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Design and evaluation of a hypoallergenic peptide-based vaccine for *Salsola kali* allergy

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ABSTRACT

Background: The *Salsola kali* (*S. kali*) pollen is one of the most important causes of allergic rhinitis in the deserts and semi-desert areas. Immunotherapy with allergen extracts remains the only available treatment addressing the underlying mechanism of allergy. However, given the low efficacy of this method, it is necessary to find more effective and alternative therapeutic interventions using molecular biology and bioinformatics tools. In this study, a hypoallergenic vaccine was designed on the basis of B-cell epitope approach for *S. kali* immunotherapy.
Methods: Using the Immune Epitope Database (IEDB), a 35-mer peptide was selected and chemically conjugated to a keyhole limpet hemocyanin (KLH) molecule. Specific IgG and IgE from immunized BALB/c mice sera against the vaccine (Sal k 1-KLH), *S. kali* extract and the recombinant protein, rSal k 1, were measured using ELISA. Also, inhibition of IgE by mouse IgG was evaluated using an inhibitory ELISA. Finally, the IgE reactivity and T-cell reactivity of the designed vaccine were evaluated by dot blot assay and MTT assay.
Results: Vaccination with the vaccine produced high levels of protective IgG in mice, which inhibited the binding of patients IgE to recombinant proteins. The result showed that the designed vaccine, unlike the recombinant protein and extract, did not induce T-cell lymphocytes response and also exhibited decreased IgE reactivity.
Conclusion: The designed vaccine can be considered as a promising candidate for therapeutic allergen-specific immunotherapy.

1. Introduction

Sensitization to *Salsola kali* (Russian thistle) pollen – a well-known species of Amaranthaceae family- has been increasing in parallel with IgE-mediated diseases in dry and temperate regions around the world particularly in the western part of USA, Southern Europe, North Africa and Middle-Eastern countries [1–4]. So far, six proteins have been identified as *Salsola kali* (*S. kali*) allergens [5], among which, Sal k 1 is the major allergen with multiple isoforms and is responsible for up to 80% sensitization in *S. kali* allergic patients [6]. Since the first allergen-specific immunotherapy (SIT) experiment by L. Noon [7], SIT, unlike symptomatic drugs is the only treatment that can interfere with the underlying immunological mechanism of the disease thus inducing tolerance even after the discontinuation of immunotherapy course [8,9].

However, SIT with natural allergen extracts has been reported to cause severe immediate and late side effects due to poor quality of the extracts [10,11]. This has led to the notion that rather than using whole allergens from biological sources, SIT can be improved using modern methods of vaccinology. The last decade has witnessed an increase in the adoption of recombinant DNA technology in modifying important allergens so as to come up with hypoallergenic immunotherapy vaccine derivatives that are safe and efficacious [12]. Ever since the debut studies on Phl p 1, the major timothy grass pollen allergen, and Bet v 1, the major birch pollen allergen, fusion proteins [13,14], B cell epitope-based strategy has received considerable attention on account of lower allergenicity and lack of late phase side effects compared to other hypoallergenic derivatives and T-cell epitope-based vaccines [15]. Consequently, based on this method, significant progress has been made toward the production of SIT vaccines using the most important

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allergens like Phl p 1,2,5,6, Fel d 1, Ole o 1, Der p 1,2, Alt a 1 [16–21].

"Top-down" techniques have been applied to identify and select genome sequences of potential vaccines using in silico tools during the initial steps of SIT vaccine production. These computational methods help to narrow down the potential candidate peptides then a series of in vitro and in vivo assays are performed in order to assess the quality and immune response toward the vaccine [22].

Interestingly despite the high prevalence of *S. kali* sensitization, there has been no study investigating the designing and production of a recombinant hypoallergenic vaccine for *S. kali* allergy treatment to date except a mutant rSal k 1 that was synthesized by direct mutagenesis of three amino acids [23]. Here, we designed and characterized a novel hypoallergenic peptide-based vaccine for *S. kali* SIT using B-cell epitope approach with the aim of reducing IgE-mediated responses together with non-IgE-mediated allergic inflammations.

2. Methods and material

2.1. Bioinformatics analysis

In order to design the hypoallergenic vaccine, first, it was necessary to assess Sal k 1 amino acid sequence. Thus, each one of the Sal k 1 allergen isoforms was obtained from the National Center for Biotechnology Information (NCBI) and UniProt databases and saved in FASTA format. These sequences were then compared with the native Sal k 1 and their homology percentage compared using CLUSTAL omega 1.2.4 software.

