ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
June 2018; 17(3):281-290.

Sublingual Immunotherapy with Sal k1 Expressing *Lactococcus lactis* Down-regulates Th2 Immune Responses in Balb/c Mice

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Received: 30 July 2017; Received in revised form: 14 November 2017; Accepted: 18 November 2017

ABSTRACT

Sublingual immunotherapy (SLIT) has been introduced as a noninvasive and safer approach for allergen-specific immunotherapies. In this study we investigated the efficacy of oral immunotherapy with recombinant Salsola kali 1 protein (Sal k 1) on Th1/Th2 balance in a mouse model of allergy.

Female Balb/c mice were intraperitoneally sensitized with rSal k1, followed by a respiratory challenge with 1% (w/v) rSal k1. The sensitized mice were subjected to SLIT using rSal K1 expressing *Lactobacillus lactis* strain for three weeks. Each week the experimental group underwent SLIT protocol twice. Finally, serum levels of specific immunoglobulins including IgE, IgG1 and IgG2a, as well as secretion of different cytokines from splenocytes including IL-2, IL-4, IL-10, IFNγ and TGFβ into culture media were measured by ELISA. Following immunotherapy, the levels of specific IgE and IgG1 in mice sera as well as IL-4 level in supernatant of splenocytes were significantly lower than allergic controls.

While serum IgG2a, IgG2a/IgG1 ratio as well as concentration of IL-2, IL-10, IFNγ, and TGFβ were higher in the SLIT group compared to the controls. The histopathological examination of intestinal tissues revealed no sign of inflammatory response following SLIT.

This study revealed that Th2 immune responses are reduced in allergic mice after feeding them with allergen expressing probiotic bacteria as a SLIT approach. Since the safety of this procedure was previously approved, thus, it seems that a similar protocol using human based probiotics could be applied for *Salsola kali* sensitive patients.

Keywords: Lactococcus lactis; Salsola kali; Sublingual immunotherapy

INTRODUCTION

Type-I hypersensitivity is an immediate allergic reaction to proteins or glycoproteins.[1,2] Most of allergens share common characteristics such as: low-to-high molecular weight (5-70 KDa), solubility, stability and foreignness to immune system.[2] Allergens may get access to the immune system through inhalation

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(aeroallergen), ingestion (food allergen), injection, and/or skin contact. Aeroallergens, including pollens, are the most common causes of respiratory allergic diseases in susceptible individuals.[3] Management of allergic diseases is a growing challenge, worldwide.

Allergen-specific immunotherapy (ASI) has increasingly been drawing attentions for desensitization in allergies to pollens, dust mites and even food components.[4,5] It is believed to be a logical way to induce tolerance to potential allergens.[6] However, the dose and route of allergen administration are two key factors which determine the efficacy of an ASI approach. While preference is still given to the subcutaneous route of allergen administration (i.e. SCIT), recently sublingual immunotherapy (SLIT) or non-injection immunotherapy methods have been introduced as noninvasive and safer approaches with no evidence of anaphylactic shock or other systemic complications.[7-9] Moreover, since a higher amount of allergen is administrated in the SLIT compared to the SCIT approach (50 to 100 folds more), the former method seems to be more efficacious than the latter one.[10]

During SLIT, a high supply of allergen is kept under the tongue for 1-2 minutes before being swallowed. This allows the allergen to be captured by Langerhans-like antigen-presenting dendritic cells (DCs) residing within the oral mucosa.[11] However, since the allergens used for SLIT are applied based on aqueous extract preparations obtained from natural allergen sources, administration of higher amounts of allergenic extracts necessitates access to more natural allergens of interest.[12] To overcome this barrier, molecularly-defined recombinant allergens can be modified in desired ways in order to reach maximum efficacy.[13] For this purpose, genetically-engineered probiotics including lactic acid bacteria (LABs), particularly *Lactococcus lactis*, possesses several advantages to be used as a mucosal delivery vector.[14,15] For example, *L. lactis* can *per se* induce Th1 responses.[16]

Salsola kali, also known as Russian thistle, is the most common species of Salsola genus belonging to Chaenopodiacea subfamily and is widely distributed in desert and semi-desert regions such as: Asia, North Africa, Europe, and USA.[17] The *S. kali* pollen contains various potentially allergenic molecules among which Sal k1, a 40-kDa pectin methylesterase, has been identified as the main allergenic part of the pollen.[18,19]

Recently, we expressed recombinant Sal k1 (rSal k1) using engineered *L. lactis* (data is under publication). Given that there was no evidence on the efficacy of the rSal k1 in vivo, in the present study, we used animal models sensitized to the rSal k1 to investigate the possible effects of this protein on Th1/Th2 balance.

