

Original article

DNA vaccine encoding Der p2 allergen down-regulates STAT6 expression in mouse model of allergen-induced allergic airway inflammation

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Keywords: DNA vaccine; *Dermatophagoides pteronyssinus* group 2; allergic airway inflammation; signal transducer and activator of transcription 6 (STAT6)

Background Activation of signal transducer and activator of transcription 6 (STAT6) plays a critical role in the late phase of Th₂-dependent allergy induction. STAT6 is essential to Th₂ cell differentiation, recruitment, and effector function. Our previous study confirmed that DNA vaccination inhibited STAT6 expression of spleen cells induced by allergen. In the present study, we determined whether DNA vaccine encoding *Dermatophagoides pteronyssinus* group 2 (Der p2) could down-regulate the expression and activation of STAT6 in lung tissue from asthmatic mice.

Methods After DNA vaccine immunization, BALB/c mice were sensitized by intraperitoneal injection and challenged by intranasal instillation of rDer p2. The levels of the cytokines IL-4 and IL-13 in BAL fluid were measured by enzyme-linked immunosorbent assay. The lung tissue was assessed by immunohistochemical staining with anti-STAT6. The protein expression of STAT6 was determined by Western blot. The activation of STAT6 binding ability was analyzed with electrophoretic mobility shift assay.

Results DNA vaccine encoding Der p2 allergen effectively decreased the levels of IL-4 and IL-13 in the asthmatic mice. Histological evidence and Western blot showed that the expression of STAT6 in the DNA treated mice was markedly attenuated. STAT6 binding to specific DNA motif in lung tissue from the gene vaccinated mice was inhibited.

Conclusion DNA vaccine encoding Der p2 prevents allergic pulmonary inflammation probably by inhibiting the STAT6 signaling pathway in mice with Der p2 allergen-induced allergic airway inflammation.

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The incidence of allergic asthma as a global common disease is increasing.¹ Evidence has shown that house dust mites are an important and commonly inhaled allergen causing asthma.² Since experimental studies have concentrated on asthma therapy,³ we once reported the effects of gene vaccination with plasmid DNA encoding Der p2 on immune response in allergic airway inflammation mice.⁴

Th₂ cytokines (IL-4 and IL-13) are considered to play a crucial role in initiating and maintaining the asthmatic airway and in the development of detrimental pathophysiologic responses.⁵ STAT6 is an obligatory transcription factor for IL-4 and IL-13, which is essential to IgE class switch recombination and contributes to the Th₂ cell differentiation.⁶ Because STAT6-deficient mice are unable to control

pulmonary inflammation after allergen challenge,⁷ STAT6 is a key transcription factor in the pathogenesis of allergic asthma. Until now, little is known about the regulation of STAT6 in the lung of asthmatic mice responded to gene vaccination with plasmid DNA. The aim of the present study was to

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assess whether plasmid DNA encoding Der p2 effectively prevents allergic pulmonary inflammation by inhibiting the STAT6 signaling pathway in the asthmatic mice.

METHODS

Construction of the eukaryotic expression vector pcDNA-Der p2

The eukaryotic expression vector pcDNA-Der p2 was constructed as described. Briefly, pBC167 was used as a template for polymerase chain reaction (PCR) with Taq polymerase with primers specific for Der p2. The amplified PCR products were then cloned into the pcDNA3.1 eukaryotic expression vector (Invitrogen, San Diego, CA, USA) and sequenced to verify the insertion of the right gene with an appropriate open reading frame. Plasmids were purified in a large scale with a QBIOMAXIprep GFII (tm) Endo-Free kit (USA) according to the manufacturer's instructions.

Immunization protocols

BALB/C mice, 6–8 weeks old, were obtained from the Guangzhou Experiment Animal Center. Thirty-two BALB/c mice were divided randomly into normal control group ($n=8$), pcDNA control group ($n=8$), DNA vaccine group ($n=8$), and asthmatic group ($n=8$). The mice were anesthetized and immunized by the injection of 100 μ l of inoculum into the tibialis anterior muscle through a 1-ml tuberculin syringe. The pcDNA control group was immunized with pcDNA3.1 plasmid (100 μ g/mice), the DNA vaccine group was treated with pcDNA-Der p2 (100 μ g/mice), and the asthmatic group was treated with phosphate-buffered saline (PBS). They were boosted 7 days later. Fourteen days after final vaccination, sensitization and challenge were performed as described. Briefly, the pcDNA control, DNA vaccine, and asthmatic groups were sensitized by intraperitoneal injection with 10 μ g rDer p2 and 10 μ g mite dust extract (Shenzhen University, China) adsorbed onto 4 mg aluminum potassium sulfate (alum) on days 23, 30 and 37. Airway challenge was induced in anesthetized animals by intranasal instillation of rDer p2 (25 μ g in 50 μ l PBS) for 7 days. The normal control group received PBS only. After 24-hour challenge, the mice were sacrificed, BAL fluids were collected, and the lungs were removed and fixed.

