

Derp1-modified dendritic cells attenuate allergic inflammation by regulating the development of T helper type1(Th1)/Th2 cells and regulatory T cells in a murine model of allergic rhinitis

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ABSTRACT

The CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) are known to regulate Th2-induced allergic rhinitis (AR). In this study, we evaluated the efficacy of *Derp1*-modified dendritic cells (DCs) in AR immunotherapy. *Derp1* was synthesized and transfected into DCs to generate *Derp1*-modified DCs. Phenotypes of *Derp1*-modified DCs were analyzed with flow cytometry using antibodies against DC markers CD11c, CD11b, CD59, CD103 and Toll-like receptor 1 (TLR1). Four groups of subject mice were formed; the controls were treated with immature DCs, while the AR mice models were sensitized with Derp1(AR) and treated with DCs(DC-AR) or *Derp1*-modified DCs (*Derp1*DC-AR). The frequency of sneezing and scratching, eosinophil cell count, and Th1/Th2 ratio in the spleen were measured for all groups. The percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in peripheral blood mononuclear cells was measured using flow cytometry; serum IgE, IgG1, and histamine were measured using enzyme-linked immunosorbent assay; expression levels of transcription factors T-bet, GATA3, Foxp3⁺ and IL-10 were analyzed using reverse transcription-polymerase chain reaction, and Western blot used in analyzed expression of Foxp3⁺ and IL-10 in nasal mucosa. Treatment with *Derp1*-modified DCs ameliorated the allergic response. The *Derp1*DC-AR group had significantly lower eosinophil cell count and the IgE, IgG1, and histamine levels than the AR and DC-AR groups, and higher mRNA levels of Th1 transcription factors T-bet, IL-10 and Foxp3 in nasal mucosa than DC-AR mice, but Th2 transcription factors GATA3 mRNA expression level has the opposite results. Furthermore, the Th1/Th2 ratio and percentage of CD4⁺CD25⁺Foxp3⁺ Tregs was significantly lower in the AR group ($p < 0.05$), but higher in the *Derp1*DC-AR group than in the control group ($p < 0.01$). Thus, the *Derp1*-modified DCs increased the percentage of CD4⁺CD25⁺Foxp3⁺Tregs and influenced the balance of Th1/Th2, showing an immunotherapeutic effect against AR.

1. Introduction

Allergic rhinitis (AR) is characterized by elevated serum immunoglobulin E (IgE), eosinophilic cell infiltration, and mucus hypersecretion (Galli et al., 2008). Numerous studies have reported that AR is associated with T-helper type 1 (Th1)/Th2 imbalance and Th2 polarization. It has also been reported that CD4⁺Th2 cells and their cytokines (interleukin [IL]-4, IL-5, and IL-13) are responsible for initiating and maintaining allergic disorders.

The CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) are known to play important roles in the regulation of Th2-induced allergic responses (Levy et al., 1996; Sandrini et al., 2015). Tregs that express the fork

head/winged helix transcription factor (Foxp3) are known to have an anti-inflammatory role and to maintain tolerance to self-components by direct contact with cells or by releasing anti-inflammatory cytokines such as IL-10 (Hsu et al., 2010). It has been reported that the levels of CD4⁺CD25⁺Foxp3⁺Tregs were significantly low in pediatric patients with AR and bronchial asthma (Lee et al., 2007; Shaoqing et al., 2016). Several studies in the past have also shown that Tregs can influence allergic responses in asthma and AR by altering the Th1/Th2 balance (Elkord, 2006).

Specific immunotherapy (SIT) has been shown to be an effective treatment with the potential to provide long-term post treatment benefits and alter the natural course of allergic disease such as AR and

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asthma. Currently, Okamoto et al. has found that the house dust mite (HDM) sublingual immunotherapy (SLIT) tablet has been proved effective and safe for patients with AR (Okubo et al., 2016; Okamoto et al., 2017). Although convenient for operation, immunotherapy is also invasive, and the absorption of HDM vaccines might be so low that the treatment may not be efficient enough. Thus, novel therapies with fewer side effects and the ability to provide long-term immunity are required (Lee et al., 2007). In recent years, dendritic cells (DCs), being dedicated antigen-presenting cells, have been considered as a tool for immunotherapy. For instance, immunotherapy with genetically modified DCs might prove to be useful for protection against allergic airway inflammation and is a potential therapeutic approach for allergic disease (Hsu et al., 2010). Although DC vaccines have been used in asthma research (Qu et al., 2013), there are still no reports about the efficacy of DC vaccines against AR.

HDM are major indoor allergens worldwide and can trigger perennial AR and asthma. *Dermatophagoides pteronyssinus* (Der p1), an allergen from the HDM, is one of the most well-studied and characterized allergens (HuangFu et al., 2006). SLIT with Der p1 is currently recognized as an effective therapy to ameliorate allergic symptoms (Cosmi et al., 2006). Based on this, we hypothesized that immunotherapy with *Derp1*-modified DCs would enhance the antigen presentation. To investigate the therapeutic effect of *Derp1*-modified DC vaccines, the effect of this vaccine on the Th1/Th2 balance and the changes in Tregs were evaluated in a mouse model. Through this study, we aimed to elucidate the underlying mechanisms of *Derp1* DC therapy in allergic disease.

2. Materials and methods

2.1. Material

The following materials were used in this study: pcDNA3.1(+) (Invitrogen Inc, USA), BamHI, NheI, GeneRuler DNA Ladder (Fermentas Inc, Canada), T4 ligase (NEB Inc, USA), MISSION™ Lentiviral Packaging Mix (Invitrogen Inc, USA), Der p1 protein (ProSpec Inc, Israel), and male BALB/c mice (SLACCAS Inc. China). All experimental procedures involving animals were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Tongji University Institutional Animal Care and Use Committee.

2.2. Construction, amplification, purification, and titration of lentivirus-based vector expressing Der p1

The *Derp1* from the dust mite was synthesized through gene synthesis, digested with NheI/BamHI, and inserted into the plasmid pcDNA3.1(+). The gene encoding the green fluorescence protein (GFP) was amplified from pEGFP using PCR. This gene was also digested with BamHI/XhoI and cloned into pcDNA3.1(+)-*Derp1*. *E. coli* bacteria were then transformed with these plasmids, which were then isolated and verified through DNA sequencing.

The gene encoding *Derp1*-GFP was amplified using PCR with the template of pcDNA3.1(+)-*Derp1*-GFP. This PCR product was digested and cloned into a lentivirus vector, pLenti6.3-MCS/V5 DEST. This vector plasmid, which contained Der p1-GFP, was transfected into 293T with a MOI of 100:1. Forty-eight hours after transfection, the constructed viral vector was harvested by multiple freeze–thaw cycles and purified by polyethylene glycol. The vector was titrated with TCID50 (tissue culture infective dose 50).

2.3. Isolation and primary culture of mouse DCs

The healthy BALB/c nude mice were decapitated, and the femur and tibia were extracted by a sterile operation. The remaining tissues were cleaned off and each end of the bone was cut off. A sterile syringe was used to fill the bone with RPMI1640 and the bone marrow was collected

by centrifugation. The enriched monocytes were suspended in RPMI1640 (with 10% FBS, 10 µg/L GM-CSF, and 3 µg/L IL-4) and incubated at 37 °C with 5% CO₂ for 24 h. After gently blowing away to retain only the adherent cells, cells were cultured in fresh complete medium with rm GM-CSF and IL-4 for 5 days.