MATERIALS AND METHODS

Reagents, Antigens and Strains

The wild type *L. lactis* strain NZ9000 was obtained from MoBiTec Company GmbH, (Germany). Previously, the recombinant-Sal k1-producing L. lactis was generated in our laboratory (unpublished data, accepted in IJAAI). Furthermore, we have previously expressed rSal k1 using PET32-Sal k1 recombinant plasmid in *E. coli* BL21.[3] The rSal k1 concentration was measured by BCA assay using a commercially available kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Alum (aluminum hydroxide) adjuvant (pH 6.5) was prepared using aluminum potassium sulfate (Merck, Germany). The IgE, IgG1, IgG2a, IL-2, IL-4, IL-10, IFNγ, and TGFβ specific ELISA kits were purchased from eBioscience (USA).

Animals

Female Balb/c mice (6-8 weeks old, 19-22 g) were purchased from Royan Research Institute (Tehran, Iran) and maintained in a standard light and temperature condition with free access to food and water. The experiment and animal handling procedures were in accordance with guidelines for care and use of laboratory animals. The ethical committee of Mashhad University of Medical Sciences (Mashhad, Iran) approved the study protocol (N.700274). All animals were subjected to euthanasia at the end of the experiment. All efforts were made to minimize the suffering of animals if observed.

Sensitization to rSal k1 Protein

For sensitization, 100 µl of the allergen suspension (5 µg rSal k1 proteins and 5 µg Alum in PBS) was intraperitoneally injected to animals on days 1, 7, 14 and 21. Then, on day 28, the animals were challenged with inhalation of 1% (w/v) rSal k1 protein on seven consecutive days (20 min. per day) using a nebulizer (Omron, Japan). The control group (n=6) was neither

sensitized nor treated. The PBS group (n=6) was intraperitoneally given rSal k1 (based on the protocol designed for all groups except for control group), and inhaled with rSal k1-free PBS. Bloodshot (red) eyes, sneezing, and severe itching were considered as symptoms of sensitization. Prior to SLIT, after sensitization, IgE level of blood samples, collected from the retro-orbital sinus, was assessed by a specific ELISA kit.

Sublingual Immunotherapy

The successfully rSal k1-sensitized animals were randomly assigned to six experiment groups (n=6/group) as follows: PBS, wild-type probiotic strain (the probiotic without protein expression), recombinant strain (the probiotic that expresses rSal k1), rSal k1 alone, wild-type strain+ rSal k1, and recombinant strain+ rSal k1.

The wild-type or recombinant bacteria (10^8-10^9 CFU), and rSal k1 protein (0.1 mg) were dissolved in PBS at a total volume of 25 µL and were then administrated under the tongue of each animal in the respective group, every two days for a period of three weeks. The PBS group was given only 25 µl of PBS. The control group received no treatment.

Measurement of Serum IgE, IgG1 and IgG2a

Twenty-four hours after the last administration, all animals were sacrificed by cervical dislocation following anesthesia by ketamine (80 mg/kg) and xylene (8 mg/kg), blood sample were collected, and serum was prepared by centrifugation at 2000 rpm, 4°C for 15 min. Serum concentrations of IgE, IgG1 and IgG2a antibodies were measured using specific ELISA kits, according to the manufacturers' instructions.

Measurement of Spleen Cytokine Production

The spleen was aseptically removed and gently suspended in a 10 mL of RPMI 1640 medium. Afterwards, the suspension-containing splenocytes, red blood cells and spleen debris- were rinsed through 70 µm cell strainers into a 50 ml tube, and then were centrifuged at 1650 rpm [4°C, 6 min.]. Next, the supernatant containing spleen debris was discarded. In order to remove the red blood cells, the pellet was re-suspended in 10 mL of ammonium chloride followed by centrifuging at 1650 rpm [4°C, 10 min.]. After the removal of the supernatant containing lysed red blood cells, the remaining mononuclear cells were re-suspended in complete RPMI 1640 medium (FBS (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL)). Viability of the cells was checked by Trypan blue (4%) exclusion.

