



Enhanced sublingual immunotherapy by TAT-fused recombinant allergen in a murine rhinitis model



Farhad Salari^a, Fatemeh Vahedi^{b,c}, Abdol-Reza Varasteh^d, Hanieh Ketabdar^e,
Jamshidkhan Chamani^f, Mojtaba Sankian^{g,*}

^a Department of Immunology, School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran

^b Department of Pathology and Molecular Medicine, McMaster Immunology Research Centre, Hamilton, Ontario, Canada

^c Biotechnology Department, Razi Vaccine and Serum Research Institute, Mashhad, Iran

^d Allergy Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

^e Department of Biochemistry and Biophysics, Faculty of sciences, Mashhad Branch, Islamic Azad University, Mashhad, Iran

^f Department of Biochemistry and Biophysics, Faculty of sciences, Mashhad Branch, Islamic Azad University, Mashhad, Iran

^g Immunobiochemistry Lab, Immunology Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

ARTICLE INFO

Keywords:

Sublingual immunotherapy (SLIT)

IgE

TAT protein transduction domain (TAT-PTD)

Recombinant allergen

ABSTRACT

Allergen-specific sublingual immunotherapy (SLIT) is well known as an effective and non-invasive route to induce allergy desensitization. The goal of this study was to investigate whether a TAT-fused recombinant allergen could enhance SLIT efficacy. BALB/c mice sensitized to the main allergen (Che a 3) of *Chenopodium album* pollen were treated sublingually either with rChe a 3 (100 µg/dose) or rTAT-Che a 3 (100 µg/dose), two times per week for eight weeks. SLIT with rTAT-Che a 3 led to significantly greater allergen-specific IgG2a than rChe a 3; however, neither rTAT-Che a 3 nor rChe a 3 affected allergen-specific IgE or IgG1 antibody levels. In addition, interleukin 4 (IL-4) levels in re-stimulated splenocytes from the rTAT-Che a 3 mice were significantly lower than in those from the rChe a 3 mice, while interferon-γ (IFN-γ) was significantly greater in the rChe a 3 mice than in the rTAT-Che a 3 mice. Furthermore, sublingual administration of rTAT-Che a 3 induced significantly greater TGF-β secretion in re-stimulated splenocytes than administration of rChe a 3. Accordingly, SLIT with rTAT-Che a 3 led to significantly greater expression of TGF-β- and Foxp3-specific mRNAs in the splenocytes than in those from the rChe a 3 mice. Our findings demonstrate that TAT-fused rChe a 3 suppressed the allergic response through preferential enhancement of systemic regulatory T-cell (Treg)-mediated immunity responses, likely by facilitating allergen capture and presentation by sublingual Langerhans-like dendritic cells.

1. Introduction

The increasing prevalence of allergic diseases has become a public health problem in many developed and developing countries [1]. Allergen-specific sublingual immunotherapy (SLIT) is well known as an effective and non-invasive method to induce allergy desensitization [2–6]; however, current vaccines for SLIT require further enhancement of allergen presentation to sublingual immune cells. Second-generation vaccines using well-defined recombinant allergens, rather than allergen extracts from natural sources, are under development [7,8]. During SLIT, the allergens are captured within the sublingual mucosa by local Langerhans-like dendritic cells (DCs) following phagocytosis, macro-

pinocytosis, or receptor-mediated endocytosis [9,10]. Currently, new vaccine formulations, which take into account the appropriate delivery systems to improve allergen targeting to the sublingual immune cells, are being investigated. In particular, muco-adhesive vaccines have been shown to increase SLIT efficacy by enhancing the contact duration of the allergen with the sublingual mucosa [11,12].

The TAT protein transduction domain (TAT-PTD), an 11-amino acid arginine- and lysine-rich peptide derived from the human immunodeficiency virus type 1 (HIV-1), has been demonstrated to cross cellular membranes, both alone and linked to therapeutic biomolecules [13,14]. Several groups have shown the effectiveness of the TAT protein transduction domain (PTD) as a delivery vehicle for a wide variety of

Abbreviation: SLIT, sublingual immunotherapy; Che a 3, main allergen of *Chenopodium album* pollen; rChe a 3, recombinant Che a 3; TAT, HIV virus-TAT protein transduction domain; rTAT-Che a 3, recombinant TAT-fused Che a 3; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HRP, horseradish peroxidase; Far- and near-UV CD, circular dichroism (CD) spectroscopy

* Corresponding author at: Immunobiochemistry Lab, Immunology Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.

E-mail addresses: f.salari@kums.ac.ir (F. Salari), vahedif@mcmaster.ca, vahedif@yahoo.com (F. Vahedi), varasteha@mums.ac.ir (A.-R. Varasteh), h.ketabdar@yahoo.com (H. Ketabdar), Chamani@ibb.ut.ac.ir (J. Chamani), sankianm@mums.ac.ir (M. Sankian).

<http://dx.doi.org/10.1016/j.intimp.2017.04.011>

Received 7 November 2016; Received in revised form 12 March 2017; Accepted 12 April 2017

1567-5769/ © 2017 Published by Elsevier B.V.

biomolecules including peptides, proteins, anti-cancer therapeutic cargos, and antisense oligonucleotides, both *in vivo* and *in vitro* [15–17]. The TAT-PTD, with six arginine and two lysine residues, is a highly cationic peptide. Mechanistic studies have shown that the positive charge of TAT plays a key role in its transduction. Various studies have suggested that an initial strong electrostatic interaction between the positively-charged TAT-PTD and the negatively-charged plasma membrane of eukaryotic cells induces endocytic pathways leading to the cellular internalization of TAT [18,19].

Herein, we hypothesized that a TAT-fused recombinant allergen might enhance SLIT *via* allergen crossing of sublingual endothelium or prolongation of the contact between the positively-charged allergen and the negatively-charged sublingual mucosa. To investigate, we used a mouse model sensitized to the main allergen (Che a 3) of *Chenopodium album* (*C. album*) pollen to compare recombinant TAT-Che a 3 (rTAT-Che a 3) to free recombinant Che a 3 (rChe a 3) in SLIT.

2. Materials and methods

2.1. Animals

Female, 6–8 weeks old BALB/c mice were purchased from the Razi Vaccine and Serum Research Institute) Mashhad, Iran. All the animal experiments and handling procedures were approved by the Institutional Animal Ethics Committee (No. 87542) of the Mashhad University of Medical Sciences.

2.2. Antigens

Recombinant Che a 3 was expressed in *E. coli* BL21 Codon Plus (DE3) RIL cells (Stratagene, USA) and purified by Ni-NTA chromatography as described previously [20]. The recombinant TAT-Che a 3 fusion protein (rTAT-Che a 3) was produced as describe below.

2.2.1. Recombinant TAT-Che a 3 plasmid construction

To produce rTAT-rChe a 3, DNA was amplified by PCR from the *pET21b⁺-rChe a 3* vector described previously [20], using the following primers: 5'-GGAAAGCTTGGCAGAAGAAGCGGAGACAGCGACGAAGAGCTATGGCTGCTGAGGATACACCTC-3' and 5'-ATTCTCGAGATGGCTGCTGAGGATACACCTC-3'; the underlined sequences are *HindIII* and *XhoI* restriction sites respectively, and italicized letters correspond to the TAT sequence. The PCR product was cloned into the *HindIII* and *XhoI* cloning sites of the *pET21b⁺* expression vector (Novagen, USA) and expressed in *E. coli* BL21 (DE3) cells. A clone containing the desired construct was verified by sequence analysis.

