

Suppression of Allergic Inflammation by Allergen-DNA-modified Dendritic Cells Depends on the Induction of Foxp3⁺ Regulatory T Cells

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Abstract

CD4⁺CD25⁺Foxp3⁺Regulatory T cells (Tregs) play important roles in regulating allergic inflammation. To analyse if allergen-DNA-modified dendritic cells (DC) can suppress allergic responses and what roles Treg cells play in DC-based allergen-specific immunotherapy. Immature DC were transfected with retrovirus encoding Der p2 DNA, and administered to mice that sensitized and challenged with Der p2 protein. After Treg cells were depleted with anti-CD25 mAb, mice were re-challenged to observe the airway inflammation, and Treg cells in spleen CD4⁺ T cells. And responses of spleen CD4⁺ T cells to Der p2 were determined. Co-culture of naïve CD4⁺ T cells with allergen-modified DC induced Foxp3⁺ Tregs. Sensitized and challenged mice developed allergic airway inflammation and Th2 responses, and decreased Foxp3⁺ Tregs. Treatment with allergen-modified-DC suppressed airway inflammation and Th2 responses, and increased IL-10 and IFN- γ production and Foxp3⁺ Tregs significantly; and eliminated the responses of CD4⁺ T cells to allergen. Administration of anti-CD25 mAb eliminated all the effects of modified-DC except for the increasing of IFN- γ . Allergen-modified DC can induce immune tolerance to allergens and reverse the established Th2 responses induced by allergen, with dependence on the induction of Foxp3⁺ Tregs.

Introduction

Allergic asthma has been considered as a disease of deficiency in immune tolerance to inhaled 'innocuous' allergens [1, 2]. The 'hygiene hypothesis' initially contributed its pathogenesis to a missing immune deviation of allergen-specific responses from a Th2 to a Th1 profile. However, it is clear that Th1 cells are pro-inflammatory and that the development of a Th1-associated inflammatory response can exacerbate asthma and allergic disease [1, 3]. More recently, the importance of the lacking of the role of Tregs has been emphasized [2, 4].

Regulatory T cells compose of two main groups [2, 4], nature-occurring Treg cells (Tregs) and inducible Treg cells. Tregs are the most studied and best characterized subset, they emerge from thymus in mature state and account for 5–10% of peripheral CD4⁺ T cells in healthy human and mice, and constitutively express interleukin (IL)-2 receptor α chain (CD25) in high levels. The forkhead transcription factor, forkhead box protein 3 (Foxp3, and in human FOXP3) is expressed in mouse

CD4⁺CD25⁺ T cells and is considered essential for the development and function of Tregs [5]. By the expression of Foxp3, Tregs are best recognized. Tregs function in a cell–cell contact manner *in vitro* and also through releasing IL-10 and transforming growth factor- β (TGF- β) *in vivo*. It has been shown that suppressive cells with similar phenotype function as Tregs, such as the expression of Foxp3 and cytotoxic T-lymphocyte antigen 4 (CTLA4) and glucocorticoid-induced tumor necrosis factor receptor (GITR), can be experimentally induced from peripheral CD4⁺CD25⁺ T cells in mice and human after antigen stimulation or co-cultured with cytokines such as TGF- β [6]. Inducible Treg cells mainly composed of two subsets, type I regulatory T cells, which mainly function through releasing of IL-10, and T helper (Th) 3 cells, which mainly function through releasing of TGF- β [2, 4].

In healthy individuals, Tregs normally suppress Th2 responses to allergens. Furthermore, these subsets were decreased in allergic individuals [7], and lack of Tregs will result in the development of allergic airway inflammation [8, 9]. Foxp3 plays crucial roles in the

development and maintenance of Tregs. Foxp3 mutations results in the loss of Tregs and underlie a homologous autoimmune lymphoproliferative disorder including allergic dysregulation syndrome [8, 10, 11]. Reagents that could augment allergic airway inflammation also function through reducing IL-10⁺ CD4⁺ T cells and Foxp3⁺ Tregs [12].

Therefore, restoring the suppression of Tregs is an attractive target for immunotherapies. It has been shown that transfer of Foxp3⁺ Tregs suppressed allergic airway inflammation [13, 14] and hyperreactivity [14]. Allergen-specific immunotherapy (SIT) with *schistosoma japonicum* egg antigen or HLA-DR-based PLA2 peptide also could inhibit antigen-induced allergic airway inflammation and Th2 responses in a CD25⁺ Treg cell- or IL-10-dependent manner [15, 16]. Strickland *et al.* [17] also shown that prevention of ensuing cycles of T-cell activation and resultant airway hyperreactivity was mediated by Foxp3⁺ Tregs. Heme oxygenase-1 [18] could suppress allergic airway inflammation and enhance Foxp3 expression. Glucocorticoids are the most effective drugs in asthma treatment. It has been demonstrated that, in asthmatic patients who were treated with inhaled glucocorticoid, Foxp3⁺ Tregs and IL-10⁺ Tregs significantly increased; and glucocorticoid had induced IL-10 and Foxp3 expression in CD4⁺ T cells in short-term and long-term cultures *in vitro* [19].

Allergen-specific immunotherapy is an old but widely used treatment of allergic diseases, and has been demonstrated to be clinically efficacious in both allergic rhinitis and asthma. SIT reduces subsequent allergic sensitization [20] and appears to reduce the incidence of asthma in later life [21]. Subcutaneous administration of increasing doses of the sensitizing allergen modifies the immune response in a variety of ways. It has been supposed that induction of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) plays crucial roles in successful SIT [15, 22–26].

Although SIT is efficient in treatment of allergic disease, the fact remains that treating allergic individuals with a sensitizing allergen molecule occasionally results in allergic adverse events. Rarely, these can be fatal. Considerable resources have been invested in developing strategies to reduce the allergenicity of immunotherapy preparations whilst maintaining their ability to modify T-cell and/or B-cell responses. One such approach is to treat patients with DNA vector that encoding the allergen. Allergen gene DNA-based SIT has been shown to be effective in modulating allergic immune response in mice models [7]. Apart from that, dendritic cells (DC) are potent tools for the allergen-specific modulation of allergic immune responses. DC under different state determine the type of immune response. When bone marrow myeloid DC are kept in an immature state and injected into the trachea, they suppress the development of airway inflammation in an IL-10-dependent fashion,

whereas, when maturation is allowed, they induce Th2 priming [27]. Immature DC present antigens to T cells in the absence of appropriate co-stimulation, as leads to T-cell anergy or deletion [28] or to the development of Treg cells [29]. DC also show remarkable functional plasticity and capacity to regulate T-cell immunity so that DC are increasingly used as adjuvants for vaccination [30]. Owing to their capacity to regulate T-cell immunity, DC are increasingly used as adjuvant for vaccination, and the immunogenicity of antigens delivered by DC has now been shown in patients with cancer [31]. Targeting of antigens to immature DC induces Treg cells and can suppress delayed-type hypersensitivity *in vivo* [32], and allergen-DNA-modified asthmatic DC can inhibit Th2 CD4⁺ responses and enhance Th1 CD4⁺ responses *in vitro* [33]. These results suggest that DC modified with allergen-DNA can be used as vaccines to reverse the Th2 responses.