Then, the cells were seeded into a 96-well plate at 25×10^4 cells/well with 100 µL of complete RPMI 1640 medium and incubated at 37°C for 48 hours in a 5% CO_2 and 95% humidified atmosphere. To elicit cytokine production, cells were further incubated either alone, with rSal k1 protein (10 µg/mL) or phytohemagglutinin mitogen (PHA, 20 ng/mL) at 37°C for 72 hours. Finally, concentrations of IL-2, IL-4, IL-10, IFNγ, and TGF-β cytokines in the culture supernatant were measured with specific ELISA kits, according to the manufacturers' instructions.

Histopathological Examination

A small specimen was taken from the intestinal duodenum tissue of each animal in formalin and was subjected to hematoxylin and eosin (H&E) staining. Each sample was pathologically examined for any probable inflammatory response.

Statistical Analysis

Data analysis was performed using the 5th version of GraphPad Prism Software for Windows (La Jolla CA, USA), and the results were expressed as Mean±Standard Deviation SD. Differences between the groups were evaluated by the one-way analysis of variance (ANOVA) test followed by Tukey's test, where applicable. Changes in serum IgE level before and after the intervention within each group were examined by paired t-test. A p value<0.05 was considered a statistically significant level of difference.

RESULTS

Serum IgE Level Changes after Sensitization and SLIT

As demonstrated in Figure 1A, sensitization of mice with intraperitoneal injection and, followed by, inhalation of rSal k1 protein caused significant elevation in the allergen-specific serum IgE level of sensitized animals compared to that of the control group (p<0.001) which was not sensitized. However, significant reductions were observed in the serum IgE level of animals receiving SLIT with rSal k1 (p<0.01), wild-type *L. lactis* strain (p<0.05), recombinant *L. lactis* strain (p<0.001), wild-type strain+ rSal k1

(p<0.001), and recombinant strain+rSal k1 (p<0.001) compared to the PBS group which received rSal k1-free PBS during the SLIT protocol (Figure 1B). Decrease in serum IgE level of PBS group was less remarkable than the other studied groups (Figure 1C).

IgG1/IgG2a Antibody Ratio Changes after SLIT

After SLIT, serum IgG1 level was significantly higher in the PBS group compared to a control group without any injections (p<0.001). However, significant reductions were seen in the serum IgG1 level in animals undergoing SLIT with rSal k1 (p<0.01), wild-type *L. lactis* strain (p<0.001), recombinant *L. lactis* strain (p<0.001), wild-type strain+ rSal k1 (p<0.001), and recombinant strain+ rSal k1 (p<0.001) compared to the PBS group (Figure 2A). On the other hand, the serum level of the protective IgG2a antibody increased in these groups compared to either the control or PBS groups (Figure 2B). Furthermore, the IgG1/IgG2a antibody ratio was significantly higher in the PBS group compared to the control group (p<0.001), while this index was significantly lower in the animals