Next, linear B-cell epitopes of Sal k 1 were then determined by submitting the Sal k 1 amino acid sequence into the Immune Epitope Database-Analysis resource (IEBD-AR) linear B-epitope prediction server with the default threshold settings [24]. For the avoidance of regions containing T-epitopes from vaccine sequence, the most common HLA class II alleles in Iran (DRB1 ∗ 11:04، 15:01، 4:01، DQA1 ∗ 05:01 and DQB1 ∗ 03:01) [25] were submitted to IEBD T- epitope prediction and predicted T-epitope regions shown to be having higher percentile rank of > 5 were not excluded from the vaccine's sequence. Accordingly, the final vaccine sequence was selected from B-epitopes and non-T-epitope regions. The final sequence comprising 35 amino acids in length was then conjugated to a Keyhole limpet hemocyanin (KLH) molecule that would act as a carrier molecule (Sellecklen Company, USA). An extra cysteine residue was attached to the N-terminal of the peptide so that KLH molecule would able to form a disulfide bridge and create the final vaccine.

2.2. Recombinant Sal k 1 (rSal k 1) and Salsola kali extract

rSal k 1 protein was obtained from prof. Varasteh AR (Mashhad University of Medical Sciences) as a gift [23].

Salsola kali extract (Russian thistle) was purchased from GREER Laboratories (NC, USA) and checked for protein content and quality under reducing condition by SDS-PAGE. All dilutions were performed with phosphate buffered saline (PBS), and the extract was stored at 4 °C to use for further experiments.

2.3. Participants

Blood samples (5 ml) were drawn from thirty patients (16 female and 14 male) with a mean age of 34.5 years (ranging from 21 to 54), and eight non-allergic individuals (as controls) after participants gave their consent. Ethical approval was sought and granted by the Tehran University of Medical Sciences (TUMS) ethics committee. Inclusion criteria were positive case history indicative of allergic rhinitis (AR), positive skin prick test to *S. kali* extract and not being undergoing any form of allergen immunotherapy. Non-allergic controls were selected among students and staffs based on their case history and specific IgE measurements.

Peripheral blood mononuclear cells (PBMCs) from eight of these patients and controls were separated by Ficoll-paque PLUS (GE healthcare, Little Chalfont, United Kingdom) gradient centrifugation as previously described [26]. The PBMCs were transferred in freezing solution and cryo-preserved while the sera were kept at −20 °C until use.

2.4. Immunization of mice

Fifteen 6-week-old female BALB/c mice were purchased from Razi Institute (Tehran, Iran) and housed at the animal care unit of the TUMS under pathogen-free, controlled temperature and light-dark cycle, according to the local guidelines for animal care. Animals were allowed to adapt for one week then divided into three groups of five randomly and received 100 μg/injection of Sal k 1-KLH vaccine, 100 μg/injection of rSal k 1 and the same amount of PBS all adsorbed to aluminum hydroxide (alum) adjuvant (Charles River, Kisslegg, Germany) in vaccine, recombinant and control groups respectively. The mice were immunized four times subcutaneously in the neck at three-week intervals and sample collected by bleeding from eye sinus a day prior to subsequent immunization. Sera were separated and stored at −20 °C for specific antibody detection. Then animals were sacrificed at the end of week 10.

2.5. Specific IgG and IgE measurement in immunized mice

Specific antibody responses during ten weeks of immunization were determined using indirect ELISA. Briefly, ELISA plates (Nunc, Roskilde, Denmark) were coated with 20 μg/ml of *S. kali* pollen extract diluted in bicarbonate (0.1 M, pH 9.6) at 4 °C overnight. To reduce nonspecific bindings, 300 μl of 3% skimmed milk (Merck, Darmstadt, Germany) diluted in PBS was added into each well at room temperature for 1 h. After blocking plates were incubated with diluent sera of immunized mice (1:100, 1:200, 1:500 for IgG and 1:50, 1:100 for IgE measurement) at 37 °C. Subsequently, bound specific IgG or IgE were detected with goat anti-mouse antibody (diluted 1:2000 in PBS-T) followed by rabbit anti-goat IgG-HRP (diluted 1:5000 in PBS-T) incubated at 37 °C. The unbound antibodies were removed from the wells by washing the plates three times with PBS-T (PBS containing Tween 20 0.05%) after each step, 3,3′5,5′-Tetramethylbenzidine (TMB) was added as a chromogenic substrate, and the reaction was stopped by adding 50 μl of H_2SO_4. Optical densities (ODs) were detected in an ELISA microplate reader at 450 nm and 652 nm as reference wavelength.