In previous study, we have shown that plasmids DNA encoding allergens, can induce Treg cells in allergic models [34]. But it is still unknown whether allergen DNA-modified DC can induce tolerance to allergens or reverse established Th2 responses, or what roles Treg cells play in this process. Therefore, the aims of this study is to determine whether immunization with allergen-DNA-modified DC could induce protection against allergen in a mouse allergic airway inflammation model, and ascertain what roles Treg cells play in the immune mechanism of protection, as may have important implications for rational design of vaccines.

Methods

Animals. Female C57BL/6 mice at age of 6–8 weeks were purchased from Chongqing Medical University. Animal care and experimental procedures were in accordance with the animal ethics regulations of the Home Office, UK.

Plasmids and modification of DC. Erythrocytes from collected bone marrow cells were lysed by ammonium chloride treatment at day 1. Cells (1.0×10^6 cells/ml) were plated in 24-well plates in RPMI1640 medium (1 ml per well) supplemented with 10% fetal calf serum (FCS), 10 mmol/L HEPES, 50 μ mol/L 2-ME, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (eBioscience Inc, San Diego, CA, USA). At day 4, the top two-thirds of the media were replaced with fresh medium. At day 6, non-adherent cells were collected and re-plated into fresh 24-well plates. Flow cytometry (FCM) with FITC-rat-anti-mouse CD11c (Serotec Ltd, Oxford, UK) showed that the obtained population was greater than 85% CD11c⁺.

The full length of Der p2, an allergen of house dust mite, were get from the plasmids pBC176 (HESKA Cor,

Loveland, CO, USA), and inserted into a pMSCV-based retroviral vector encoding an enhanced GFP (a gift from Probst-Kepper M, MD, Hannover Medical School). The amphotropic PT67 packaging cell line was transfected as described elsewhere [35]. Filtered (0.45 μ m) virus-containing supernatant supplemented with 8 mg/ml sequabrene (Sigma–Aldrich Int., St Louis, MO, USA) was applied to DC, established as described above. GFP expressing cells were sorted 1 week later using a FACS-Vantage (BD Biosciences, Franklin Lakes, NJ, USA), the resulting DC were termed allergen-DC. The expression of Der p2 was detected with SDS-PAGE and western blotting using anti-Der p2 mAb 7A1 (Indoor Biotechnologies Inc., Manchester, UK), and retrovirus with GFP and without Der p2 modified DC (termed GFP-DC) were used as negative control.

Also at day 7, immature DC were pulsed with 10 μ g/ml Der p2 (termed pulsed-DC). And 48 h after stimulation, the allergen-pulsed DC were collected.

The expressions of CD40, CD80, CD86 on DC were detected with FCM using PE-coupled anti-CD40, CD80, and CD86 monoclonal antibody (mAb) (eBioscience Inc.).

Induction of Foxp3⁺Tregs in naïve CD4⁺ T cells by modified DC in vitro. Autologous CD4⁺ T cells were obtained from spleen using antibody-coated paramagnetic MultiSort MicroBeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers protocol. After detaching, CD4⁺ T cells were stained with CD25 MicroBeads (Miltenyi Biotec) and CD4⁺CD25⁺ T cells were positively and CD4⁺CD25[−] T cells were negatively selected. Separation was controlled by FCM (purity >98% CD4⁺ T cells, >98% CD4⁺CD25[−] T cells and >95% CD4⁺CD25⁺ T cells).

CD4⁺CD25[−] T cells at 4×10^5 cells/well were incubated in the presence of 4×10^4 allergen-DC at 37 °C in 5% CO₂-humidified air. After 72 h of incubation, the cells were harvested to examine the expression of CD25 and Foxp3 using Mouse Regulatory T cell Staining Kit (eBioscience Inc.).

Antigen-presenting cell isolation. Antigen-presenting cells (APC), obtained by isolating the fraction of the CD4[−] magnetic sort after depleting CD8⁺ T cells from spleen cells, were irradiated with 30 Gy.

Depletion of CD25⁺ Treg cells. To determine the efficacy of depletion of CD25⁺ Treg cells with anti-CD25 mAb (PC61, ATCC), the antibody (1 mg) was administered intra-peritoneal injections (i.p.) at day −7, −4, 0, and an isotype control (rat IgG1) Ab used as control. At day 4, 7, 10, CD4⁺CD25⁺Foxp3⁺ T cells of spleen were determined by FCM.

Animal models and study design. The allergen Der p 2 was affinity-purified from spent mite medium using the anti-Der p2 mAb, allergens did not contain detectable amounts of LPS and were >99% pure. C57BL/6 mice

were primed and sensitized by a single subcutaneous injection (day 1) followed by seven i.p. of Der p2 (10 μ g in 100 μ l 0.1 mol/L PBS) at interval of 48 h (day 3–15). Airway inflammation was induced in animals anaesthetized with ether by three intra-tracheal instillation of Der p2 at interval of 24 h (days 29, 30 and 31) (we named them model mice). Control mice received 0.1 mol/L PBS.

Mice were randomly divided into seven groups. Primed mice received one intravenous injection of allergen-DC or GFP-DC or pulsed-DC or untransfected DC (1×10^6 cells per animal) at day 45. At days 52, 56 and 59, some allergen-DC treated mice were interfered by three i.p. of anti-CD25-mAb (PC61, American Type Culture Collection [ATCC]). At day 66, 67 and 68, all of the groups except the control mice were re-challenged with Der p 2. (Fig. 1).

Histological analysis of lung tissues. Twenty-four hours after allergen re-challenging, the excised lungs were inflated under constant pressure and fixed with neutral buffered 4% formalin and finally embedded in paraffin. Sections were assessed for general morphology and cellular infiltration using haematoxylin and eosin (haematoxylin and eosin staining, HE).

Measurement of eosinophil and lymphocyte and cytokine levels in bronchoalveolar lavage. To investigate the cellular infiltration in the airways, 24 h after allergen challenge, the tracheae were exposed and cannulated using 27-gauge silicon tubing, and the lungs were gently instilled two times with total 600 μ l cold 0.1 mol/L PBS. The volume, total cell number and composition of bronchoalveolar lavage (BAL) samples were determined. Samples were centrifuged at 700 g for 5 min at 4 °C and re-suspended at 1×10^5 cells in 100 μ l (PBS/10% FCS) to make cyto-spin by cyto-centrifugation (300 g for 3 min) onto poly(L-lysine)-coated slides that were air-dried, fixed in methanol and stained by Diff/Quick using standard procedures (Merck, Darmstadt, Germany). Differential cell counts were performed in duplicate on coded slides for 300 cells from each sample. BAL fluid was stored at −70° for determining levels of the cytokines IL-4, IL-5, IL-10 and IFN- γ using specific ELISA according to the user's manual (ELISA kits, eBioscience).