2.2.2. Expression and purification of rTAT-Che a 3

100 μ L aliquots of overnight cultures of BL21 cells carrying the plasmids were aliquoted into 100 mL of LB medium containing 50 μ g/mL of ampicillin. The cultures were grown at 18 °C and induced at OD₆₀₀ = 0.5 with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 12 h. To purify the recombinant fusion protein, the cells were harvested by centrifugation at 9000 $\times$ g at 4 °C for 10 min and then resuspended in 10 mL of lysis buffer containing 50 mM Tris-HCl, pH 7.8, 5 mM imidazole, and 500 mM NaCl. The bacterial cell walls were disrupted by three freeze-thaw cycles in liquid nitrogen and 37 °C. The supernatants and insoluble fractions were separated by centrifugation. Ni-NTA sepharose columns (Amersham, UK) were used to purify the recombinant proteins according to the manufacturer's instructions. The elution fractions were electrophoresed by SDS-PAGE to identify the fraction(s) containing the fusion protein. The fractions containing rTAT-rChe a 3 were dialyzed overnight against phosphate buffer (pH 7.4), using 12 kDa molecular weight cut-off dialysis membranes (Sigma, Germany).

2.3. SDS-PAGE and western-blotting

Allergen purity was analyzed by 15% SDS-PAGE under non-reducing conditions. The acrylamide gels were stained with Coomassie Brilliant Blue G-250 (Merck, Germany). For western blotting, following SDS-PAGE, purified proteins were transferred onto PVDF membranes (Immobilon P, Millipore Corp., Bedford, USA) at 0.3 A, 100 V (using Bio-Rad System, USA), for 15 min at 4 °C. After blocking in 2% BSA, pH 7.4, overnight at 4 °C, the membranes were incubated overnight at 4 °C with pooled sera from five patients allergic to *C. album* pollen, diluted 1:3 in blocking buffer. Human-specific IgE was detected by a biotinylated goat anti-human-IgE antibody (KPL, USA) diluted 1:1000 in blocking buffer for 3 h at room temperature, and then with HRP-conjugated streptavidin (Bio-Rad, USA) diluted 1:20,000 for 1 h at room temperature, followed by development with a Chemiluminescence Western Blotting kit (ParsTous, Iran). A Syngene (UK) apparatus was used for image acquisition. A pooled sera from non-allergic individuals was applied as a negative control.

2.4. Circular dichroism

Far- and near-UV CD analyses were performed on a Jasco-815 spectropolarimeter (Jasco, Tokyo, Japan) as previously described [21]. Briefly, spectra were determined from samples with concentrations of 0.01% in 20 mM phosphate buffer, pH 7.4, using a 1 mm path length cuvette. Far- and near-UV CD spectra were obtained over wavelength ranges of 190–240 and 250–350 nm. The secondary structures of the proteins were analyzed by SELCON3, CONTIN, and CDSSTR to estimate the relative proportions of α -helices, β -sheets, turns, and random coils [22,23].

2.5. Fluorescence spectroscopy

The fluorescent spectra were determined using an F-2500 spectrofluorimeter (Hitachi, Japan) as previously described [21]. Briefly, slit widths were set at 5 nm, and sample emission spectra were determined using a 1.0-cm quartz cuvette. The excitation wavelengths were 280 and 295 nm at 5 nm steps and emission scans were performed at wavelengths of 220 to 500 nm.

2.6. Experimental procedure

2.6.1. Sensitization phase

The effect of the TAT-fused recombinant allergen on SLIT efficacy was tested in a therapeutic *in vivo* model of rChe a 3-sensitized mice, according to a previously-described protocol [11] (Fig. 1). Briefly, BALB/c mice were sensitized to rChe a 3 by means of two intraperitoneal injections with 10 μ g of rChe a 3 adsorbed onto 2 mg of aluminum hydroxide (Sigma, Saint Quentin Fallavier, France) in 150 μ L of PBS on days 0 and 14. Starting on day 21, the mice were challenged for 20 min with 1% (w/v) rChe a 3 on four consecutive days using an aerosol delivery system (Omron CX3 nebulizer, Netherlands). Control mice were sensitized with PBS and 2 mg of aluminum hydroxide. On day 25, to evaluate sensitization, blood samples were collected through the retro-orbital sinus, and based on rChe a 3-specific IgE and IgG1 levels, the mice were stratified into treatment groups ($n = 5$).

2.6.2. Sublingual immunotherapy

SLIT was started on day 28, twice a week for eight weeks by applying 100 μ g/dose of rChe a 3 or rTAT-Che a 3, in a total volume of 25 μ L, under the tongue. The sham-treated group received PBS. Furthermore, a not-sensitized, not-treated group was included as the control group. On days 90 and 91 all groups were challenged for 20 min with aerosolized rChe a 3 (1%) as described previously. The blood samples from the retro-orbital plexus were collected weekly during the sensitization and immunotherapy stages. Mice were euthanized at the

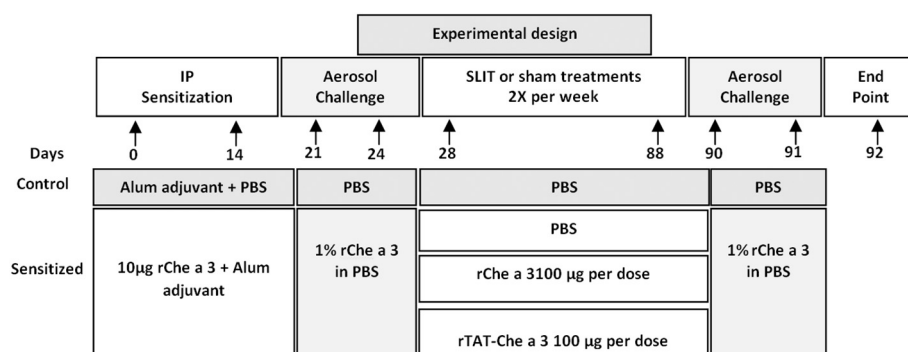


Fig. 1. Experimental design: BALB/c mice were sensitized to rChe a 3 by two intraperitoneal injections of 10 µg of allergen adsorbed onto aluminum hydroxide followed by an aerosol challenge. Control mice received PBS. SLIT was conducted for eight consecutive weeks with either the soluble rChe a 3 or rTAT-Che a 3 with the doses shown in the figure or PBS (sham groups) whereas control mice received PBS. At the end of experiments mice groups ($n = 5$) were euthanized and blood, bronchoalveolar lavage fluid, and spleens were collected for further analyses.

end of experiment (day 92) and blood and spleens were collected.

