Derp2-mutant gene vaccine inhibits airway inflammation and up-regulates Toll-like receptor 9 in an allergic asthmatic mouse model

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Summary

Background: A DNA vaccine encoding the whole segment of the Derp2 allergen could prevent allergic airway inflammation in a Derp2 allergen-induced allergic airway inflammation mouse model.

Objective: This study investigated the effect of DNA vaccine encoding Derp2-mutant gene in which an IgE epitope was deleted on airway inflammation and the role of TLR9 in the asthmatic mouse model.

Methods: A Derp2-mutant DNA vaccine was constructed. Mice were immunized, sensitized and challenged. Airway inflammation, airway hyper reactivity (AHR) and serum antibody were tested. The expression of Toll like receptor9 (TLR9) was detected with western-blot and immune-histochemistry.

Results: We demonstrated that the Derp2-mutant-DNA induced IgG2a and inhibited IgE production, inhibited airway allergenic inflammation and AHR. Derp2-mutant-DNA vaccine induce TLR9 expression in lung tissue.

Conclusions: The data indicate that allergen DNA vaccine deleted IgE epitope could prevent allergenic airway inflammation, AHR, and up-regulate lung TLR9 express. ***(Asian Pac J Allergy Immunol 2010;28:287-93)***

Key words: *DNA vaccine, TLR9, asthma, airway inflammation*

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Introduction

Asthma is a common allergic disorder associated with Th2 cytokines, allergen-specific IgE and airways hyper responsiveness[1]. Allergens are an important trigger factor for asthma attacks. Dermatophagoides pteronyssinus is the most important cause of inhaled allergen induced asthma[2]. Allergen specific immunotherapy is a major treatment for allergic asthma at present with allergen crude extract, but it is associated with many problems, such as difficulty in standardizing the allergen, the risk of inducing an allergic reaction mediated by IgE production in response to the treatment and difficulty in determining the dosage[3]. All of these problems are confined to the allergen crude extract used widely in clinical practice. This has stimulated the search for a vaccine with high quality and safety.

We reported that a DNA vaccine encoding the whole segment of Derp2 allergen prevents allergic pulmonary inflammation, probably by inhibiting the STAT6 signaling pathway in mice with Derp2 allergen-induced allergic airway inflammation[4]. It was reported that amino acid residues 21-27, 73-78, 118-119 of DerP2 were important allergens for IgE epitopes[5]. TLR play a very important role in recognizing the invasion of pathogenic microorganisms in innate immunity.

The DNA vaccine activation of the immune system was associated with Toll-like receptors (TLR) and CpG motif in immune cells[6,7]. However, it is not clear whether allergen DNA vaccine deleted IgE epitope is effective in preventing allergenic airway inflammation. This study investigated the effect of DNA vaccine encoding Derp2-mutant gene, in which the IgE epitope was deleted, on airway inflammation and the role of TLR9 in the asthmatic mouse model.

Methods

Reagents and mice

pCDNA3.1 plasmid was obtained from Invitrogen (USA). A Cycle-Pure Kit(100) was obtained from OMEGA (USA).Mite dust extract and recombinant Derp2(rDerp2) were stored in our lab. Peroxidase-conjugated anti-mouse IgE, IgG1 and IgG2a antibodies were obtained from Southern Biotech (USA) and BALB/c mice (6-8 weeks old) were obtained from the Hua Xi Experiment Animal Center, China.

Amplification of Derp2-mutant by polymerase chain reaction

Amino acid residues 21-27, 73-78,118-119 of Derp2 allergen were deleted by gene synthesis technology (HD Biosciences, Shuanghai, China). pBC167 encoding Derp2 cDNA was the template for polymerase chain reactions (PCR) with Pfu polymerase and primers specific for Derp2 (5'-GCGGCATATGGATCAAGTCGATGTCAAAG -3'and5'-GCGGGAATTCATCGCGGATT TTAGCATGAG -3'). The primers included Nde l and EcoR I sites for cloning into PET32a. The total reaction volume was 50ul and the condition was as follows: an initial denaturation for 5 min at 94 degrees, denaturation for 30sec at 94 degrees C,annealing for 1min at 57 degrees C,extention for 90sec at 72 degrees C.There were 30 cycles in all. An extention for 10min at 72 degrees C was included to be sure that the polymerase has completely finished its work. The product of PCR was recycled by QIA quick Gel Extraction Kit after agarose gel electrophoresis to obtain the desired gene.