Determination of allergen-specific IgE levels in sera. After the mice were killed, the blood was collected for preparation of sera. Levels of Der p2-specific IgE were measured with a slightly modified solid-phase ELISA [36] (ELISA kits, Bethyl Lab, Montgomery, USA). Briefly, 96-well microtiter plates were incubated with 50 μ l Der p2 (25 μ g/ml) overnight at 4 °C. The antigen-coated plates were washed three times with 0.05% PBS-Tween 20 buffer (washing buffer) and incubated with five-fold diluted mice sera overnight at 4 °C. Plates were washed five times with washing buffer and incubated with peroxidase-conjugated antimouse IgE antibody overnight at

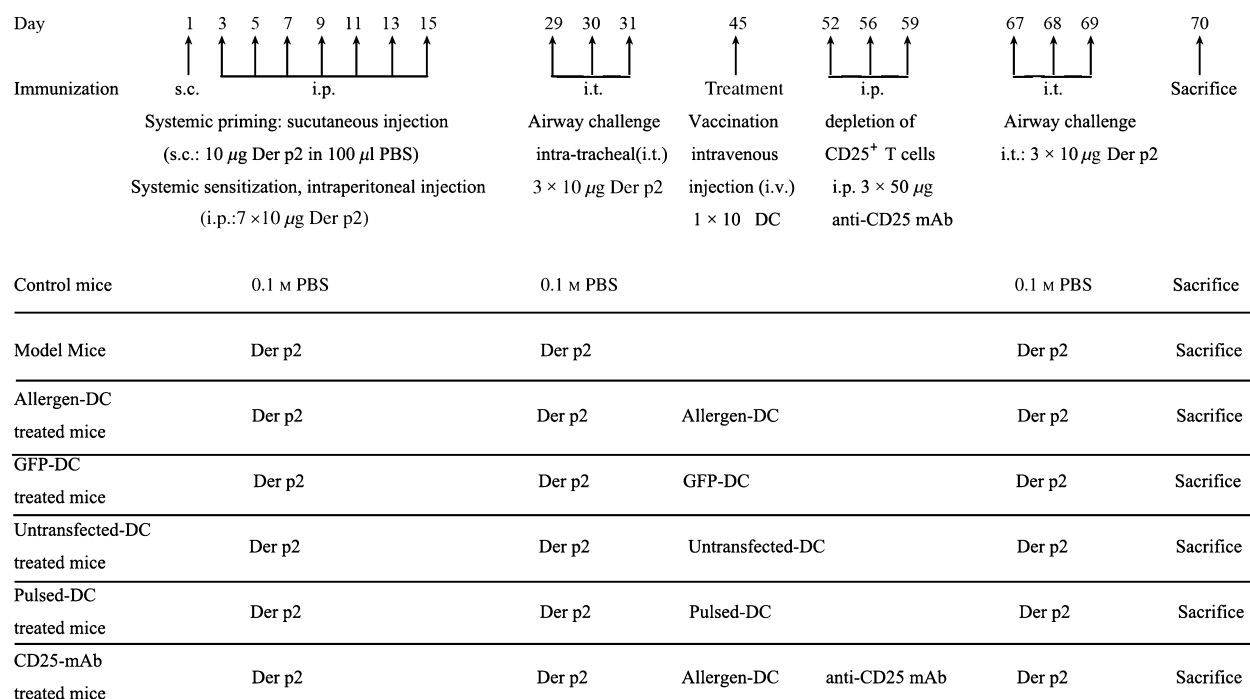


Figure 1 Study design. Animals were randomly divided into 7 groups, control group, model group, allergen-DNA-modified DC treated group (allergen-DC group), GFP-modified DC treated group (GFP-DC group), untransfected-DC treated group (untransfected-DC group), pulsed-DC treated group (pulsed-DC group) and anti-CD25 mAb treated group (anti-CD25 group). All the animals except the control group were sensitized and challenged with Der p2, and mice of control group received 0.1 M PBS. Mice of allergen-DC group and anti-CD25 group received allergen-DNA modified DCs, and GFP-DC group, untransfected-DC group, pulsed-DC group and received GFP-DC, or untransfected-DC or pulsed-DC respectively at day. And at day, mice of anti-CD25 group received anti-CD25 mAb (PC61) for three times. Then all of the mice were rechallenged with Der p2 or 0.1 M PBS, and sacrificed.

4 °C, and then washed a further five times and added citric acid-phosphate buffer (pH 5.0) containing 0.15 mg/ml of O-phenylenediamine (Sigma, St Louis, MO, USA). Colour was developed at room temperature and the reaction stopped with 2.5 M sulphuric acid and measured at 492 nm. The level of Der p2-specific IgE was compared to the standard serum pooled from six mice that were immunized with 100 µg of Der p2 twice, and that had nasally inhaled 10 µg of antigen six times. The standard serum is expressed as 100 ELISA units/ml.

Measurement of proliferation and cytokine production of CD4⁺ T cells stimulated with allergen in vitro. To assay T-cells proliferation, 1×10^5 spleen CD4⁺ T cells were cultured in 96-well plates (Gibco, Carlsbad, CA, USA) with APCs (1×10^5) and 10 µg/ml Der p2 in 200 µl of PRMI 1640 medium supplemented with 10% FCS. After 5 days, 37 kBq/well of tritiated thymidine ([methyl-³H] thymidine;) were added to the media. 6 h later, tritiated thymidine incorporation was evaluated in a beta counter (MicroBeta, Perkin-Elmer, Waltham, MA, USA).

To assay cytokine production, 5×10^5 CD4⁺ T cells were cultured with with APCs (5×10^5) in 48-well plates in 1 ml of PRMI 1640 medium supplemented with 10% FCS and 10 µg/ml Der p2. On day 3,

supernatants were collected and cytokines were determined with specific ELISA.

Detection of Foxp3⁺ Tregs in spleen CD4⁺ T cells. Spleen CD4⁺ T cells were labelled with Mouse Regulatory T cell Staining Kit to detect the expression of CD25 and Foxp3 in spleen CD4⁺ T cells.

Allergen-induced degranulation of mast cells. Mast cell degranulation was assessed in 1-µm Toluidine blue-stained plastic sections as previously described [37]. The granules of normal, non-degranulated mast cells were identified by their characteristic dark blue-purple colour. Degranulated mast cells were identified by the altered staining properties of the granules resulting in the appearance of large pink granules. For each mast cell, the numbers of normal and altered granules were identified and the percentage of altered granules was calculated (on average 1100 granules were analysed from each sample).

Statistical analysis. Data were expressed with means ± SD, with eight mice per group. Difference between groups was analysed using software SPSS for windows (version 8.0) by unpaired two-tailed parametric Student's *t*-test or ANOVA test. *P*-values below 0.05 were considered statistically significant.