2.4. Transfection of lenti-*Derp1*-GFP into DCs

On the 5th day, the immature DCs were collected. The lenti-*Derp1*-GFP was transfected into DCs with MOI of 100:1. Five hours post-transfection, the medium was replaced with RPMI1640 with 20% FBS, 10 µg/L GM-CSF, and 3 µg/L IL-4. After 48 h, the DCs were collected and observed under a microscope for evaluating the transfection efficiency. By estimating the amount of green fluorescence emitted from the cells, the transfection efficiency of the *Derp1*-modified DC vaccine was determined. On the 10th day, phenotypes of mature DCs were analyzed with flow cytometry using antibodies against the DC markers CD11c, CD11b, CD59/Ly6c, CD103 and Toll-like receptor 1 (TLR1) (eBioscience Inc. USA), and then conjugated with secondary antibodies labeled Alexa Fluor488 (Bioss Inc. Beijing, China). Cell surface staining was performed per standard flow cytometry procedures.

2.5. Immunofluorescence staining of DCs

DCs were collected with centrifuge (1000 rpm for 5 min) and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature (RT). Then, the cells were permeabilized with 0.2% Triton X-100 in PBS for 20 min at RT and blocked with 5% BSA in PBS for at least an hour at RT. Rabbit polyclonal primary antibody was diluted 200-fold by PBS, and incubated with the cells overnight at 4 °C followed by washing PBS 5 times. The cells were then further incubated with mouse anti-rabbit IgG/Cy3 secondary antibody at RT for an hour. Finally, the cells were washed, had their nucleus stained with DAPI (Vectashield), and dropped on the slides for microscopic observation. The primary antibody against B7-H1 and B7-H2 and the secondary antibody were all purchased from Bioss Inc (Beijing, China). The slides were examined with a LSM780 laser-scanning microscope (Nikon C2 Plus. Japan). Typically, 100 cells were examined in each experiment. Representative images were collected and processed using Image J.

2.6. Sensitization and treatment of the AR mice

Twenty-four 5-week old BALB/c mice, each weighing around 20–30 g, were used as experimental animals. The mice were randomly divided into 4 groups, each with 6 mice. Purified natural Der p1 allergens (Indoor Biotechnologies Inc. USA) had been dissolved in PBS and stored in a 4 °C refrigerator. Animal models of AR were prepared according to the method described by Mo et al. (2006). Mice in all the groups, except the control group, were sensitized with 1.5 µg of Der p1 in PBS by subcutaneous injection on days 1, 3, 5, 8, and 10; by intraperitoneal injection on days 12, 15, and 17; and by nasal inhalation on days 19, 22, and 24. The mice in the control group were sensitized with PBS instead of Der p1. On day 24, all the mice were challenged with Der p1 via intranasal instillation, and the nasal symptoms, nose rubbing, and sneezing were observed for 6 consecutive days.

The injections were performed in the four groups as follows: (1) for group A (negative control group), 100 µl of PBS; (2) for group B (AR group), the untreated AR model; (3) for group C (DC-AR group), AR model treated with 5×10^6 units of DC by intraperitoneal injection; and (4) for group D (*Derp1*DC-AR group), AR model treated with 5×10^6 units of *Derp1*-modified DCs.

All mice received the treatments twice a week, for 4 weeks. Two hours after the last nasal instillation of the allergen or the modified DC was administered, the frequency of nasal scratching and sneezing were estimated for 10 min.

2.7. Enzyme-linked immunosorbent assay (ELISA) for detecting histamine, IgG1, and IgE in mouse serum

All mice were sacrificed 2 h after the final treatment. The serum was isolated from a blood sample by eyeball extirpation and stored at -80°C . Total IgG1 (Thermo Scientific Pierce Inc. USA), IgE, and histamine (Market Design Inc. USA) levels were measured using ELISA, according to the manufacturer's instructions.

2.8. Eosinophil cell count

Blood samples were obtained and were allowed to clot for 30 min at 37°C . The total counts were performed, and the blood smears were used for estimating the differential counts (Liu's stain), where a minimum of 100 cells were counted and classified as eosinophil cells, based on standard morphological criteria.

2.9. Assay of the Th1/Th2 ratio

The spleen was removed from each sacrificed mouse and placed in a tube containing the RPMI1640 medium. A single cell suspension was prepared by disrupting the spleen using a cell disrupter (Beckman Coulter, Inc.). The cells were then equally distributed in the tubes and washed once with PBS. The cells were then collected, centrifuged to remove the supernatant, mixed to an even state, and then incubated in the dark at 20°C for 30 min to generate rat anti-mouse CD4-FITC (BD Biosciences Inc, USA) antibodies. The samples were washed twice with PBS, centrifuged to remove the supernatant, mixed to an even state, and then incubated in the dark at room temperature for 30 min with $10\text{ }\mu\text{g/ml}$ of rat anti-mouse IL-4-APC (BD Biosciences, USA) and $10\text{ }\mu\text{g/ml}$ of rat anti-mouse IFN- γ -PerCPcy5.5 (BD Biosciences, USA). The samples were again washed twice with PBS and adjusted to a concentration of 1×10^6 cells/ml. T lymphocyte cell antibodies against the CD4 antigen (anti-CD4-FITC, Becton Dickinson Immunocytometry Systems) was used to set a gate for the Th cells, and anti-IL-4 and anti-IFN- γ antigen antibodies (Becton Dickinson Immunocytometry Systems) were used to distinguish between Th1 and Th2 cells. The percentage of Th1 and Th2 cells and the ratio of Th1/Th2 were calculated.

2.10. Assay of the percentage of CD4⁺CD25⁺Foxp3⁺Treg

To detect CD4⁺CD25⁺Foxp3⁺Treg cells, peripheral blood mononuclear cells were isolated from fresh blood and stained with immunofluorescent antibodies against Treg surface markers. Cells were incubated in the dark at room temperature for 30 min with the rat anti-mouse CD4-FITC (BD Biosciences Inc.USA) and CD25-PE (BD Biosciences Inc.USA) antibodies. Samples were washed twice to remove the supernatant, fixed with a fixative for 30 min, and then washed once with PBS. A permeation fluid was applied for 5 min, and the samples were incubated in the dark at room temperature for 30 min with the anti-mouse Foxp3-PE-Cy5 (BD Biosciences Inc.USA) antibody. FACS was used to detect the labeled cells. The percentage of CD4⁺CD25⁺Foxp3⁺Tregs in CD4⁺ cells was calculated according to the formula: (Heuer et al., 2005; Marazuela et al., 2006)

$$\text{Percentage of CD4}^+\text{CD25}^+\text{Foxp3}^+\text{ Treg} = \frac{\text{CD4}^+\text{CD25}^+\text{Foxp3}^+}{\text{CD4}^+} \times 100\%.$$

2.11. Quantitative PCR of transcription factors T-bet, GATA3, Foxp3+ and IL-10

Fluorescent quantitative reverse transcription-PCR (qRT-PCR) was performed to determine the expression levels of IL-10 and Foxp3+ mRNAs in the nasal mucosa. The total RNA was extracted and cDNA was synthesized using a cDNA synthesis kit (PrimeScript RTase, TaKaRa

Table 1

Pairs of primers used for quantitative reverse transcription-polymerase chain reaction.