Construction of Derp2-mutant-DNA vaccine

After purification by gel, Derp2-mutant and pET32a were double digested with Nde l and EcoR I. Then ligation was carried out at 16 for 16h by T4 DNA ligas. 5ul of the ligated product was transformed into competent E.coli TOP10.Asingle transformed colony was selected to extract plasmid,using PET32a containing Derp2-mutant gene as a template for PCR. This primer (5'-CTG GAT CCG ATA TGG ATC AAG TCG ATG-3' and 5'-CGG GAA TTC TTA ATC GCG GAT TTT AGC-3') includes BamH I and EcoR I sites for cloning. The product of PCR was first identified by double digestion with BamH I and Eco R I .Then it was sequenced to verify that the gene with an appropriate open reading frame was correctly inserted. The right recombinant gene was cloned into pCDNA3.1 which is an eukaryotic expression vector (Invitrogen, USA) and sequenced. Large-scale purification of plasmid was manipulated using a Cycle-pure kit (100) (OMEGA gene Inc, USA) according to the manufacturer's instructions.

Immunizing protocols

BALB/c mice (8-week-old, female, 20-25 g) were maintained under standard conditions with free access to water and animal feeds. Forty mice were divided randomly into asthma group, rDerp2 group, Derp2 -mutant-DNA vaccine group and normal control group. The mice were anesthetized and immunized by the injection of 100ul of inoculums into the tibialis anterior muscle using a 1ml tuberculin syringe at days 0 and 8. The

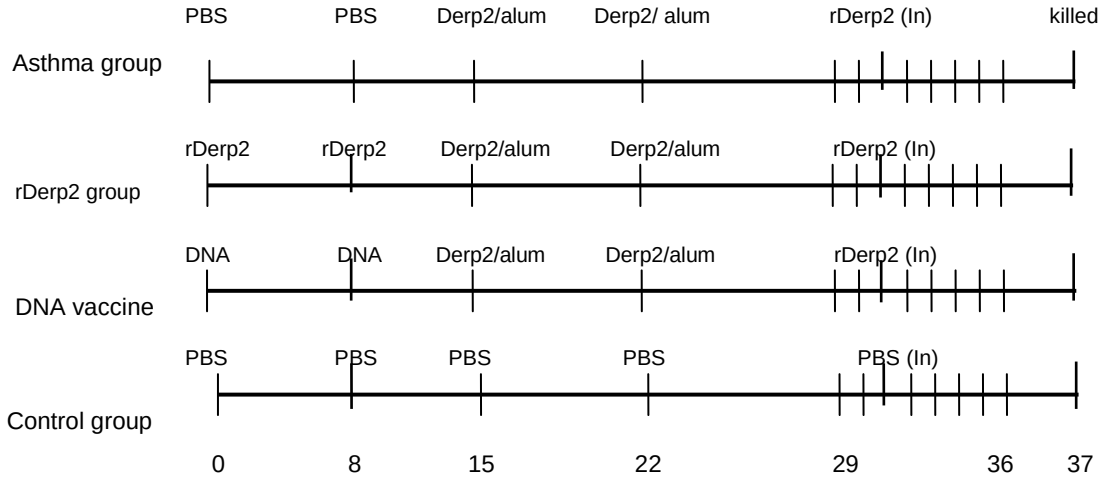

Figure 1. Experiment scheme for immunotherapy. On day 0 and 8, mice were immunized in tibialis anterior muscle. Mice were intraperitoneally sensitized at 15, 22 day. From day 22 to 29, mice were boosted with intranasal instillation allergen.

asthma group, rDerp2 group and Derp2 -mutant-DNA vaccine group were pretreated respectively with PBS (100ul), rDerp2 (100ug) and Derp2 -mutant-DNA vaccine (100ug). Then the mice were intraperitoneally sensitized by 10ug recombinant Derp2 and 10ug house dust mite crude extract plus 4mg aluminum hydroxide at days 15 and 22. After sensitization the four groups were intranasal challenged by 25ug/50ul recombinant Derp2 from day 29 for seven consecutive days. 24 hours after the last intranasal challenge, the following checks were made. (Figure 1) The control group only received PBS.