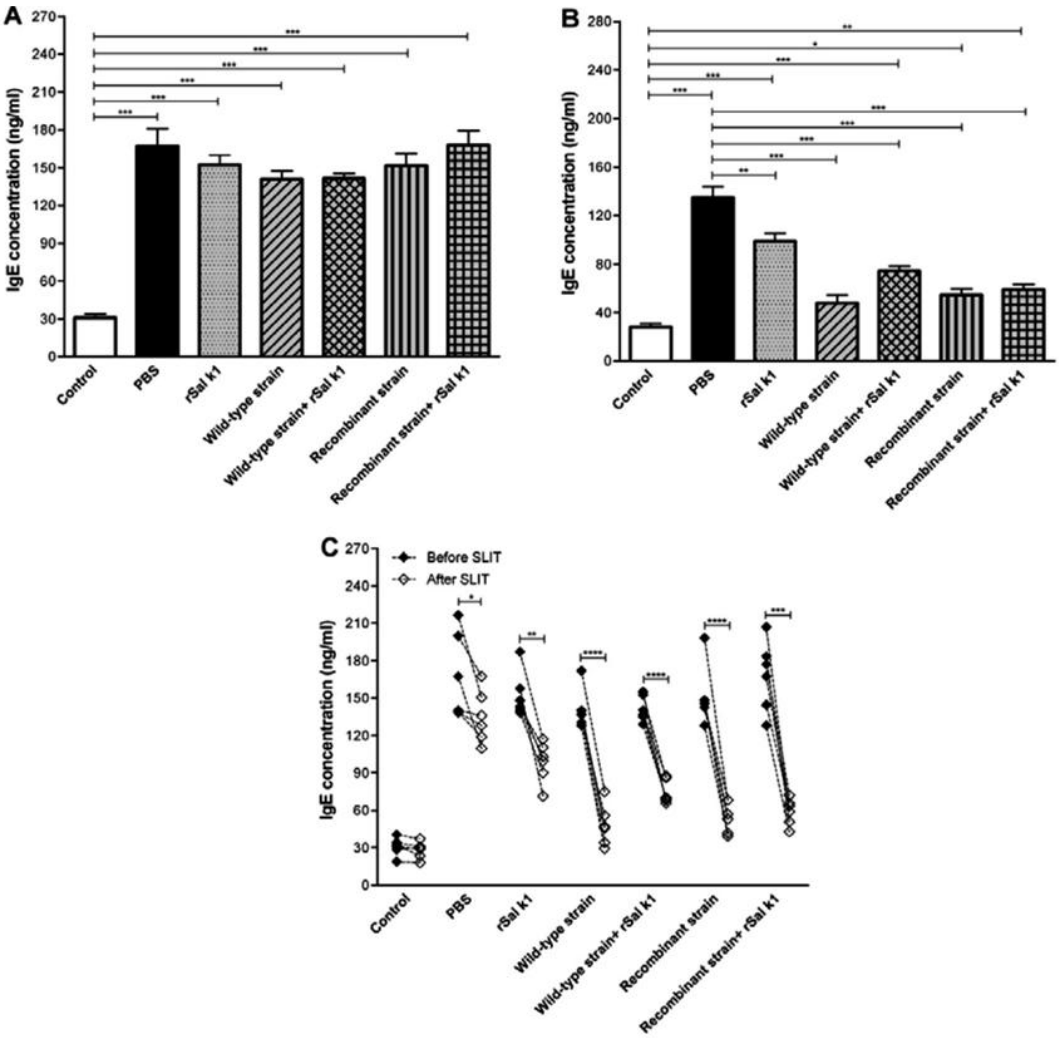

Figure 1. Changes in serum level of IgE in rSal k1-sensitized mice; assessed by a specific ELISA kit: (A) Prior to sublingual immunotherapy, blood was collected from the retro-orbital sinus of all animals one day following rSal k1 challenge. The control group was neither sensitized nor treated. The PBS group was intraperitoneally given rSal k1, and was subjected to the SLIT protocol with only PBS. (B) Blood was collected 24 hours after the last sublingual immunotherapy with rSal k1, wild-type or recombinant *L. lactis*. The PBS group was given only PBS as SLIT. (C) Serum IgE decreased significantly in all groups receiving SLIT: n= 6 mice per group. Results are expressed as Mean ± standard error (SEM). $^{*}p$<0.05, $^{**}p$<0.01, $^{***}p$<0.001, and $^{****}p$<0.001.

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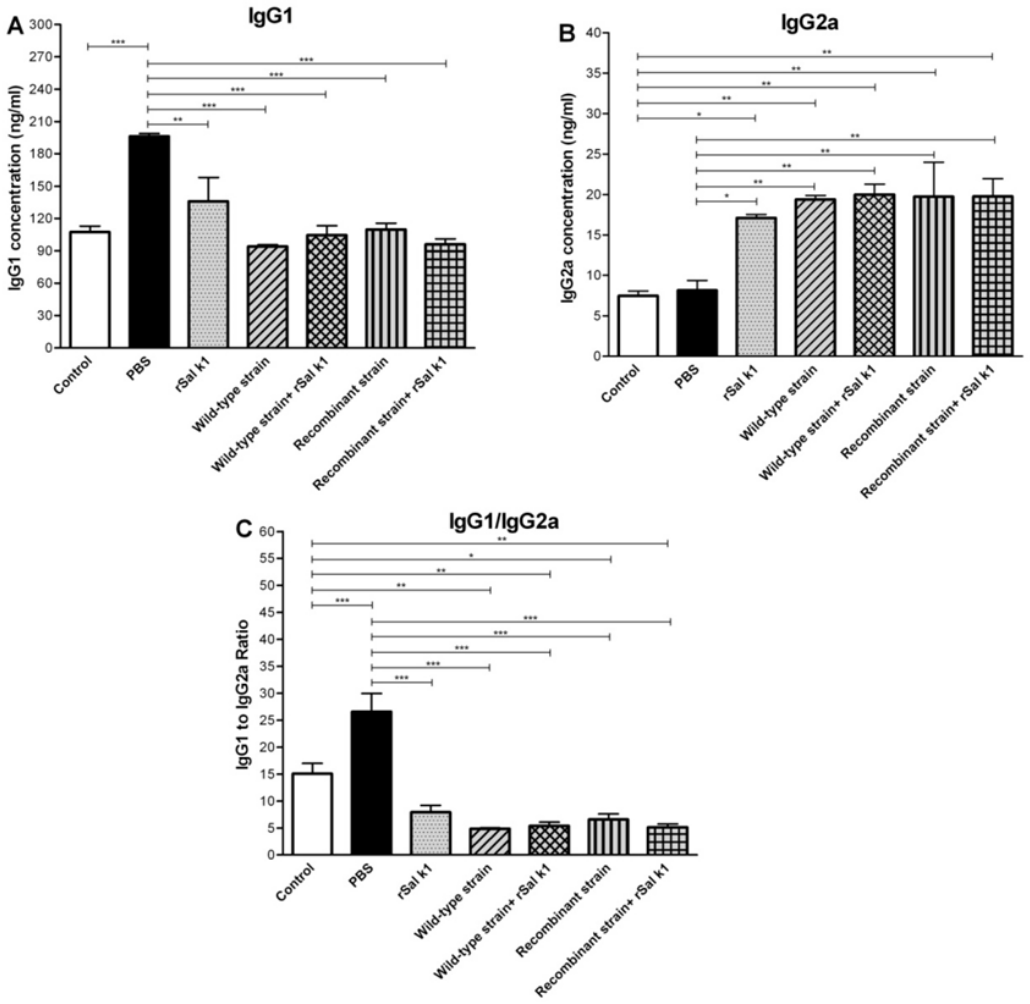