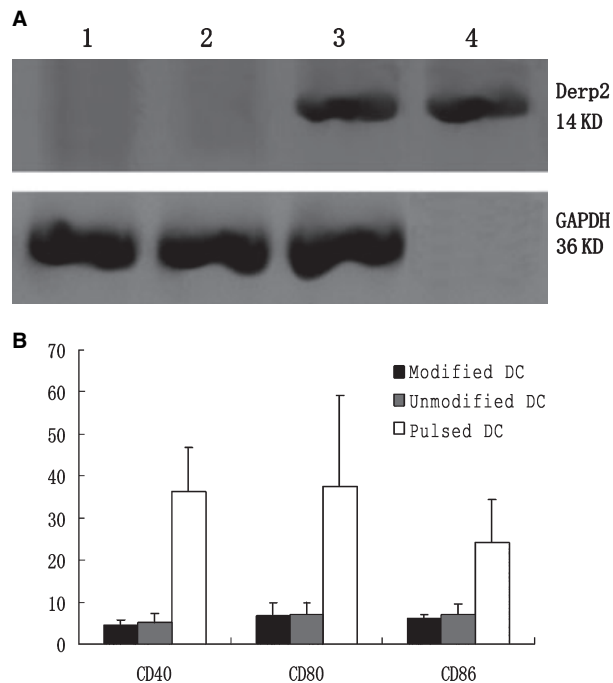


Figure 2 (A) The expression of the protein Der p2 by transfected DCs. The cells were lysed 48 hours after transfection. Der p2 (0.5ng) was used as a positive control, and untransfected-DC and GFP-DC used as negative controls. 1, untransfected-DC; 2, GFP-DCs; 3, allergen-DC; and 4, positive control. (B) Marker molecules expressed on the surfaces of DCs after transduced with Der p2. All of the molecules we detected, CD40, CD80, CD86, were examined with flow cytometry and expressed in low levels in modified and untransfected-DC, but in high level in pulsed-DC (percentage of positive cells).

Results

Expression of the protein Der p2 in immature DC transfected with Der p2 DNA

Expression of Der p2 in allergen-DC was assessed with Western blotting. DC were lysed 2 days after transfection. Der p2 protein (0.5 ng) was used as positive

control. As shown in Fig. 2A, allergen-DC expressed the protein Der p2, while the untransfected DC and GFP-DC did not express any amount of Der p2 protein. The protein-loaded control actin was expressed at similar amounts by all transfected and untransfected DC.

Both Allergen-DC and untransfected DC expressed similar low levels of the costimulatory molecules CD80 and CD86 and CD83. These data suggest that transfection with DNA has no influence on the maturity of DC, the resulting DC was immature. (Fig. 2B).

Depletion of CD25⁺ Treg cells

To determine the efficacy of depletion of CD25⁺ Treg cells with anti-CD25 mAb, we examined CD25⁺Foxp3⁺ Treg cells at day 4, 7, 10 after anti-CD25 mAb treatment. As shown in Fig. 3, i.p. administration of PC61 to depleted CD4⁺CD25⁺Foxp3⁺ T cells in spleen sufficiently, and this depletion effect of PC61 persisted at least 10 days. Rat IgG1 that used as control mAb have no effect on CD25⁺Foxp3⁺ T cells.

Allergen-DC treatment induced regulatory T cells from CD4⁺CD25⁺ T cells *in vitro*

Induction of Tregs plays key roles in conventional SIT. To determine whether allergen-DC could induce Tregs *in vitro* or not, we examined the effect of allergen-DC on naïve CD4 T cells *in vitro*. Naïve CD4⁺T cells were isolated from spleen, and co-cultured with allergen-DC. After 3 days of culture, Foxp3⁺T cells increased significantly. This result suggested that allergen-DC could induce Tregs (Fig. 4).

CD4⁺T cells isolated from allergen-DC treated mice exhibited low proliferation and cytokine production profiles *in vitro*

To determine the proliferation and cytokine production of CD4⁺ T Cells in response to allergen, we cultured

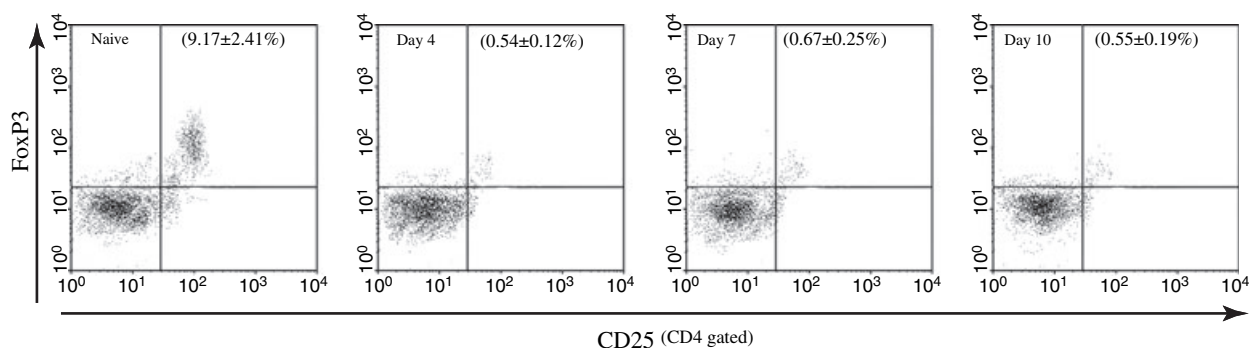


Figure 3 Depletion of CD4⁺CD25⁺ Tregs. To evaluate the depletion efficacy of PC61 on CD4⁺CD25⁺ Tregs, we examined the percentage of Tregs in spleen CD4⁺ T cells. As shown in Fig. 3, PC 61 could thorough deplete CD4⁺CD25⁺ Tregs, and the effects persisted for 10 days at least.

Figure 4 Co-culture of CD4⁺CD25⁻T cells with allergen-DCs induced Foxp3⁺CD25⁺ T cells. In CD4⁺CD25⁻ T cells, Foxp3⁺CD25⁺ T cells could hardly be detected. Co-culture allergen-DCs induced Foxp3⁺CD25⁺ T cells from CD4⁺CD25⁻ T cells.

