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Sublingual Immunotherapy for Experimental Allergic Conjunctivitis in a Murine Model Induced by *Dermatophagoides farinae* Allergen

Li-lin Liu Dan-dan Guo Qiao-xia Liang Shan Ding Jing-ya Chen Bing Wu Qin Li

Division of Research and Development, Wolwo Bio-Pharmaceutical Inc., Shanghai, PR China

Key Words

Murine model · Allergic conjunctivitis · Sublingual immunotherapy · *Dermatophagoides farinae* · Immunoglobulin E · Immunoglobulin G · Interleukin-4 · Interferon-γ

Abstract

Background: Sublingual immunotherapy (SLIT) is a clinically effective treatment in allergic conjunctivitis (AC); however, the mechanism of the underlying pharmacodynamics remains unclear. Here, we investigate the efficacy and the mechanism of a sublingually administered *Dermatophagoides farinae (Der f)* vaccine in a murine AC model. ***Methods:*** A murine model of AC caused by *Der f* extract was developed in BALB/c mice by repeated application of allergen. Sensitized mice were SLIT treated by *Der f* drops and subsequently analyzed for AC symptoms, histopathological and immunological parameters. ***Results:*** In this study, *Der f* extract successfully induced the symptoms of AC in BALB/c mice. In these sensitized mice, clinical symptoms (scratching behavior, lacrimation, conjunctival hyperemia and edema), immunological and histopathological findings (inflammatory cell infiltration) were very similar to those in human AC. SLIT treatment of sensitized mice markedly reduced the clinical and histopathological symptoms and decreased the expression levels of total immunoglobulin E (IgE), *Der f*-specific IgE and T helper cell 2 (Th2) cytokine interleukin-4, with a significant increase in *Der f*-specific IgG4 and Th1 cytokine interferon-γ. ***Conclusions:*** SLIT with *Der f* drops is a potentially effective means of immunotherapy for *Der f*-induced AC by modulating the Th2-biased allergic immune response.

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Introduction

Allergic conjunctivitis (AC), which is a common allergic eye disease, is caused by direct exposure of the ocular mucosal surfaces to environmental allergens. Over the past decades, the prevalence and incidence of allergic diseases worldwide has dramatically increased [1, 2]. AC is clinically characterized by itching, hyperemia and chemosis in the early-phase hypersensitivity response and is followed by cellular infiltration into the conjunctiva after exposure to an allergen [3, 4]. Numerous studies have demonstrated that this is a type I hypersensitivity reaction, with participation of T lymphocytes that secrete cytokines. Chemotactic factors released by mast cells are responsible for accumulation of eosinophils and neutrophils at the site of allergic inflammation. Eosinophils contain granules in which preformed cytokines are rap-

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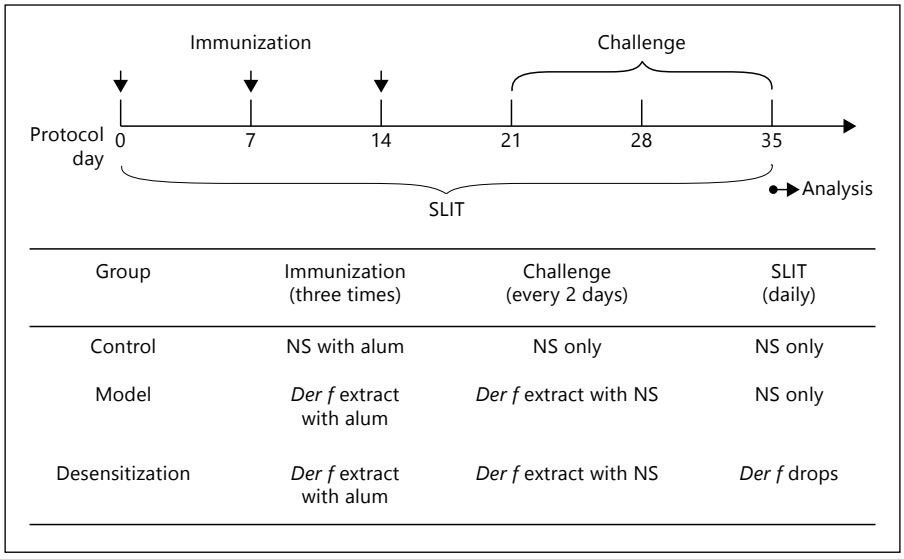
Correspondence to: Dr. Qin Li
Division of Research and Development, Wolwo Bio-Pharmaceutical Inc.
5th Floor, 40# Building, No. 333 Qinjiang Road
Shanghai 200233 (PR China)
E-Mail liqin@wolwobiotech.com



