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Role of miR-146a in Enforcing Effect of Specific Immunotherapy on Allergic Rhinitis

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ABSTRACT

Allergic rhinitis (AR) is one of the common disorders in airway allergic inflammation. The pathogenesis of AR is unclear. It is accepted that immune deregulation is associated with the pathogenesis of AR. Recent reports suggest that a large number of micro RNAs (miR) can regulate immune functions. This study aims to investigate the role of miR-146a in an enforcing immunotherapy of AR. In this study, a mouse AR model was created. The levels of miR-146a in the mouse nasal mucosa were assessed by real time RT-PCR. A specific immunotherapy was performed in AR mice. The results showed that the AR mice had an AR-like inflammation in the nasal mucosa. Compared with naïve mice, markedly lower levels of miR-146a were detected in AR mice. The co-administration with miR-146a significantly enforced the effect of ovalbumin (OVA)-specific immunotherapy on inhibition of AR inflammation in the nasal mucosa. Further analysis showed that miR-146a induced transforming growth factor- β in dendritic cells; the latter induced naïve CD4⁺ T cells to differentiate into regulatory T cells. In conclusion, miR-146a can enforce OVA-specific immunotherapy via inducing antigen-specific regulatory T cells. miR-146a may have therapeutic potential to be used in the immunotherapy of allergic diseases.

KEYWORDS

Allergy; immunotherapy;
micro RNA; nasal mucosa;
vaccine

Introduction

Allergic rhinitis (AR) is an IgE-mediated allergic inflammation of the nasal mucosa (Xu et al., 2014). Clinical symptoms of AR include rhinorrhea, sneezing, nasal congestion and nasal pruritus (Tharpe & Kemp, 2015). The prevalence of AR varies from place to place; it has reached around 10% in the whole population worldwide (Tharpe & Kemp, 2015). Although AR did not cause a fatal threat in human life, it compromises the life quality of human beings. Furthermore, it is involved in the pathogenesis of hypersensitivity of lower airway (Gaspar-Lopez et al., 2014). Thus, it is necessary to find innovative remedies to treat AR.

It is recommended by the World Health Organization that the antigen-specific immunotherapy (SIT) is the unique specific remedy to treat allergic disorders (Simons et al., 2013). Important characteristic of SIT is to inhibit the IgE-mediated immune response in the local tissue. However, the mechanism of SIT remains to be further investigated; the therapeutic efficiency of SIT is to be further improved.

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The regulatory T cells (Tregs) are an important sub-population of T lymphocytes. Tregs regulate the immune system, maintain tolerance to self-antigens to prevent autoimmune diseases (Coe et al., 2014; Feng et al., 2015). In general, Tregs suppress or down regulate the induction and proliferation of effector T cells, and suppress immune responses of other cells (Zheng et al., 2014). The most well-understood Tregs are those that express CD4, CD25, and Foxp3 (CD4⁺CD25⁺ Tregs) (Ouaguia et al., 2014). Additional Treg types include Tr1, Th3, CD8⁺CD28⁻, and Qa-1 restricted T cells. The CD4⁺CD25⁺ Tregs naturally occur in the body; these Tregs are designated as the natural Tregs (Ngalamika et al., 2015). Tregs can also be induced after birth; these Tregs are inducible Tregs (Abdoli & Najafian, 2014). Yet, the mechanism of the generation of antigen-specific Tregs has not been fully appreciated.

The micro RNA (miR) has been put in place to the research area in the recent years. The miR-146a is one of the miRs having the anti-inflammatory ability (Baldeon et al., 2014). Building on the information that the pathological feature of allergic diseases is immune inflammation, Tregs are capable of inhibiting allergic inflammation (Yang et al., 2014), we hypothesize that miR-146a may facilitate the generation of Tregs to suppress allergic inflammation. To test the hypothesis, we created a mouse model of allergic rhinitis (AR). The AR mice were treated with a nanovaccine containing miR-146a or/and ovalbumin (OVA, using as an antigen). The study aims to test the effect of miR-146a-laden nanovaccine on induction of Tregs and the inhibition of AR inflammation.

