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Original Article

DGKα DNA vaccine relieves airway allergic inflammation in asthma model possibly via induction of T cell anergy

Yan Wang, Qiao Zhang, Qianli Ma, Youlan Zhang, Zhiwei Li, Changzheng Wang

Institute of Respiratory Diseases, Xinqiao Hospital, Third Military Medical University, Chongqing, 400037, China

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Abstract: Induction of T cell immune tolerance is thought to be a good method for treatment of asthma. Diacylglycerol kinases alpha (DGKα), enzymes that catalyze phosphorylation of diacylglycerol to produce phosphatidic acid, could inhibit diacylglycerol (DAG)-mediated signaling following T-cell receptor engagement and prevent T cell hyperactivation, thus playing important roles in the induction of T cell anergy. In the present study, we aimed to investigate the effects of DNA vaccine encoding DGKα gene administration on allergen-induced airway allergic inflammation in the murine model of asthma. Animal models were created and plasmid containing DGKα were constructed. Cytokine production was detected after the administration of DGKα gene plasmid. Immunization of mice with alum-adsorbed ovalbumin (OVA) followed by challenged with inhalation of aerosolized OVA resulted in the development of airway allergic inflammation. Administration of DGKα gene before the aerosolized OVA challenge significantly decreased the allergic airway inflammation and eosinophil infiltration in bronchoalveolar lavage fluid (BALF). Immunization with DGKα DNA vaccine decreased OVA-specific IgE and interleukin 13 (IL-13) levels in sera, and increased the IFN-γ level in BALF. The results of the present study provide evidence for the potential utility of the administration of DGKα DNA vaccine as an approach to gene therapy for asthma.

Keywords: Asthma, diacylglycerol kinases alpha, airway inflammation, DNA vaccine

Introduction

Asthma is a common disease. In recent years, its prevalence and incidence have increased in many developed and developing countries. More than 300 million people worldwide suffer from this disease and the number of affected people grows steadily [1]. In healthy subjects, the respiratory confrontation with an innocuous antigen first leads to a short-lived induction of a local immune response to this antigen, followed by long-term peripheral tolerance [2]. In asthmatic patients, harmless antigens can provoke an unwanted Th2 sensitization to these aeroallergens and cause Th2 responses [3]. The cytokines, such as interleukin (IL)-4, IL-5 and IL-13, released by activated CD4+ Th2 cells on exposure to allergen are responsible for the recruitment and activation of inflammatory cells and the release of pro-inflammatory towards airway allergic inflammation [4, 5]. Allergic asthma therefore has been found to be characterized by a pathological expansion of at least Th2 cells.

Induction of the immune tolerance to allergen is thought to provide the possibility of protecting against and controlling the occurrence of asthma. Previous evidence showed that the administration of Fas-ligand-expressing adenovirus-transfected dendritic cells could decrease allergen-specific T cells and inhibit airway inflammation in a murine model of asthma [6]. Antigen-specific Th2 cells are anergized by IL-10 and Th2 cell tolerance may suppress eosinophilic inflammation in allergic asthma [7]. Specific allergen immunotherapy could induce the production of IL-10 that elicits anergy in T cells by selective inhibition of the CD28 co-stimulatory pathway and controls suppression and development of antigen-specific immunity and allergic airway inflammation in asthma [8-10]. The

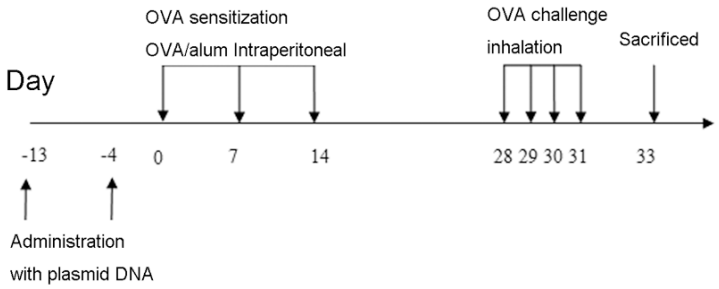

Figure 1. Experimental design Animals were randomly divided into 4 groups. All the animals except for the normal group were sensitized and challenged with OVA, while mice in the normal group received normal saline. Plasmid DNA of DGKα or control plasmid was intramuscularly injected twice on day -13, -4.

above evidence indicates that the induction of T cell anergy to allergen might provide an approach for the prevention and treatment of asthma.