Levels of IL-4 and IL-13 in bronchoalveolar lavage

To investigate the Th₂ cytokines in the airway, the trachea was exposed and cannulated and the lung was gently instilled twice with 500 μ l cold PBS. After centrifugation of samples (500 r/min for 5 minutes at 4°C), BAL fluid was removed, stored at -70°C and the levels of IL-4 and IL-13 were determined by enzyme-linked immunosorbent assay (R&D systems, USA; sensitivity >2 pg/ml).

Detection of STAT6 in the lung by immuno-histochemical staining

The mice were sacrificed 24 hours after the last allergen challenge, their lungs were removed and fixed overnight in 10% formalin and embedded in paraffin. Sections of 5- μ m thickness were heated for 1 hour at 60°C, rehydrated, and incubated for 30 minutes with 0.3% H₂O₂/0.2 mmol NaN₃, followed by blocking with 1% bovine serum albumin (BSA) in TTBS (0.1% Tween, 100 mmol/L Tris-HCl, 0.9% NaCl). The sections were stained for 1 hour with rabbit anti-STAT6 (1:500, labvision, USA). After two washes in TTBS, horseradish peroxidase (HRP)-labeled anti-rabbit IgG_{2a} antibody was applied for 1 hour at room temperature. The sections were washed again and incubated for 4 minutes in medium containing 50 mmol/L Tris-HCl buffer (pH 7.6), 0.02% DAB and 0.01% hydrogen peroxide. Chromogenic reaction was terminated with water followed by hematoxylin counter staining.

Detection of expression of STAT6 in the lung by Western blot

The lung tissue of the mice was homogenized in cold PBS supplemented with 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor, and 100 μ g/ml of phenylmethylsulphonylfluoride (PMSF). The lysate was clarified by centrifugation (12000 r/min, 4°C, 5 minutes). From the supernatant, 15 μ g protein was separated by 12% SDS-PAGE and electrotransferred into a nitrocellulose membrane for Western blotting. The membrane was then blocked with 1% BSA in washing buffer for 1 hour, incubated with anti-STAT6 (1:500, labvision, USA) overnight, washed with TTBS for 3 times, incubated with HRP-labeled secondary antibody (1:1000) for 1 hour, and washed with washing buffer for 3 times. Chemiluminescent substrate (KPL, USA) was added and the membrane was finally subjected to

autoradiography.

Detection of STAT6-DNA complex in the lung by EMSA

The nuclear extracts were prepared by nuclear extract kit (Activemotif, USA) according to the modified method of Deryckere and Gannon.⁸ After the last 24-hour challenge, the lung tissue was removed and washed with ice-cold PBS supplemented with phosphatase inhibitors. Nuclear extracts were prepared from nuclear pellets after hypotonic lysis in complete lysis buffer. Measured by BCA, the protein was stored at -80°C for use. Gel shift oligonucleotides were labeled with terminal transferase and DIG-11-dUTP (Roche, USA). DNA probes 5'-CTTCTTCCCAAGAAACAG-3', 5'-CTGTTTCTTGGGAAGAAG-3' were at the high-affinity STAT6-binding site with N4 motif "TTCnnnnGAA" in both human and murine germline ϵ promoters.⁹ EMSA was performed according to DIG gel shift kit instruction manual (Roche, USA). Nuclear extracts (20 μg) and DNA probes (15–30 fmol) were added to the binding buffer including poly[d(I-C)] and incubated for 15 minutes at 15–25°C. The reaction mixture was resolved on 5% nondenaturing polyacrylamide gel at 4°C in 0.5xTBE buffer (90 mmol/L Tris-borate, 2 mmol/L EDTA), and transferred by electro-blotting for 60 minutes at 400 mA. The nylon membrane was rinsed briefly in washing buffer, and incubated for 30 minutes in anti-digoxigenin-AP (1:10 000) for 30 minutes after being blocked, equilibrated for 5 minutes in detection buffer. The membrane was placed on hybridization bag, and CSPD working solution was applied. Finally, the membrane was exposed to X-ray film for 20 minutes at 15–25°C.