Group	Immunization (three times)	Challenge (every 2 days)	SLIT (daily)
Control	NS with alum	NS only	NS only
Model	*Der f* extract with alum	*Der f* extract with NS	NS only
Desensitization	*Der f* extract with alum	*Der f* extract with NS	*Der f* drops

Fig. 1. Outline of the experimental design. NS = Normal saline; alum = aluminium hydroxide.

idly secreted depending on the cytokine stimuli, T helper cell 1 [Th1; interleukin (IL)-12, IL-27 and interferon (IFN)-γ] and Th2 (IL-4 and IL-13) [5].

Seasonal and perennial AC are the most prevalent forms of ocular allergy. Perennial AC exhibits the classic immunoglobulin E (IgE)/mast cell-mediated hypersensitivity to common perennial household allergens such as dust mites, molds and animal dander, rather than to grass or weed pollens as in patients with seasonal AC. Eighty-nine percent of patients with perennial AC had specific serum IgE for house dust mite compared with only 43% of patients with seasonal AC [2]. Generally, the current recommended treatments for AC constitute allergen avoidance and pharmacotherapy. These treatments only affect the symptoms and have short-lasting efficacy. Allergen-specific immunotherapy offers advantages of specific treatment with long-lasting efficacy and the ability to modify the natural course of the disease [6]. Sublingual immunotherapy (SLIT) is recommended as a new promising and safe method of allergen-specific immunotherapy [6, 7]. In the past, the efficacy of SLIT in both house dust mite-induced asthma or conjunctivitis and pollen-induced rhinoconjunctivitis has been documented clinically [5, 8–10]. However, studies resolving the efficacy and underlying mechanisms of action of SLIT in mite-induced AC patients are rare.

Murine models are the most common and useful supplement to clinical and in vitro studies of allergic diseases [1] and may provide valuable information associated with the effect and therapeutic mechanisms of new treatments in allergic diseases [11]. The majority of AC experimental models were sensitized by pollen allergens [11]. The animal AC model induced by house dust mite allergen is scarce and has not yet been used for investigating the mechanisms of AC treatment [12]. The possible immunological mechanisms of SLIT described are mostly based on data from experiments in allergic rhinitis or asthma animal models or in humans. There are also researches about the clinical usefulness of SLIT in the treatment of AC, but no report which focuses on the histopathological improvement and therapeutic mechanism in the AC mouse model. In summary, we aim to solve the efficacy and underlying mechanism of SLIT in mouse AC induced by *Dermatophagoides farinae (Der f)*.

In the present study, the efficacy and the underlying mechanism of SLIT with *Der f* drops for AC has been investigated in a murine AC model sensitized by *Der f* extract.

Materials and Methods

The protocol of immunization and challenge was improved according to the methods of Takada et al. [13]. Mice were randomly allocated into three groups: the control group, the model group and the desensitization group. As shown in figure 1, all were immunized on day 0, 7 and 14. Then, ocular challenge was carried out every 2 days from day 21 and continued for 2 weeks. SLIT was performed once a day from day 0, following the standard protocol, as recommended by the manufacturer and the Chinese medical authority, for patients with allergic asthma or rhinitis. Analyses and the collection of material for laboratory analysis were conducted 24 h after ocular challenge and SLIT. Comparison between the control group and the model group was performed to assess

whether or not the animal model was established successfully. The efficacy of SLIT was evaluated by comparison between the model group and the desensitization group. Data in the study were representative of three independent experiments. The outline of the experimental design is shown in figure 1.

Animals

Female BALB/c mice, 8–10 weeks old, were purchased from Shanghai SIPPR-BK Laboratory Animal Co. Ltd. These mice were reared in a temperature-controlled room at 23 ± 2°C, with 50 ± 10% humidity and a 12-hour light, 12-hour dark cycle. All experimental animal care and treatments followed the guidelines set up by the National Science Council of the People's Republic of China.