Both diacylglycerol (DAG) and phosphatidic acid (PA) are important second messengers involved in signaling transduction from many immune cell receptors and can be generated and metabolized through multiple mechanisms. Recent studies indicated that diacylglycerol kinases (DGKs), enzymes that catalyze phosphorylation of DAG to produce PA, play critical roles in regulating the functions of multiple immune cell lineages [11-15]. DGKα is a subtype of DGK, I-type DGK, which is also highly expressed in thymocytes and peripheral T cells [16]. Expression of DGKα is regulated by T-cell activation status. DGKα is expressed at high levels in naive T cells and down-regulated after T-cell activation. In anergic T cells, DGKα expression is elevated with relative to that in naive T cells [13, 14, 17]. Therefore, elevated DGKα expression appears to be critical for T-cell anergy. However, the issue of whether DGKα could inhibit allergic airway inflammation by inducing the T-cell anergy is unclear. In the present study, we aimed to constructed DGKα DNA vaccine and further explore its possible effects on the development of airway allergic inflammation in a mouse model of asthma.

Materials and methods

Animals

Male BALB/c mice were purchased from the medical laboratory animal center of Guangdong Province. All mice were used at 4-6 wk of age. All animals were maintained under specific pathogen-free conditions. Animal care and experimental procedures were conducted in accordance with the animal ethics regulations of the Home Office, UK.

Plasmid construction

The fusion gene encoding mouse IL-2 signal peptide and full-length mouse DGKα were synthesized (Shanghai Qinglan Biotech Company, China), and the fusion gene was cloned to pEGFP-N3 vector to obtain the DGKα plasmid. The clone was sequenced by double-stranded sequencing (Sangon Scientific Co. Shanghai, China). Endotoxin-free plasmid DNA was prepared and purified with the Endotoxin-free Plasmid Maxi Kit (Beijing Tiangen Biotech Company, China).

Induction of murine model of asthma

Mice were sensitized and challenged by OVA (Sigma, MO, America) according to a modification of the method of Krinzman et al [18]. Briefly, BALB/c mice were intraperitoneally immunized with 20 ug of OVA mixed with aluminum hydroxide (Sigma, MO, America) in 200 ul volume on days 0, 7, 14. On days 28-31 after the first immunization, mice were challenged by inhalation of aerosolized 1% OVA in normal saline in a chamber using an nebulizer (Pari, Germany) for 35 min. Normal saline instead of OVA protein was used in the normal group (**Figure 1**).

Experimental design and administration of DNA

Experimental design was summarized as **Figure 1**. Mice were randomly divided into four groups (twelve mice each group): model group, normal group, DGKα plasmid group, control plasmid group. DGKα plasmid was dissolved in normal saline with the concentration of 2000 ug/ml. Each mouse of DGKα plasmid group was injected intramuscularly with 100 ug of DGKα plasmids in 50 ul volume on day -13, -4 before the first immunization. An empty plasmid (pEGFP-N3) vector was used as a control plasmid (**Figure 1**) in the control plasmid group.

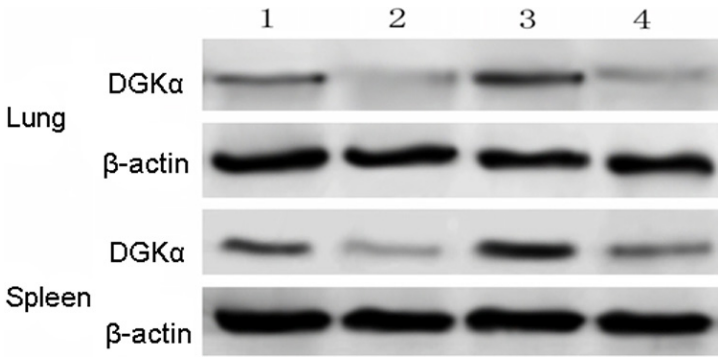

Figure 2. The expression of DGKα protein in spleen and lung. Mouse tissues were prepared and proteins were assessed by SDS-PAGE and western blotting. The blot was probed with anti-DGKα Ab. 1. Normal group, 2. Model group, 3. DGKα plasmid group, 4. Control plasmid group.

Detection of DGKα protein expression by western blotting

Mouse DGKα protein expression in lung and spleen was detected using Western blotting. Total cell lysates were resolved in SDS-PAGE and transferred to nitrocellulose membranes for Western blot analysis. Proteins were visualized with anti-DGKα monoclonal antibody (sc-271644, Santa Cruz, America).