Gene	Sequences (5' → 3')	Product length (bp)
GAPDH	F- GGCAAAITCAACGGCACAGT R-CTCGTGGTTCACCCATCA	255
T-Bet	F-AACCAGTATCCTGGTCCAGC R-TGTGCGCCACTGGAAGGATAG	439
GATA-3	F-GAAGGCATCCAGACCCGAAAC R-ACCCATGGCGGTGACCATGC	255
IL-10	F-CAGTACAGCCGGGAAGACAA R-CCTGGGGCATCACTTCTACC	229
FOXP3	F- GCGCCCTCTGGACAGC R- TTCTTTCTGGGCACACTGG	181
GFP	F-TTCGGGATCCGTGAGCAAGGGCGAGGAGC R-ATTACTCGAGTTACTTGTACAGCTCGTCCATGCC	737

Inc.). The mRNA of the transcription factors GATA3, T-bet, Foxp3+ and cytokines IL-10 were analyzed using the SYBR Green detection system. The primer sequences used are listed in Table 1. The mRNA levels were normalized relative to the mRNA levels of GAPDH.

2.12. Western blot for analysis of IL-10 and Foxp3⁺

The total protein content of IL-10 and Foxp3⁺ was measured using a Bradford assay. Equal amounts of the protein were electrophoresed on a 10%-SDS gel and then transferred onto a PVDF membrane. The membranes were blocked, incubated for 4 h with primary anti-IL-10 and Foxp3⁺ antibodies at 1:1000 (Santa Cruz Inc. USA) at room temperature, washed, and then incubated with secondary antibodies for 2 h at 4°C . The blots were visualized using chemiluminescence detection reagents. GAPDH was used as an endogenous control for protein normalization. The experiment was performed in duplicate.

2.13. Statistical analysis

All statistical analyses were performed using the SPSS 19.0 software package. Student's *t*-test and one-way ANOVA were used to determine the differences between the mean values. The differences were considered significant if $p < 0.05$. The data was shown as mean $\pm$ SD in all indices.

3. Results

3.1. The successful expression of Derp1 in DC vaccine

Upon agarose gel electrophoresis and double enzymatic digestion, pcDNA3.1(+)-Der p1 showed two bands at around 1700 and 5000 bp (Fig. 1A). This GFP gene was amplified by polymerase chain reaction (PCR) and ligated into pcDNA3.1(+)-Der p1. This was then transfected into *E. coli* and then isolated. The fused product of Der p1 and GFP was then subjected to PCR. Fig. 1B shows the obtained band at around 4000 bp. Sequence alignment by BLAST showed that Der p1 and the GFP gene were successfully cloned into pcDNA3.1(+).

Lentiviral vectors carrying Derp1-GFP and GFP were then constructed with viral titers of 1.9×10^8 TU/mL and 2×10^8 TU/mL, respectively. After a 48-h infection on 293T cells, the expression of GFP was observed in cells infected with lenti-Derp1-GFP and lenti-GFP, but not in the control cells, which indicated that 293T cells could efficiently express Der p1.

DCs were then infected with these recombinant viruses to generate the genetically modified DCs, which were observed under a fluorescence microscope. As shown in Fig. 1C and D, green fluorescence was observed in these DCs, which indicated the successful expression of Der p1 in DCs.

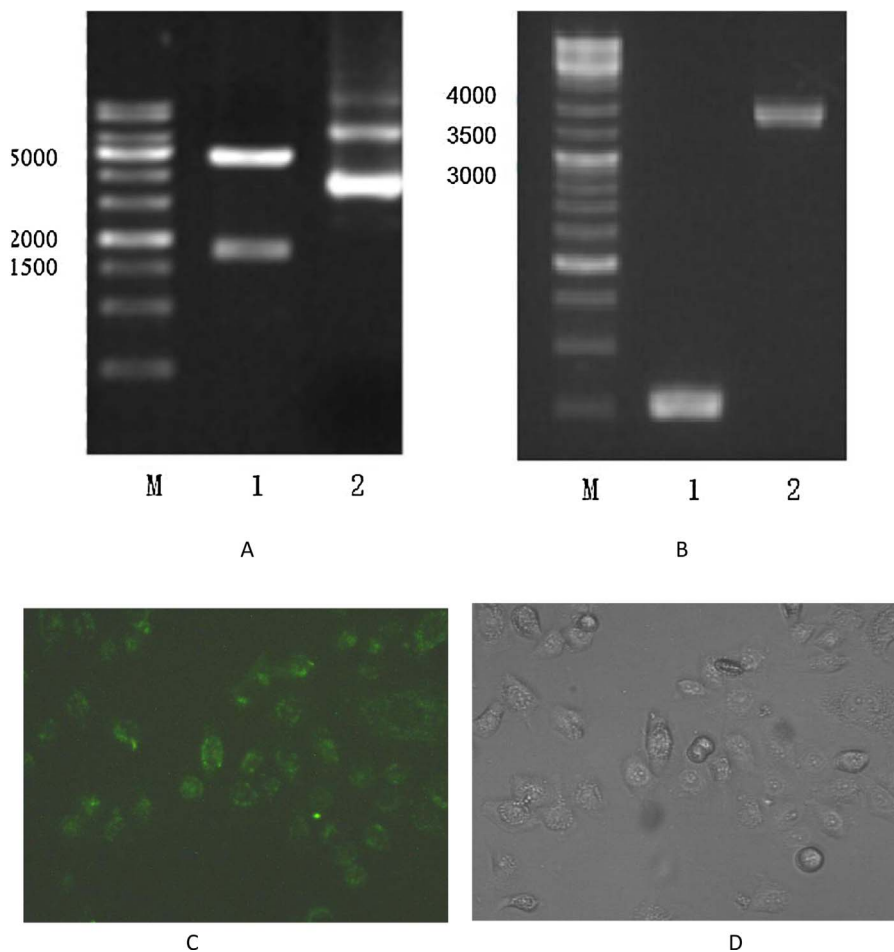


Fig. 1. Cloning and characterization of the *Derp1* and the fused product of *Der p1* and GFP. (A) Restriction enzymatic digestion results of pcDNA3.1(+)-*Der p1* (M: DNA Marker; 1: pcDNA3.1(+)-*Der p1*; 2: undigested pcDNA3.1(+)-*Der p1*); (B) polymerase chain reaction of the fused product of *Der p1* and GFP (M: DNA Marker; 1: pcDNA3.1(+)-*Der p1*; 2: pcDNA3.1(+)-*Der p1*-GFP). GFP expression by *Der p1* modified DCs in fluorescence (C) and bright light microscopy (D) showed the successful infection of Lenti-*Derp1*-GFP into DC. (original magnification: $\times 40$).

3.2. Characteristics of DC vaccine

DCs were studied using monoclonal antibodies against maturation markers CD11c, CD11b, CD59/Ly6c, CD103 and TLR1 (Fig. 2A). imDCs and DCs infected with lenti-GFP expressed low amounts of maturation markers. In the case of *Derp1*-modified DCs, an increase in expression levels of five maturation markers CD11c, CD11b, CD59/Ly6c, CD103 and TLR1 was observed. Maturation markers B7-H1 and B7-H2 were also used in studying DC phenotype. DCs infected with lenti-GFP expressed low amounts of maturation markers and their phenotypes were close to immature. Plenty of expressions of two maturation markers B7-H1 (Fig. 2B) and B7-H2 (Fig. 2C) were observed localized in the DC cytoplasm in the *Derp1*-modified DCs. All these indicated that DCs stimulated by lenti-*Derp1*-GFP expressed some maturation markers and most probably corresponded to matured DCs.