2.6. Inhibition assay

To investigate the ability of immunized mice IgG antibodies to inhibit the binding of allergic patients' IgE to rSal k 1, inhibitory ELISA was employed [19]. In short, plates were coated with 2 μg/ml of rSal k 1 (diluted in PBS) and incubated at 4 °C overnight. After blocking (skim milk 3%) wells were incubated with mice sera (diluted 1:100 in PBS) at 37 °C. Next, sera from patients and non-allergic participants (as control) were diluted 1:100 and incubated for 2 h. Bound human IgE Abs were detected with HRP-coupled goat anti-human IgE and ODs were measured at 450 nm. Lastly, the percentage reduction of IgE binding was calculated according to the formula: $100 - (ODs / ODp) \times 100$. Where ODs and ODp represent the absorbance after pre-incubation with the mice sera and pre-immune sera, respectively.

2.7. Dot blot assay

Patients' IgE reactivity toward the peptide vaccine, rSal k 1 and *S. kali* extract was evaluated using dot blot assay and the procedure was as follows: in order to activate two strips of polyvinylidene difluoride (PVDF) paper (Roche, Basel, Switzerland), membranes were soaked in methanol for 5 min followed by 3 min into transfer buffer. Sal k 1

peptide, rSal k 1 and *S. kali* extract were blotted in dots of 10 μl in triplicate on the two semi-dried PVDF membranes. Next, membranes were air dried completely and then blocked with skim milk 3% (diluted in Tris-buffered saline- tween20 (TBS-T) for 1 h. This was followed by washing three times with TBS-T. The membranes were then incubated for 2 h with allergic patients' pooled sera and that of non-allergic individuals. After washing, HRP-coupled goat anti-human IgE Abs (diluted 1:5000 with TBS) were added to the membrane and incubated for 1 h. The bound IgE Abs were detected using 3, 3′-diaminobenzidine tetrahydrochloride (DAB, Roche, Switzerland) reagent for 1 min. All incubations were done at room temperature while shaking slowly on the shaker.

2.8. PBMC proliferation assay

PBMCs obtained from blood samples were thawed and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium enriched with 10% fetal bovine serum (Gibco, California, USA) and 1 × streptomycin at a density of 50,000 cell/well in 96-well plate (Nunc, Roskilde, Denmark). After 24 h cells were stimulated in triplicate with 2 μg/ml of Sal k 1 peptide, rSal k 1, *S. kali* extract or medium alone (as the negative control) and incubated at 37 °C in 5% CO_2 for 48 h. 10 μg/ml phytohaemagglutinin (PHA) was added as a positive control and the plates were further incubated for another 24 h at 37 °C in 5%. Prior to adding 10 μl of MTT solution (5 μg/ml) to each well and incubating for 4 h, 100 μl of supernatant were collected for IFN-γ analysis. To solubilize the formazan compound, 100 μl of $DMSO_4$ (Sigma-Alderich, USA) was added to each well and absorbance was measured at 570 nm in ELISA microplate reader [27].

2.9. Measurement of IFN-γ in PBMC supernatant

The amount of IFN-γ cytokine in PBMC cell culture was evaluated using an ELISA kit (R&D Systems, MN, USA) according to the manufacturer instruction. The OD values measured at 450 nm were then converted to absolute values using a standard curve prepared with serial dilutions of recombinant IFN- γ standards.

2.10. Statistical analysis

Differences in the levels of antibodies induced by immunization of mice were determined using the Mann–Whitney *U* test using on GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif). To compare the groups, one-way ANOVA with multiple comparisons test was used for comparing the differences between controls and vaccination groups. Data were expressed as mean ± standard error of mean and p-values < 0.05 were considered significant.

3. Results

3.1. Sal k 1 B-cell epitopes vaccine

Sal k 1 (Genebank: accession no. ACO34813.1) reported by Assarehzadegan et al. [23] was selected as a native source for designing the vaccine. After the identifying linear B and T epitopes using IEBD-AR server as described earlier, the best scoring peptide containing B-epitopes and lacking any T epitopes was chosen. The Sal k 1 peptide comprised 35 amino acids a sufficient enough size to elicit antibody responses while minimizing the chance of recognition by IgE antibodies as a result of conformational disruption at position 275–310 on Sal k 1 amino acid sequence (Fig. 1).