Bronchoalveolar lavage and histopathological examination

Mice were sacrificed 48 hours after the last OVA challenge. After retro-orbital bleeding under anesthesia, the serum was collected and stored at -70°C. Eight animals were used for BAL fluid while another four for lung histopathological examination.

BAL collection and lung fixation were performed essentially as previously described [19]. A 16-gauge catheter was inserted into the exposed trachea of mice and secured with ligatures. The ribcage was opened, and the lung was lavaged with 0.8 ml of saline for three times. The lavage fluid was centrifuged at 1500 rpm for 10 min. After washing, the BAL cells were resuspended in 1 ml PBS, and the total cells were counted with hemocytometer. Cytocentrifuged preparations were stained with hematoxylin and eosin (HE) for differential cell counts. A minimum of 600 cells were counted and classified as Eos, lymphocytes, macrophages, neutrophils and macrophages, according to the standard morphological criteria. Supernatants of BAL were also collected, stored at -70°C.

The lung was fix-inflated with 10% buffered formalin. The fixed lung was then excised and fixed in formalin overnight. The tissues were subsequently embedded in paraffin and cut into 5 μm thick sections. These sections were stained with HE. Lung histology were examined and photographed with an Olympus microscope equipped with a digital camera. The degree of inflammation and Eos infiltration was quantified by image analysis.

After retro-orbital bleeding under anesthesia, lungs were lavaged three times with 0.8 ml PBS and the BAL fluid was collected. The supernatants were removed and stored at -20°C. Cell pellets were resuspended in 1 ml PBS and total cells were counted with a hematocytometer. For histopathological examination, the right and left lungs were sectioned from top to bottom, with four-to-five cross-sectional pieces taken from each lung.

Enzyme-linked immunosorbent assay (ELISA) for cytokine production

Supernatants of BAL were assayed for IFN-γ, IL-4 and IL-13 by ELISA (eBioscience, America). The assay inter-well variances were <10% for cytokine concentrations ranging 5-10 pg/ml.

Quantitation of OVA-specific IgE

OVA-specific IgE levels in sera were measured using ELISA kits according to the procedure recommended by the manufacturer (Chondrex, America).

Statistical analysis

Data are expressed as mean±SD. The significance of differences between experimental groups was analyzed using analysis of variance (ANOVA). A P value <0.05 was considered significant.

Results

Expression of the DGKα protein

After the mice were killed, DGKα protein expression in murine lung and spleen tissues was