3.3. Behavioral observation of AR mouse models

The frequency of sneezing and nose rubbing in the sensitized AR group were found to be significantly higher than in the non-sensitized group ($p < 0.01$), and there was no significant difference between the DC-treated group and the AR group ($p > 0.05$). The frequency of sneezing and nose rubbing were significantly decreased in the *Derp1*DC group (Fig. 3A) ($p < 0.05$), indicating that *Derp1*-modified DCs could relieve symptoms of AR inflammation.

3.4. Eosinophil cell count in blood and histamine, IgG1, and IgE levels in serum

The eosinophil cell count in the circulating blood was significantly

higher in the AR group (42.6 ± 8.4) than in the control group (20.3 ± 3.6). The count was found to be decreased significantly in the *Derp1*DC-AR group (28.1 ± 8.8), compared to the AR group ($p < 0.05$), while no significant changes were found between the AR and DC-AR groups (43.5 ± 13.4 , $p > 0.05$) (Fig. 3B). The histamine, IgG1, and IgE levels in serum were higher in the AR group than in the control group ($p < 0.05$), and lower in the *Derp1*DC-AR group than in the AR group (Fig. 3C and D). This result indicated that *Derp1*-modified DCs could alleviate allergic inflammation of mice.

3.5. Observation of percentage of Th1 and Th2 cells and Th1/Th2 ratio

The percentage of Th1 and Th2 cells was higher in the AR group than in the control group ($p < 0.05$), while it was lower in the *Derp1*DC-AR group than in the control group (Fig. 4A). The Th1/Th2 ratio in the spleen was significantly lower in the AR group than in the control group (0.40 ± 0.18 vs. 0.60 ± 0.14 , $p < 0.05$), and the mice in the AR group had significantly higher percentages of Th2 (as shown in Fig. 4B and C). In addition, the Th1/Th2 ratio in the *Derp1*DC-AR group was 0.64 ± 0.18 , which was significantly higher than that in the AR group ($p < 0.05$), but there was no significant difference between the Th1/Th2 ratios in the AR and DC-AR groups (0.39 ± 0.15 , $P > 0.05$). Thus, the *Derp1*-modified DCs increased the percentages of both Th1 and Th2 cells, but more Th1 cells were increased and that causing Th1 polarization leads to Th1/Th2 ratio increased.

3.6. *Derp1*-modified DCs increased percentage of $CD4^+ CD25^+ Foxp3^+$ Tregs

Fig. 5A shows the individual cells that were stained positive as

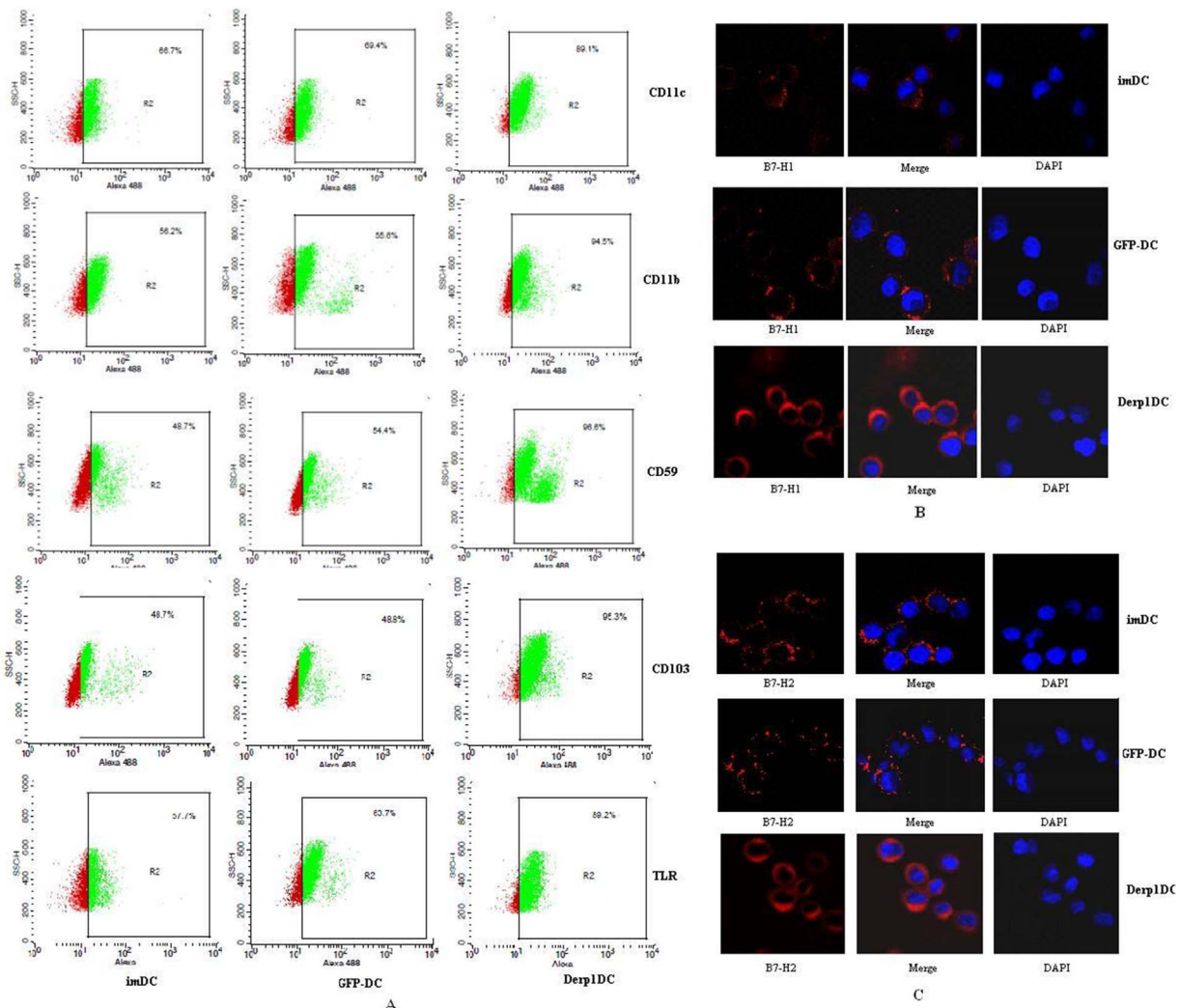


Fig. 2. Characteristics of *Derp1*-modified DC. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
 A. Surface expression of CD11c, CD11b, CD59/Ly6c, CD103 and TLR1 by DCs after transfection with lenti-GFP and lenti-*Derp1*. DCs without any stimulation served as negative controls. DCs were stained with Alexa Fluor488 and were analyzed by flow cytometry. Ten thousand cells were counted for each sample. Data are presented as percentage of fluorescent cells of whole population of DCs. Immune staining of B7-H1 (B) and B7-H2(C) expression in DCs. Top: DCs without any stimulation; middle: DCs transfection with lenti-GFP; bottom: DCs transfection with lenti-*Derp1*. Left: B7-H1 or H2; right: 4',6-diamidino-2-phenylindole (DAPI); middle: merged DAPI + B7-H1 or H2. B7-H1 and B7-H2 were observed expression in the DC cytoplasm in the *Derp1*-modified DCs (red). (Original magnification $\times 400$)

CD4⁺CD25⁺Foxp3⁺ Tregs. The results of the flow cytometric analysis of the CD4⁺CD25⁺Foxp3⁺ Tregs from the different groups are illustrated in Fig. 5B. The percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in the AR group ($0.54 \pm 0.14\%$) was significantly lower than that in the control group ($7.25 \pm 1.93\%$, $p < 0.01$), while that in the *Derp1*DC-AR group was much higher ($5.22 \pm 1.53\%$) than that in the AR group ($p < 0.01$). Additionally, there were no significant differences between the percentages of CD4⁺CD25⁺Foxp3⁺ Tregs in the DC-AR ($0.47 \pm 0.14\%$) and AR groups ($p > 0.05$). These results indicated that *Derp1*-modified DCs could influence the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in mice with AR.