2.7. Measurement of humoral response against rChe a 3

Serum allergen-specific IgE, IgG1, and IgG2a antibodies were measured by ELISA. Briefly, ELISA plates (Nunc, Roskilde, Denmark) were coated with purified rChe a 3 (20 µg/mL) for 1 h at 3 °C. After washing and blocking, diluted mouse sera were incubated on the plates overnight at 4 °C. Mouse sera dilutions were 1:10, 1:2500, and 1:320 for IgE, IgG1, and IgG2a determinations, respectively. For IgE determination, 100 µL of 1:2000 dilutions of biotinylated rat anti-mouse IgE (AbD Serotec, Inc., USA) were added to each well and incubated for 2.5 h at room temperature. Then, 1:20,000 dilutions of horseradish peroxidase (HRP)-conjugated streptavidin (1 mg/mL) were added to each well and the plates were incubated for 1 h at room temperature. For IgG1 and IgG2a measurements, HRP-conjugated rat anti-mouse IgG1 or biotinylated rat anti-mouse IgG2a (Zymed Laboratories Inc., USA and BD Biosciences, San Diego, CA, USA, respectively) were added to the plates according to the manufacturer's instructions. Finally, samples were developed by addition of a chromogenic substrate. Optical density (OD) was read at 450 nm using an ELISA microplate reader (Stat Fax® 2100 Microplate Reader, Awareness Technology, USA).

2.8. Cytokine assay

Spleens from individual mice were harvested, minced, and filtered through a stainless steel mesh. Red blood cells were lysed with ammonium chloride (180 mM NH₄Cl, 17 mM Na₂EDTA). Splenocytes were washed with PBS and resuspended in RPMI medium (Gibco Ltd., Paisley, Scotland) supplemented with 10% inactivated fetal calf serum (FCS) and 100 U/mL of penicillin and 100 µg/mL of streptomycin. Cells were plated in 24-well flat-bottomed culture plates (Nunc, Denmark) in duplicate at 1×10^6 cells/mL, and stimulated with rChe a 3 (20 µg/mL), PHA (1 µg/mL), or media, at 37 °C for 72 h. Supernatants were analyzed for IL-4, IFN-γ, IL-10, and TGF-β using mouse ELISA kits (eBioscience, San Diego, California) according to the manufacturer's instructions.

2.9. Proliferation assay

Splenocytes from each group were pooled, adjusted to 2.5×10^5 cells per well in 96-well round-bottomed culture plates in triplicate (Nunc, Denmark), and stimulated with rChe a 3 (20 µg/mL) or rPla o r 3 (20 µg/mL) as the control allergen, PHA (1 µg/mL) as a positive control, or medium alone, for 72 h at 37 °C. Cell proliferation in response to the rChe a 3 was determined using a colorimetric immunoassay based on the WST-1 proliferation assay kit (Roche Diagnostics, Germany) following the manufacturer's instructions. Cell

proliferation was expressed as the stimulation index (SI) and calculated as a ratio of the mean OD at 450 nm of stimulated vs. unstimulated cultures.

2.10. Evaluation of gene expression by real-time PCR

Total RNA was isolated from splenocytes stimulated with rChe a 3 using a Total RNA Extraction Kit (ParsTous, Iran). Reverse transcription was performed with a First Strand cDNA Synthesis Reverse Transcriptase Kit (ParsTous, Iran) using random hexamers according to the manufacturer's instructions. Primers for mouse cytokines and transcription factors were designed with Beacon Designer™ Software (Primer Biosoft, USA) (Table 1). Quantitative real-time PCR was performed using SYBR® Real-Time PCR Master Mix (ParsTous, Iran) on a Rotor Gene Q Series II (Qiagen, Germany). The mRNA expression levels for specific genes were normalized with GAPDH as a reference gene. Relative mRNA expression ratios were calculated with a mathematical model, $R = (E_{\text{target}})^{\Delta Ct \text{ target (control - sample)}} / (E_{\text{Ref}})^{\Delta Ct \text{ Ref (control - sample)}}$, as described by Pfaffl [24].

2.11. Statistical analysis

Statistical analyses were performed using the GraphPad Prism® 5.0 package for Windows (GraphPad Software Inc., San Diego, CA, USA) and p values < 0.05 were considered significant. Results are expressed as means ± standard deviations (SDs). Comparisons between control, sham, and treatment groups were evaluated by one-way ANOVA and Tukey's tests.

3. Results

3.1. Characterization of recombinant allergens

3.1.1. SDS-PAGE analysis and immunoblotting

IPTG (1 mM) was added to the recombinant *E. coli* BL21 (DE3) cells at 16 °C to induce expression of rChe a 3 and rTAT-Che a 3. The recombinant allergens were visualized by SDS-PAGE (not shown). To investigate the reactivity of the purified recombinant allergens with IgE, the proteins were immunoblotted under reducing conditions with

Table 1

The secondary structure contents of rChe a 3 and rTAT-Che a 3 obtained by far-UV CD analysis.

Protein	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Unordered coil (%)
rChe a 3	45.13	11.72	6.22	36.93
rTAT-Che a 3	51.08	11.36	6.50	31.06

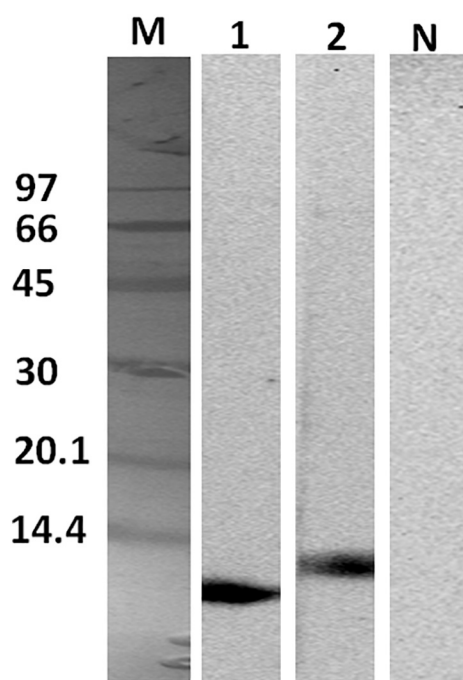


Fig. 2. Purified rChe a 3 and rTAT-Che a 3 were analyzed by western blot using pooled sera from *C. album*-allergic patients. Lane M, MW marker; lane 1, rChe a 3; lane 2, rTAT-Che a 3; lane N, rChe a 3 blotted with pooled sera from non-allergic subjects as negative control.

pooled sera from five *C. album* allergic patients and a secondary goat anti-human IgE antibody. Anti *C. album* IgE reacted with rChe a 3 (13 kDa) and rTAT-Che a 3 (14 kDa) (Fig. 2, lanes 1 and 2). No bands were seen in the negative control lane incubated with non-allergic sera (Fig. 2, lane N).

3.1.2. Circular dichroism (CD) spectroscopy analysis

The secondary structures content of the recombinant allergens were analyzed by CD spectroscopy (Fig. 3 and Table 1). The far-UV spectra for rChe a 3 and rTAT-Che a 3 displayed the basic features of properly-folded α -helical proteins, with characteristic negative maxima at 223 and 208 nm and a positive maximum near 195 nm. The absolute ellipticities at 223 nm were similar. No detectable differences in the ellipticity in the near-UV spectra were observed between the recombinant allergens (Fig. 4).

