, the control group had the highest degree of inflammation. The results of the study showed that the co-administration of OVA and NIC (both in 10 and 1 mg/kg doses) significantly decreased the inflammation score compared to the control group. Nevertheless, only 10 mg/kg of NIC (the OVA-NIC10 group) could significantly reduce inflammation score compared to the OVA group, and the difference between the OVA-NIC1 and OVA groups was insignificant. Moreover, there was insignificant difference between the OVA-NIC10 and OVA-NIC1 groups. Furthermore, there was an insignificant difference between the OVA and control groups. The microscopic image of each group represented in Fig. 1B-1F.



**Fig. 1.** (A) The effect of NIC plus OVA administration on inflammation score in the lung tissue. The administration of OVA plus NIC at 1 and 10 mg/kg doses could significantly reduce the inflammation in the lung tissue compared to the control group (\*\* $P \leq 0.01$ ) OVA group. # Indicates significant differences compared to the OVA group  $P \leq 0.05$ . B, Control; C, OVA; D, OVA-NIC1; E, OVA-NIC10; and F, normal group indicate digital images of the lung tissue from different groups. The inflammation significantly reduced in the lung tissue of mice, which received NIC plus OVA compared to the control and OVA groups. The co-administration of 10 mg/kg OVA and NIC significantly reduced the inflammation score compared to the OVA group. NIC, Nicotine; OVA, ovalbumin.



**Fig. 2.** (A) The effects of co-administration of NIC and OVA on the percentage of eosinophils in the bronchoalveolar lavage fluid. Ten and 1 mg/kg of NIC plus OVA significantly reduced the immigration of eosinophils to the lung tissue compared to the (\*\*\*)  $P \leq 0.001$ ). Although both doses of NIC were effective in inflammation suppression, it seems that 10 mg/kg NIC had more suppressive effects on the recruitment of eosinophils. #  $P \leq 0.05$  and ##  $P \leq 0.01$  indicate significant differences between groups; (B) indicates digital image of eosinophil. Peripheral smear of the bronchoalveolar lavage fluid stained by the Wright-Giemsa staining method; eosinophils marked by the black arrows. NIC, Nicotine; OVA, ovalbumin.

#### Analysis of the bronchoalveolar lavage fluid

Figure 2A shows that co-administration of OVA and NIC (both in 10 and 1 mg/kg doses) could significantly decrease the eosinophils

number in BAL fluid compared to the control and OVA groups. In addition, administration of OVA alone could not significantly decrease the eosinophils number in the BALF compared

to the control group. Furthermore, 10 mg/kg dosage had a significantly more suppressive effect on eosinophil recruitment into the lung compared to 1 mg/kg dose. An example of mouse eosinophils illustrated in Fig. 2B.

**Measurement of transforming growth factor-β and interleukin-4**

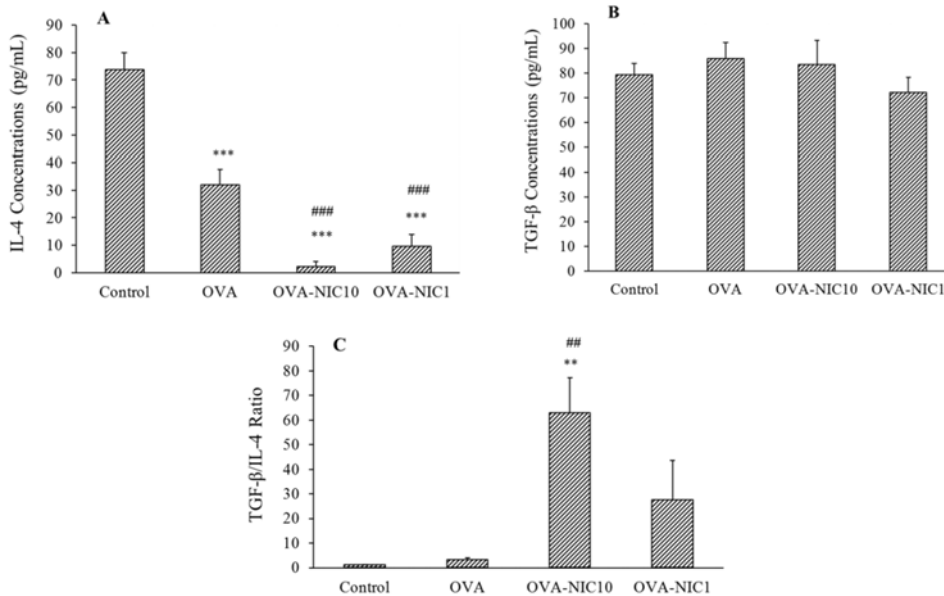
As demonstrated in Fig. 3A, splenocytes of the groups receiving OVA plus NIC produced a significantly less amount of IL-4 when exposed to OVA (40 μg/mL) *in vitro*. However, both doses of NIC effectively decreased IL-4 production; the group treated with the greater dose of NIC (10 mg/kg) plus OVA (the OVA-NIC10) produced much less amounts of IL-4. Furthermore, the OVA group produced significantly less amount of IL-4 compared to the control group.