Statistical analysis

The data were analyzed by the SPSS software to assess the statistical difference and expressed as mean $\pm$ standard deviation (SD). $P < 0.05$ was defined statistically significant.

RESULTS

Inhibition of IL-13 and IL-4 in BALF by DNA vaccine

The cytokine profile within the lung after DNA vaccination was studied in the BAL fluid. The mice receiving DNA vaccine showed a lower level of IL-4 ($P < 0.01$) than those receiving pcDNA or PBS (Fig. 1). The trend of IL-13 secretion was similar to that of

IL-4. DNA vaccinated mice produced less IL-13 than PBS and pcDNA groups, both of which showed significant levels of IL-4 and IL-13. DNA vaccine markedly decreased the production of Th₂ cytokine in the pathogenesis of allergic asthma, thus down-regulating allergic inflammatory response in the airway.

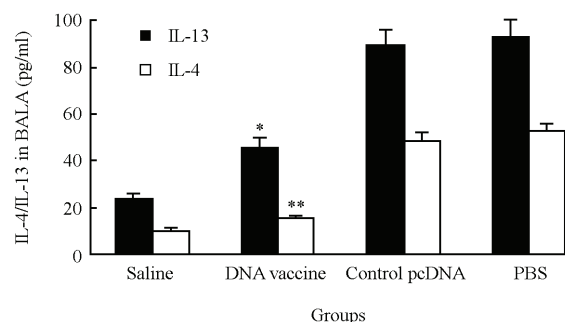


Fig. 1. The levels of IL-4 and IL-13 determined by enzyme-linked immunosorbent assay in the BAL fluid. Compared with pcDNA and PBS groups, * $P < 0.01$. ** $P < 0.01$.

Histopathologic features of STAT6 in the lung tissue after DNA vaccination

Since histopathologic distribution of STAT6 is directly related to the induction of an allergic peribronchial inflammation of Th₂-mediated cytokine responses, we investigated histologically the efficacy of pcDNA vaccines in modulating the expression of STAT6 on allergen challenged mice. Twenty-four hours after the last challenge, the mice were sacrificed and the lung was examined histologically by anti-STAT6 staining. After allergen challenge, the expression of STAT6 was markedly increased in the asthmatic group (Fig. 2). A strong but scattered staining of STAT6 was detected in all biopsy specimens, predominantly in the inflammatory tissues of the lung. In contrast, DNA vaccinated mice exhibited a poor STAT6 in the response as compared with pcDNA and PBS asthmatic groups, indicating the decreased production of a Th₂-associated transcription factor. The attenuated vestige of STAT6 in the DNA vaccinated mice was similar to that in the lung of the saline-treated mice. This evidence revealed that allergen challenge induced a strong expression of STAT6 in the lung tissue, but it was considerably lower in the DNA vaccinated mice.

Western blot in detecting the expression of STAT6 in the lung

The STAT6 expression in the asthmatic group and

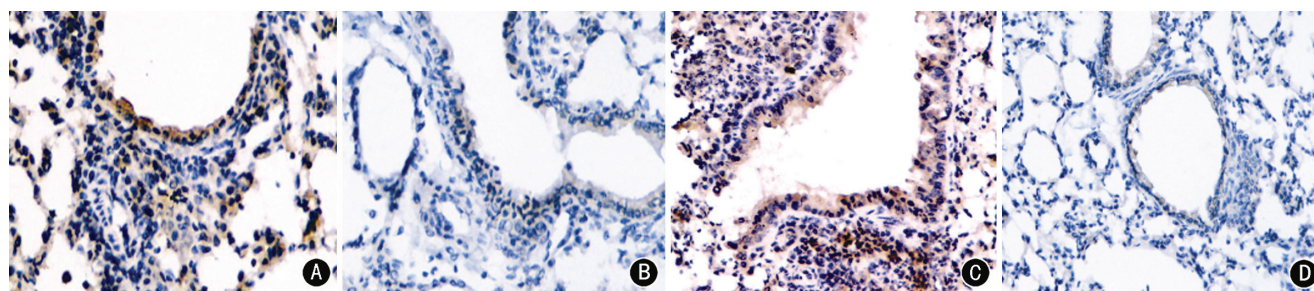


Fig. 2. Immunohistological analysis of representative sections of the lung tissue obtained from different treatment groups 24 hours after allergen challenge. The expression of STAT6 detected morphologically and cellular distribution shown by staining with anti-STAT6. **A:** asthmatic group; **B:** DNA vaccine group; **C:** pcDNA group; **D:** control group. (original magnification $\times 200$)