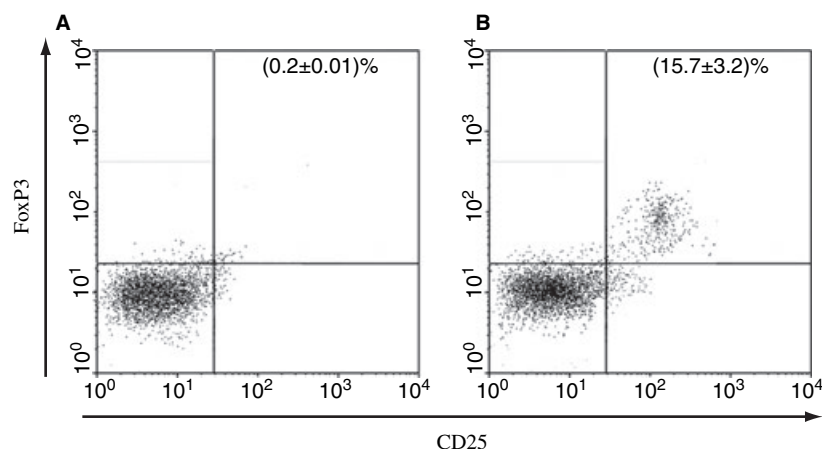
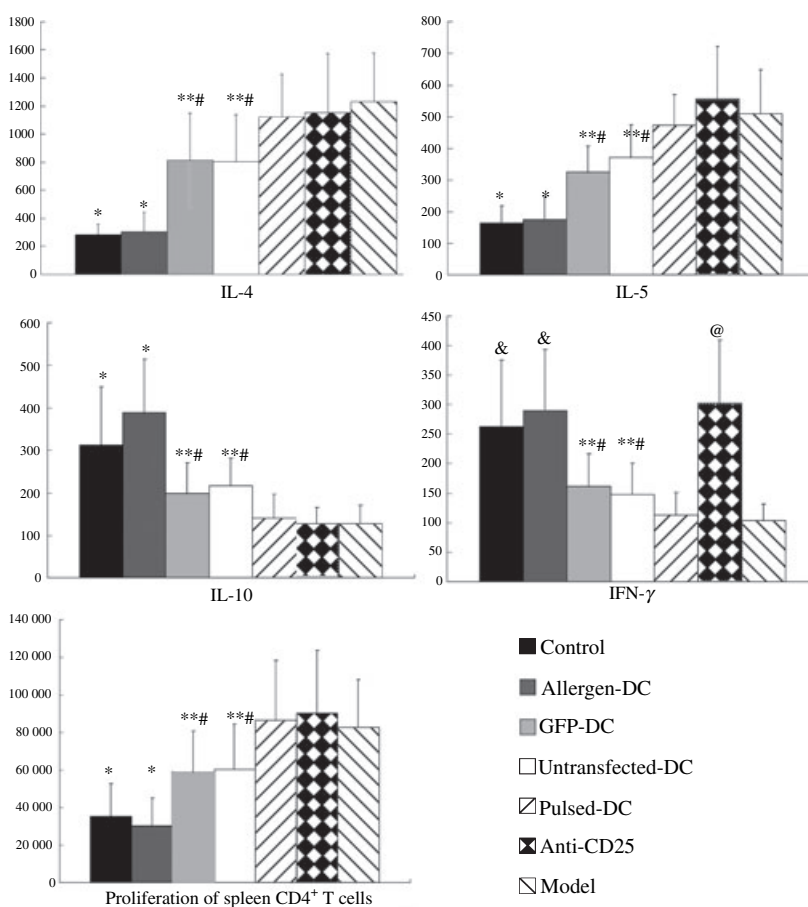


Figure 5 Responses of spleen CD4⁺ T cells to Der p2. In allergen-DC group, proliferation (in cpm) and Th2 cytokine production (all in pg/ml) of CD4⁺ T cells were lower, and IL-10 production (in pg/ml) were higher as compared with model mice. In anti-CD25 group, the proliferation and Th2 cytokine and IL-10 production of CD4⁺ T cells were similar with model group, and Th1 cytokine production (pg/ml) still were in high levels. *: $P < 0.01$, vs model mice and anti-CD25 group. **: $P < 0.01$, vs control group and allergen-DC group. #: $P < 0.05$, vs model mice. &: $P < 0.01$, vs model mice. @: $P < 0.01$, vs allergen-DC mice.



spleen CD4⁺ T Cells supplemented with allergen. Results shown CD4⁺ T Cells from model mice had higher proliferation and higher Th2 cytokine productions as compared with control mice. In CD4⁺ T cells from allergen-DC group, proliferation and Th2 cytokines productions were lower than model mice, and had no difference with control mice. This suggested treatment with allergen-DC induced immune tolerance to allergens. Untransfected-DC and GFP-DC had similar but weaker effects with allergen-DC (all $P < 0.01$) (Fig. 5).

Treatment with allergen-DC increased regulatory T cells *in vivo*

Induction of Tregs, plays crucial role in successful SIT, hence we detected Tregs in spleen CD4⁺ T cells and found CD25⁺Foxp3⁺ in model mice was lower than that in control mice. Treatment with allergen-DC significantly increased CD25⁺Foxp3⁺ ($P < 0.001$). GFP-DC and untransfected DC and pulsed-DC served as controls. In GFP-DC or untransfected-DC group, the proportion of