Pulmonary function assessment

The AHR of the mice was measured using the double-chambered whole body plethysmograph (Buxco, USA). AHR was expressed as an enhanced minute pause (Penh), which reflects pulmonary resistance. Each mouse was placed in an unrestrained whole body plethysmograph without anesthetization and exposed to nebulized physiologic saline for 3 minutes. Increasing concentrations of nebulized methacholine (0, 1.5, 3, 6, 12, 24, 48) were administered by an ultrasonic nebulizer. Measurements were obtained for 3 min after each nebulization. Penh were measured during this period, which were averaged and expressed as absolute Penh values. Data from regular ventilation was collected and recorded for each mouse. After the data were obtained from the Penh System they were automatically analyzed using Fine Point software.

Determination of specific antibody levels of Derp2 in serum

Peripheral blood was collected to determine specific IgE, IgG1 and IgG2a of Derp2 by ELISA. The method was performed as described previously[8]. 96 microtiter plates were coated with 100ul Derp2 (10ug/ml in 0.1mol/L carbonate buffer, PH9.6) overnight at 4 °C. The Derp2-coated plates were washed five times with PBST (0.5% Tween-20 in PBS). 1:100 Diluted mouse serum (1ul serum with 100ul sample dilution) was added to the Derp2-coated wells (100ul/ well) and incubated for 2 hours at 37°C. The plates were then washed with PBS five times. Peroxidase-conjugated anti-mouse IgE (1:1000), IgG1 (1:4000) and IgG2a (1:4000) antibody (Southern Biotech, USA) was added to the to the plates (100ul/well) and incubated for 2 hours and washed as above. 3, 3′, 5 and5′-Tetramethylbenzidine(TMB), 100 µl, were added to the plates and incubated for 15min at 37°C, away from light. The reaction was stopped with sulfuric acid and measured at 450nm.

Histological evaluation and immunohistochemistry

The lung tissue weas fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections (5um) of specimens were put onto 3-amino propyltriethoxy silane-coated slides. The tissues were assessed for general morphology and cellular infiltration with haematoxylin and eosin (H&E) staining. The lung tissues were also used to carry out immunohistochemistry to detect the expression of TLR9 on the airway epithelium. Formalin-fixed and paraffin-embedded lungs were deparaffinated and hydrated. The antigen was repaired with microwaves. Confining liquid was added for 20 minutes at room temperature and the redundant liquid was discarded. The sections were incubated with anti-TLR9 monoclonal antibody (Abcam) at 37 degrees for 2h (1:500). The slides were washed with PBS for 3x5min and incubated with HRP-labeled goat anti-mouse immunoglobulin antibody (Beyotime) at 37 degrees for 1h (1:1000). The slides were washed with PBS for 3x5min and coloured with DAB finally.

Detection of expression of TLR9 in the lung tissue by Western-blot

150mg lung tissue was weighed from each group and homogenated with lysing buffer (1000ul RIPA with 10 ul PMSF, Beyotime). Suspensions were centrifuged at 10000 rpm/min at 4 °C for 30 minutes and then the supernatant was collected. The protein levels of the sample from the four groups were measured using a BCA concentration measurement kit (P0012, Beyotime). 15ug of protein was loaded from each group with 2xSDS to equalise final volume. A rainbow pre-stained marker was used for reference. The protein was separated by 8% SDS-PAGE (100V 1.5h) and electrophoresis and transferred into nitrocellulose membranes with mini trans-blot electrophoretic transfer cells, BIO-RAD (300mA 30min). The membrane was blocked with western confining liquid (Beyotime) at 37°C for 1hour. Dividing the membrane at 72kd into two pieces and incubating them with anti-TLR9 monoclonal antibody (Abcam) and anti-β-actin monoclonal antibody (Beyotime)