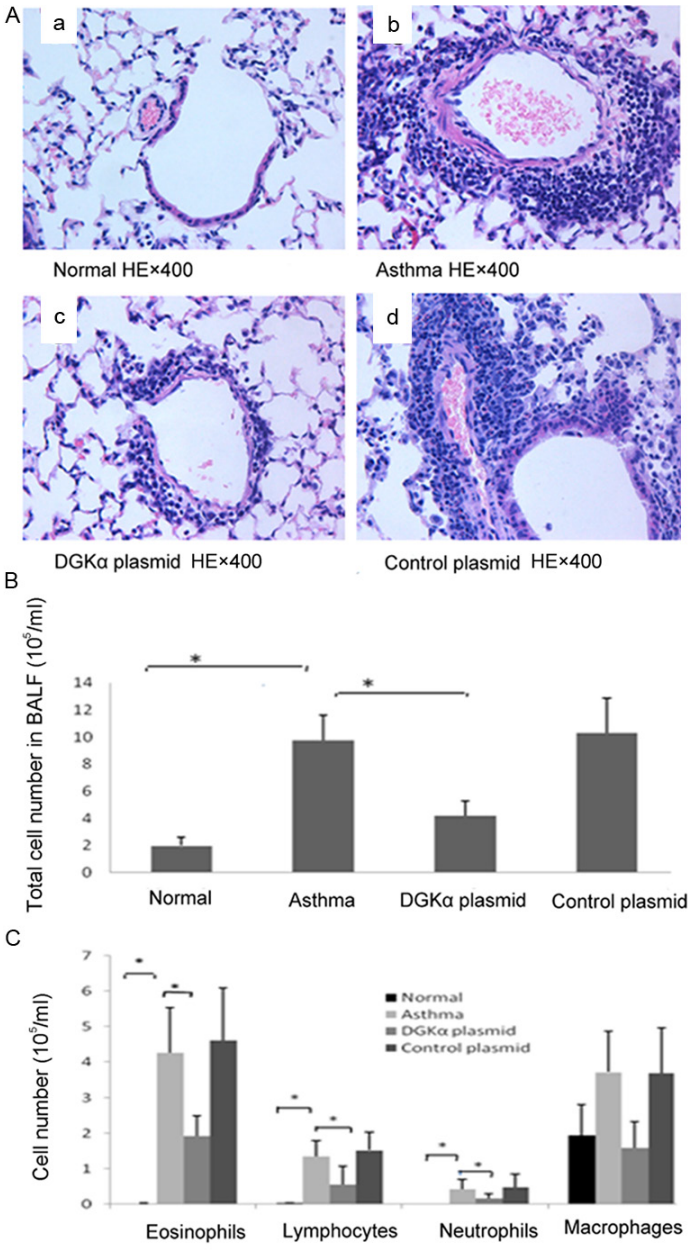

Figure 3. Immunization with DGKα DNA could prevent airway eosinophilic inflammation. Lung tissues and BALF were collected 48 hours after the last OVA challenge. A. Representative histological examination of lung section stained with hematoxylin and eosin (×400 magnification). a. Normal group, b. Model group, c. DGKα plasmid group, d. Control plasmid group; B. Total cell numbers in BALF were assessed. C. Differential cell counts in BALF were analyzed. Results are expressed as mean±SD pg/ml for eight mice in each group. Results are expressed as mean±SD pg/ml for eight mice in each group (*p<0.05).

analyzed by Western blot assay using a monoclonal antibody against DGKα. Mouse DGKα monoclonal antibody was raised against amino acids 242-298 mapping within an internal region of DGKα of human origin. As shown in **Figure 2**, in spleen and lung tissue, the expression of the DGKα protein in the model group was lower than that in the normal group. Administration with DGKα plasmid DNA significantly increased the expression of DGKα protein in lung and spleen tissue. We also found that DGKα was highly expressed in spleen and lowly in lung. It was coincidently with the previous study that DGKα is abundant in T lymphocytes and highly expressed in spleen and thymus [15].

Immunization with DGKα DNA protected mice from airway eosinophilic inflammation

Since administration of the DGKα plasmid could increase the expression in vivo, we tested whether DGKα DNA vaccine could protect mice from the development of asthma. We constructed a murine model of asthma sensitized and challenged by OVA. DGKα plasmid or control plasmid was intramuscularly injected into the mice on the 13th, 4th before the first sensitization. In the model group, eosinophil infiltration in the bronchial interstitium, particularly in the peribronchiolar and perivascular area, and damaged epithelial cells lining was observed. No inflammation was observed in the normal group. Lung histology showed that the administrations of DGKα plasmid decreased the infiltration of inflammatory cells in the airway, especially eosinophil, while administration of control plasmid did not (**Figure 3A**).

To further investigate the effects of DGKα DNA on allergen-induced airway inflammation, we examined cell counts in BALF (**Figure 3B**, **3C**). Consistent with the histological data, results of cell counts showed that the total number of cells and the number of eosinophils in BALF was significantly increased in the model group compared with those in the normal group. In the DGKα plasmid