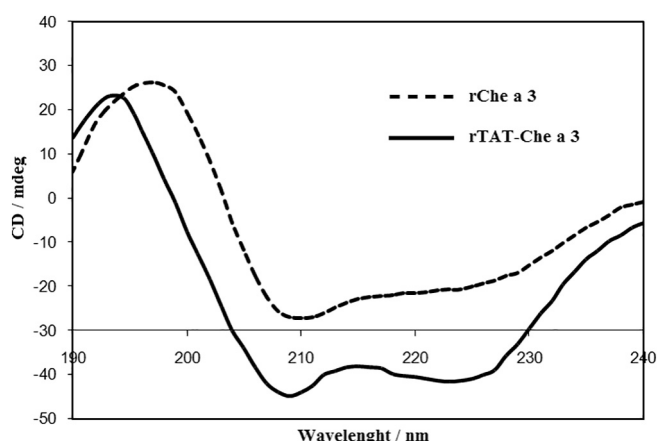


Fig. 3. Far-UV CD spectroscopy. Secondary structures of rChe a 3 and rTAT-Che a 3 were analyzed in 20 mM phosphate buffer (pH 7.4) at 25 °C. Spectra revealed properly-folded α -helical structures for both recombinant allergens.

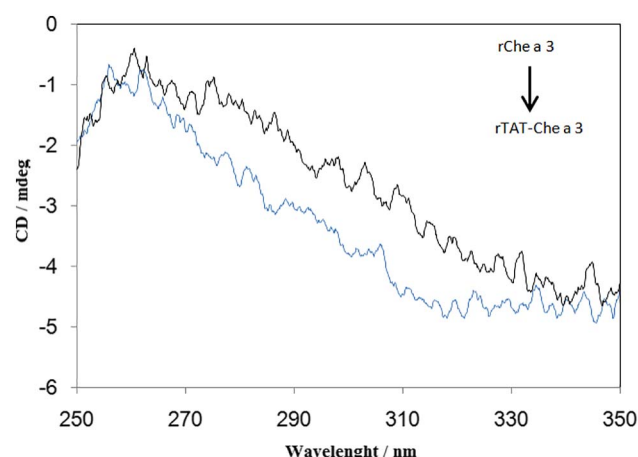


Fig. 4. Near-UV CD spectroscopy. Conformational changes analyses around the aromatic amino acid chains and disulfide bonds of rChe a 3 and rTAT-Che a 3 between 250 and 350 nm indicate the tertiary structures were highly similar.

3.1.3. Fluorescence spectroscopy analysis

To identify the tertiary structures and conformational changes of the recombinant allergens fluorescent spectroscopy was used. Fluorescence spectra related to tyrosine residues in the excitation wavelength of 280 nm showed that rChe a 3 and rTAT-Che a 3 had similar emission maxima (Fig. 5).

3.2. High level of specific IgE induced by intraperitoneal sensitization

To analyze the allergic state in the mouse model, the allergen-specific IgE, IgG1, and IgG2a were measured. Mice sensitized to rChe a 3 in alum adjuvant exhibited significantly greater specific IgE and IgG1, but not IgG2a, levels than control mice that received PBS in alum adjuvant (Fig. 6).

3.3. Effects of SLIT on allergen-specific IgE and IgG levels in the serum

To investigate the effect of immunotherapeutic approaches on antibody responses, the rChe a 3-specific serum IgG1, IgG2a, and IgE were measured by ELISA. In both the rChe a 3 and rTAT-Che a 3-treated mice, rChe a 3-specific IgG2a was significantly greater than in the sham-treated group, and allergen-specific IgG2a was greater in the

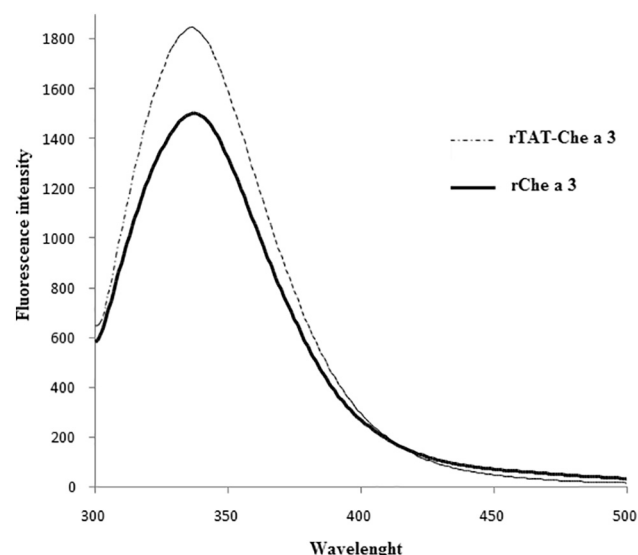


Fig. 5. Fluorescent emission spectra of rChe a 3 and rTAT-Che a 3. The tyrosine residues in rChe a 3 and rTAT-Che a 3 were excited at 280 nm. Similar spectral features were observed.

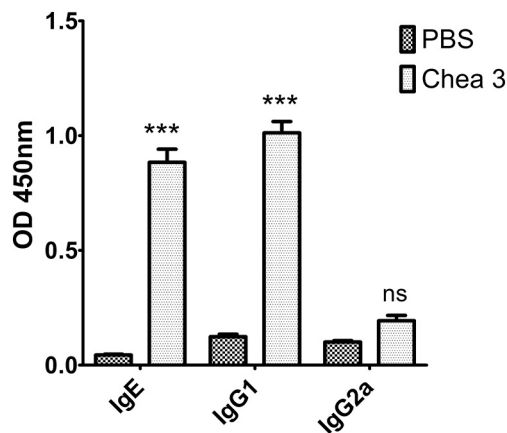


Fig. 6. Serum levels of rChe a 3-specific IgG1, IgG2a, and IgE in sensitized mice: Mice were sensitized as described in Fig. 1, blood samples were collected on the day after aerosol challenge (day 25), and antibodies were measured by ELISA. *** $p < 0.001$ and ns: not significant.

rTAT-Che a 3-treated mice than in the rChe a 3 mice (Fig. 7a). In contrast, no significant differences in allergen-specific IgG1 (not shown) or IgE were observed between the rChe a 3 and rTAT-Che a 3-treated groups. Although allergen-specific IgE levels were increased during the first week of SLIT, at the end of the study specific IgE was slightly less in the allergen-treated than in the sham-treated mice; however, the differences were not statistically significant (Fig. 7b).

3.4. High level secretion of the suppressive cytokine TGF- β by stimulated splenocytes

The rChe a 3-specific IFN- γ , IL-4, IL-10, and TGF- β cytokine levels in supernatants of re-stimulated spleen cells of control and SLIT-treated mice are shown in Fig. 8a, b, c, and d respectively. Splenocytes from mice that received rChe a 3 produced significantly greater levels of IFN- γ than the mice in all other groups, including those from the mice that received rTAT-Che a 3 (Fig. 8a). The rChe a 3-specific IL-4 production was significantly less in splenocytes from mice treated with r-Che a 3 or rTAT-Che a 3 than in sham-treated mice ($p < 0.001$). However IL-4 was less in the rTAT-Che a 3 mice than in the rChe a 3 mice ($p < 0.05$) (Fig. 8b). Similar IL-10 levels were observed in both the rTAT-Che a 3-

and rChe a 3-treated mice (Fig. 8c). Splenocytes from mice treated with rTAT-Che a 3 produced significantly greater levels of TGF- β in response to rChe a 3 as the stimulus than rChe a 3 or control mice (Fig. 8d).