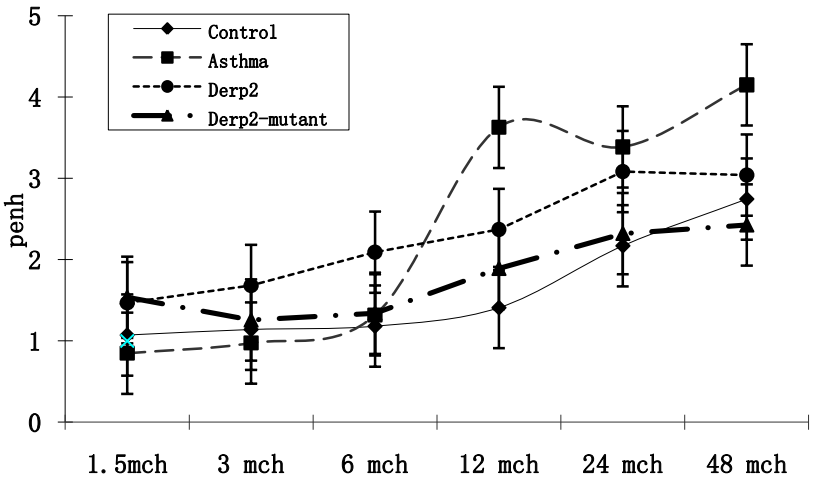

Figure 2. Assessment of airway hyper-responsiveness following allergen sensitization and challenge. The AHR of mice was measured by the double-chambered whole body plethysmograph. AHR was expressed as Penh, Penh was analyzed in asthma group, rDerp2 group, Derp2-mutant-DNA vaccine group and control group after last stimulation by Meh. ▲$P < 0.05$ compared with asthma group (12, 24, 48mch).

respectively (1:500) at 37°C for 2h. The membrane was washed with PBS for 3x10min and incubated in HRP-labeled goat anti-mouse immunoglobulin antibody (Beyotime) with 1 : 1000 ratio at 37°C for 1hour. It was then washed with PBS for 3x10min. The chemiluminescent substrate (Beyotime) was added to the membrane and exposed strips were obtained from a Kodak Image Station 4000MM（USA）.

Statistical analysis

Data were expressed as mean ± standard error. Statistical analysis was performed by ANOVA, and the significant level was defined as $P < 0.05$.The data were analyzed by SPSS statistical software.

Results

Effects of Derp2-mutant-DNA vaccine on airway hyper reactivity

Mice were challenged with aerosol control followed by increasing dosages of methacholine and the baseline reading of Penh was collected. Penh was analyzed by Fine Point software. (Figure 2) Mice in control group did not show an alteration in their baseline Penh values or airway responses to increasing concentrations of methacholine. The asthma group showed significant airway hyper-responsiveness compared with the Derp2-mutant-DNA vaccine group which displayed lower Penh values ($P < 0.01$). The rDerp2 group showed higher Penh than Derp2-mutant-DNA vaccine group.

Allergen-specific IgE, IgG1 and IgG2a in serum

Allergen-specific IgE, IgG1 and IgG2a in the serum were detected by ELISA. The levels are shown in Figure 3. The results indicated that the Level of IgE and IgG1was inhibited by Derp2-mutant-DNA vaccine, compared with the asthma group and the rDerP2 group ($P<0.05$). The level of IgE in the rDerP2 group was higher than that in Derp2-mutant-DNA vaccine group ($P < 0.05$). The level of IgG2a in Derp2-mutant-DNA vaccine group was increased compared with the control and asthma groups ($P < 0.01$), but there was no difference with the rDerP2 group ($P > 0.05$).The results indicate that Derp2-mutant-DNA could induce IgG2a and inhibite IgE.