Preparation of Solutions for Immunization and Challenge

Lyophilized *Der f* extract was provided by Wolwo Bio-Pharmaceutical Inc. It was freeze dried from 3 liters of liquid extract with 3.86 kUA/l total potency, which was extracted from the *Der f* polypides. Briefly, mites were defatted with acetone and extracted in normal saline (NS) overnight. The extract was dialyzed for 48 h, filtered and stored at 4°C. Extracts prepared for immunization were weighed and diluted in 10% aluminium hydroxide to a final 4-mg/ml concentration. A solution of 10% aluminium hydroxide compounded with NS was used as a placebo. The *Der f* extract for challenge was diluted in NS to a final 500-mg/ml concentration, and NS was used as a placebo for ocular challenge.

Animal Immunization and Challenge

Mice were separated into three groups (the control group, the model group and the desensitization group) so that each group contained at least 12 animals. As shown in figure 1, the control group was immunized with placebo and the other two groups, i.e. the model group and the desensitization group, were immunized with the immunization solution containing 2 mg *Der f* allergen with aluminium hydroxide. The same volume of sensitization solutions or placebo was subcutaneously injected at multiple sites on day 0, 7 and 14. From day 21, mice in the model group and the desensitization group were administered *Der f* extract solution for ocular challenge in each eye (5 mg *Der f* extract/10 μl/eye/time) every 2 days; meanwhile, the same volume of NS was used as placebo in the control group.

SLIT Administration

The *Der f* drops (Chanllergen), provided by Wolwo Bio-Pharmaceutical Inc., was the first standardized *Der f* vaccine for SLIT officially approved by the Chinese State Food and Drug Administration for allergic rhinitis or asthma patients. According to the instructions, it is a liquid vaccine derived from the *Der f* culture medium, containing major allergens Der f 1 and Der f 2 and other allergic proteins. The production process comprises: extraction, separation, concentration and stabilization. Concentrations are described in table 1. As shown in figure 1, only mice in the desensitization group received *Der f* drops for SLIT, whereas the same volume of NS was applied as placebo for mice in the control group and the model group. Sublingual administration of *Der f* drops or placebo was conducted after sensitization in accordance with the same procedure (table 1). Sublingual administration was performed by holding the scruff of the mice and carefully applying different specifications of *Der f* drops solution under the tongue.

Table 1. SLIT procedure of *Der f* drops

Specification	Dose incremental phase, ml/day			Dose constant phase, ml/day	
	No. 1	No. 2	No. 3	No. 4	No. 5
Week	1st	2nd	3rd	4th	5th
Day 1	0.05	0.05	0.05		
Day 2	0.10	0.10	0.10		
Day 3	0.15	0.15	0.15		
Day 4	0.20	0.20	0.20	0.15	0.10
Day 5	0.30	0.30	0.30		
Day 6	0.40	0.40	0.40		
Day 7	0.50	0.50	0.50		

Specification of *Der f* drops (antigen/volume): No. 1, 1 μg/ml; No. 2, 10 μg/ml; No. 3, 100 μg/ml; No. 4, 333 μg/ml; No. 5, 1,000 μg/ml.

The mice were held by the scruff for an additional 20–30 s to prevent the animal from swallowing the solution [14]. They were treated with increasing doses of *Der f* drops for 3 weeks and a constant dose of *Der f* drops for 2 weeks.

Clinical Evaluation

After challenge, mice were examined in order to clinically verify the occurrence and severity of conjunctivitis. Four clinical signs were observed: scratching, conjunctival edema, conjunctival hyperemia and lacrimation. In an adaptation of the scoring system developed by Brimnes et al. [14], scratching behavior was observed 0–20 min after challenge and estimated as follows: 0, never scratched; 1, scratched once; 2, scratched twice in 5 s; 3, scratched for more than 5 s. Ocular conjunctiva symptoms were evaluated according to Takada et al. [13]. The severity of hyperemia and edema was estimated as follows: 0, no symptoms; 1, slight hyperemia; 2, severe hyperemia; 3, severe hyperemia and slight edema; 4, severe edema. All scores were evaluated before and after 1, 3 and 5 weeks during the treatment period.

Histopathological Study

Conjunctiva from the lesional and the normal site was fixed with 10% neutral formalin and embedded in white paraffin. Serial paraffin sections, 5 μm thick, were prepared and stained with hematoxylin-eosin. The number of eosinophils in conjunctiva in a field of 1,000 × 1,000 μm was counted by an ophthalmologist trained in ocular pathology, blinded as to the origin of the tissues, under a microscope at a magnification of ×400. The total number in 5 representative fields of 6 sections from each group was accumulated.