Materials and methods

Reagents

The ELISA kits of OVA-specific IgE and IL-4 were purchased from Shanghai Ximei Chem (Shanghai, China). The miR-146a primers, miR-146a and its detection kit were provided by GeneScript (Nanjing, China). The reagents of real time RT-PCR and Western blotting were purchased from Invitrogen (Shanghai, China). The fluorochrome-labeled antibodies for flow cytometry were purchased from BD Biosciences (Shanghai, China). The magnetic cell sorting kits were purchased from Miltenyi Biotec (Shanghai, China). The anti-TGF- β antibody was purchased from Santa Cruz (Shanghai, China).

Preparation of nanoparticle vaccine (NPV)

The NPV of OVA or/and miR-146a was synthesized following published procedures (Rosalia et al., 2015). The resulted products were NPVo (NPV-OVA), NPVm (NPV-miR146a) and NPVom (NPV-OVA/miR-146a).

Allergic rhinitis mouse model

Following published procedures (Hong et al., 2014; Yang et al., 2015) with a minor modification, male BALB/c mice (6–8 week old) were purchased from the Shanghai Xinmao Experimental Animal Center (Shanghai, China). The mice were kept in a pathogen-free environment with freely accessing to food and water. Ovalbumin (OVA) was used as a specific antigen. Each mouse was subcutaneously injected OVA

(0.2 mg/mouse in 0.1 ml alum) to the back skin on day 0 and day 7. The mice were received nasal drop (10 μ l OVA/nostril at 1 mg/ml saline) daily from day 10 to day 19. From day 20 to day 26, the mice were treated with vaccines of OVA or/and miR-146a (10 μ l OVA/nostril daily). The mice were challenged with OVA on day 27 (10 μ l OVA/nostril at 1 mg/ml saline). The sneezing-like sound and nasal secretion were observed for each mouse in the period of 30 min after the challenge. Two hours after the challenge, the mice were sacrificed. The blood, nasal mucosa and the spleen were collected and analyzed in further experiments.

Enzyme-lined immunoassay (ELISA)

The serum cytokine levels were determined by ELISA with commercial reagent kits following the manufacturer's instructions.

Isolation of CD4⁺ T cells from the nasal mucosa

The nasal mucosal specimens were collected from each mouse. Specimens from six mice were pooled to one sample. The mucosa was incubated in 0.25% EDTA (Ethylenediaminetetraacetic acid) at 37 °C for 20 min with mild agitation to remove mucus. The mucosa was then cut into small pieces and incubated with collagenase IV (0.5 mg/ml) for 30 min. The cells were filtered through a cell strainer (40 μ m). CD4⁺ T cells were isolated by MACS (magnetic cell sorting).

T cell proliferation assay

CD4⁺ T cells from nasal mucosa or spleen were labeled with CFSE (Carboxyfluorescein succinimidyl ester) and cultured with DCs (T cell:DC = 5:1) in the presence of the specific antigen OVA (5 μ g/ml) for 3 days. The cells were collected at the end of culture and analyzed by flow cytometry.

Collection of human nasal epithelial samples

The nasal epithelial samples were collected from 10 patients with AR and 10 healthy subjects. The diagnosis and treatment of AR were carried out by their physicians. Briefly, AR was diagnosed based on: 1, the patients had AR symptoms more than 2 years; 2, had a positive response in the allergen skin test; 3, serum levels of antigen-specific IgE were greater than 30 IU/ml. Patients having sinusitis were excluded. The epithelial samples were gently scrapped on the surface of the inferior nasal turbinate using a plastic curette. On the other hand, nasal epithelial samples were collected from the mouse nasal mucosa. After sacrifice, the nasal cavity was opened; the nasal epithelial samples were collected by gently scrapped on the surface of the nasal mucosa using a plastic curette.

Ethic statement

The usage of human and animal tissues in experimental procedures were approved by the Research Ethic Committee at Shandong University.