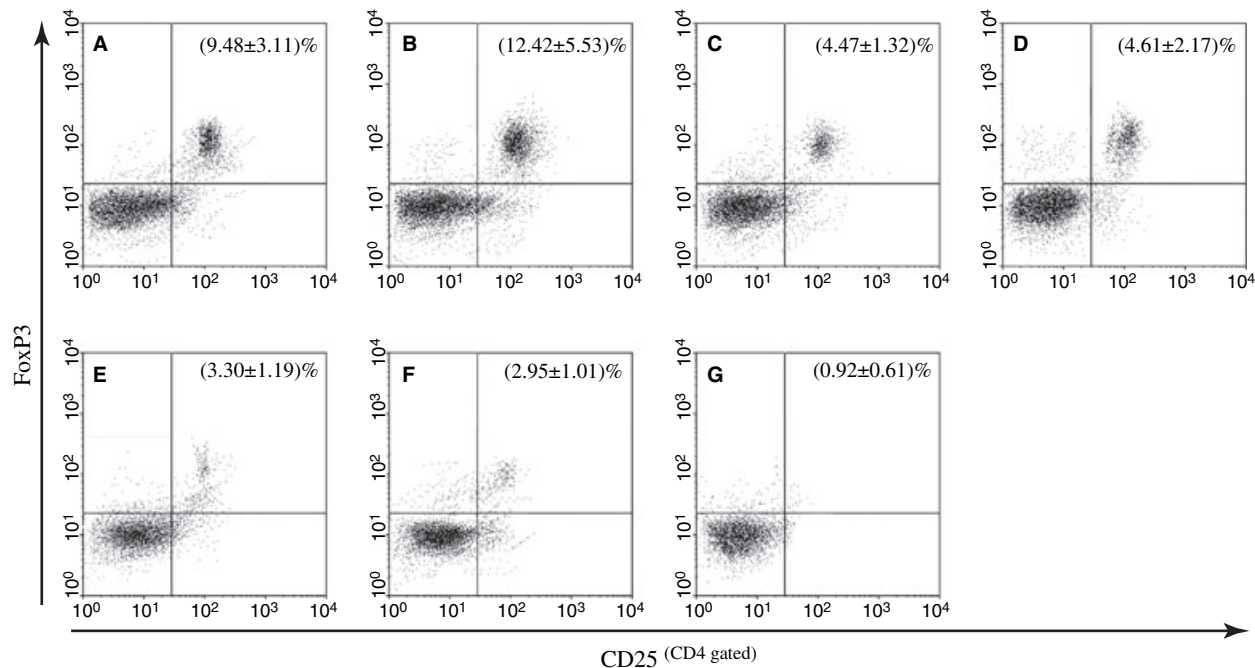


Figure 6 CD25⁺Foxp3⁺ in spleen CD4⁺ T cells. (A) control mice; (B) allergen-DC group; (C) GFP-DC group; (D) untransfected-DC group; (E) pulsed-DC group; (F) anti-CD25 group; (G) model group. In model mice, the proportions of CD25⁺Foxp3⁺ in spleen CD4⁺ T cells were lower than control mice. Allergen-DC elevated the proportion of CD25⁺Foxp3⁺ T cells. Treatment with anti-CD25 mAb depleted CD25⁺Foxp3⁺ T cells thoroughly. Control mice *vs* model mice: $P < 0.01$; allergen-DC group *vs* model mice, and *vs* anti-CD25 group: $P < 0.01$; GFP-DC and untransfected-DC group *vs* model mice: $P < 0.05$, and *vs* allergen-DC group: $P < 0.01$.

CD25⁺Foxp3⁺ T cells also were higher than model mice ($P < 0.05$), but lower than allergen-DC treated mice ($P < 0.01$); and pulsed-DC had no effect on Tregs (Fig. 6).

Treatment with allergen-DC reduced tissue pathology and decreased inflammatory cells in BALF

Lung histological sections were stained with HE to determine whether the suppressed airway cellular infiltration represented a more general down-modulation of pathology. In untreated model mice, airway re-challenge led to a dense inflammatory infiltrate of lymphocytes, mononuclear and eosinophils as well as to epithelial shedding. However, in treated mice, tissue inflammation after re-challenge was greatly reduced (Fig. 7A) with significantly less cellular infiltration.

Similar to lung histological sections, airway re-challenge led to more inflammatory cells, especially eosinophils and lymphocytes in BALF of allergic animals. Treatment with allergen-DC greatly reduced inflammatory cells (Fig. 7B).

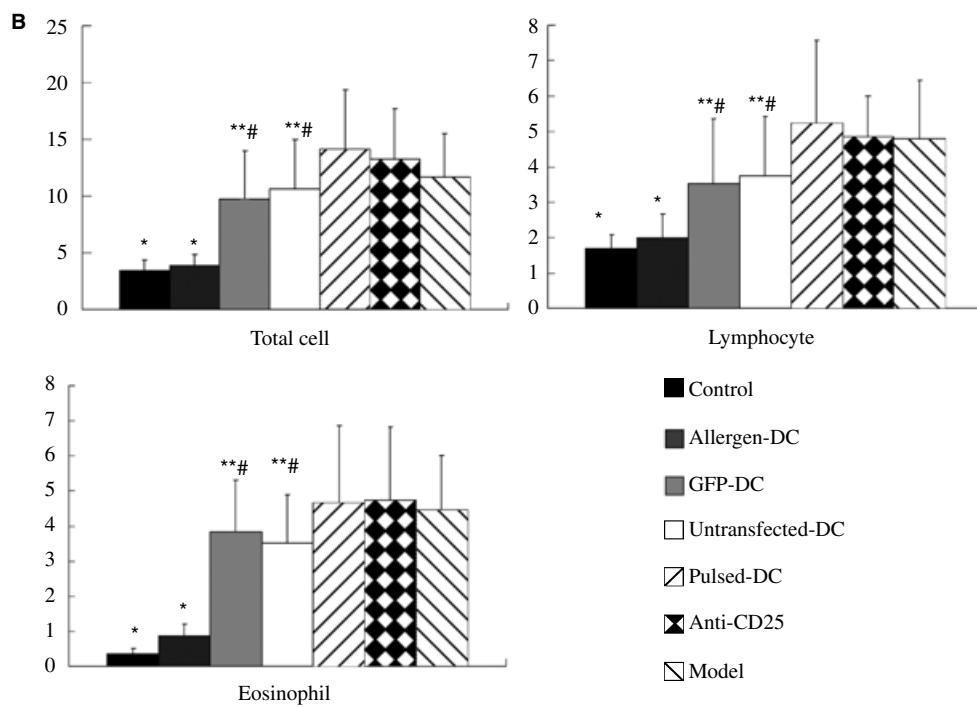
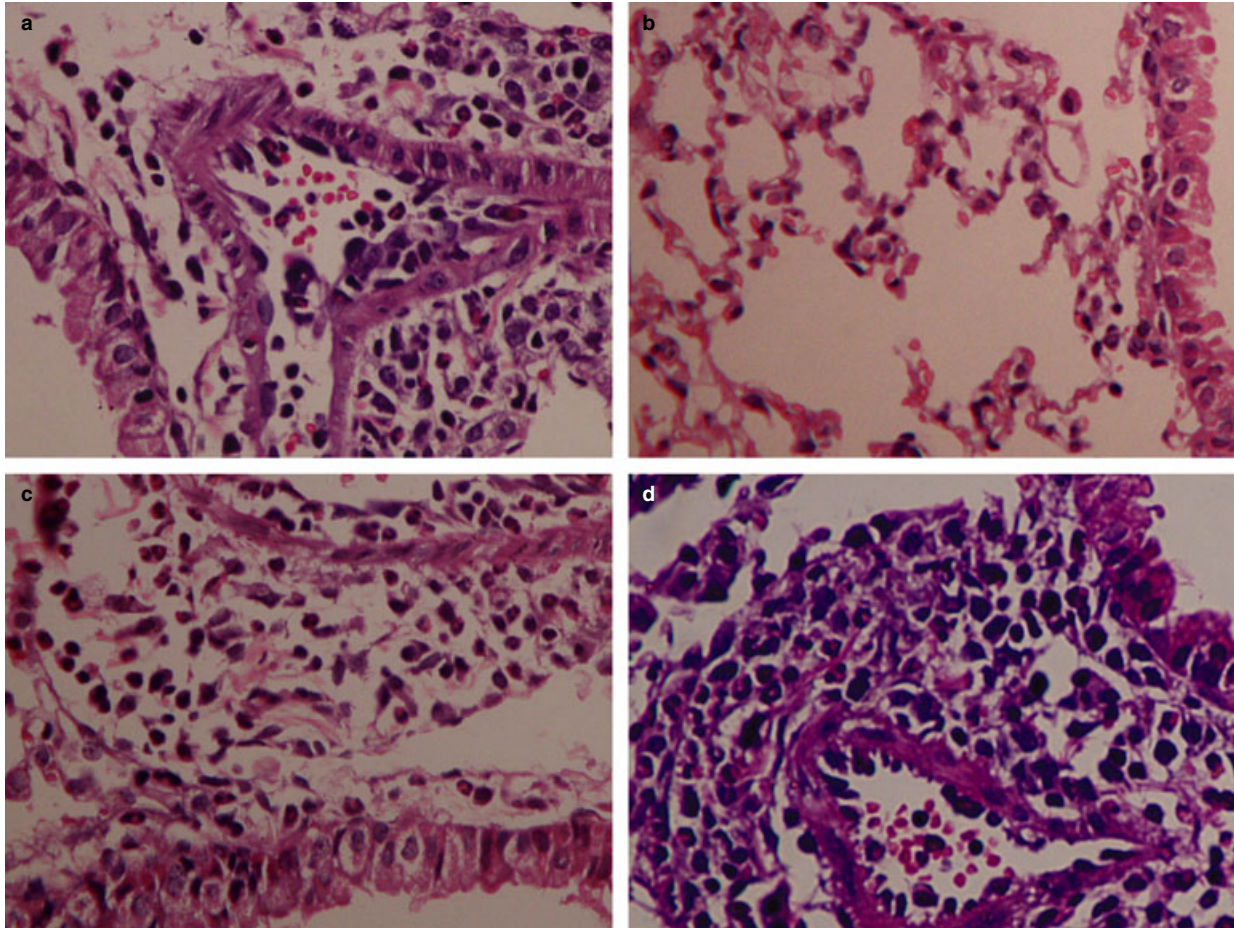
Allergen-DC suppressed local Th2 cytokines and serum IgE production and degranulation of lung mast cell