the pcDNA group (lanes 1–2) was significantly increased as compared with that in the saline group. In contrast to the augmentation, the STAT6 expression in the asthmatic group after gene vaccination with plasmid encoding Der p2 DNA was weaker. No difference was observed in the expression of STAT6 between saline and DNA vaccinated mice (Fig. 3, lanes 3–4). The marked down-regulation of STAT6 expression in the DNA vaccinated mice suggested that the effect of the DNA vaccine on the asthmatic group was marked in STAT6 inhibition during allergen-induced Th₂ cytokine expression. Possibly the down-modulation of IL-4 and IL-13 was reversely responsible for the inhibitory action of STAT6 expression.

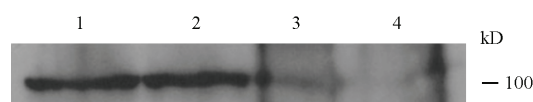


Fig. 3. Detection of STAT6 expression in mouse lung by Western blot. Lane 1: PBS group; lane 2: pcDNA group; lane 3: DNA vaccine group; lane 4: saline group.

DISCUSSION

after last challenge and the general state of serious airway inflammation. In contrast, STAT6 binding the nuclear extracts of the lung in the asthmatic mice after gene vaccination with plasmid encoding Der p2 DNA was inhibited (Fig. 4, lane 3). This result and the cytokine evidence of IL-4 and IL-13 demonstrated that IL-4 and IL-13 production was inhibited by the suppression of STAT6 activation after the administration of DNA vaccine.

Asthma is a complex disease with an increasing incidence and mortality worldwide. Pathologically, asthma has a complicated interaction of multiple cell populations and diverse mechanisms, characterized at a molecular level by chronically increased expression of multiple inflammatory proteins.¹⁰ Cytokines secreting predominantly from Th₂ cell like IL-4 and IL-13 are a kind of proteins which involve in the asthmatic process, playing an integral role in exuberant CD₄⁺ Th₂-cell activation, while activating inflammatory and residential effector pathways directly or indirectly.¹¹ The effects of gene vaccination with plasmid DNA encoding Der p2 on the mice with allergic airway inflammation are mostly based on the therapeutic strategy of rebalancing Th₁/Th₂ immune responses.⁴

Detection of STAT6-DNA complex in the lung by EMSA

To assess whether the efficacy of DNA vaccine on the asthmatic group was possibly via the STAT6 signaling pathway as a potential mechanism for the down-regulated production of Th₂ cytokines, we measured the nuclear translocation and DNA binding of STAT6 in lung extracts. In the groups of the asthmatic mice treated with PBS and control pcDNA (Fig. 4, lanes 1–2), there was a plenty of STAT6 in the nuclear extracts of the lung, indicating the allergen-induced STAT6 activation in the lung

We found that gene vaccine encoding Der p2 generates immunologic protection in recombinant Der p2 allergen-induced allergic airway inflammation mice model, and that DNA vaccination inhibits STAT6 expression of spleen cells induced allergen. The important function of STAT6 in allergic airway disease