3.7. Th1/Th2-transcription factors and related cytokine IL-10 expression in nasal mucosa

The expression levels of key transcription factors for Th1 (T-bet), Th2 (GATA3), and Treg (Foxp3) were directly analyzed on tissue biopsies by means of qRT-PCR. This revealed that GATA3 mRNA expression level was higher in AR group compared with control group ($p < 0.05$), but decreased significantly in *Derp1*DC-AR group compared with AR group ($p < 0.05$) and DC-AR group ($p < 0.05$)

(Fig. 4D). The expression of T-bet, Foxp3 and IL-10 was significantly lower in AR group compared with control group ($p < 0.05$). However, in *Derp1*DC-AR group, T-bet, Foxp3 and IL-10 mRNA expressions were higher compared with AR ($p < 0.05$) and DC-AR group ($p < 0.05$) (Figs. 4 D, 6 A and B). In addition, there is no significant mRNA difference between AR group and DC-AR group ($p > 0.05$).

3.8. Expression levels of IL-10 and Foxp3 protein in nasal mucosa

As shown in Fig. 6C and D, the expression levels of IL-10 and Foxp3 protein in the nasal mucosa were significantly decreased in the AR group, compared to the control group ($p < 0.05$). In addition, no significant difference in these levels was found between the AR and DC-AR groups ($p > 0.05$). However, the protein expression levels of IL-10 and Foxp3 in the *Derp1*DC-AR group was significantly higher than in the AR group ($p < 0.05$). These results confirmed that *Derp1*-modified DCs could up-regulate the IL-10 and Foxp3 expression during allergic inflammation.

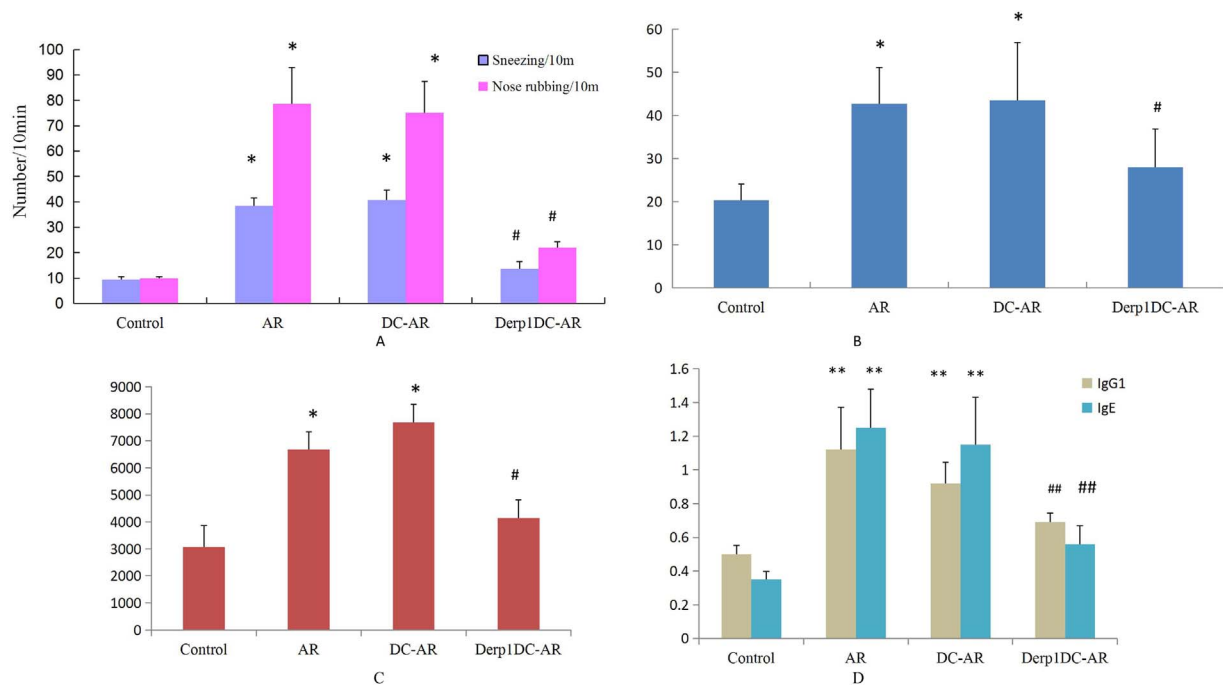


Fig. 3. Symptoms and inflammatory indicators of AR in the different groups A. Sneezing and nose rubbing. B. Eosinophil cell count in circulating blood. C. Serum histamine, D. IgG1, and IgE levels. Each column and vertical bar represents the mean $\pm$ S.D. *, **: Significantly different from the control group ($p < 0.05$ and $p < 0.01$, respectively); #, ##: Significantly different from the AR group ($p < 0.05$ and $p < 0.01$, respectively).

4. Discussion

This is the first research to investigate the therapeutic efficacies of *Derp1*-modified DCs on AR by regulating the development of Th1/Th2 cells and Tregs in a mouse model. In this study, a *Derp1*-modified DC vaccine was successfully constructed. As a result, the *Derp1*-modified DCs could relieve nasal symptoms of AR inflammation such as nose rubbing and sneezing, and ultimately lead to Th1 polarization. Our research also indicated that *Derp1*-modified DCs could influence the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in mice with AR, and up-regulate the IL-10 and Foxp3 expression during allergic inflammation.

Although the etiology of AR is not fully understood, it has become clear that DCs have an important role, both in the sensitization phase and in the maintenance of the disease. DCs play an important role as primary antigen-presenting cells by initiating and maintaining T-cell responses. In this study, we designed an *in vivo* model to estimate the effect of a therapeutic vaccine based on *Derp1*-modified DCs against AR. The results of our study showed that genetically modified DCs could induce immune responses and inhibit the production of serum levels of histamine, IgE, IgG1 and eosinophils in the circulating blood in mice with AR. Suppression of IgE synthesis has been associated with elevated levels of Th1-derived cytokine, IFN- γ . Unlike human serum, IgG1 plays a major role in promoting inflammation in the mouse serum, and its synthesis is known to be regulated by Th2 cytokines. Thus, genetically modified DCs could alleviate allergic inflammation in mice with AR.

Today, AR has become a serious health problem worldwide. Although immune therapies have been widely used to treat AR, these treatments have low efficiency and take a high amount of time; it is therefore difficult to achieve the desired therapeutic effect because of poor patient compliance. Recently, DNA-based immunization was found to be effective for modulating allergen-specific IgE responses and may provide a novel therapeutic approach against allergic diseases (Lombardi et al., 2010). Although DNA vaccines show great promise for preventing and treating allergic disease in mouse models (Ou et al., 2014), the clinical potency is limited, particularly because of a lack of efficient antigen presentation.