Inhibition of allergen-induced airway inflammation by Derp2-mutant-DNA vaccine

Histological analysis was performed to investigate the efficacy of Derp2-mutant-DNA vaccine in airway inflammatory. Extensive infiltration of inflammatory cells around the central bronchi, lung alveolus and blood vessels were inhibited by Derp2-mutant-DNA vaccine, compared with the asthma group. However in the rDerp2 group there was more extensive infiltration of inflammatory cells than in the lung tissue of the Derp2-mutant-DNA vaccine group.

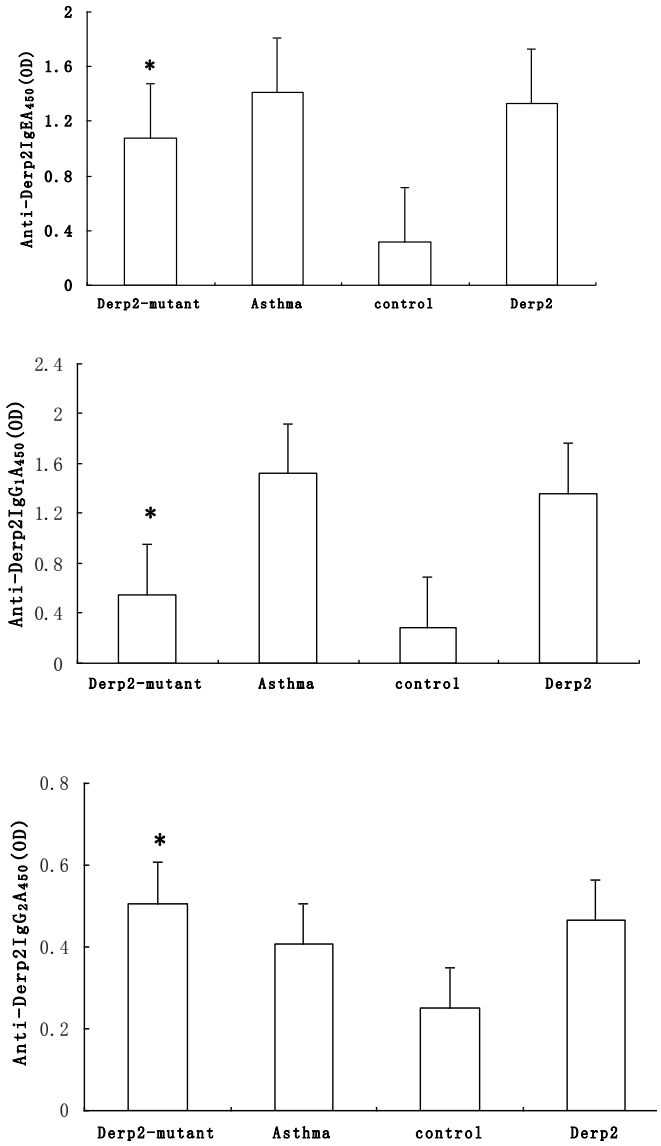

Figure 3. Level of IgE, IgG1 and IgG2a in sera of the tested mice. IgE (A), IgG1(B) and IgG2a(C) levels in the sera of the tested mice were determined by ELISA. 1:100 Diluted mouse serum (1ul serum with 100ul sample dilution) were used. The peroxidase-conjugated anti-mouse IgE was diluted to 1:1000, and peroxidase-conjugated anti-mouse IgG1and IgG2a were diluted to 1:4000. Level of IgE and IgG1was inhibited by Derp2-mutant-DNA vaccine , compared with rDerp2 group and asthma group ($P < 0.01$. However, the level of IgG2a in Derp2-mutant-DNA vaccine group was increased compared with control and asthma groups ($P < 0.01$).