As demonstrated in Fig. 3B, there was an insignificant difference between the groups in TGF-β production rate, while TGF-β/IL-4 ratio (Fig. 3C) revealed that administration of OVA and NIC at the both concentrations

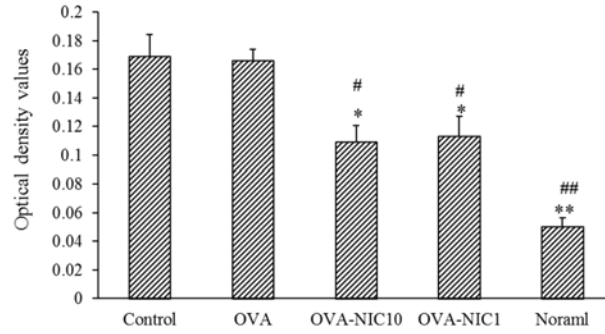
increased the ratio compared to the control and OVA groups. However, both concentrations of NIC increased the ratio, but increment was only significant in the OVA-NIC10 group.

**Assessment of ovalbumin-specific immunoglobulin E titers**

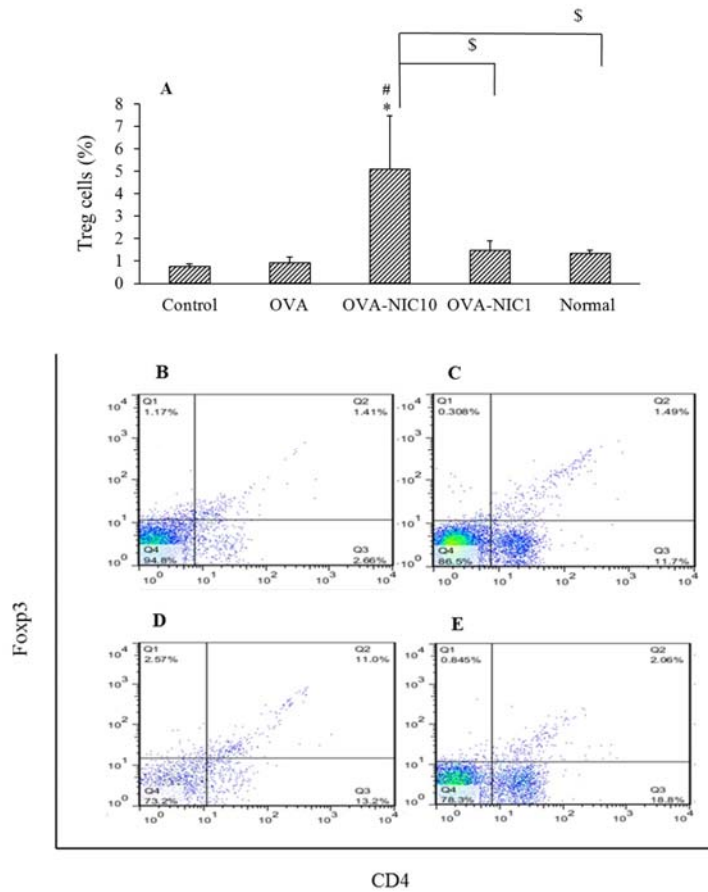
The serum titer of OVA-specific IgE was measured using an indirect ELISA technique. As shown in Fig. 4, the co-administration of OVA and NIC at both dosages significantly reduced the serum titer of OVA-specific IgE and its production compared to the control and OVA groups. Although both doses of NIC reduced the OVA-specific IgE levels, no significant difference was observed between the applied doses. Furthermore, the administration of OVA alone did not reduce the allergen-specific IgE production significantly compared to the control group.