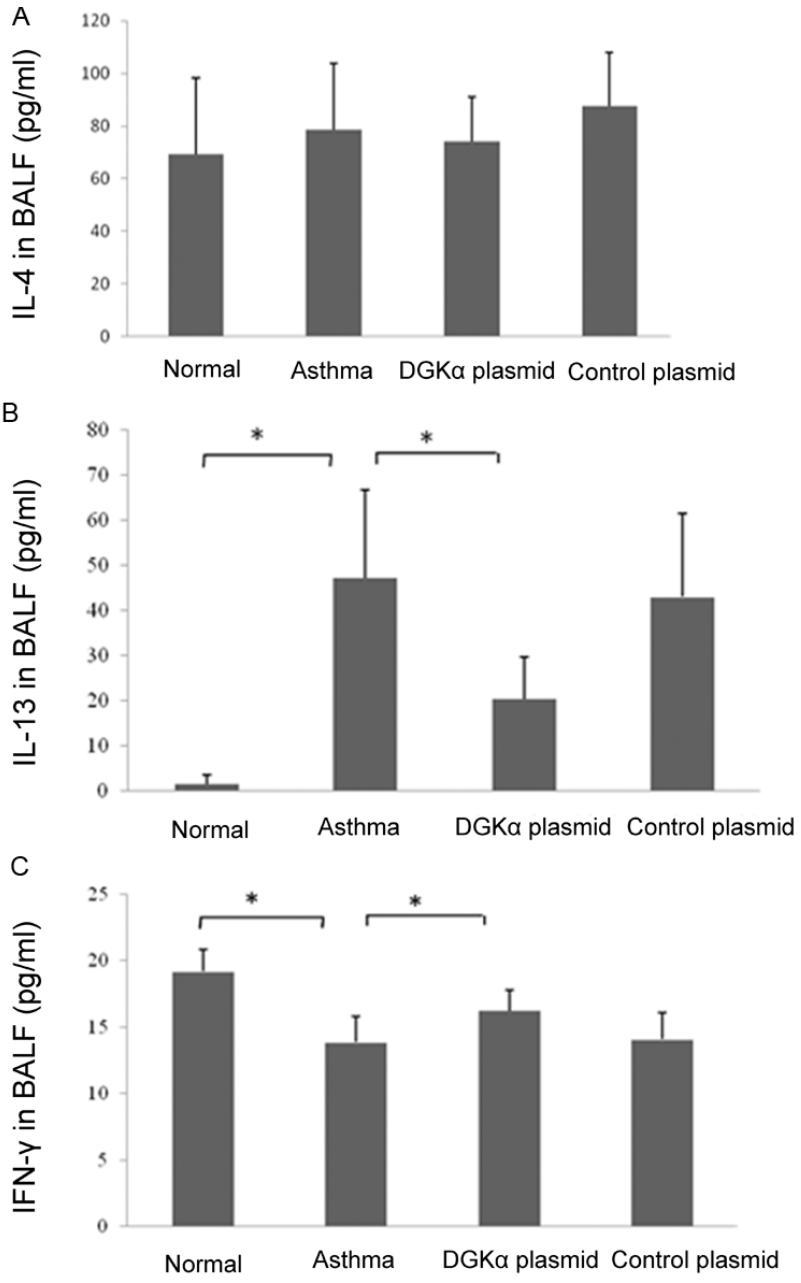

Figure 4. Immunization with DGKα DNA decreased the level of IL-5 and IL-13, and increased the level of IFN-γ in BALF. Results are expressed as mean±SD pg/ml for eight mice in each group (*p<0.05).

group, airway inflammation was inhibited with a 56.86% decrease in the total number of cells and a 54.93% decrease in the number of eosinophils compared with those in the model group. Nevertheless, there was no difference between the control plasmid group and the model group. These results revealed that administration of the DGKα DNA could protect mice from airway eosinophilic inflammation.

Immunization with DGKα DNA changed the cytokine production in BALF

Increase of Th2 cytokines and imbalance between Th1 and Th2 cells is the key feature in the murine model of asthma. IL-4, IL13 and IFN-γ levels in BALF were analyzed in the present study. As shown in **Figure 4**, compared with the normal group, the level of IL-13 was increased and IFN-γ was decreased in the model group. However, there was no difference among the four groups for IL-4. The results showed that the intramuscular injection of DGKα significantly reduced the level of IL-13 and improved the level of IFN-γ in BALF, but could not reduce the level of IL-4.

Immunization with DGKα DNA suppressed the production of serum OVA-specific IgE antibody

The evaluation of the efficacy of immune response immunized by DGKα DNA vaccine was performed by measuring the serum OVA-specific IgE antibody level in all groups by ELISA assay. We observed a strong OVA-specific IgE response in the model group. Nevertheless, OVA-specific IgE could not be detected in the normal group. Intramuscular injection of DGKα plasmid prior to the sensitization significantly decreased the level of serumal OVA-specific IgE antibody. The results showed that administration with DGKα plasmid elicited the IgE immune response (**Figure 5**).

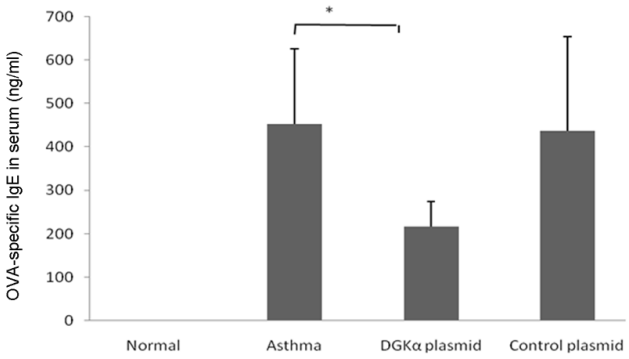

Figure 5. Immunization with DGKα DNA decreased the level of serum OVA-specific IgE. Results are expressed as mean±SD pg/ml for twelve mice in each group (*p<0.05).