Figure 2. Changes in serum level of IgG1 and IgG2a; assessed by a specific ELISA kit, in rSal k1-sensitized mice 24 hours after the last sublingual immunotherapy with rSal k1, wild-type or recombinant *L. lactis*. The control group was neither sensitized nor treated. The PBS group was intraperitoneally given rSal k1, and was subjected to the SLIT protocol with only PBS. n=6 mice per group. Results are expressed as Mean±standard error (SEM). $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

receiving SLIT with either rSal k1, or wild-type or recombinant *L. lactis* strains compared to that of either the control or PBS groups (Figure 2C).

Changes in ex vivo Cytokine Production by Splenocytes after SLIT

The rSal k1-elicited production of IL-2, IL-4, IL-10, IFNγ, and TGFβ cytokine production in the splenocytes culture supernatant was evaluated by ELISA test. As illustrated in Figure 3A, after SLIT, IL-2 production augmented in wild-type *L. lactis* strain+ rSal k1 ($p<0.05$), recombinant *L. lactis* strain and recombinant strain+ rSal k1 ($p<0.001$) groups compared to the control group. The IFNγ (Figure 3B) and TGFβ levels (Figure 3C) increased significantly, while IL-4 decreased (Figure 3D) in all groups receiving various SLIT protocols compared to either the control or PBS groups (for all $p<0.001$). In addition, the IL-10 production in all groups receiving the SLIT, except in the PBS and wild-type groups, was significantly greater compared to the control group (Figure 3E).

Intestinal Inflammatory Response after SLIT

As depicted in Figure 4, the histopathological examination of H&E-stained intestinal tissues from mice revealed no sign of inflammatory or pathologic responses due to the SLIT administration.