Real time quantitative RT-PCR (RT-qPCR)

The total RNA was extracted from the samples using TRIzol reagents. The cDNA was synthesized using a reverse transcription kit. The cDNA of miR-146a was synthesized by GeneScript (Nanjing, China). Real-time PCR was carried out in a Real time PCR device (MiniOpticon, Bio-Rad, Shanghai, China) with the SYBR Green Master Mix. The results were calculated with the $2^{-\Delta\Delta C_t}$ method using the healthy or naïve samples (or U6) as the controls, and normalized to a percentage of the internal control β -actin gene.

Western blotting

The proteins were fractioned with SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred onto a PVDF membrane. After blocking in 5% skim milk for 30 min, the membrane was incubated with the primary anti-TGF- β antibody (300 ng/ml) overnight at 4°C and followed by incubation with the second antibody (conjugated with peroxidase) for 1 h. After incubation, the membrane was washed with TBST (Tris buffered saline Tween 20). The immune blots on the membrane were developed with ECL (the enhanced chemiluminescent). The results were recorded by photographing with the KODAK Image Station 4000Pro (KODAK, Shanghai, China).

Generation of bone marrow-derived DCs

Mouse bone marrow (BM) cells from BALB/c mouse femurs were prepared and cultured for 10 d in medium supplemented with GM-CSF (40 ng/mL). As analyzed by flow cytometry, more than 95% cells were CD11c positive. The DCs were exposed to NPVm or NPVom in the culture for 6 d. As analyzed by flow cytometry, more than 90% DCs showed a tolerogenic feature as expressing TGF- β (data not shown).

Generation of Tregs by NPV

CD4⁺ CD25⁻ T cells were isolated by MACS from the mouse spleen and cultured with the tolerogenic DCs (induced by NPVm or NPVom) at a ratio of 1:1 for 6 d. The frequency of CD25⁺ Foxp3⁺ Treg was determined by flow cytometry. The Tregs were isolated by MACS based on the criterion of CD4⁺ CD25⁺ CD127⁻.

Flow cytometry

The cells were fixed with 2% paraformaldehyde (in case of the intracellular staining, the fixative contained 0.1% Triton $\times$ -100) for 2 h. After washing with PBS, the cells were blocked by 1% BSA for 30 min, and then incubated with the fluorochrome-labeled antibodies. The cells were analyzed by flow cytometry (FACSCanto II; BD Biosciences).

Adoptive transfer of Treg

The AR mice were adoptively transferred with Tregs via tail vein injection at 10^6 Tregs/mouse.

Assessment of cell viability

The cell viability was assessed by the Trypan blue exclusion assay.

Statistics

The data are presented as mean $\pm$ SD. The difference between groups was determined by Student's t-test or ANOVA if more than two groups. A $p < 0.05$ was set as a significant criterion.

Results

The levels of miR146a are suppressed in the AR nasal mucosa

Published data indicate that miR-146a is capable of suppressing inflammation (Baldeon et al., 2014). We wondered if miR-146a also played a part in suppressing allergic inflammation. Therefore, we collected nasal epithelia from 10 AR patients, 10 healthy subjects. The samples were analyzed by RT-qPCR. The results showed that levels of miR-146a were detected in the normal nasal epithelia while the levels of miR-146a were significantly lower in the nasal epithelia from patients with AR (Figure 1A). The results suggest that AR nasal epithelia have lower levels of miR-146a. To enforce the consequences, we created an AR mouse model. The nasal mucosa of mice was analyzed by RT-qPCR. The results showed that levels of miR-146a were also significantly lower in AR mice than normal controls (Figure 1B).