Since the rationale behind our vaccine designing strategy was to achieve a hapten-carrier effect described by B. Benacerraf [28,29], the vaccine was chemically conjugated to a KLH molecule, which has been used carrier for several antigens in mice and human [11]. This should be part of the methodology. You are describing what you did and not giving results.

Further analysis using the Karplus and Schulz, Chou and Fasman, Parker and Emini algorithms (Fig. 2) demonstrated that the selected region contained B-cell epitopes.

3.2. Sal k 1-KLH vaccine induced higher level of specific IgG antibodies compared to rSal k 1

Immunization experimental results showed that a significant ($p < 0.05$) amounts of IgE Abs were detected after the first injection during the first 3-weeks (Fig. 4). Although specific IgG increased three weeks after the first immunization in both groups, the amount only increased significantly (p) after nine weeks. Interestingly, there was a comparable difference within the group in 9th and 10th week (Fig. 3).

3.3. Specific IgG Abs obtained from immunized mice sera inhibited the binding of allergic patients' IgE

ELISA inhibition experiments were used to examine the ability of IgGs in competing with the patients' IgE for binding to the plate-bound rSal k 1. Results demonstrated that sera from immunized mice (Sal k 1-KLH and rSal k 1 groups) were able to inhibit the most sensitized patients' IgE reactivity to rSal k 1 in a dose-dependent manner. In a within-group comparison, at given serum dilutions, anti-Sal k 1-KLH Abs inhibited more effectively than anti-rSal k 1 Abs. After using the formula to calculate the inhibition percent, it was shown that rSal k 1 inhibited 53% while Sal k 1-KLH inhibited > 65% of IgEs from binding to the allergen and difference was significant ($p < 0.05$) in both concentrations (Table 1).

3.4. Sal k 1-KLH peptide revealed low IgE reactivity

We used pooled sera of 6 patients who had the highest level of specific IgE to evaluate the IgE reactivity of Sal k 1 vaccine by means of non-denaturing dot blot assay. Pooled sera showed weak IgE reactivity toward the Sal k 1-KLH the vaccine. Apparently, IgE binding to the whole *S. kali* extract was highest while no IgE reactivity was detected when non-allergic sera (negative control) were incubated with the dotted membrane (Fig. 5).

A more detailed analysis of the IgE-binding of the Sal k 1-KLH peptide was evaluated by comparing the binding of patients' sera to plate-bound Sal k 1-KLH peptide, rSal k 1 and *S. kali* extract. Results shown in Fig. 5 confirmed with those shown in Table 2.

3.5. Sal k 1-KLH peptide elicited no patients T-cell response

Finally, PBMCs proliferation was used to indicate the ability of the Sal k 1 vaccine, rSal k 1 and the extract to induce late-phase reactions. As for controls, treatment of PBMCs with PHA showed positive proliferation ($p < 0.001$), while no proliferation was recorded from the negative control from RPMI media alone. The specificity of the proliferation was validated by stimulation of non-*S. kali* allergic patients PBMCs with same three allergens, which no proliferation was detected at 570 nm by ELISA reader. Unlike rSal k 1 and *S. kali* extract which were able to stimulate the proliferation of the PBMCs while there was no significant proliferation PBMCs with Sal k 1 vaccine (Fig. 6).

3.6. No comparable differences were observed in IFN-γ profile of S. kali, rSal k 1 and Sal k 1 peptide supernatant

IFN-γ as a proinflammatory cytokine of TH1 lymphocytes was measured from the supernatant of patients' PBMC culture that had been stimulated with *S. kali*, rSal k 1 and Sal k 1 peptide in triplicate using an ELISA kit. We observed no statically significant differences between three stimulators as shown in Fig. 7. The highest level of IFN-γ was induced when rSal k 1 added to the cell culture.

QPIPPNPAELESWFQGAVKPVSEQKGLEPSVVQAESGGVETIEVRQDGSGKFK
TISDAVKHVKVGNTKRVIITIGPGEYREKVKIERLHPYITLYGIDPKNRPTITFAGTA
AEFGTVDSATLIVESDYFVGANLIVSNSAPRPAGKRKGAQASALRISGDRAAFYN
CKFTGFQDTVCDDKGNHLFKDCYIEGTVDLIFGEARSLYLNTELHVVPGDPMA
MITAHARKNADGVGGYSFVHCKVTGTGGTALLGRAWFEAARVVFSYCNLSDA
VKPEGWSDNNKPAAQKTIFFGEYKNTGPGAAADKRVPYTKQLTEADAKTFTSL
EYIEAAKWLPPPKV