3.5. Splenocyte proliferation assays

Splenocyte proliferation was assessed to determine whether SLIT affects allergen-specific T cell responses through *in vitro* re-stimulation with rChe a 3. The stimulation indexes of both rChe a 3- and rTAT-Che a 3-treated groups were significantly less than those of the sham group. No significant differences in splenocyte proliferation were found between the rChe a 3 and rTAT-Che a 3 groups (Fig. 8e).

3.6. High level expression of Foxp3, T-bet, TGF- β , and IFN- γ genes following SLIT

mRNA expression of selected Th1, Th2, and Treg-related transcription factors and cytokines from splenocytes of sublingually treated mice are shown in Fig. 9. The rChe a 3- and rTAT-Che a 3-treated mice expressed significantly less of the Th2 cytokines IL-4 and IL-13, and the transcription factor GATA-3, than the sham-treated group (Fig. 9a, b, and f). Splenocytes were also examined for the expression of IFN- γ and T-bet, a Th1 transcription factor and Th2-suppressive cytokine. Sublingual administration of rChe a 3 led to significantly greater expression of IFN- γ and T-bet-specific mRNAs than that seen in the rTAT-Che a 3- and sham-treated groups (Fig. 9c and g). In addition, the induction of Treg cells, considered to be one of the key mechanisms of allergen-specific SLIT, was analyzed via IL-10, TGF- β , and Foxp3 expression. No significant differences were seen between the rChe a 3- and rTAT-Che a 3-treated groups in IL-10 expression (Fig. 9d); however, TGF- β and Foxp3 expressions were significantly greater in the rTAT-Che a 3-treated than in the rChe a 3-treated mice (Fig. 9e and h).

4. Discussion

The efficient delivery of allergens to sublingual tolerogenic DCs to enhance tolerance induction during SLIT is the goal of many studies. In this context, those in which increasing the contact time between the allergen and the sublingual mucosa, thereby presumably facilitating allergen uptake by sublingual Langerhans-like dendritic cells, is an interesting research area [11,12,25]. In the present study, we evaluated

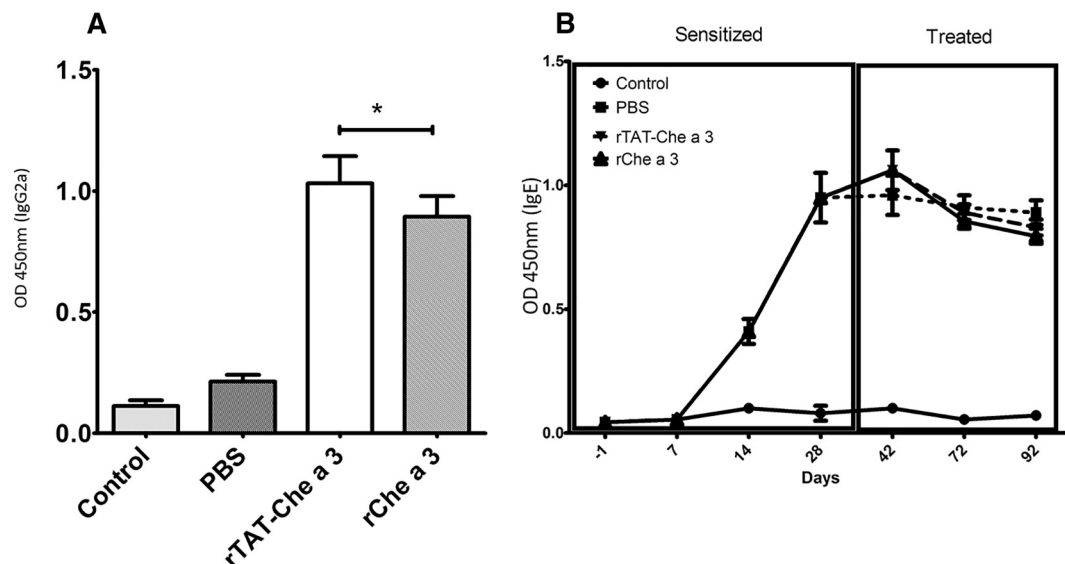


Fig. 7. Serum levels of rChe a 3-specific IgG2a and IgE levels in mice by ELISA: (A) Effect of SLIT on allergen-specific IgG2a levels was measured on day 92. (B) To measure serum IgE, blood samples taken on days 0, 7, 14, 25, 42, 72, and 92, from day 0 to day 42 the results of pooled serum ($n = 35$), and after day 42 individual serum are shown. SLIT had no significant effect on specific IgE levels. Results are shown as mean $\pm$ SD. * $p < 0.05$ and ns: not significant.