Co-administration of miR-146a enforces specific immunotherapy on allergic rhinitis (AR)

We then created a mouse model of AR. The mice showed AR-like symptoms after challenging with the specific antigen, OVA, including sneezing like sound, increase in nasal secretion

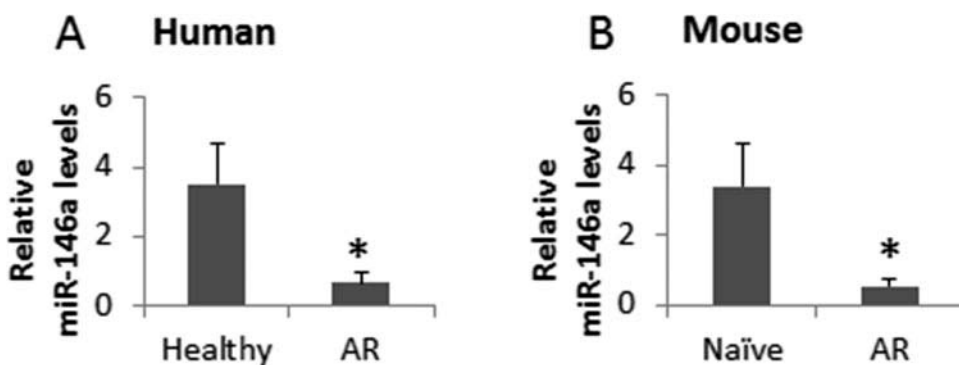


Figure 1. AR suppresses the levels of miR-146a in the nasal mucosa. Nasal epithelial samples were collected from patients with allergic rhinitis (AR; $n = 10$), healthy subjects ($n = 10$), naïve mice ($n = 6$), and AR mice ($n = 6$). The total RNA extracts of the samples were analyzed by RT-qPCR. The bars indicate the levels of miR-146a. The data are presented as mean $\pm$ SD. *, $p < 0.01$, compared with healthy group (A), or naïve group (B). Samples from individual human subjects and individual mice were processed separately.

Table 1. AR symptoms.

	Naïve	Saline	NPVo	NPVm	NPVom	Treg	Treg2	CD4 ⁺ T cell
Sneezing	-	+	+	+	-	-	+	+
Secretion	-	+	+	+	-	-	+	+

The symptoms were observed and recorded between 0 and 30 min after nasal challenge with the specific antigen (OVA). “-”, none symptom. “+”, the symptoms were observed. Each group consists of six mice. NPV: Nano particle vaccine, NPVo: OVA-laden NPV, NPVm: miR-146a-laden NPV, NPVom: OVA/miR-16a-laden NPV. Sneezing: Mice give rise to “head shake-like retraction” after challenge with the specific antigen. Secretion: There was visible water-like discharge in or around the nostril.

(Table 1), high levels of specific IgE (Figure 2A), IL-4 (Figure 2B) in the serum and the nasal mucosa, and OVA-specific spleen T cell proliferation (Figure 2C–J). Grouped AR mice were treated with saline (using as a control group), NPVo, or NPVm, or NPVom for one week. The mice were sacrificed; the AR-related responses were assessed. The results showed that exposure to NPVo exacerbated the AR-related responses (Figure 2A, B, E); treatment with NPVm did not significantly alter the AR-related responses (Figure 2A, B, F). Treatment with NPVom markedly suppressed the AR-related responses (Figure 2A, B, G).

Nanovaccines containing specific antigen and miR-146a induce antigen-specific Tregs

Based on that antigen-specific Tregs are capable of suppressing allergic inflammation (He et al., 2011), we inferred that the inhibition of AR symptoms as shown in Figure 2 might