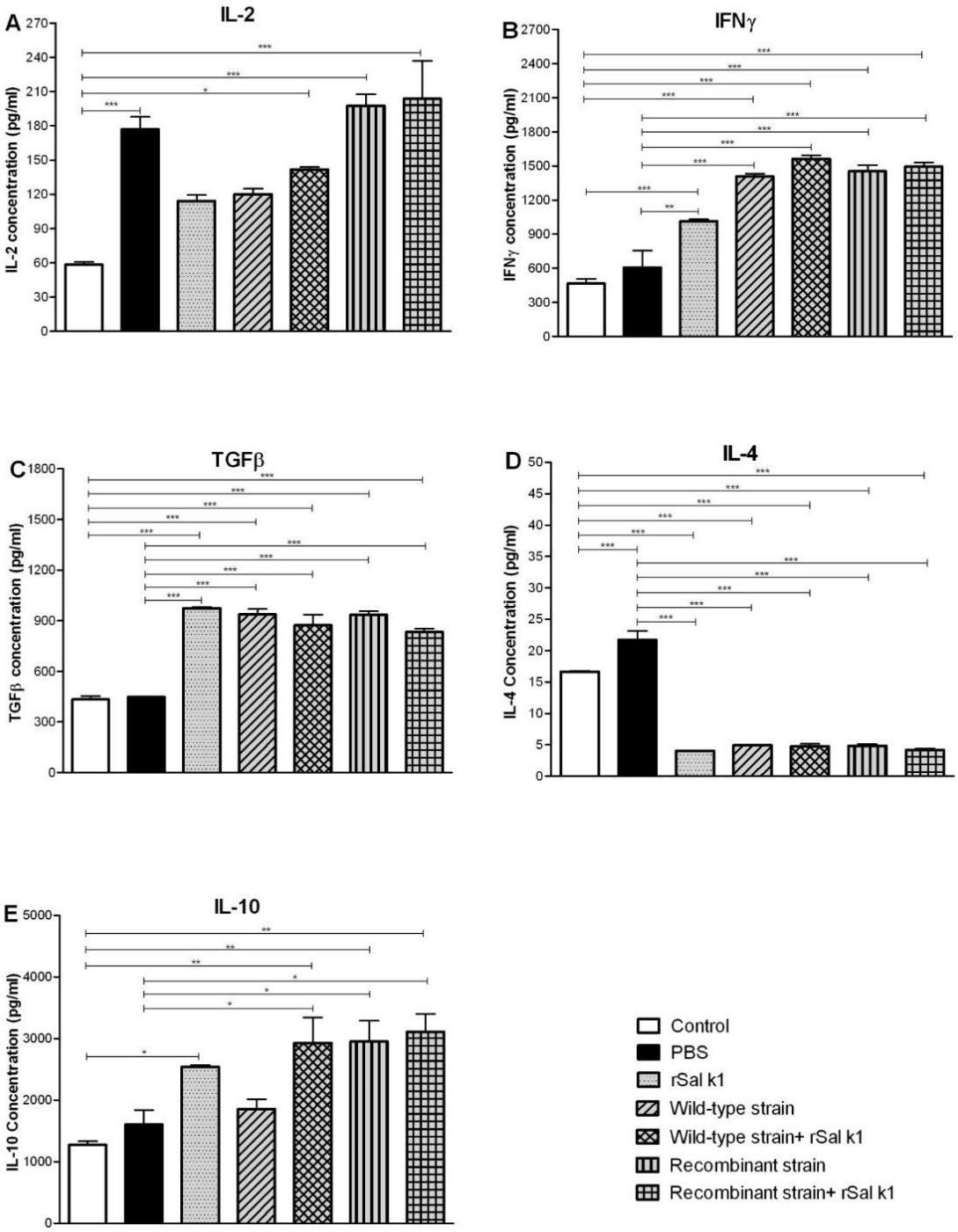

Figure 3. Changes in cytokine production by splenocytes from mice, 24 hours after the last sublingual immunotherapy with rSal k1, wild-type or recombinant *L. lactis*. Splenocytes were re-stimulated with rSal k1 protein (10 µg/mL) ex vivo at 37°C for 72 hours. Concentrations of IL-2, IL-4, IL-10, IFNγ, and TGFβ cytokines in the culture supernatant were measured using specific ELISA kits, n= 6 mice per group. Results are expressed as Mean ± standard error (SEM). $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