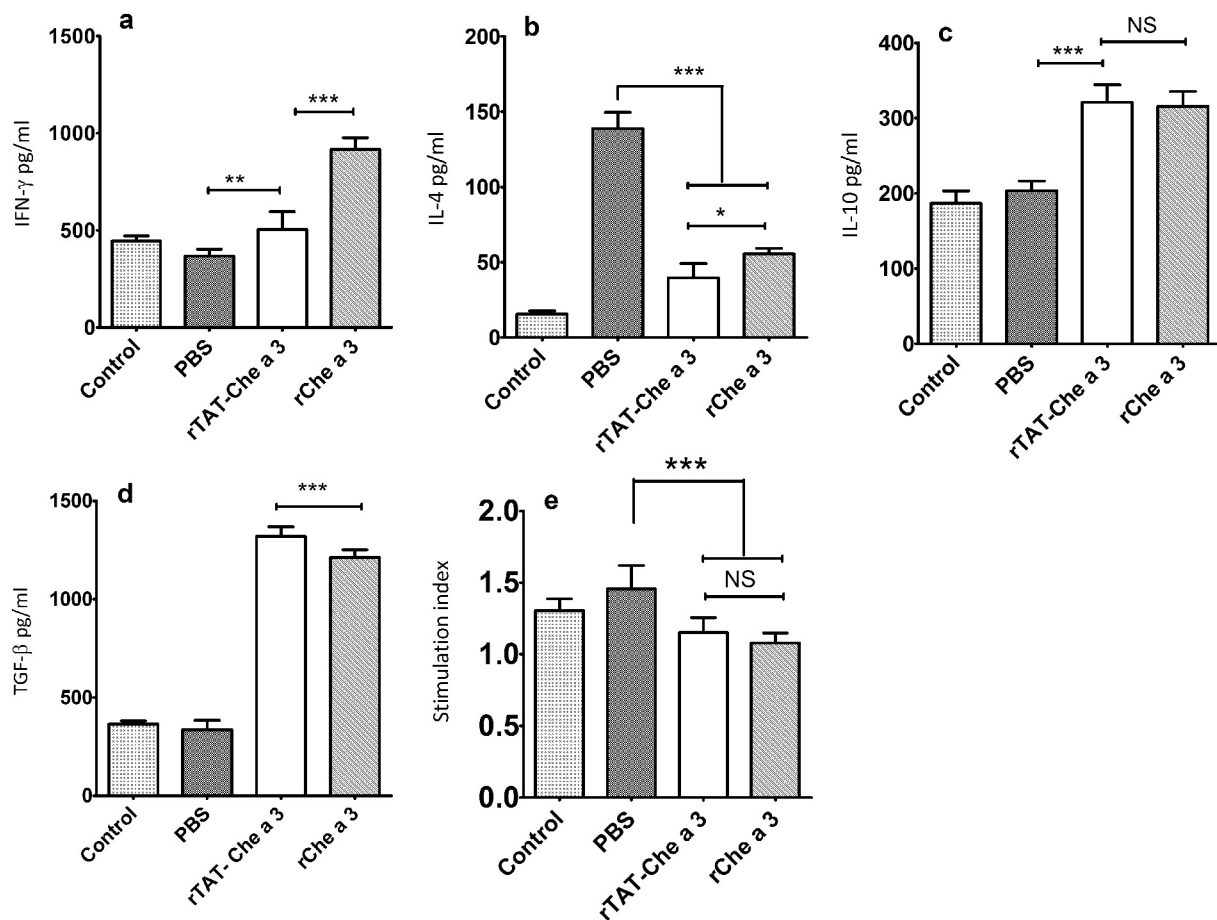


Fig. 8. T-cell proliferation and cytokine production in spleen cell cultures after SLIT: (a-d) Splenocytes from treatment groups were re-stimulated *in vitro* with 10 μ g/mL rChe a 3 for 72 h. IFN- γ , IL-4, IL-10, and TGF- β cytokine concentrations (pg/mL) were measured in culture supernatants by ELISA. Results are presented as mean values $\pm$ SDs. (e) rChe a 3-specific proliferation of splenocytes in the treatment groups. Values represented means of stimulation index $\pm$ SD. * p < 0.05, ** p < 0.01, *** p < 0.001, and ns: not significant.

whether a TAT-fused allergen could enhance SLIT efficacy over free allergen in an animal model. To this aim, TAT-fused rChe a 3 was administered sublingually to previously rChe a 3-sensitized BALB/c mice and its immunomodulatory effects were compared to those of soluble rChe a 3.

To date, TAT-PTD has been used to enhance translocation of fused proteins across membranes, within both cells and tissues [26]. It has been demonstrated that TAT fusion proteins enter cells directly through the membrane, either by macropinocytosis or endocytic pathways [27–31]. It seems the first step in transduction is the binding of TAT-fused proteins to the cell surface, likely *via* an ionic interaction between the positively-charged PTD and negatively-charged cell surface proteoglycans [27,32]. Indeed, loss of a single basic residue (lysine or arginine) out of eight charged residues in TAT has been shown to be sufficient to reduce macropinocytosis. Moreover, substitutions of two basic residues with neutral ones resulted in a reduction to near background levels of macropinocytosis [18,19,32]. Therefore, in this experiment, using a TAT-fusion allergen, we relied on the TAT-PTD to first prolong allergen contact time with sublingual mucosa through an electrostatic interaction with the negatively-charged sublingual membrane, and then aid in the translocation of the fusion allergen into the sub-mucosa where the DCs reside.

In SLIT, the allergen is generally held under the tongue for 1–2 min and then swallowed (sublingual-swallow). Oral Langerhans cells are known to express constitutively both the high- (Fc ϵ RI) and low- (CD23) affinity Fc receptors for IgE [44]. The preferred approach for SLIT relies on allergens presented in conformations similar to those that trigger the allergic state. In this way targeting and capture of allergens through IgE-dependant epitopes and the type I Fc receptor for IgE (Fc ϵ RI) on

dendritic cells in the oral mucosa of atopic patients would be maximal [44]. Thus, in the present study the folding of rChe a 3 and rTAT-Che a 3 was determined by physicochemical and immunological assays.

The far-UV CD spectra results demonstrate that the secondary structure was conserved despite the introduction of additional amino acids at the rChe a 3 N-terminus. Analysis in the near-UV spectral region found no detectable conformational differences between rChe a 3 and rTAT-Che a 3 (Fig. 5). These results suggest that no disruption of the configuration around the aromatic amino acids (tyrosine and phenylalanine) occurred and the tertiary structures of rChe a 3 and rTAT-Che a 3 were similar. In addition, the fluorescent spectra of the recombinant proteins were also similar, with emission maxima at 330 nm (Fig. 5), indicating conservation of the overall tertiary structure, in agreement with the near-UV CD spectra results. Furthermore, immunologic characterization revealed that both recombinant allergens were recognized by IgE from *C. album* allergic patients' sera. As a result, we conclude that the fusion tag had no influence on the IgE-binding capacity of the recombinant allergen.

In the mouse model of allergic rhinitis used in this study, a systemic immune response against rChe a 3 developed, which was characterized by a marked elevation of serum rChe a 3-specific IgE and IgG1 levels and Th2 cytokine production (IL-4) from spleen cell cultures. IgG2a expression was significantly greater following sublingual treatment with rTAT-Che a 3 than with rChe a 3. It is believed that the IgG2a may effectively act as blocking antibodies to antagonize allergic inflammation resulting from allergen binding to the IgE bound to mast cells [33]. We consider this IgG-subclass shift as a modulation of the predominant allergic Th2-response, even though no changes in the allergen-specific IgE and IgG1 levels were seen in any of the mouse groups, likely due to