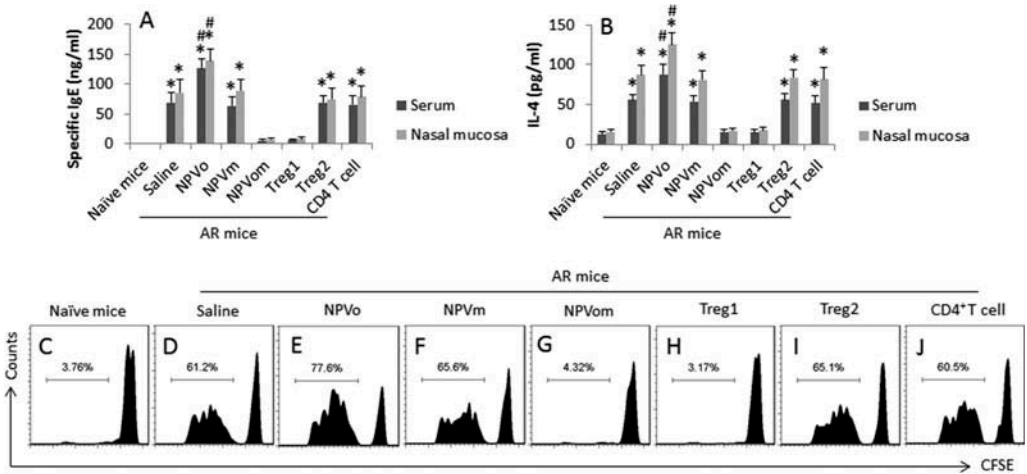


Figure 2. Nanovaccination modulates specific Th2 response in AR mice. AR mice were treated with NPV, or adoptive transfer with an OVA-specific Tregs (Treg1), or non-specific Tregs (Treg2), or naïve CD4⁺ T cells, at 10⁶ cells/mouse on day 1 of the vaccination. The mice were sacrificed on day 10 of the vaccination. The blood and the spleen were collected from each mouse and analyzed by ELISA and flow cytometry. (A) The bars indicate the levels of OVA-specific IgE in the serum. (B) The bars indicate the levels of IL-4 in the serum. (C–J) The histograms indicate the frequency of CD4⁺ T cell proliferation. NPV: Nano particle vaccine, NPVo: OVA-laden NPV, NPVm: miR-146a-laden NPV, NPVom: OVA/miR-16a-laden NPV. The data are presented as mean ± SD. *, $p < 0.01$, compared with the naïve mouse group. #, $p < 0.01$, compared with the saline group. Each group consisted of six mice (48 mice in total in this figure). Samples from individual mice were processed separately.

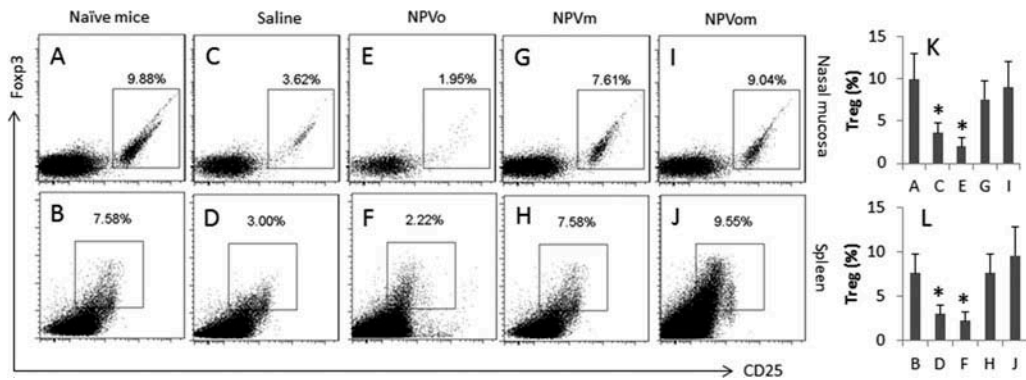


Figure 3. Assessment of Tregs. The treatment of AR mice was denoted above the dot plot panels. Single cells were prepared from the nasal mucosa and the spleens. $CD4^+$ T cells were isolated by MACS and analyzed by flow cytometry. The gated dot plots indicate the frequency of $CD25^+$ $Foxp3^+$ Tregs. The bars indicate the summarized data of the dot plots. NPV: Nano particle vaccine, NPVo: OVA-laden NPV, NPVm: miR-146a-laden NPV, NPVom: OVA/miR-16a-laden NPV. Specimens from six mice were pooled as one sample; each experiment was carried out three times (90 mice in total in this figure).