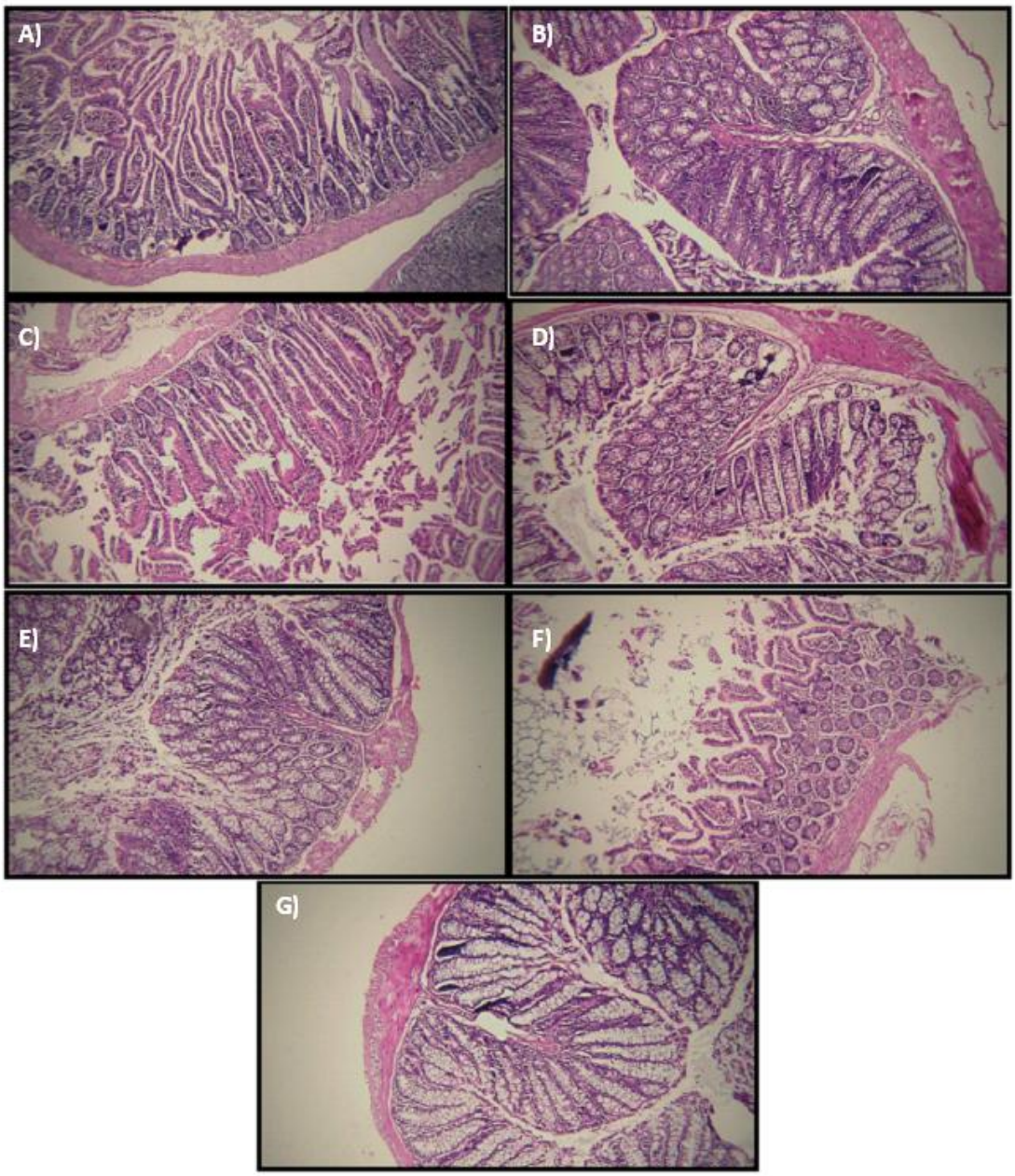

Figure 4. Histopathological examination of intestinal tissue of mice 24 hours after the last sublingual immunotherapy with rSal k1, wild-type or recombinant *L. lactis*. Tissues were stained with Hematoxylin and Eosin and examined for any probable inflammatory response. A) Control; B) PBS; C) rSal k1; D) wild-type strain; E) wild-type strain+ rSal k1; F) recombinant strain; G) recombinant strain+ rSal k1. The intestinal incision of a mouse was shown randomly from each group.

DISCUSSION

In the present study, we investigated the effects of SLIT using previously engineered rSal k1 pollen allergen into *L. lactis* strain on the Th1/Th2 immune responses in animal models sensitized to the allergen. Our results demonstrated that the recombinant rSal k1 protein, when sublingually administered either alone or in combination with wild-type or recombinant *L. lactis* strain, seemingly shifted the Th1/Th2 balance towards the Th1 responses. To the best of our knowledge, this is the first study to report the induction of allergen-

specific Th1 responses by means of SLIT with recombinant rSal k1 pollen allergen in vivo.