Recently, some researchers have confirmed the therapeutic effect of

DC vaccines modified by transfection with allergens on allergic diseases of the respiratory pathway, just as asthma (Rohrbach et al., 2005; Rolland et al., 2000; Knutson and Disis, 2005). Targeting antigens using DCs could also improve the efficacy of allergen presentation (Cusi et al., 2004; Ruffini et al., 2004), and because of this, DC vaccination has sparked considerable interest. To design more effective DC vaccines, the viral gene transfer methods were given priority for their generally highly efficient gene transferring abilities. In contrast to the non-viral gene delivery systems, the viral gene transfer transduced DCs elicited immune responses more efficiently than the peptide-pulsed DCs (Arthur et al., 1997). The lentivirus-based vector was chosen as the gene transfer tool in our research due to its advantages of having high titers, stable transgenic expression, high transduction efficiencies and improved safety (Kikuchi, 2006).

Der p1 is a cysteine protease, and its full-length cDNA encodes a precursor of 320 amino acid residues, which comprises a signal peptide (18 amino acids), a proenzyme region (80 amino acids), and a mature enzyme (222 amino acids) (Chua et al., 1988). Der p1 has been shown to have sequence homology with different variants of the dust mite allergen, and vaccination against Der p1 may provide protection against allergy to other antigens such as Der p2 or Der f1, and even the whole dust mite as well. Based on these observations, we decided to clone a codon-optimized cDNA that encodes only the mature Der p1 to design a safe Der p1-based DNA vaccine with hypoallergenic properties. We then transferred this cDNA into DCs to generate a genetically modified DC vaccine. Our results showed that Der p1 could be intracellularly expressed in DCs. Treatment with this vaccine drastically reduced IgE levels and ameliorated allergic reactions in mice with AR.

In our research, five maturation markers CD11c, CD11b, CD59/Ly6c, CD103 and TLR1 were observed in high expressions in *Derp1*-modified DCs. Under inflammatory conditions, DCs recruited to the conducting airways parenchyma as exposure to allergens is called inflammatory DCs. Inflammatory DCs are classically defined as CD11b⁺CD11c⁺ and Ly6C⁺ cells, and are the only DC subtype that expresses the high-affinity receptor for IgE (the Fc ϵ R1) in mice (Froidure et al., 2016; Lambrecht and Hammad, 2012). Additionally, CD103(+) DCs were found to be the prime Th2 responses to inhaled allergens, and

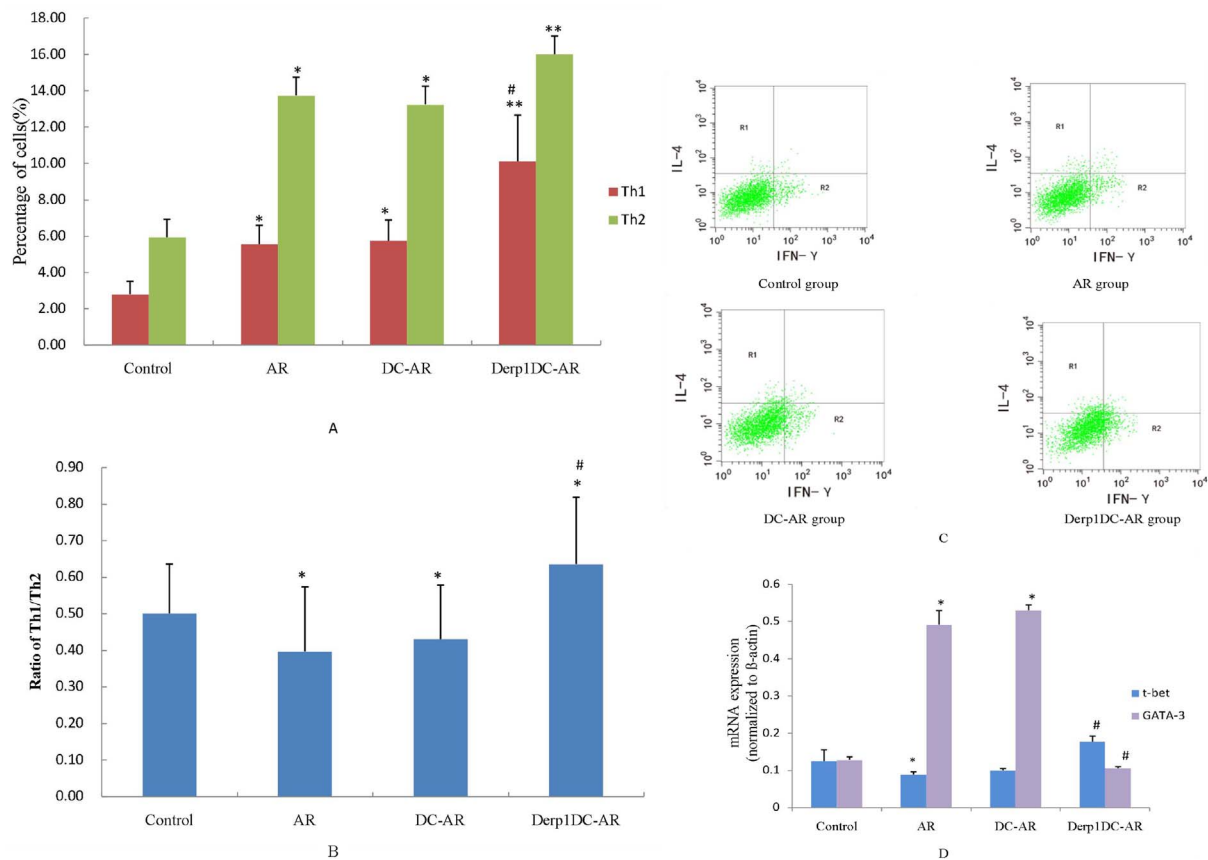


Fig. 4. Assay of the Th1 and Th2 cells in spleen and expression of T-bet and GATA-3 mRNA in nasal mucosa of the different groups. (A) Percentage of Th1 and Th2 cells in the different groups. (B) Ratio of Th1/Th2. (C) Percentage of Th1 and Th2 cells of the different groups, measured by flow cytometry. T lymphocyte cell antibody against CD4 antigen was used to set a gate for Th cells, and anti-IL-4 and anti-IFN- γ antigen antibodies were used to distinguish between Th1 and Th2 cells. A live gate was set using forward and sideways scatter characteristics, and the ratio of Th1 and Th2 cells was determined. (D) There was an decrease in mRNA expression of T-bet in AR group compared with controls and an increase expression in Derp1DC-AR group compared with AR and DC-AR group. Expression of GATA-3 mRNA increased in AR group, compared with the control, and they decreased in the Derp1DC-AR group. Each column and vertical bar represents the mean $\pm$ S.D. *, **: Significantly different from the control group ($p < 0.05$ and $p < 0.01$, respectively); #, ##: Significantly different from the AR group ($p < 0.05$ and $p < 0.01$, respectively).

TLR1 expression on DCs has been shown to induce maturation of DCs in influencing immune responses (Nakano et al., 2012; Dearman et al., 2009). All these indicated that Derp1-modified DCs were mature and have the priming potentials for divergent immune responses. Key costimulatory molecules B7-H1 and B7-H2 (also called CD80 and CD86) are proteins found on dendritic cells, activated B cells, and monocytes that provides a costimulatory signal necessary for T cell activation and survival. B7-H1 works in tandem with B7-H2 to prime T cells (Csillag et al., 2010). The expression of B7-H1 and B7-H2 in Derp1-modified DCs suggests that lenti-Derp1 can successfully promote DCs maturation and differentiation and prepare for further immune responses.