Measurement of Antibodies

All the mice were sacrificed to collect sera at the end of treatment. Sera were analyzed by ELISA to measure the levels of total IgE, *Der f*-specific IgE and IgG. The sera were diluted with PBS containing 1% BSA and 0.05% Tween-20, pH 7.4. The concentration of total IgE in mouse serum was measured using a mouse IgE EIA kit (Bethyl), and the levels of total IgE in each serum were es-

Table 2. Sequences of primers in RT-PCR

Gene	Forward primer	Reverse primer
IL-4	5′-ACGGAGATGGATGTGCCAAACGTC-3′	5′-CGAGTAATCCATTTGCATGATGC-3′
IFN-γ	5′-TACTGCCACGGCACAGTCATTGAA-3′	5′-GCAGCGACTCCTTTTCCGCTTCCT-3′
β-Actin	5′-GTGGGCCGCTCTAGGCACCAA-3′	5′-CTCTTTGATGTCACGCACGATTTC-3′

timated according to the standard protocols from the manufacturer's kit. *Der f*-specific antibody was measured using a modified antigen-specific ELISA [15]. Briefly, 96-well microtiter plates (Costar) were coated with antigen *Der f,* which was diluted to 5 g/ml with 0.05 M carbonate buffer, pH 9.5, and overnight incubation at 4°C. The plates were blocked with PBS containing 3% BSA for 3 h. After overnight incubation with serum samples prepared as described above for 2 h at room temperature, the plates were developed using horseradish peroxidase-conjugated goat anti-IgG subclass-specific antibodies (Southern Biotech). In these assays, the concentration of anti-*Der f* antibody was estimated using standard curves constructed by coating wells with anti-Ig antibody against the appropriate isotype and by adding polyclonal Ig standards of the pertinent isotype. In the IgE assays, the same procedure described for the IgG measurements was used, except that samples were absorbed on plates coated with anti-mouse IgG, IgM and IgA to remove *Der f*-specific IgG, IgM and IgA that might compete with *Der f*-specific IgE, and thus, cause us to underestimate *Der f*-specific IgE. After overnight incubation, the plates were washed, and horseradish peroxidase-conjugated anti-IgE (Southern Biotech) was added. Finally, substrate solution (TMB) was added to each well. After 15 min at room temperature, 2 M H_2SO_4 was added to the wells in order to terminate the reaction, and then, absorbance at 450 nm was measured.

mRNA Expression Level of IL-4 and INF-γ in Conjunctiva

Total RNA was isolated from 50–100 mg of tissue of lesional or normal conjunctiva with Trizol reagent (Invitrogen, Carlsbad, Calif., USA). Target RNA obtained from the tissue was reverse transcribed to cDNA using Revert™Aid First Strand cDNA Synthesis Kit (Invitrogen). To minimize variations in reverse transcriptase efficiency, all samples were transcribed simultaneously. The primers used to amplify cDNA for murine IL-4, IFN-γ and β-actin were based on the sequences published by Overbergh et al. [16] and are shown in table 2. The cDNA was used for PCR reactions. PCR was carried out for 35 cycles by the following conditions: denaturation at 94°C for 30 s, annealing at 62°C for 30 s and elongation at 72°C for 5 min. Samples were run on 1.5% agarose gel in the presence of ethidium bromide. After gel separation, equal ethidium bromide band intensities of both fragments were quantified by the NIH image software and used to determine the amount of cellular cDNA. The results were expressed as a ratio of cytokine cDNA to cDNA of the constitutively expressed β-actin gene.

Statistical Analysis

SPSS 11.5 was used for statistical analysis. Differences between treatment groups were examined using a paired Student t test. The criterion for statistical significance was set at either $p < 0.05$ or $p < 0.01$.

Results

Clinical Symptoms and Scores

We succeeded in inducing AC triggered by allergen of *Der f* extract in BALB/c mice. Mice sensitized and challenged as described above displayed clear clinical symptoms of conjunctivitis (fig. 2a, c), as obvious hyperemia and edema in the conjunctiva was observed compared to the control mice. In addition, the scratching point was increased more than thirty times after 2 weeks of induction (fig. 2b). Having established a mouse model of conjunctivitis, we applied this model to study the effect of SLIT on the course of conjunctivitis. As can be seen in figure 2, SLIT treatment of mice sensitized with *Der f* extract led to a significant reduction in the clinical signs of conjunctivitis. Figure 2c shows that conjunctivitis scores were reduced by more than 50%, and figure 2b shows that also the scratching point was significantly decreased in *Der f* SLIT-treated mice. These results demonstrated that SLIT treatment of sensitized mice with *Der f* extract led to a significant reduction in the clinical signs of conjunctivitis.