be mediated by OVA-specific Tregs. To test the inference, we assessed the frequency of Tregs in the nasal mucosa and the spleens of the mice. As shown in flow cytometry data, the frequency of Tregs in AR nasal mucosa and the spleen was significantly less than normal controls (Figure 3A–D, K, L); treatment with either NPVo or NPVm did not alter much of the Treg frequency (Figure 3E, F, K, L) while treatment with NPVom significantly increased the frequency of Tregs in the nasal mucosa and the spleen of AR mice (Figure 3G, H, K, L). Further analysis showed that the Tregs in mice treated with NPVom were OVA-specific. In addition, the treatment with NPVom significantly suppressed the antigen-specific Th2 cells in the nasal mucosa and the spleen of AR mice.

NPVm or NPVom induces TGF- β expression in DCs

The above results implicate that miR-146a contributes to the generation of Tregs. Since DCs are the key cell type in the generation of Tregs (Luo et al., 2014), we firstly observed the effect of NPV on inducing TGF- β expression in DCs. The results showed that exposure to either NPVm or NPVom markedly increased the expression of TGF- β in DCs while exposure to NPVo did not (Figure 4A–B). The results imply that the NPVm or NPVom-primed DCs may be capable of inducing Tregs. To test the inference, we cultured the NPV-primed DCs with naïve $CD4^+$ T cells. The cells were then analyzed by flow cytometry. The results showed that NPVm or NPVom markedly converted the naïve $CD4^+$ $CD25^-$ T cells to $CD4^+$ $CD25^+$ $Foxp3^+$ TGF- β^+ Tregs (Figure 4C–E). The Tregs induced by the NPVom proliferated markedly after exposure to OVA in the culture while the NPVm-induced Tregs did not (Figure 4F–H). To strengthen the results, in separate experiments, the Tregs induced by NPVom-primed DCs were exposed to BSA in the culture, which did not induce the Treg proliferation (Figure 4I).

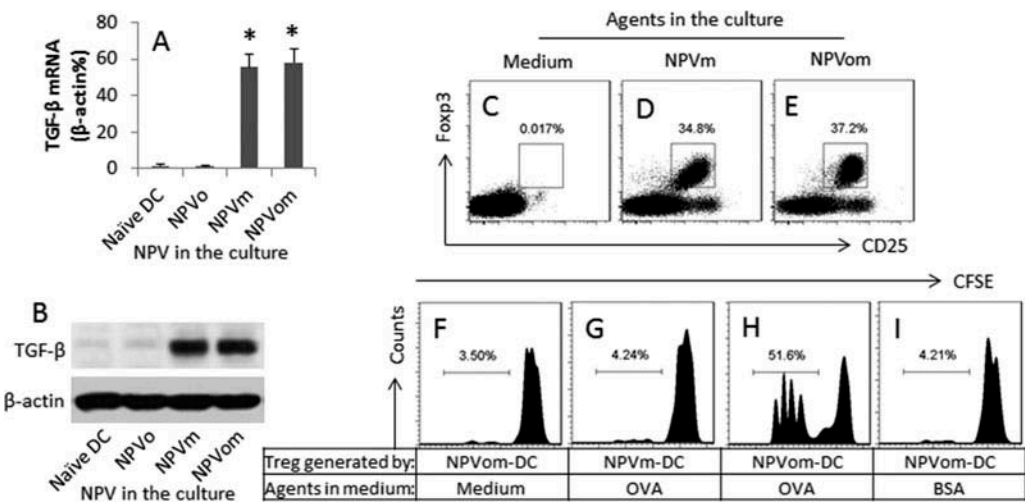


Figure 4. OVA/miR-146a generates OVA-specific Tregs. (A–B) bone marrow-derived DCs were prepared in the presence of agents as denoted in the figure. The DC extracts were analyzed by RT-qPCR and Western blotting. (A) The bars indicate the mRNA levels of TGF-β. (B) The Western blots indicate the protein levels of TGF-β. (C–E) The DCs primed by the procedures of A and B were cultured with CD4⁺ CD25⁺ T cells at a ratio of 1:1 for 6 days. The dot plots indicate the frequency of CD25⁺ Foxp3⁺ Tregs. (F–I) The Tregs of C–E were isolated by MACS based on a criterion of CD4⁺ CD25⁺ CD127⁺. The Tregs were cultured with DCs (T cell:DC = 5:1) in the presence of agents as denoted in the table below the histograms. NPV: Nano particle vaccine, NPVo: OVA-laden NPV, NPVm: miR-146a-laden NPV, NPVom: OVA/miR-146a-laden NPV. The data are representatives of three separate experiments.