Fig. 1. Selection of final peptide sequence from *Sal k 1* allergen. T-epitopes are highlighted in yellow and B-epitope are underlined. The final selected sequence is shown as a boxed sequenced. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

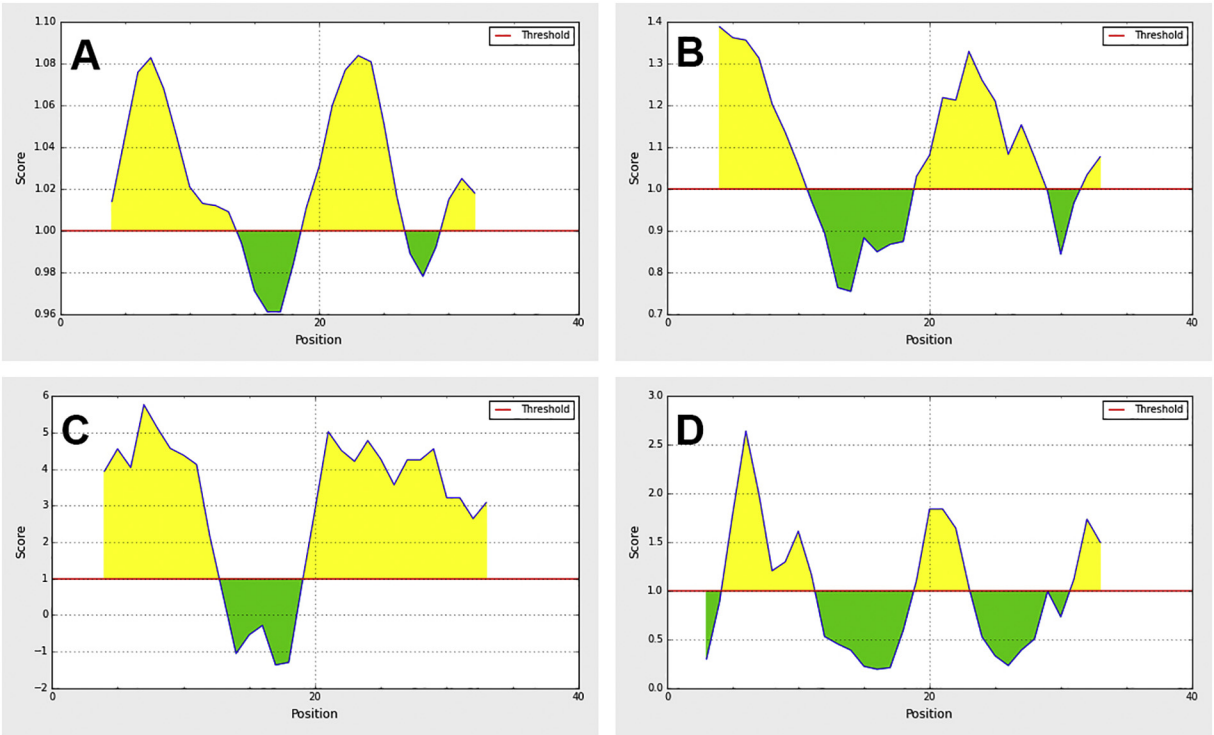

Fig. 2. Analysis of the selected hypoallergenic region. Diagrams shows analysis of Sal k 1 peptide using (A) Kaplus & Schulz flexibility prediction, (B) Chou and Fasman β turn prediction, (C) Parker hydrophilicity prediction scale, (D) Emini surface accessibility prediction. The x-axis reveals amino acid residues' position while the y-axis shows the corresponding score for each amino acid residue.

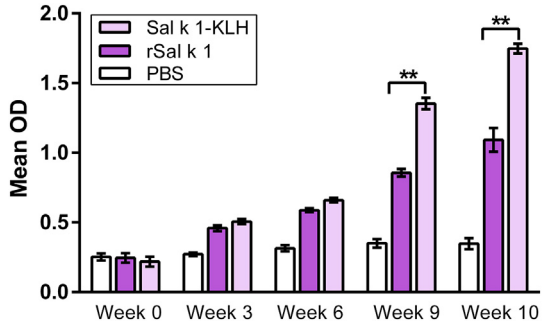

Fig. 3. Comparison of specific IgG titers in two groups of immunized mice over a 10 week period. Groups of 5 mice were immunized with PBS, rSal k 1 and Sal k 1-KLH in three-week intervals. Sera samples were obtained before each immunization and tested for specific IgG using indirect ELISA (sera dilution 1:500). Y-axis shows the mean level of calculated optical density (mean OD) at 450 nm. There was a significant difference between rSal k 1 and Sal k 1-KLH groups in two last weeks. (** $p < 0.01$).