Histopathological Analysis

Histopathological study showed an inflammatory process in the conjunctiva and cornea, with epithelial lesions, edema and infiltrates, with a predominance of eosinophils in the model mice compared with the controls (fig. 3a). In addition, an important number of lymphocytes and a discrete increase in mast cells in the substantia propria of the conjunctiva in model mice have been observed (data not shown). The numbers of goblet cells, eosinophils and neutrophils in the conjunctival lesion were analyzed, whereas only the number of eosinophils in the desensitization model group was significantly less than that in the model group (fig. 3b). Influx of eosinophils was essential in allergic inflammation and in profound changes in the conjunctival mucosa [17]. These results indicated that SLIT leads to a clear reduction in local allergic inflammation.

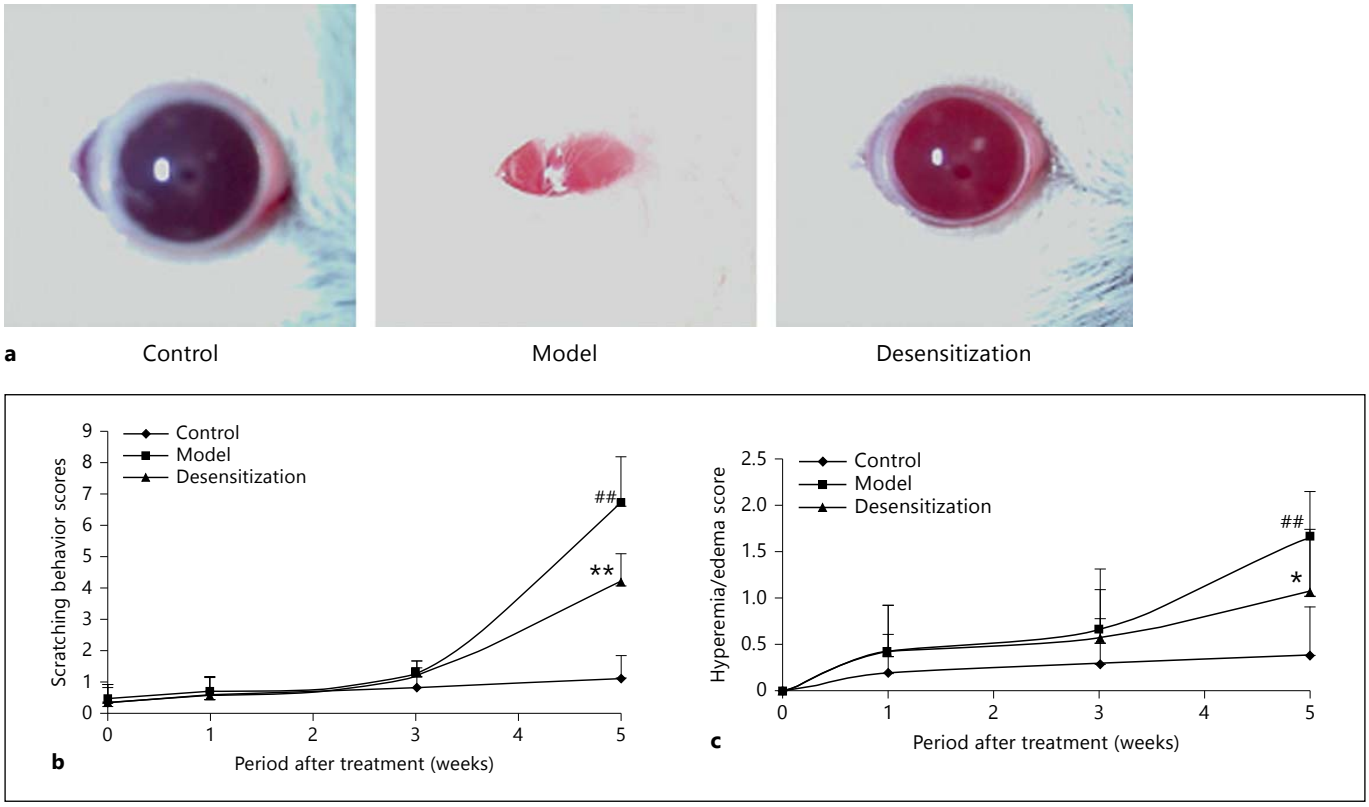


Fig. 2. Clinical symptoms and scores in mice under different treatments. **a** Eye features. **b** Quantitative analysis of scratching behavior. **c** Evaluation of conjunctivitis. Data represent the mean ± SD. ## $p < 0.01$, compared with mice in the control group; * $p < 0.05$ and ** $p < 0.01$, compared with mice in the model group. Data are representative of three independent experiments.