We injected animals with and exposed them to rSal k1 protein, which resulted in systemic elevation of serum allergen-specific IgE and IgG1 antibodies, in association with sensitization symptoms. We observed that after application of SLIT using rSal k1, wild-type *L. lactis*, recombinant *L. lactis*, or a combination of the protein and bacteria, the allergen-specific IgE and IgG1 levels restored to their normal points. In addition, the protective IgG2a was elicited by the SLIT so that a lower IgG1 to IgG2a ratio was achieved in all SLIT mice. IgE class switching induced by IL-4 and IL-13 cytokines, and thereby, increased production of IgE is definitely emblematic of an inappropriate Th2 immune response during type-1 allergies.[20,21] In murine, elevation of allergen-specific IgG1, associated with IgE, has been implicated with unfavorable allergic immune responses.[16] Moreover, induction of allergen-specific IgG2a is considered as an indicator for a successful and effective immunotherapy approach, as it is likely involved in blocking IgE or IgG1 functions as well as in modulating allergic inflammatory responses.[22,23] Furthermore, a lower IgG1 to IgG2a ratio has been suggested as an indicator for Th1/Th2 biased responses in immunotherapy approaches.[24] Therefore, in our study, the reduced IgE and IgG1 production together with pronounced IgG2a production may represent an improvement of immune responses from Th2 towards Th1 in the SLIT models. However, unlike the present study, SLIT has shown limited impact on IgE production in previous investigations.[12,25] In addition, an increased IgE, following SLIT, has been reported in other cases.

ASI has been shown to inhibit the expansion of IL-4, IL-5, and IL-13; produce Th2 phenotypes; decrease IgE production and increase IgE blocking antibodies.[26,27] In this view, allergen-specific CD4+CD25+ regulatory T-cells (T_{reg}) seem to play a critical role. Indeed, these cells are potent suppressors of both Th1 and Th2 responses through various mechanisms.[28] Nevertheless, inhibitory impacts of T_{reg} on Th2 responses are even greater than on Th1 responses.[29] IL-10 and TGFβ, produced by T_{reg} and dendritic cells, can increase the production of protective IgG2a antibodies, while decreasing the production of allergen-specific IgE antibodies as well as IL-4 and IL-5 cytokines associated with Th2 phenotypes.[25] In addition, IL-2 is highly required for the maintenance of T_{reg} function.[30] In the present study, secretion of IFNγ, IL-2, IL-10, and TGFβ from spleen cell cultures increased, while IL-4 cytokine significantly decreased in the SLIT models compared to the control group. These observations suggest a predomination of IL-10 and TGF-β producing T_{reg} cells, which might be induced by SLIT; although the population of these cells remained to be investigated in the present study. This is consistent with the results obtained from studies performed on human.[31] Moreover, reduction of IL-4 (a Th2 cytokine) concomitant with the elevation of IFNγ and IgG2a support the concept of reorientation of immune responses towards the Th1 phenotype by the applied SLIT approaches.

In addition to successful immunomodulation by the co-application of probiotic LABs and allergens,[32] mucosal application of non-recombinant LABs stains, *per se*, has also been proved to potentially improve the Th1/Th2 balance.[33] This is in accordance with our results, herein; we observed quite similar changes in the antibody and cytokine response in all SLIT mice receiving the rSal k1, wild-type and recombinant *L. lactis*, either alone or co-administrated. However, the beneficial effects of simultaneous administration of rSal k1 and wild-type/recombinant *L. lactis* on modulation of cytokine production seemed to be superior to that of the allergen or bacteria alone, since the latter failed to induce a considerable change in IL-2 and IL-10 secretion.

Another compelling finding of the present study was that the SLIT was well tolerated by animals and none of the different SLIT approaches, neither rSal k1 protein nor wild-type *L. lactis* nor recombinant *L. lactis*, were shown to result in usual local or rare systemic adverse effects including gastrointestinal irritation, oral itching, IgE-mediated reactions, and accumulation of inflammatory cells in the gut.[10] This evidence further supports rSal k1, wild-type *L. lactis*, and recombinant *L. lactis* potential to be safely used for SLIT against *S. kali* pollen allergen.

Despite the strengths of the present study, there are some points to be considered in future studies; the mechanism(s) by which the SLIT approach screws allergic responses from Th2 towards Th1 phenotypes are to be elucidated. Especially, the role of T_{reg} populations needs to be investigated in this context. Also, the optimal dosing in order to achieve the highest efficacy should be determined. Additionally, the

efficacy of SLIT using rSal k1 remains to be compared with that of SCIT approaches.

The present study indicated the efficacy of SLIT using distinct rSal k1 allergen protein, wild-type *L. lactis* and engineered *L lactis* strains or combination of the recombinant protein and bacteria in mice sanitized to the allergen. The study provided data on the induction of Th1 responses concomitant with down-regulation of Th2 responses by various SLIT approaches. However, the underlying mechanisms remain to be elucidated in the future.

ACKNOWLEGEMENTS

This study was supported by grant number 900948 provided by Mashhad University of Medical Sciences, Mashhad, Iran. The results were derived from Ziba Ghasemi's dissertation.

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