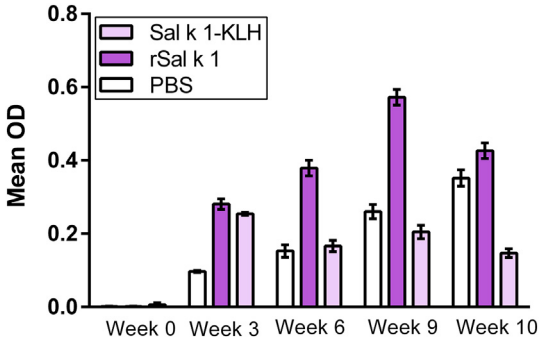

Fig. 4. Comparison of specific IgE Abs in mice injected with rSal k 1 and Sal k 1-KLH over the 10-week period. Sera were obtained from immunized mice before each injection and analyzed with ELISA for specific IgE titers in 10-week course (x-axis). The mean levels of the allergen-specific IgE levels in each group (n = 5) are displayed on the y-axis as optical densities (OD) measured at 450 nm.

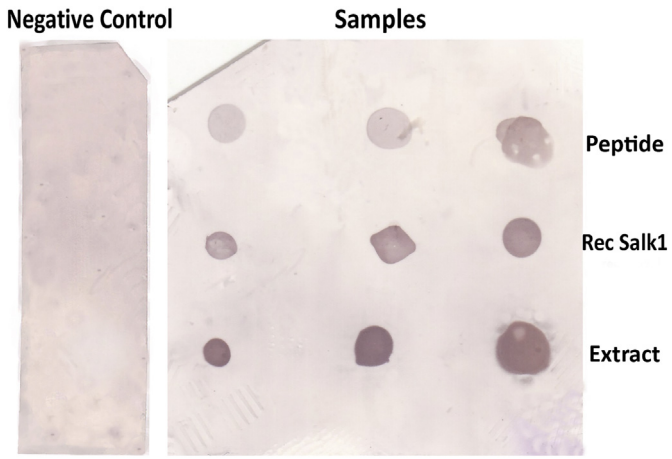

Fig. 5. Display of IgE reactivity toward *S. kali* extract, rSal k 1 and Sal k 1 peptide. Membranes were dotted with *S. kali* extract, rSal k 1 and *Sal k 1* peptide and exposed to patients' and non-allergic pooled sera (n = 6) for IgE reactivity. Bound IgE Abs were detected by HRP enzymatic reaction with 3,3-diaminobenzidine substrate (DAB).

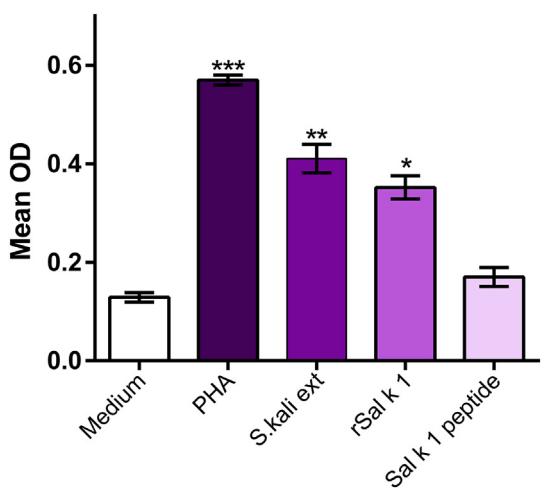

Fig. 6. Proliferation of patients' PBMCs stimulated with *S. kali* extract, rSal k 1 and Sal k 1 peptide. Peripheral blood mononuclear cells (PBMCs) from *S. kali* patients (n = 5) were cultured with 10 μg of *S. kali* extract, rSal k 1 and Sal k 1 peptide in complete RPMI media. Phytohemagglutinin (10 μg/ml) was used as positive control for stimulation, while cells in media alone served as negative control. The proliferation was measured by MTT assay and mean OD at 570 nm displays in y-axis. Significant differences are shown with star (*). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