**Fig. 3.** The effects of co-administration of NIC and OVA on IL-4 and TGF-β production and TGF-Beta/IL-4 ratio. (A) Both doses of NIC (1 and 10 mg/kg) significantly reduced the IL-4 production compared to the OVA (###*P* ≤ 0.001) and control (\*\**P* ≤ 0.001) groups; (B) the co-administration of NIC and OVA did not increase the TGF-β production by splenocytes in RPMI 1640; and there was an insignificant difference between the groups; and (C) the TGF-β/IL-4 ratio in the mice from the OVA-NIC10 group was significantly higher than that of observed in the OVA (##*P* = 0.008), and control (\*\**P* = 0.010) groups. NIC, Nicotine; OVA, ovalbumin; TGF, transforming growth factor; IL, interleukin.



**Fig. 4.** The effect of co-administration of NIC and OVA on the serum level of allergen-specific IgE. Administration of NIC (1 and 10 mg/kg) plus OVA decreased the allergen-specific IgE production compared to the control ( $*P \leq 0.05$  and  $**P \leq 0.01$ ) and OVA ( $\#P \leq 0.05$  and  $\#\#P \leq 0.01$ ) groups. However, the different doses of nicotine had almost the same effects on allergen-specific IgE production. NIC, Nicotine; OVA, ovalbumin; Ig E, immunoglobulin E.



**Fig. 5.** (A) The effect of co-administration of OVA and NIC on the population of Treg-cells. Seven days after the last challenge, splenocytes cultured in RPMI 1640 containing the allergen and 72 h later, the cells were stained with anti-CD4 and anti-Foxp3 antibodies. The population of Treg cells from the OVA-NIC10 group significantly increased compared to the control ( $*P \leq 0.05$ ) and OVA ( $\#P \leq 0.05$ ) groups. The population of Treg cells in the OVA-NIC1 group was more than that detected in the control and OVA groups; however, the difference was insignificant. <sup>S</sup> Indicates significant ( $S P \leq 0.05$ ) differences between groups. (B-E) The control, OVA, OVA-NIC10, and OVA-NIC1 groups, respectively, indicate the gating strategy and expression of CD4 and Foxp3 in splenocytes of BALB/c mice. FL3 indicated Foxp3 (PE-Cy5) and FL1 specified CD4 (FITC). Data were analyzed with FlowJo software. NIC, Nicotine; OVA, ovalbumin; Ig E, immunoglobulin E; Treg-cell, regulatory T-cell.



### Assessment of Treg cells population

The splenocytes were co-cultured with OVA (200 µg/mL) in order to evaluate the Treg cell population changes between the groups. Moreover, to minimize the spillover between PE-CY5 and PE fluorochromes, Foxp3<sup>+</sup> and CD4<sup>+</sup> cells (PE-CY5<sup>+</sup> and FITC<sup>+</sup>) were considered as Treg cells. As illustrated in Fig. 5A, Treg cells population in the OVA-NIC10 group was much higher than that of the control and OVA groups. However, the population of Treg cells in the OVA-NIC1 group was greater than those of the OVA and control groups, but the difference was insignificant. An example from each group for gating strategy represented in Fig. 5B.

## DISCUSSION

The present study examined the ability of NIC to improve the efficacy of immunotherapy in a mouse model of allergic asthma. The study aimed at evaluating the tolerogenic activity of NIC in combination with the allergen in a mouse model of immunotherapy. In the current study, the dose of OVA was lower than that required for the suppression of allergic inflammation. This low dose contributed to the evaluation of the efficacy of NIC in immunotherapy process. To the best of our knowledge, the current study for the first time examined co-administration of NIC, as a tolerogenic adjuvant, and the allergen to increase immunotherapy efficacy.