Mice in rDerp2 group showed more infiltration of inflammatory cells around the central bronchi, lung alveolus and blood vessels. (Figure 4)

Expression of TLR9 in the lung tissue

Expression of TLR9 in the lung tissue was determined by immunohistochemistry and western-blot. The results of immune-histochemistry showed that TLR9 was located in airway and lung alveolus. The expression of TLR9 in Derp2-mutant-DNA vaccine group was significantly increased as compared with that in other three groups. The results were also found in western-blot analyses. This means that the DNA vaccine could induce TLR9 express in lung tissue. (Figure 5 and Figure 6)

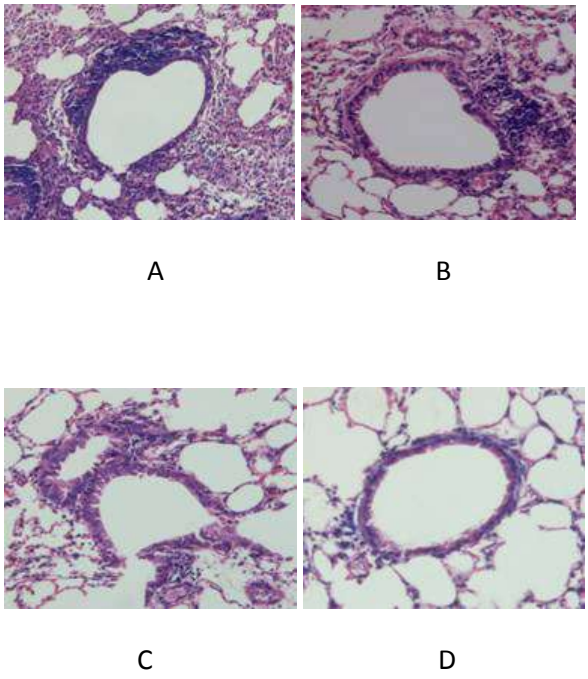

Figure 4. Histological evaluation of lung tissue. Lung samples were stained with hematoxylin and eosin (HE). Mice in asthma group showed extensive infiltration of inflammatory cells around the central bronchi, lung alveolus and blood vessels. Derp2-mutant-DNA vaccine had the role of inhibition on Infiltration of inflammatory cells. (A): Asthma group; (B); rDerp2 group; (C) Derp2-mutant-DNA vaccine. (D) Control group (original magnification ×200).

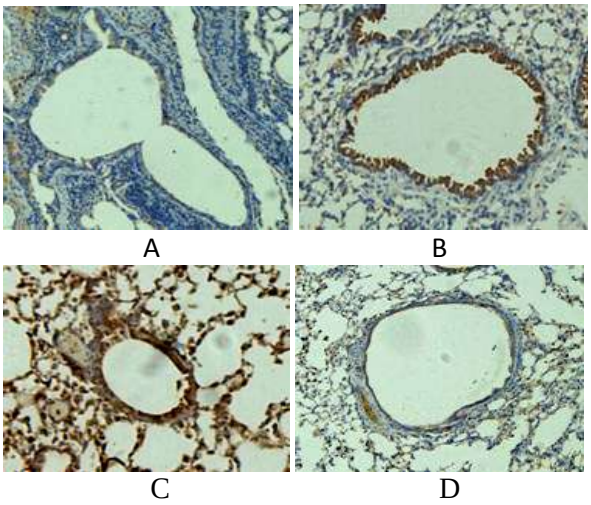

Figure 5. Detection of TLR9 expression in lung by immunohistochemistry. The location of TLR9 was showed by immunohistochemistry. The expres-sion of TLR9 in Derp2-mutant-DNA vaccine group (C) was significantly increased as compared with that in other three groups (A: Asthma group; B: rDerp2 group; D: Control group) .Original magnification ×100.