Table 3. Sera antibody levels of total IgE, *Der f*-specific IgE and *Der f*-specific IgG in mice under different treatments

Groups	n	*Der f*-specific IgE ng/ml	*Der f*-specific IgG ng/ml	n	Total IgE, ng/ml
Naïve[1]	9	3.546±0.203	10.912±0.987	9	9.13±8.20
Control	12	8.931±1.764	31.905±3.237	10	29.32±10.16
Model	12	30.519±2.276*	98.798±7.958*	12	102.01±11.89*
Desensitization	14	23.862±3.078***	103.102±5.916**	12	58.88±9.85***

Data are representative of three independent experiments and represent the mean ± SD. * $p < 0.01$, compared with the control group; ** $p < 0.05$ and *** $p < 0.01$, compared with the model group.

[1] Sera measured as the naïve group was derived from the normal BALB/c mice.

Antibody Levels in Sera

Significantly elevated levels of total IgE, *Der f*-specific IgE and *Der f*-specific IgG were found in the model mice compared with the control group (table 3). This confirms that AC in the model mice is *Der f* allergen induced. After SLIT treatment with *Der f* drops, the levels of total and specific IgE in the desensitization mice were remarkably downregulated as compared with those of the model mice. In contrast, there was a significant increase in the level of specific IgG (table 3). These results demonstrated that SLIT treatment could downregulate the expression of *Der f*-specific IgE in desensitized mice, with upregulation of *Der f*-specific IgG.

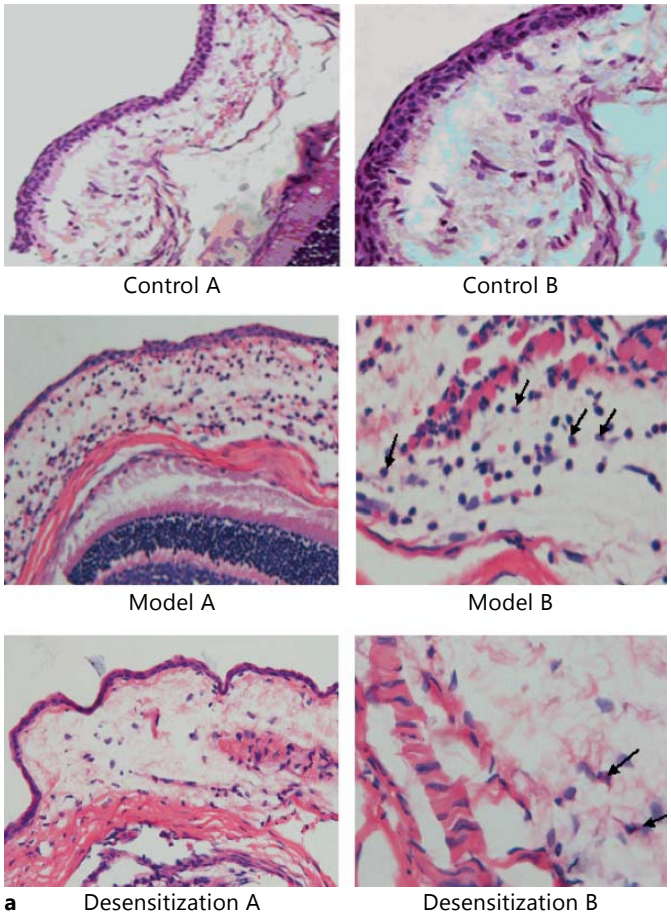


Fig. 3. Histopathological evaluation of the conjunctiva inflammatory process under different treatments. **a** Micrographs of conjunctiva for mice. ×200 (control A, model A and desensitization A). ×400 (control B, model B and desensitization B). Eosinophils (arrows) are marked in model B and desensitization B. **b** Quantitative analysis of eosinophil infiltration into conjunctiva. Data represent the mean ± SD. $^{\#\#}$ $p < 0.01$, compared with mice in the control group; ** $p < 0.01$, compared with mice in the model group. Data are representative of three independent experiments.