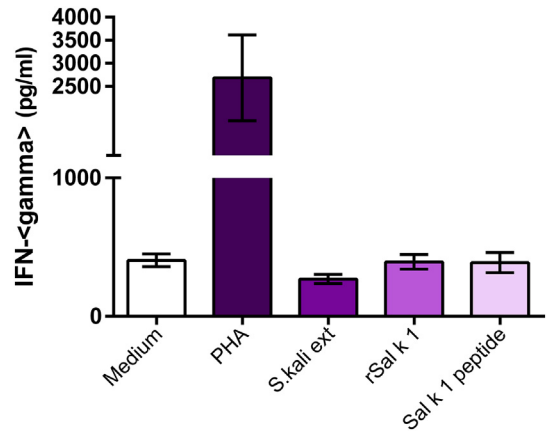

Fig. 7. Comparison of IFN-γ cytokine in supernatant of PBMCs stimulated with *S. kali* extract, rSal k 1 and Sal k 1 peptide. Diagram shows the concentration amount of IFN-γ in *S. kali* extract, rSal k 1 and Sal k 1 peptide groups, where PHA was added as a positive control and cells with no stimulation (only medium) used as a negative control.

Table 1
Inhibition of allergic patients' IgE binding to rSal k 1 with specific IgG Abs from immunized mice. Displayed are percentage of inhibition for 21 patients with mice sera of rSal k 1 or Sal k 1-KLH groups in two dilutions (1:25 or 1:50).

Patients' no.	Sal k 1 inhibition (%)		Sal k 1-KLH inhibition (%)	
	1:25	1:50	1:25	1:50
1	59.95	33.73	77.90	33.70
2	67.15	46.56	80.23	53.12
3	69.66	38.83	44.66	28.29
4	29.57	12.82	53.72	25.52
5	52.67	21.28	61.85	25.96
6	61.80	33.90	75.45	50.19
7	56.42	31.30	63.33	46.83
8	55.22	34.57	74.94	53.02
9	33.71	16.06	34.80	19.13
10	21.08	7.48	51.96	24.62
11	43.46	27.66	45.12	21.77
12	46.51	29.48	48.32	32.61
13	33.30	11.16	81.48	46.08
14	71.13	38.63	54.45	22.64
15	42.69	27.71	55.12	33.94
16	50.00	32.72	79.18	51.64
17	66.59	38.92	67.09	36.28
18	71.08	54.35	74.56	43.86
19	66.11	40.70	81.58	46.89
20	68.36	47.87	80.20	39.44
21	57.80	33.28	79.97	57.79
Mean ± SEM	53.49 ± 3.24	31.38 ± 2.67	65.04 ± 3.24	37.27 ± 2.53

4. Discussion

In this study, we developed a hypoallergenic vaccine for the treatment of *S. kali* pollen allergy, which is a predominant cause of allergy in most parts of Iran [30–32] and has been increasing worldwide due to the climate changes and greening programs [33,34]. To date, few studies investigating *S. kali* immunotherapy have been performed. Garde and co-workers showed that subcutaneous administration of standard *S. kali* extract was safe and well-tolerated in both conventional and cluster schedule and later on, Colas and colleagues concluded that immunotherapy with chemically modified extract is safe and can improve the symptoms of allergic rhinitis and asthma. However, both clinical trials were accompanied by mild local and systemic reactions, and there were no follow-up studies to determine the long-lasting effect of AIT in *S. kali* sensitized patients [35,36]. In order to design a safer vaccine, we used major B-epitopes which we identified using bioinformatics tools from *S. kali* allergen [37,38]. Using in silico tools, a 35-mer peptide was derived from Sal k 1 B-epitope rich regions lacking T-cell epitopes. In a study conducted by Assarehzadegan et al. [23], it was noted that the selected peptide was one of the most dominant B-cell epitopes using ABCpred software and homology modelling. To render immunogenicity, the selected peptide was conjugated to a KLH molecule. As demonstrated by similar studies, T-cell help provided by the carrier protein aided the induction of allergen-specific IgG antibodies upon immunization [16].

Immunological characterization of the Sal k 1-KLH vaccine revealed that it had low IgE-reactivity compared to rSal k 1 and *S. kali* extract probably due to the disruption of the three-dimensional structure of the allergen as described in [39], the IgE antibody response to respiratory allergens is mainly associated with conformational epitopes. Due to lack of conformation, it can be speculated that during SIT patients' specific IgE Abs would not bind to this vaccine thus basophil and mast cell degranulation would be prevented. Besides, the Sal k 1-KLH had

Table 2
IgE reactivity of *S. kali* extract, rSal k 1 and Sal k 1-KLH. OD values corresponding to IgE levels specific for *S. kali* extract, rSal k 1 and Sal k 1-KLH are shown for 20 patients (P1-P20) and 3 non-allergic subjects (NA1-NA3).