Local cytokine levels were measured 24 h after the final allergen challenge *in vivo* in the BALF. The Th2 cytokine IL-4 and IL-5 were both elevated in the BALF of model mice after airway re-challenge. However, IL-4 and IL-5 were significantly diminished by allergen-DC. In contrast, production of Th1 cytokines, IFN- γ decreased in the BALF of model mice after airway re-challenge but significantly elevated by allergen-DC (Fig. 8).

We also examined the regulatory cytokine IL-10. In contrast to Th2 cytokines, IL-10 diminished in model mice as compared with control mice, but treatment with allergen-DC increased IL-10 (Fig. 8).

Similar to Th2 cytokines, levels of Der p2-specific IgE in sera and de-granulation of lung mast cells increased in model mice but administration of allergen-DC significantly decreased Der p2-specific IgE and de-granulation of mast cells (Fig. 8).

Figure 7 (A) Infiltration of inflammatory cells into the lung of mice. a: Lung tissue of model group, there were a great number of inflammatory cells. b: Lung tissue of allergen-DC group. c: Lung tissue of pulsed-DC group. d: Lung tissues of mice treated with anti-CD25 group. HE $\times 400$. (B) Number of inflammatory cells mice ($\times 10^4/\text{mL}$). Sensitization and challenging with Der p2 resulted in inflammatory cells, include lymphocytes and eosinophils infiltration to the lung. Treatment with allergen-DC decrease the cell number, and the effects of allergen-DC were abolished by depletion of Treg cells with anti-CD25 mAb. *: $P < 0.01$, *vs* model group and anti-CD25 group; **: $P < 0.05$, *vs* model group and anti-CD25 group; #: $P < 0.01$, *vs* allergen-DC group.

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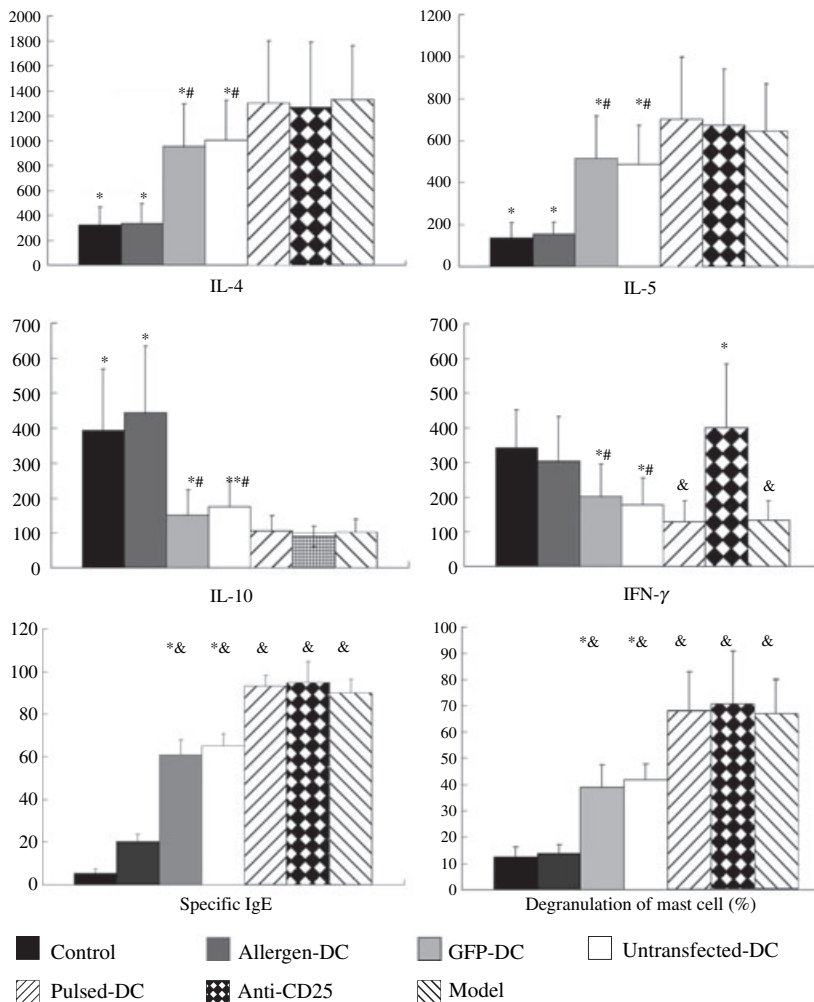


Figure 8 Levels of cytokines in BALF (pg/ml), and Der p2-specific IgE in sera (pg/ml). Similar to the inflammatory cells in BALF, the levels of Th2 cytokines in BALF and IgE in sera of allergen-DC group were lower than model group and anti-CD25 group. As levels of IFN- γ , were higher in allergen-DC group than model group, but were higher than anti-CD25 group. The levels of IL-10 in model group were lower than control group. In allergen-DC group, the levels of IL-10 were similar with control mice, but were higher than model group and anti-CD25 group. There were no significance difference between the levels of IL-10 in model and anti-CD25 group. *: $P < 0.01$, vs model group. #: $P < 0.05$, vs model group. &: $P < 0.01$, vs control group and allergen-DC group.

We used untransfected-DC and GFP-DC and pulsed-DC as controls. We found untransfected-DC and GFP-DC also had similar effects with allergen-DC on allergic inflammation, but weaker than allergen-DC. And pulsed-DC have no effects on allergic inflammation.