Changes in Cytokines

To examine whether *Der f* sublingual drops treatment could suppress ongoing Th2 responses (as shown by increased IL-4 level in the model mice), the cytokine levels in the conjunctiva were determined. As shown in figure 4, untreated model mice displayed a predominant Th2 reaction, while mice SLIT treated with *Der f* sublingual drops showed a decreased level of IL-4 and an increased level of IFN-γ, which indicated a switch from Th2 to Th1 or Th0 reaction.

Discussion

So far, guinea pigs, rats and mice have been used for the establishment of animal models of ocular allergy using a variety of antigens [11]. All three species have been used in our pre-experiment (data not shown). We have found that the guinea pig is the most sensitive, whereas it is hard for the ELISA kits to detect the antibody level of guinea pigs or rats. Meanwhile, the severity of conjunctivitis-like symptoms in mouse has been equivalent to that in rat. To analyze the effect and the mechanism of SLIT as complete as possible, we have used mice to develop the AC model. The clinical and histopathological parameters of AC have been successfully induced by *Der f* extract in BALB/c mice, without adverse reaction. The clinical symptoms and histopathological changes in the BALB/c mice model corresponded to the immediate and late phases of the allergic process, as described in humans [18, 19]. In addition to ocular disease, there was also a systemic immune response induced by the mite, characterized by an increase in both total serum and *Der f*-specific IgE and IgG levels and in the ratio of Th2 cytokine IL-4/Th1 cytokine IFN-γ, which parallels findings in humans [5]. The present model, as a good mimic of human AC, indicates that it is an accurate instrument for studying the pathogenesis of ocular allergy and for testing therapies for AC. Up to now, we found lots of studies about AC mouse models induced by various pollen allergens, but only one research in which the AC murine model triggered by another most common house dust mite *Dermatophagoides pterronyssinus (Der p)* was established [12]. Despite the differences in the kind of allergens, the quality and potency of the allergen extract, and the route and schedule of sensitization, they are consistent in several aspects. In accordance with the *Der p*-induced AC model, BALB/c mice also needed a large amount of mite allergens to induced similar AC symptoms in our study. Moreover, in addition to similar AC symptoms, a significant increase

in specific IgE and IgG levels and in Th2 cytokine profiles are shown in the two studies. However, in addition to more clinical symptoms which were observed in this study, more detailed analyses of clinical symptoms and histological changes were performed, and we further evaluated the effect of SLIT using the established AC model.

In this study, we have demonstrated that SLIT with *Der f* is able to reduce AC symptoms in the above-described mouse model. To our knowledge, this is the first time that the effect of SLIT has been studied in an animal model of AC, making a comparison with similar studies difficult. However, the results are in accordance with what has been observed in humans, as SLIT treatment has been demonstrated to efficiently reduce both the patient dropout rate and symptom scores in AC patients [8, 20]. In addition, the allergen dose in combination with the administration frequency executed in our study is the same as for *Der f* drops applied clinically, which has been approved effectively for SLIT treatment in human allergic asthma or rhinitis. We believe that it is likely that the dose and frequency dependency also holds true for clinical SLIT treatment in AC patients. However, this issue needs to be addressed in well-designed clinical studies in the future.

Regarding our understanding of the underlying immunological mechanism of SLIT treatment, it is now well established that SLIT treatment leads to a systemic increase in allergen-specific IgG4, whereas systemic, allergen-specific IgE is either increased or not affected [10, 21]. Following treatment with *Der f* drops, SLIT significantly reduced the level of allergen-specific IgE and led to an increase in specific IgG, which is in accordance with the results in asthma or rhinitis animal model studies [14, 22, 23]. We propose that the therapeutic effects of *Der f* vaccine were achieved by inhibition of IgE and elevation of allergen-specific IgG production, since the shift in balance between IgE and IgG is crucial to successful allergen-specific immunotherapy [10, 21]. Furthermore, in the desensitization of mice, a decreased level of IL-4 and an increased level of IFN-γ mRNA were observed, indicating that *Der f* sublingual vaccine downregulated the Th2 response in the murine AC model of dust mite hypersensitivity. It is believed that a shift from Th2 to Th1 plays an important role in SLIT [14, 22, 24, 25]. Our experiments first confirm that SLIT in AC with *Der f* antigen could make a switch in the Th1/Th2 balance, which might be the most important success in immunotherapy of AC induced by *Der f* allergen. Therapeutic mechanism researches in allergic respiratory diseases indicate that SLIT could regulate the Th2 [26] and