Subject's no.	*S. kali* extract	rSal k 1	Sal k 1-KLH
P1	0.747	0.286	0.146
P2	0.743	0.412	0.097
P3	0.961	0.268	0.200
P4	0.577	0.472	0.140
P5	0.528	0.366	0.023
P6	0.640	0.356	0.131
P7	0.732	0.377	0.221
P8	1.190	0.430	0.213
P9	0.844	0.532	0.367
P10	0.638	0.327	0.053
P11	0.578	0.283	0.142
P12	0.552	0.324	0.089
P13	0.843	0.147	0.065
P14	0.767	0.276	0.102
P15	0.934	0.234	0.115
P16	0.768	0.257	0.145
P17	0.827	0.184	0.028
P18	0.714	0.349	0.108
P19	0.776	0.317	0.194
P20	0.648	0.187	0.131
Mean ± SEM	0.750 ± 0.035	0.319 ± 0.021	0.135 ± 0.017
NA1	0.043	0.028	0.017
NA2	0.054	0.035	0.027
NA3	0.022	0.041	0.011
Mean ± SEM	0.039 ± 0.01	0.034 ± 0.003	0.018 ± 0.004

considerably reduced ability to stimulate the proliferation of patients' T-cell when compared to its recombinant and extract; as a result late-phase reactions are mostly avoidable. Data from in-vitro experiments of the BM32 fusion protein in cultured with patients' PBMCs, confirmed this result as well [16].

Put it together, the safety profile of this vaccine can lead to not only high dose injection but also a significant reduction in the number of injections needed to induce tolerance. This assumption is confirmed by the BM32 vaccine "a biomay product consist of four allergens of timothy grass pollen", which was tolerated even at high doses in patients who participated in phase IIb study conducted by Valenta et al. and encouraging results were observed after 3 injections in grass pollen allergic patients [40,41]. Immunization of BALB/c mice with Sal k 1-KLH vaccine resulted in the production of large amounts of specific IgG Abs. This was observed in similar studies with respiratory allergens, which showed that peptides with a length of approximately 30 amino acids coupled to a carrier molecule could induce a robust IgG Ab response against the intact wild-type allergen [11,13,42]. Interestingly the vaccine could inhibit IgE-binding more effectively than rSal k 1, suggesting that Sal k 1-KLH induced specific IgG against the major IgE-binding sites of allergen covering the epitopes more efficiently. Considering similar result obtained in a study involving B-cell epitope derived from Phl p 1 allergen conjugated to the VP1 carrier [42], this can be explained by a higher titer of anti-Sal k 1-KLH IgG compared with the anti-rSal k 1 IgG. In fact, these blocking Abs are the key element of B-epitope vaccines SIT mechanisms, which include I) avoiding the cross-linking of IgE antibodies on mast cell and basophils, by capturing free allergens thus inhibiting IgE-mediated side effects, II) preventing the stimulation of IgE memory cells and consequently secondary IgE responses, III) competing with IgE Abs residing on antigen presenting cells for antigen presentation to T-cells, therefore decreasing late-phase reactions [12]. In week 10th, the titer of specific IgE Abs decreased significantly in immunized mice; same results were obtained in our previous study with Der p 1 hypoallergen vaccine [19] and other experiments [16], suggesting that therapy-induced allergen-specific IgG could downregulate allergen-specific IgE production. Therefore, one can consider the Sal k 1-KLH as a prophylactic vaccine in future clinical studies. In addition, these specific IgG Abs could induce the tolerance by the activation of the inhibitory pathway via binding to FcγRIIB [43].

Recently it has been shown that tetanus toxoid, as well as PreS protein, can be used as a carrier molecule for allergy vaccines [19]. A similar strategy might also be used for Sal k 1 peptide described in this study to obtain antiviral immunity as well as SIT.

In conclusion, we characterized a novel hypoallergenic vaccine for treating *S. kali* allergy that is based on the B-cell epitope-carrier strategy, which revealed encouraging results in reducing both immediate and delayed-type side effects in comparison with rSal k 1 and *S. kali* extract that are currently used for AIT. Moreover, these peptide-based vaccines can be produced in a large amount as recombinant proteins in the bacterial expression systems in a standardized manner to avoid the drawbacks associated with allergen extracts [44].

Conflict of interest

The authors have no conflict of interest to declare.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. Also, for investigations involving human subjects, informed consent was sought and granted from the participants.

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