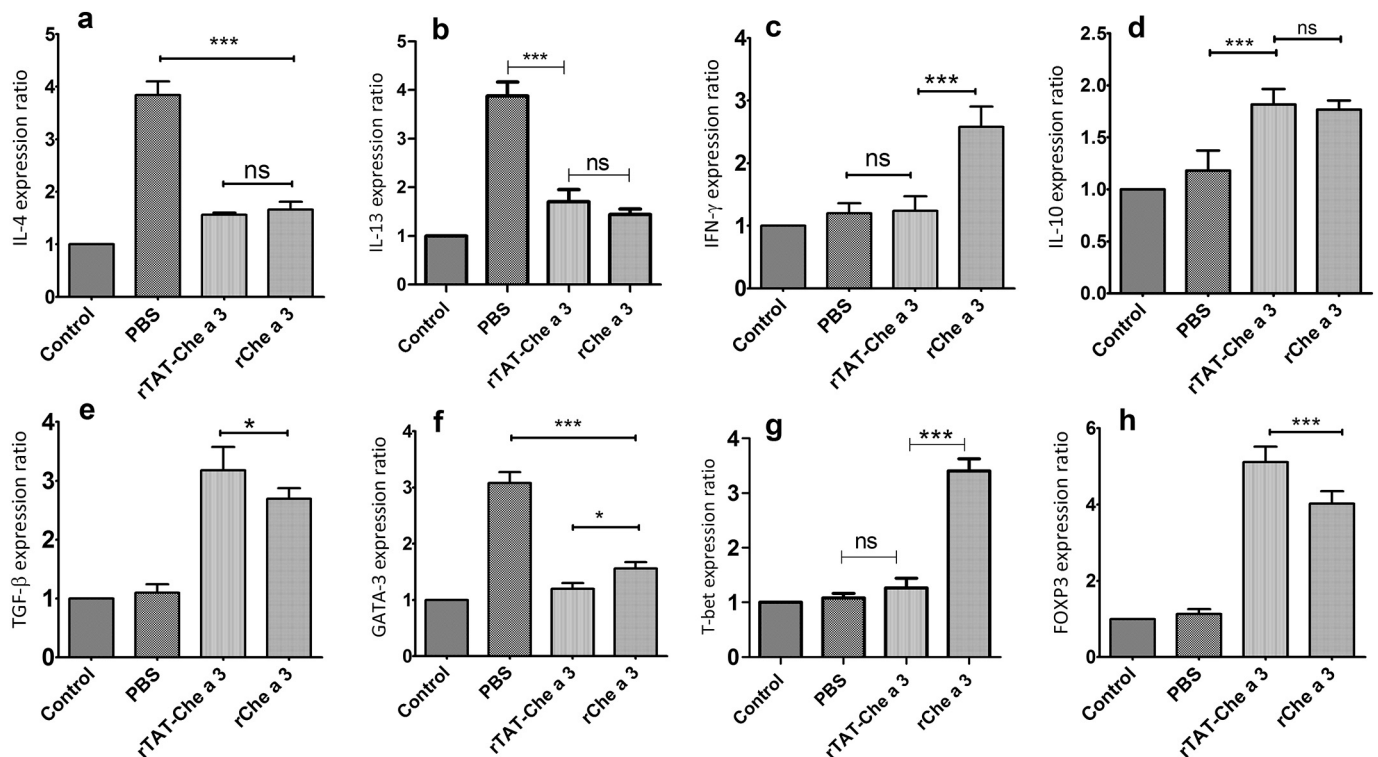


Fig. 9. Relative mRNA expression of selected Th1-, Th2- and Treg-associated cytokines in splenocytes of sublingually treated mice. mRNA expression of IL-4, IL-13, IFN-γ, IL-10, TGF-β, GATA-3, T-bet, and Foxp3 was determined by quantitative real-time PCR. Expression was normalized to GAPDH. Each data point was expressed as a ratio relative to the mean expression level in control mice. * $p < 0.05$, *** $p < 0.001$, and ns: not significant.

the presence of long-lived plasma cells [34]. Indeed, consistent with our data, most studies observed that allergen-specific IgE is either increased or not affected by SLIT [35].

In our experimental model, decreased expression of the TH2-related cytokines IL-4 and IL-13 in spleen cell cultures was observed. It is believed that a shift from Th2 to Th1 has an important role in SLIT [36–38]. However, SLIT with rChe a 3, but not rTAT-Che a 3, significantly enhanced IFN-γ production in splenocytes of treated mice, likely due to distinct immunomodulatory properties of these two antigens. Actually, some studies showed SLIT did not always lead to increased IFN-γ, a Th1-related cytokine, even though the TH2 immune responses had been strongly down-regulated [39,40]. In addition, SLIT with rTAT-Che a 3 induced significantly greater mRNA expression of TGF-β and Foxp3 transcription factor than rChe a 3 alone, in splenocytes. Tregs play a central role in down-regulation of allergic responses through cell-to-cell contact-dependent inhibition and by the secretion of suppressive cytokines such as IL-10 and TGF-β [41]. Indeed, IL-10 and TGF-β are strongly associated with inhibition of the production of Th2 cytokines such as IL-4 and IL-5 [42].

Interestingly, in our study sublingual administration of both rChe a 3 and rTAT-Che a 3 led to decreased allergen-specific splenocyte proliferation. In other studies, which also enhanced tolerance via the sublingual route, increased or unchanged T cell proliferation in spleen cells was observed [36,43]. Herein, we speculate that the decreased proliferation in our study might be due to suppressive activity of induced Foxp3+ and IL-10+ Treg cells through secretion of suppressive cytokines such as IL-10 and TGF-β or by cell-cell contact in SLIT.

In conclusion, the present study indicates that TAT-fusion may help rational development of SLIT. Indeed, this study suggests that rTAT-fused allergen, compared to free allergen, might suppress the allergic state through preferential enhanced systemic Treg-mediated immunomodulation, likely by facilitating allergen capture and presentation by sublingual Langerhans-like dendritic cells. However, it will need to be addressed in well-designed clinical studies in future.

Conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript.

Acknowledgment

This manuscript was funded by grant No. 89845 from the Research Administration Department of Mashhad University of Medical Sciences, Iran.

References

- [1] R. Pawankar, Allergic diseases and asthma: a global public health concern and a call to action, *World Allergy Organ J.* 7 (1) (2014) 12.
- [2] J. Bousquet, H.J. Schunemann, B. Samolinski, P. Demoly, C.E. Baena-Cagnani, C. Bachert, et al., Allergic rhinitis and its impact on asthma (ARIA): achievements in 10 years and future needs, *J. Allergy Clin. Immunol.* 130 (5) (2012) 1049–1062.
- [3] A. Bufer, P. Eberle, E. Franke-Beckmann, J. Funck, M. Kimmig, L. Klimek, et al., Safety and efficacy in children of an SQ-standardized grass allergen tablet for sublingual immunotherapy, *J. Allergy Clin. Immunol.* 123 (1) (2009) 167–173 (e7).
- [4] G.W. Canonica, J. Bousquet, T. Casale, R.F. Lockey, C.E. Baena-Cagnani, R. Pawankar, et al., Sub-lingual immunotherapy: world allergy organization position paper 2009, *Allergy* 64 (Suppl. 91) (2009) 1–59.
- [5] R. Dahl, A. Kapp, G. Colombo, J.G. de Monchy, S. Rak, W. Emminger, et al., Sublingual grass allergen tablet immunotherapy provides sustained clinical benefit with progressive immunologic changes over 2 years, *J. Allergy Clin. Immunol.* 121 (2) (2008) 512–518 (e2).
- [6] U. Wahn, A. Tabar, P. Kuna, S. Halken, A. Montagut, O. de Beaumont, et al., Efficacy and safety of 5-grass-pollen sublingual immunotherapy tablets in pediatric allergic rhinoconjunctivitis, *J. Allergy Clin. Immunol.* 123 (1) (2009) 160–166 (e3).
- [7] A. Didier, Future developments in sublingual immunotherapy, *Allergy* 61 (Suppl. 81) (2006) 29–31.
- [8] P. Moingeon, Sublingual immunotherapy: from biological extracts to recombinant allergens, *Allergy* 61 (Suppl. 81) (2006) 15–19.
- [9] J.P. Allam, T. Bieber, N. Novak, Dendritic cells as potential targets for mucosal immunotherapy, *Curr. Opin. Allergy Clin. Immunol.* 9 (6) (2009) 554–557.
- [10] P. Moingeon, T. Batard, R. Fadel, F. Frati, J. Sieber, L. Van Overtvelt, Immune mechanisms of allergen-specific sublingual immunotherapy, *Allergy* 61 (2) (2006) 151–165.
- [11] A. Razafindratsita, N. Saint-Lu, L. Mascarell, N. Berjont, T. Bardon, D. Betbeder,