Effects of allergen-DC on the de-granulation of airway mast cells

Mast cell de-granulation plays important role asthma. As shown in Fig. 8, an increase in mast cell de-granulation, compared with control mice, was evident in model mice. In allergen-DC treated mice, a reduction of mast cell de-granulation was seen as compared with model mice.

Depletion of CD4⁺CD25⁺T cells with anti-CD25 mAb blocks the effect of allergen-DC

Tregs could be depleted *in vivo* by using anti-CD25 mAb, with possibility influence on the activated CD25⁺ effector cells. Thus, the enhanced responsiveness after

CD25⁺ cell depletion was likely to reflect the loss of Treg cell activity, while the diminished responses might result from depletion of effector cell.

Administration of anti-CD25 antibody to allergen-transfected DC-treated model mice showed that the percentage of CD4⁺CD25⁺Foxp3⁺ in modified DC-treated mice decreased greatly and airway infiltration recovered after airway re-challenge, and the production of Th2 cytokines and IgE and mast cell de-granulation increased and that production of IL-10 decreased. Out of our expectation, the Th1 cytokines, IFN- γ showed no change, compared with the allergen-mice that did not receive anti-CD25 mAb (Figs 5–8).

Discussion

In this study, we demonstrated that bone marrow derived-DC could be modified by allergen-DNA and also synthesize the corresponding protein. The transfection did not influence the surface marker expression. Allergen-DC induced Foxp3⁺Tregs *in vitro* and *in vivo*. As expected, allergen-DC could efficiently reduce allergic airway

inflammation and inhibit the established Th2 responses induced by allergens, and these effects depended upon the induction of Tregs. The present study has confirmed a key role for Tregs as Th2 immune negative regulators and key inducers of immune tolerance to inhaled allergens as well as role of DC as regulators of immune responses.

Dendritic cells play crucial roles both in the induction of T-cell immunity, as well as tolerance [38], and have become an attractive cell type for therapeutic manipulation of the immune system in order to enhance insufficient immune responses in infectious diseases and cancer or to attenuate excessive immune responses in autoimmunity and transplantation. The success of immunotherapeutic vaccination with DC loaded with tumour-associated antigens (TAA) for induction of protective antitumour responses have been demonstrated in clinical trials [39]. In animal models, DC has been successfully used as tools and targets for induction of transplant tolerance [40]. DC could also be used to inhibit fully developed autoimmunity in animal models [41].

Dendritic cells may also be used in the treatment of allergy diseases. Although there were very few reported about this, allergen-DNA-modified asthmatic DC could inhibit Th2 CD4⁺ responses and enhance Th1 CD4⁺ responses *in vitro* [33], and DC activated by cross-linking B7-DC could block inflammatory airway disease [42].

In this study, we found that the allergen-DNA-modified DC can reverse the established Th2 responses in allergic mice models. Allergen-DC upregulated the productions of Th1 and suppressing cytokines and decreased Th2 cytokines production and inflammatory cells infiltration and allergen-specific IgE. These results were similar to the results from conventional SIT [43], and consistent with former study [33], and suggested that the allergen-DC may be useful in SIT.

Intracellular proteins, such as those from infecting viruses or expressed in the cells, are mainly presented to CD8⁺ T cells through MHC class I molecules. Therefore, delivery into DC of nucleic acid-encoded antigen favours the generation of MHC class I-restricted CD8⁺ T cell responses [44, 45]. But recent study has demonstrated that infection of mouse DC with recombinant adenovirus vectors leads to presentation of encoded antigen by both MHC class I and class II molecules [46]. This may be the reason why allergen-DNA modified DC in favour to Th1 responses.

Our results also showed Tregs decreased in model mice as compared with control mice. Administration of the allergen-DC restored Tregs, suggested that the allergen-DC can induce Tregs. *In vitro* study indicated that co-culture of naïve CD4⁺T cells with allergen-DC induced the generation of Foxp3⁺ Tregs. And treatment with allergen-DC also increased IL-10 in BALF. Allergen-DC may function in two ways. First, they function as immature DC. It has been documented that immature

DC could in the steady state are able to prime Treg cells in order to maintain tolerance to self-antigens [47]. Moreover, immature DC can also induce Treg specific for foreign peptides [29, 48]. Second, allergen DC may function in a way similar with convention allergen immunotherapy. Our data have shown that allergen could be expressed by allergen-DC, and conventional allergen immunotherapy has also been shown to function through induction Treg cells [43]. This may also be the reason why untransfected DC or GFP-transfected DC could also induce similar but weak Th1 and Treg cell responses.

As mentioned above, Treg cells play crucial roles in pathogenesis and SIT. In this study, we also shown when Tregs-depleted mice re-challenged with Der p2, they showed Th2 responses again. IL-10 may be produced by Tregs or Tr1. As mentioned above, Tregs function through producing IL-10 *in vivo*. It has also been shown that CD4⁺CD25⁺ Tregs can also convert CD4⁺ cells to become Tr1 cells, so-called 'infectious tolerance' [49, 50]. Yang *et al.* [16] had shown that depletion of CD25⁺ T cells resulted in the decrease of IL-10.

Nevertheless, after depletion of Tregs, IFN- γ , a Th1 cytokine, did not decrease and even increased at certain degrees, as indicates that not only lacking of Tregs plays a key role in pathogenesis of asthma, but also the induction of Tregs was a key factor in successful SIT. It has been shown that Th1 responses are more susceptibility to the regulation by Tregs than Th2 responses [51]. But in our study, treatment with allergen-DC upregulates Th1 responses. This effect may be due to the effects of allergens produced by allergen-DC and the CpG motifs of the vector. All the data supported that without Tregs, enhancement of Th1 immune responses could not counter-balance the Th2 responses alone, and SIT would not function.

In all, our study indicated that allergen-gene-modified DC could suppress allergen-induced airway inflammation and established Th2 response, and these effects depended on the induction of Tregs. Thus, the induction of Tregs played a key role in successful SIT. Therefore, if done selectively, allergen-gene-modified DC could be a very effective strategy for regulating allergen-specific immune responses in a local environment, such as the lung. More recently, it had been reported that activated non-regulatory T cells also express Foxp3 in high levels, and acquire hyporeactivity properties for short time [52, 53, 54], and then downregulate Foxp3 expression, and lost hyporeactivity. These cells were called 'transient regulatory T cells'. Hence are there still more things to be elucidated, such as the distribution of allergen-DNA-modified DC in mice after injection? Where are they functioning to induce Tregs, thymus, peripheral blood, spleen or the lung? In what ways are they functioning? Are they effective in big mammals? Were these cells naturally occurring Tregs or 'transient' Tregs? What about the

dynamics of Foxp3 expression and hyporeactivity and suppression activity of these cells? Airway hyperresponsiveness is a key feature of asthma, but we cannot test animal pulmonary function, we did not examine the effect of allergen-DC on airway responsiveness. If possible, we will do some work on this point in future.

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