The imbalance of Th1/Th2 cells, especially Th2 polarization, has long been known to be a characteristic of AR. Anti-inflammatory factors IFN- γ and IL-4 have been used in cell transformation between Th1 and Th2 (Webster and Sternberg, 2004). Our results also showed increased percentages of both Th1 and Th2 cells in the spleens of mice treated with Derp1-modified DCs. These results demonstrated that the production of Th1-type cytokines, as well as the Th1/Th2 ratio, was significantly increased in the Derp1DC-AR group, compared to the DC-AR group. An increased percentage of Th2 cells was also observed in the Derp1DC-AR group; this maybe related to persistent allergic reaction in these mice. Taken together, our results indicated that treatment with Derp1-modified DCs influenced the Th1/Th2 balance and thus attenuated the allergic response.

In our research, expression of transcription factor T-bet and GATA-3 of nasal mucosa were studied. We report a decrease in mRNA expression of T-bet in AR group compared with controls and an increase

expression in Derp1DC-AR group compared with AR and DC-AR group. T-bet is a Th1-specific T-box transcription factor and expression correlates with IFN- γ expression in Th1 cells (Kanhere et al., 2012; Tantisira et al., 2004). Expression of GATA-3 is higher in AR group compared with controls and decrease significantly in Derp1DC-AR compared with AR and DC-AR group. GATA-3 has been shown to promote the secretion of IL-4, IL-5, and IL-13 from Th2 cells, and induces the differentiation of Th0 cells towards this Th2 cell subtype while suppressing their differentiation towards Th1 cells (Yagi et al., 2011). All these results indicated that Derp1-modified DCs reverse Th1/Th2 balance of AR through influence expression of Th1 cell-specific transcription factor T-bet and Th2 cell-specific transcription factor GATA-3 simultaneously.

Another important regulator of the immune system, Tregs, causes a major inhibitory effect on cellular immunity through a complicated cellular immune regulation network (Saito et al., 2008). CD4⁺CD25⁺Tregs play a critical role in regulating peripheral tolerance to self-antigens and preventing allergic diseases such as rhinitis, atopic dermatitis, and asthma. Tregs can exert their immunosuppressive functions on a broad range of cell types, including innate immune cells. There has also been some evidence to demonstrate that Tregs control the immediate hypersensitivity response by acting directly on mast cells and inhibiting their degranulation (Ling et al., 2004; Gri et al., 2008). After allergen sensitization, triggering mucosal mast cells through the high-affinity receptors for IgE could induce the release of preformed mediators and elicit an IgE-mediated hypersensitivity response (Galli and Tsai, 2012).

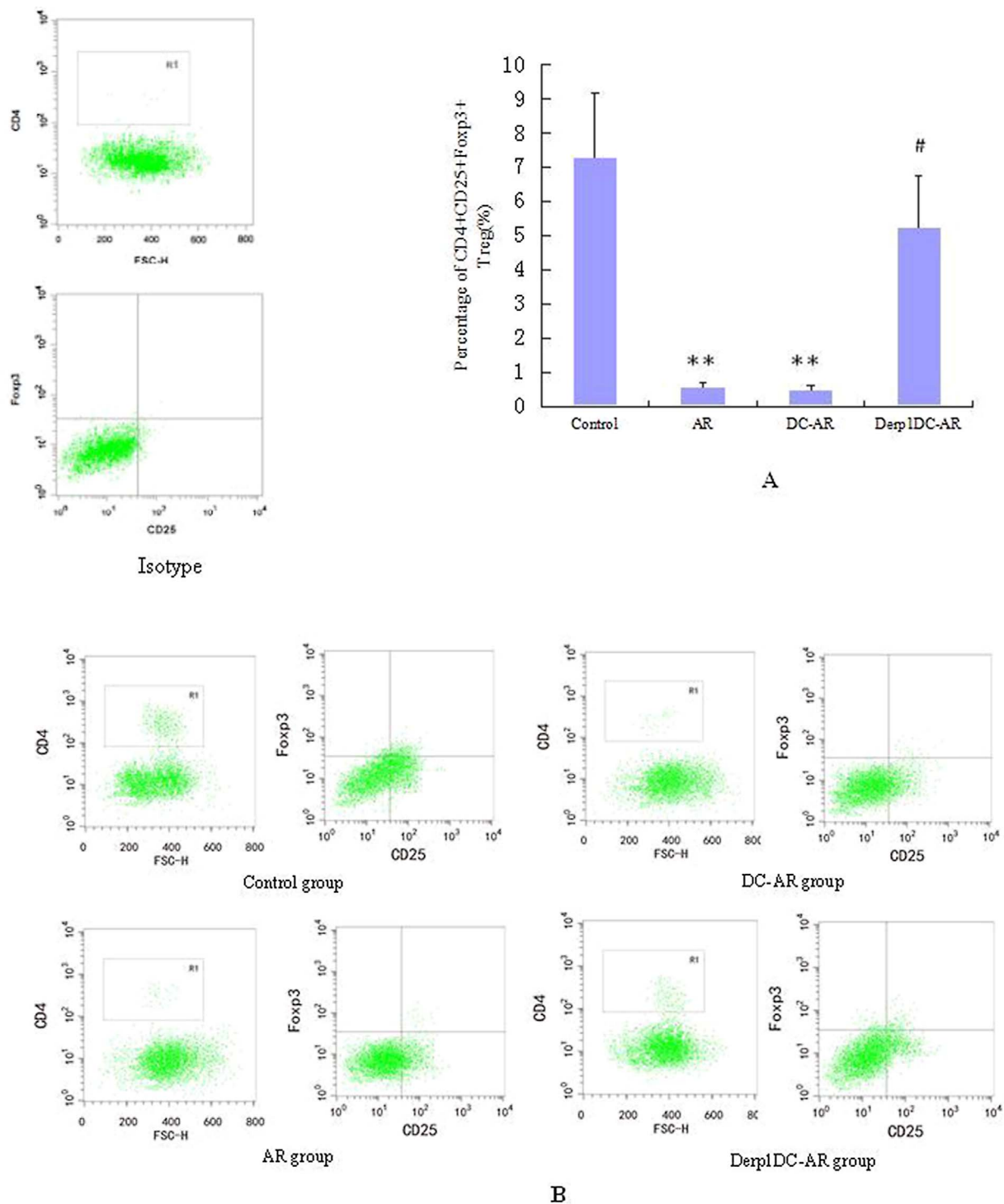


Fig. 5. The percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in the different groups. (A) Percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in the different groups. (B) Flow cytometric analysis of Tregs, Peripheral blood mononuclear cells were isolated from fresh blood and stained with immunofluorescent antibodies against Treg surface markers (PE-conjugated anti-CD25 and FITC-conjugated anti-CD4 antibodies and PE-Cy5-conjugated anti-Foxp3). Dots in the right upper quadrants show individual cells that stained positive for CD4⁺CD25⁺Foxp3⁺ Tregs. Each column and vertical bar represents the mean $\pm$ S.D. *, **: Significantly different from the control group ($p < 0.05$ and $p < 0.01$, respectively); #, ##: Significantly different from the AR group ($p < 0.05$ and $p < 0.01$, respectively).