- et al., Improvement of sublingual immunotherapy efficacy with a mucoadhesive allergen formulation, *J. Allergy Clin. Immunol.* 120 (2) (2007) 278–285.
- [12] N. Saint-Lu, S. Tourdot, A. Razafindratsita, L. Mascarell, N. Berjont, H. Chabre, et al., Targeting the allergen to oral dendritic cells with mucoadhesive chitosan particles enhances tolerance induction, *Allergy* 64 (7) (2009) 1003–1013.
- [13] M.A. Lindsay, Peptide-mediated cell delivery: application in protein target validation, *Curr. Opin. Pharmacol.* 2 (5) (2002) 587–594.
- [14] J.S. Wadia, S.F. Dowdy, Modulation of cellular function by TAT mediated transduction of full length proteins, *Curr. Protein Pept. Sci.* 4 (2) (2003) 97–104.
- [15] E.L. Snyder, S.F. Dowdy, Cell penetrating peptides in drug delivery, *Pharm. Res.* 21 (3) (2004) 389–393.
- [16] G. Cao, W. Pei, H. Ge, Q. Liang, Y. Luo, F.R. Sharp, et al., In vivo delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis, *J. Neurosci.* 22 (13) (2002) 5423–5431.
- [17] S.R. Schwarze, A. Ho, A. Vocero-Akbani, S.F. Dowdy, In vivo protein transduction: delivery of a biologically active protein into the mouse, *Science* 285 (5433) (1999) 1569–1572.
- [18] A. Ziegler, X.L. Blatter, A. Seelig, J. Seelig, Protein transduction domains of HIV-1 and SIV TAT interact with charged lipid vesicles. Binding mechanism and thermodynamic analysis, *Biochemistry* 42 (30) (2003) 9185–9194.
- [19] E. Goncalves, E. Kitas, J. Seelig, Binding of oligoarginine to membrane lipids and heparan sulfate: structural and thermodynamic characterization of a cell-penetrating peptide, *Biochemistry* 44 (7) (2005) 2692–2702.
- [20] H.R. Nouri, M. Sankian, F. Vahedi, D. Afsharzadeh, L. Rouzbeh, M. Moghadam, et al., Diagnosis of *Chenopodium album* allergy with a cocktail of recombinant allergens as a tool for component-resolved diagnosis, *Mol. Biol. Rep.* 39 (3) (2012) 3169–3178.
- [21] F. Salari, F. Vahedi, J. Chamani, A. Varasteh, H. Ketabdar, M. Sankian, Efficient expression of a soluble lipid transfer protein (LTP) of *Platanus orientalis* using short peptide tags, and structural comparison with the natural form, *Biotechnol. Appl. Biochem.* (2014).
- [22] Y.H. Chen, J.T. Yang, H.M. Martinez, Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion, *Biochemistry* 11 (22) (1972) 4120–4131.
- [23] N. Sreerama, R.W. Woody, Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set, *Anal. Biochem.* 287 (2) (2000) 252–260.
- [24] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic Acids Res.* 29 (9) (2001) e45.
- [25] P. Moingeon, V. Lombardi, N. Saint-Lu, S. Tourdot, V. Bodo, L. Mascarell, Adjuvants and vector systems for allergy vaccines, *Immunol. Allergy Clin. North Am.* 31 (2) (2011) 407–419 (xii).
- [26] S.M. Farkhani, A. Valizadeh, H. Karami, S. Mohammadi, N. Sohrabi, F. Badrzadeh, Cell Penetrating Peptides: Efficient Vectors for Delivery of Nanoparticles, Nanocarriers, Therapeutic and Diagnostic Molecules, Peptides, (2014).
- [27] I.M. Kaplan, J.S. Wadia, S.F. Dowdy, Cationic TAT peptide transduction domain enters cells by macropinocytosis, *J. Control. Release* 102 (1) (2005) 247–253.
- [28] J.S. Wadia, R.V. Stan, S.F. Dowdy, Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis, *Nat. Med.* 10 (3) (2004) 310–315.
- [29] J.P. Richard, K. Melikov, H. Brooks, P. Prevot, B. Lebleu, L.V. Chernomordik, Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors, *J. Biol. Chem.* 280 (15) (2005) 15300–15306.
- [30] A. Ferrari, V. Pellegrini, C. Arcangeli, A. Fittipaldi, M. Giacca, F. Beltram, Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time, *Mol. Ther.* 8 (2) (2003) 284–294.
- [31] A. Fittipaldi, A. Ferrari, M. Zoppe, C. Arcangeli, V. Pellegrini, F. Beltram, et al., Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins, *J. Biol. Chem.* 278 (36) (2003) 34141–34149.
- [32] L.R. Wright, J.B. Rothbard, P.A. Wender, Guanidinium rich peptide transporters and drug delivery, *Curr. Protein Pept. Sci.* 4 (2) (2003) 105–124.
- [33] C.A. Akdis, I.B. Barlan, N. Bahceciler, M. Akdis, Immunological mechanisms of sublingual immunotherapy, *Allergy* 61 (Suppl. 81) (2006) 11–14.
- [34] E.O. Luger, V. Fokuhl, M. Wegmann, M. Abram, K. Tillack, G. Achatz, et al., Induction of long-lived allergen-specific plasma cells by mucosal allergen challenge, *J. Allergy Clin. Immunol.* 124 (4) (2009) 819–826 (e4).
- [35] D.H. Han, C.S. Rhee, Sublingual immunotherapy in allergic rhinitis, *Asia Pac. Allergy* 1 (3) (2011) 123–129.
- [36] J. Brimmes, J. Kildsgaard, H. Jacobi, K. Lund, Sublingual immunotherapy reduces allergic symptoms in a mouse model of rhinitis, *Clin. Exp. Allergy* 37 (4) (2007) 488–497.
- [37] M. Majori, A. Caminati, M. Corradi, E. Brianti, S. Scarpa, A. Pesci, T-cell cytokine pattern at three time points during specific immunotherapy for mite-sensitive asthma, *Clin. Exp. Allergy* 30 (3) (2000) 341–347.
- [38] P. Kidd, Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease, *Altern. Med. Rev.* 8 (3) (2003) 223–246.
- [39] T. Yamada, M. Tongu, K. Goda, N. Aoi, I. Morikura, T. Fuchiwaki, et al., Sublingual immunotherapy induces regulatory function of IL-10-expressing CD4(+)CD25(+) Foxp3(+) T cells of cervical lymph nodes in murine allergic rhinitis model, *J. Allergy (Cairo)* 2012 (2012) 490905.
- [40] L. Mondoulet, V. Dioszeghy, M. Ligouis, V. Dhelft, E. Puteaux, C. Dupont, et al., Epicutaneous immunotherapy compared with sublingual immunotherapy in mice sensitized to pollen (*Phleum pratense*), *ISRN Allergy* 2012 (2012) 375735.
- [41] J. Verhagen, A. Taylor, K. Blaser, M. Akdis, C.A. Akdis, T regulatory cells in allergen-specific immunotherapy, *Int. Rev. Immunol.* 24 (5–6) (2005) 533–548.
- [42] D.R. Wilson, M.T. Lima, S.R. Durham, Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis, *Allergy* 60 (1) (2005) 4–12.
- [43] B.J. Kildsgaard, H. Jacobi, K. Lund, Sublingual immunotherapy in sensitized mice, *Ann. Allergy Asthma Immunol.* 4 (98) (2007) 7.
- [44] P. Moingeon, Update on immune mechanisms associated with sublingual immunotherapy: practical implications for the clinician, *J. Allergy Clin. Immunol. Pract.* 1 (3) (2013) 228–241.