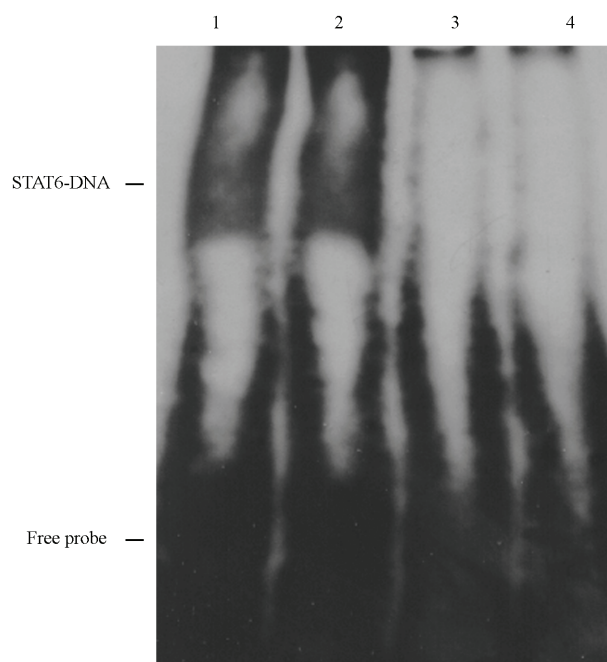


Fig. 4. Effect of DNA vaccine on activation of STAT6 detected by EMSA. Lung nuclear extracts were obtained at 24 hours after last challenge. Lane 1: PBS group; lane 2: pcDNA group; lane 3: DNA vaccine group; lane 4: saline group.

has been found in human and genetically-manipulated mice.¹² STAT6-deficient (STAT6^{-/-}) mice fail to generate IgE and a Th₂ cytokine response, and consequently have no characteristic markers of airway inflammation in the soluble ovalbumin-induced allergic airway disease,¹³ suggesting that STAT6 plays a crucial role in the pathogenesis of allergen-induced airway inflammation. Recent studies revealed that STAT6 is essential in the late phase of Th₂-dependent allergy induction¹⁴ and is responsible for the action of IL-4 and IL-13 because both cytokines share common elements in their receptors^{15,16} IL-4 is an important mediator of allergic inflammatory responses characterized by eosinophil and lymphocyte recruitment. IL-13 contributes to the development of asthma by acting directly on resident airway cells. IL-4 and IL-13 may also increase the secretion of airway mucus while exacerbating the airway responsiveness.¹⁷

In this study we assessed the effects of DNA vaccine on the regulation of STAT6 in the lung tissue of mice. The levels of IL-4 and IL-13 increased significantly in the airway of the asthmatic group with reaction to rDer p2 challenge. The result was

consistent with that IL-4 and IL-13 are produced at elevated levels in the asthmatic lung.¹⁸ In the DNA vaccinated mice, no aggressive increase was seen in the levels of IL-4 and IL-13 as compared with the saline control. This finding confirmed that DNA vaccine might decrease the production of Th₂ immune factors while releasing the allergic inflammatory response in the airway. Reports demonstrated that both IL-4 and IL-13 mediate allergic inflammatory responses via the phosphorylation and activation of STAT6.^{19,20} In our studies, Th₂-dominant asthmatic mice showed higher expression of STAT6, which may be related to the overproduction of IL-4 and IL-13 after allergen challenge. Histologically, a large amount of STAT6 was present in the lung tissue of the asthmatic group. Western blot analysis showed that STAT6 expression in mice vaccinated with plasmid encoding Der p2 DNA was weaker than that in the asthmatic group. The data indicated that the expression of STAT6 in the DNA treated group was markedly attenuated.

STAT6 plays a major role in the initiation of signals from activated Th₂ cells, specifically through IL-4 and IL-13 receptors. IL-4 receptor-mediated phosphorylation of STAT6 leads to its dimerization and nuclear localization, where it binds to the promoter elements of immunoglobulin gene and causes the expression of the ϵ -transcript.²¹ A direct effect of IL-13 on epithelial cells has been demonstrated through the selective expression of STAT6 under an epithelial-specific promoter in STAT6-null mice. Epithelial STAT6-positive mice showed significant increases in AHR and mucus overproduction after activation of an IL-13 transgene.²² In our study, STAT6 binding to DNA response element was increased in the asthmatic mice, but inhibited after gene vaccination. The effects of DNA vaccine in the asthmatic mice was produced possibly via the STAT6 signal pathway as a potential mechanism for the down-regulated production of Th₂ cytokines.

In conclusion, DNA vaccine down-regulates STAT6 expression, and inhibits STAT6 binding to specific DNA motif in cell nucleus. This effect is related to the lower levels of IL-4 and IL-13 in mice vaccinated with DNA vaccine. The down-regulation STAT6 expression may be one of the mechanisms

of DNA vaccine encoding Der p2 allergen generating immunologic protection in the mice with allergen-induced allergic airway inflammation. But the underlying mechanism of DNA vaccine for the regulation of STAT6 in asthmatic mice remains to be discussed.

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