Discussion

Allergic inflammation is orchestrated by Th2 cells, leading to IgE production and eosinophil activation. The important role of the allergen-specific Th2 cells in the immunologic and pathologic process of allergic asthma makes them potential target cells for therapy [4, 5]. In the present study, we demonstrated the effectiveness of DGKα DNA administration as a new type of immunogene therapy. The results showed that administration of DGKα plasmid prior to the sensitization can significantly decrease the total cell and eosinophil number, suppress the level of IL-5 and IL-13, and increase the level of IFN-γ in BALF. Administration of DGKα plasmid could strongly reduce the level of OVA-specific IgE and inhibit IgE immune response. Lung histological examination also showed that DGKα plasmid administration markedly decreased the infiltration of inflammatory cells in the airway, especially eosinophil.

T cell anergy and active suppression by regulatory T cells (Tregs) represent two important mechanisms for peripheral T-cell tolerance. Anergic T cells were incapable of proliferating after rechallenge with antigen, at least in part because of their inability to produce IL-2 [20, 21]. Understanding of the signaling networks regulating T cell anergy remains incomplete. However, several studies demonstrated that alterations in DAG metabolism could regulate the adoption of an anergic versus an activated T cell fate [13, 22, 23]. DAG activates many classic types of protein kinases and RasGRPs, and plays a critical role in T cell development and activation [24]. DGKα is a kinase that phosphorylates DAG to form PA and has been suggested to associate with T cell anergy [25]. DGKα were upregulated in anergic T cells, with its protein expression five to ten times of resting T cells [14]. Nevertheless, the mechanism by which DGKα induces T cell anergy has been unclear. Evidence indicates that DGKα might inhibit translocation of RasGRP1 to the cytoplasm membrane, and overexpression of DGKα might inhibit TCR-induced activation of the RasGRP1 related signal pathway [13]. Conversely, T cell lacking of DGKα produced more IL-2 and proliferated in response to T cell receptor ligation, and DGKα-deficient T cells are resistant to anergy induction [14]. In the present study, DGKα gene was used to prevent allergic eosinophilic inflammation for the first time, on the basis of its role in inducing T cell anergy.

Previous studies reported that immunotherapeutic DNA-based vaccines were used to treat asthma in experimental models [26-28]. The application of DNA vaccine-based immunotherapeutics prior to or shortly after allergen challenge, by directly modulating the cytokine milieu at the time of induction of localized responses, could influence the phenotype, functional activity and recruitment of effector T cells into the airways [29-33]. The results of the present research showed that immunization with DGKα DNA vaccine could inhibit Th2 response and the production of OVA-specific IgE, thus protecting mice from airway eosinophilic inflammation.

Th2 cytokines, such as IL-4 and IL-13, derived initially from activated CD4+ Th2 cells, are critical for the induction of Th2-dependent allergic responses and allergic airway inflammation [34, 35]. The results showed that immunization of DGKα vaccine could decrease the level of IL-13 but not IL-4. There was no difference for IL-4 level in BALF between asthma mice and normal mice, in line with previous studies [36, 37]. Nevertheless, the underlying mechanisms still remain unknown and further studies are needed.

Several limitations might exist in the present study. It was unclear whether the inflammatory

inhibition was due to the direct effect of the DGKα vaccine that induces T cell anergy or indirect effect of improving the imbalance Th1/Th2. To investigate the mechanism, further studies of the effect of the DGKα vaccine are required using DGKα deficient mice. Moreover, whether DGKα vaccine suppresses the proliferation of antigen-specific T cells and induces T cell anergy to specific antigen are needed to be clarified. However, the results of this study showed that DGKα vaccine administration effectively relieve airway inflammation in vivo.

In conclusion, in this study, we found that OVA-induced airway inflammation was inhibited after DGKα gene administration; meanwhile, Th1 cytokine production was increased while the Th2 cytokine production was decreased, suggesting that DGKα DNA-based immunotherapeutics might have therapeutic applications in the treatment of allergen-induced asthma and DGKα vaccine administration might be a potential approach to asthma gene therapy.

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Disclosure of conflict of interest

The authors declare that they have no competing interests.

Address correspondence to: Dr. Changzheng Wang or Dr. Yan Wang, Institute of Respiratory Diseases, Xinqiao Hospital, Third Military Medical University, Chongqing, 400037, China. E-mail: czwang@netease.com (Changzheng Wang); wangyansyd@yahoo.com (Yan Wang)

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