Foxp3⁺Tregs are CD4⁺ T cells with immunosuppressive functions, and have been well studied. These cells, which have a strong suppressive function and are capable of suppressing the broadest spectrum of cells, can prevent auto reactive cell activation and inhibit autoimmune and allergic diseases, thereby playing an important role in maintaining autoimmune tolerance (Stelmaszczyk-Emmel et al., 2013). Several independent reports have suggested that the number and functions of

CD4⁺CD25⁺Foxp3⁺ Tregs are impaired or altered in patients with allergies (Ling et al., 2004; Karagiannidis et al., 2004). There has also been evidence to associate reduced frequency of Tregs with allergic diseases; (Lloyd and Hawrylowicz, 2009) a reduction in the absolute number or a defect in the function of CD4⁺CD25⁺Foxp3⁺Tregs in circulation and/or in the nasal mucosa of AR patients have also been reported (Lee et al., 2007; Shaoqing et al., 2016). Anti-allergic therapies

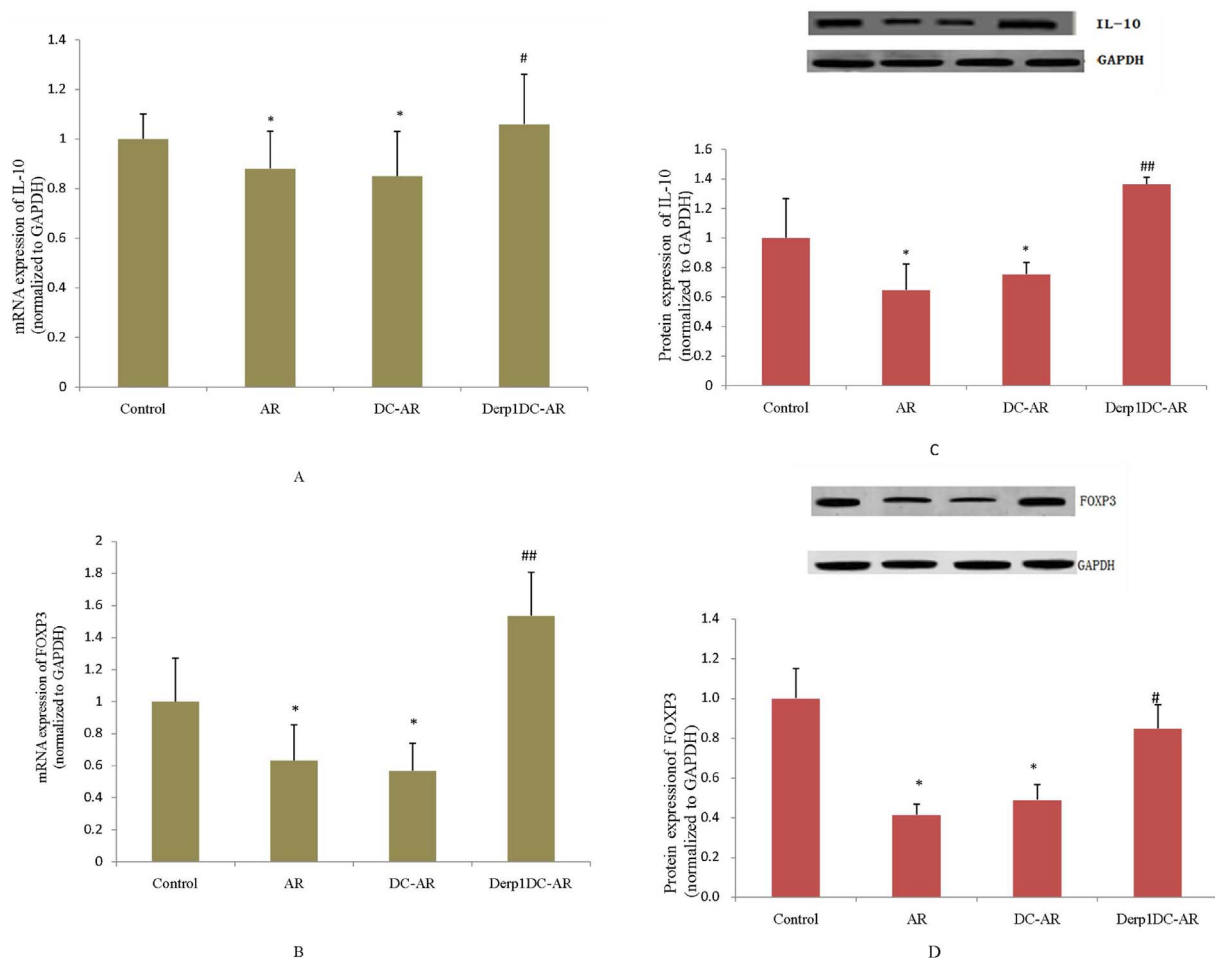


Fig. 6. Expression of IL-10 and Foxp3 mRNA and protein in nasal mucosa.

RT-PCR analysis showed that the relative mRNA expression of IL-10(A) and Foxp3(C). There was a decrease in IL-10 and Foxp3 mRNA in AR group, compared with the control, and they decreased in the Derp1DC-AR group (* $p < 0.05$ versus control, # $p < 0.05$ versus AR), whereas there was no change in GAPDH mRNA. Western blot analysis showed that the relative expression of IL-10(B) and Foxp3(D) proteins in the AR group was much higher than that in the control group, and that it was decreased in the Derp1DC-AR group, (* $p < 0.05$ versus control, # $p < 0.05$ versus AR). No significant difference in the protein expression was found between AR and DC-AR groups.

such as administration of glucocorticoids and special immune therapies have been reported to increase the expression of Foxp3 in the mucosa of patients with asthma and AR. Thus, the number of $CD4^+CD25^+Foxp3^+$ Tregs partly reflects the extent of respiratory inflammation, and can also be used to evaluate the effectiveness of the therapy.

In our study, the percentage of $CD4^+CD25^+Foxp3^+$ Tregs in Derp1-sensitized and challenged mice was found to be decreased in AR, suggesting that Tregs were deficient during AR. However, treatment with Derp1-modified DCs effectively increased the percentage of Tregs, indicating that the percentage of Tregs was related to the extent of inflammation and Derp1-modified DCs might be helpful for inducing Tregs to inhibit the Th2 responses and airway inflammation.

IL-10 has immunosuppressive functions and can modulate the activity of a key cell subset involved in allergic reactions, including mast cells, Th2 cells, eosinophils, and DCs (Noval Rivas et al., 2015; Akdis et al., 2004; Wing et al., 2008). IL-10 also plays an important role in the induction of immune tolerance in patients with allergic airway inflammations (Rubtsov et al., 2008). It is known that the suppressive functions of Tregs are mediated by the release of the inhibitory IL-10. Corticosteroid therapy has been reported to induce the up-regulation of IL-10 expression in $CD4^+$ T cells (McHugh et al., 2002; Grossman et al., 2004). Thus, Tregs can influence the response of Th1 and Th2 cells during allergic inflammation through the secretion of high levels of IL-10. In this study, up-regulation of IL-10 was observed in the Derp1DC group, indicating that treatment with the modified DCs could induce

the up-regulation of IL-10, similar to other therapies.

In conclusion, we constructed a Derp1-modified DC vaccine, and through animal experiments, demonstrated that it had an anti-inflammatory effect in mice with AR and that it maybe associated with the Th1/Th2 ratio change and an increase in the percentage of $CD4^+CD25^+Foxp3^+$ Tregs.

Competing interests

The authors declare no conflict of interest in the publication of this paper.

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