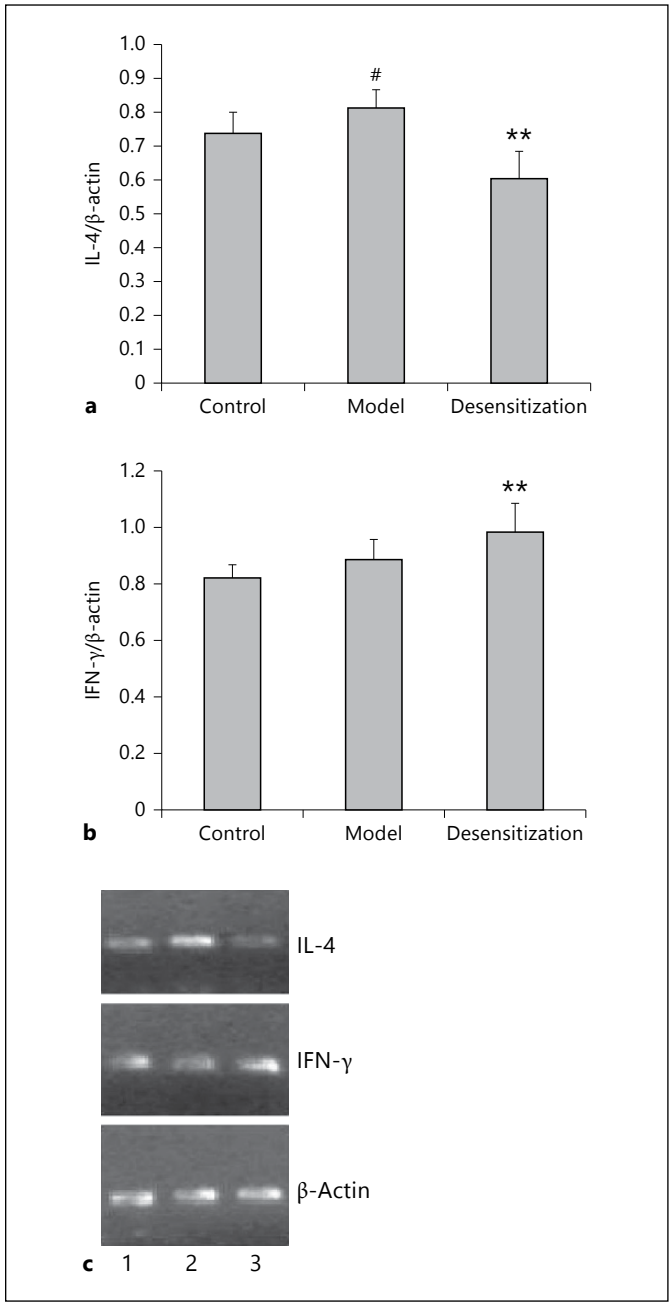


Fig. 4. Relative expression levels of IFN-γ and IL-4 mRNA in the conjunctiva after different treatments. Total RNA was isolated from the conjunctiva of mice and evaluated by RT-PCR. β-Actin is shown as a positive control. **a** Resulting electrophoretic bands (**c**) of IL-4 mRNA were semiquantitively evaluated using NIH image software. **b** Resulting electrophoretic bands (**c**) of IFN-γ mRNA were semiquantitatively evaluated using NIH image software. # $p < 0.05$, compared with mice in the control group; ** $p < 0.01$, compared with mice in the model group. **c** Expression of IL-4 and IFN-γ mRNA in conjunctiva of mice with different treatments. Lane 1 = control group; lane 2 = model group; lane 3 = desensitization group. Data are representative of three independent experiments.

Th1 responses [23] and induce the change in the level of IgE and IgG. The results indicate that SLIT in AC may involve a mechanism similar to that in allergic respiratory diseases [23, 26].

In conclusion, in the present study, we have demonstrated that it is possible to establish a murine model of AC in which the mice recognize the clinically relevant allergen *Der f* and which displays the hallmarks of human AC. Using this model, we have demonstrated that SLIT treatment leads to a significant improvement in clinical and histological symptoms of AC as well as in serological and immunological changes by modulating the Th2-biased allergic immune response.

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