NPVom-induced Tregs inhibit OVA-specific AR

The results of Figure 3 implicate that the induced Tregs by NPVom may be capable of suppressing the AR symptoms. To test the inference, we generated the Tregs as indicated above. The Tregs were adoptively transferred into the AR mice. As shown by the data in Figure 1, the transfer of Tregs generated by NPVom significantly inhibited the AR-related symptoms in AR mice while the NPVm-induced Treg or naïve CD4⁺ T cells did not.

Discussion

Although a number of therapeutic remedies have been employed for the treatment of allergic diseases, including AR, the effect of them is unsatisfactory currently. Based on the remedy of SIT, the present data show that co-administration of miR-146a and the specific antigen in a nanovaccine significantly enhances the therapeutic effect of SIT on AR in a mouse model. The results suggest that the miR-146a may be a novel remedy in the facilitating the treatment of allergic diseases.

miR-146a is involved in the maintenance of the immune homeostasis. Suppression of miR-146a is found in several inflammatory diseases. Baldeon et al. (2014) indicate that decreased serum level of miR-146a is a discriminating marker of failure of glucose control occurring on the background of obesity and dyslipidemia. Gu et al. (2015) indicate that miR-146a reduces IL-1 dependent inflammatory responses in the intervertebral disc. Our

data are consistent with those previous studies by showing that markedly lower levels of miR-146a in the nasal mucosa of AR patients and AR mice. Others have also reported that miR-146a is suppressed in allergic disorders, such as asthma (Comer et al., 2014) and dermatitis (Rebane et al., 2014). The information suggests that miR-146a is an anti-inflammation molecule.

Tregs are an important immune cell population. The abnormality of Treg number or Treg function is linked to a large number of immune disorders, such as rheumatoid arthritis (Miyara et al., 2014). Some heritable discriminatory immune diseases result from defects affecting Treg development, function, or both. They include immune deregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which is caused by mutations in *Foxp3* (Charbonnier et al., 2014). Our data demonstrate that, in AR mice, the frequency of Tregs is much less than normal control mice. The results implicate that the decrease in Treg number may be involved in the development of AR. Subsequent data support our inference by showing that adoptive transfer with Tregs significantly suppresses the AR response in AR mice. Others also point out that adoptive transfer of CD4⁺CD25⁺ Tregs from healthy to diseased animals corroborated and provided further evidence of the vital role of these populations in the prevention or cure of certain autoimmune conditions (Nouri-Aria, 2009). It is to be noted that, in the Treg adoptive transfer experiments, only the transfer with antigen-specific Tregs showed inhibitory effects on AR response while the non-specific Treg transfer did not show any suppressing effect on AR response.

SIT is a common remedy using in the treatment of allergic diseases. The therapeutic effect of SIT is supposed to be further improved. The present data show that using the specific antigen OVA-containing NPV did not inhibit the AR response in the AR mice. Instead, treatment with NPVom showed a satisfactory inhibitory effect on AR symptoms of the AR mice. The results suggest that the NPVom may have the therapeutic potential for the treatment of allergic diseases.

On the other hand, some investigators suggest that mice are not a good model of human airway disease such as asthma (Persson, 2002). In fact, no model can hundred percent mimic human disease when we have sufficient indisputable knowledge about the human disease itself. Although the murine model we used in the present study mimicked most of the allergic symptoms of human allergic rhinitis, we do think a better animal model can be developed in the future to be used in the study of allergic rhinitis.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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