The results of the current study showed that administration of NIC plus OVA increased the immunotherapy efficacy through decreasing the allergic inflammation in the lung tissue, decreasing the number of eosinophils in the BALF, as well as reducing the serum level of IL-4 and titer of OVA-specific IgE. Furthermore, while administration of OVA alone did not increase Treg cells population, co-administration of NIC and OVA significantly increased the Treg cells population. Moreover, co-administration of OVA and NIC augmented TGF-β/IL-4 ratio. These results suggested that administration of

NIC plus OVA improved immunotherapy efficacy. The results of the current study were in agreement with those of the current study in which administration of NIC, as a therapeutic agent, could ameliorate allergic responses in a mouse model of allergic asthma (35).

The results of the current study showed that the co-administration of NIC (1 and 10 mg/kg) and OVA could significantly reduce the allergic inflammation in the lung tissue. The results of the current study also showed that, although the administration of both doses of NIC could reduce the inflammation score, the greater dose (10 mg/kg) had better anti-inflammatory effects. On the other hand, although the administration of OVA alone reduced the allergic inflammation, it could not significantly suppress the inflammation compared to the control group. Furthermore, when encountered with the allergen, splenocytes from the groups receiving NIC plus OVA (the OVA-NIC1 and OVA-NIC10 groups) secreted significantly less amount of IL-4 compared to the ones from the control and OVA groups.

Eosinophilia is an important marker of some allergic diseases including the allergic asthma. Eosinophils immigrate to the target tissue, in which allergen is introduced, and start releasing pro-inflammatory cytokines (IL-1, IL-6, IL-12, and TNF-alpha) and mediators that contribute to the allergic inflammation (36). The results of the current study also showed that while the administration of OVA alone slightly reduced eosinophilia, the co-administration of NIC and OVA significantly decreased the immigration of eosinophils to the lung tissue.

The results of the current study indicated that the administration of OVA could not reduce the allergen-specific IgE production compared to the control group; while co-administration of NIC and OVA significantly decreased the IgE production. However, the dose of co-administered NIC did not affect the IgE production level; and the effects of both doses (1 and 10 mg/kg) on IgE production were almost the same. A study conducted by Mishra *et al.* showed that NIC administration decreased the serum titers of

the allergen specific IgE, while it could not reduce the serum titers of total IgE in the allergic mice (37). The results of the current study agreed with the results obtained by Mishra *et al.*, and administration of NIC in the two concentrations decreased the serum titers of the allergen specific IgE.

The results showed an insignificant difference between the groups in the secretion of TGF- $\beta$  by splenocytes. A study indicated that stimulation of Treg cells by NIC could not increase TGF- $\beta$  production in the media. They supposed that treatment with NIC may augment Treg cells suppression capacity (31). Anyway, the current study results agreed with their results; and no significant difference was detected between the groups. Although TGF- $\beta$  production by splenocytes did not increase significantly, our results indicated that TGF- $\beta$ /IL-4 ratio was significantly higher in the OVA-NIC10 group. Hence, it can be assumed shifting of the immune response onto the tolerance may occur by the co-administration of NIC and OVA. On the other hand, flow cytometry results showed that when exposed to the allergen, co-administration of NIC at 10 mg/kg and OVA dramatically increased the population of Treg cells compared to those of the control and OVA groups. However, 1 mg/kg of NIC did not significantly increase the population of Treg cells. According to the obtained results, the possible suggested mechanism includes indirect and direct effects of NIC on immune responses. Administration of NIC together with OVA may provide an anti-inflammatory microenvironment through stimulating the corresponding receptors on the surface of antigen presenting cells and other innate immune cells (11,12,38) which in turn, ultimately divert the specific immune response into anti-inflammatory responses; *i.e.* inducing more antigen-specific Treg and less antigen-specific effector cells.

It is believed that the current study had some limitations such as the limited number of the measured cytokines, the lack of a long-term monitoring, and the lack of measurement of blood levels of NIC. It is hoped that such limitations are addressed

in the further studies. On the other hand, it is thought that the current study has some advantages; for example, studying the efficacy and mechanism of action at the same time and testing different doses of NIC.

## CONCLUSION

Collectively, the results of the current study suggested that the administration of NIC, as an adjuvant, can enhance the therapeutic effects of SCIT in a mouse model of allergic asthma. To the best of authors' knowledge, the current study was the first of its kind; therefore, follow-up studies are needed to confirm the adjuvant effect of NIC in SCIT on allergic diseases and also determine the limitations of this application of NIC.

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