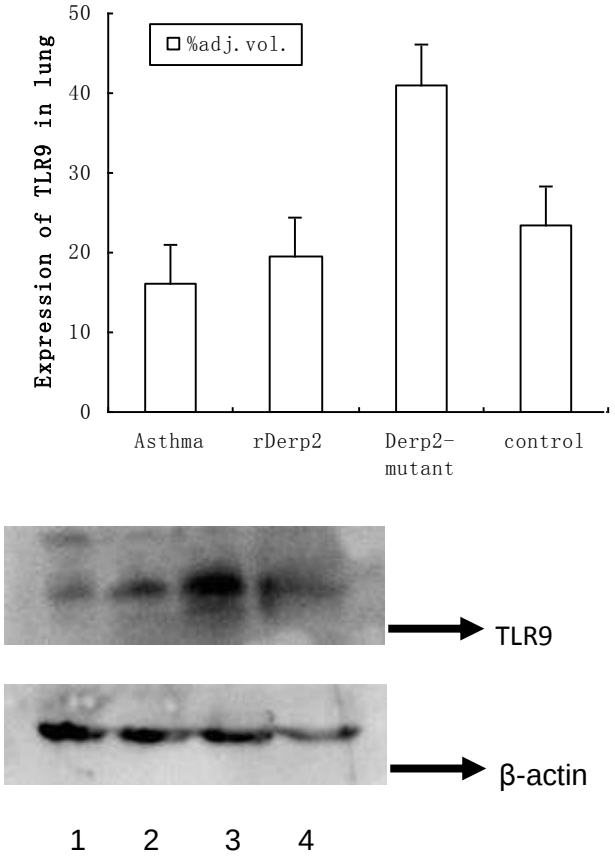

Figure 6. Detection of TLR9 expression in lung by western-blot. Lung tissue was collected and analysed by western-blot. Quantitation was completed by software of Quantity One. %adj.vol indicates the gray value of protein after subtracting the background. The figure shows that the expression of TLR9 in Derp2-mutant-DNA vaccine group (3) was significantly increased, compared with that in other three groups. Lane 1, Asthma group; Lane 2, rDerp2 group; Lane 3, Derp2-mutant-DNA vaccine group; Lane 4, control group.

Discussion

Allergic asthma is one of the most common health problems. The morbidity has increased in recent years. Allergic asthma is a kind of chronic airway inflammation and is associated with exposure to allergens. Dermatophagoides pteronyssinus is the most important one. Dermatophagoides pteronyssinus is one of the ideal candidates for vaccine development. In our past studies, it was confirmed that DNA vaccine encoding Derp2 allergen generates immunologic protection in recombinant Derp2 allergen-induced allergic airway inflammation mice model with regulation of the immune response towards a Th1-type reaction[8]. DNA vaccine encoding Derp2 allergen could be used for therapy of allergen-induced allergic airway inflammation in our mouse model, and DNA vaccination inhibited STAT6 and NF-kappaB expression in lung tissue[9]. It was reported that this amino acid residue was associated with the IgE epitope. In the present study, amino acid residues 21-27, 73-78, 118-119 were deleted in Derp2 allergen encoding gene. Our results showed that Derp2-mutant-DNA vaccine could also inhibit airway allergenic inflammation, AHR and IgE. In contrast, rDerp2 allergen immunization induced production of specific IgE. rDerp2 immunization showed more infiltration of inflammatory cells and airway hyper-responsiveness.

The process of the DNA vaccine activated immune response was associated with Toll-like receptors (TLRs) and CpG motif. CpG motif exists in the DNA of procaryotes and was

suppressed in eucaryon. Oligonucleotide containing the CpG motif (CpG ODN) can imitate specific DNA. CpG ODN can induce a Th1-bias response and prevent or even reverse the airway inflammation in animals and may become a brand-new treatment for atopic asthma.[10,11] 13 kinds of TLR have been discovered at present.[12] TlL9 can recognize a lot of the CpG ODN sequence. Binding of the CpG motif and TLR9 can activate multiple signaling cascade reactions in immune response cells. A restricted pattern of TLR9 expression to certain dendritic cells and B cells appears to provide relative specificity in responses. TLR9 activation induces a Th1-like pattern of cytokine release which has led to interest in the use of synthetic CpG oligodeoxynucleotides (CpG ODN) for the prevention and treatment of Th2-associated atopic disorders such as asthma.[13] However, it is not clear what the role of TLR9 is in lung tissue. In our study, the data indicated that TLR9 was located in airway and lung alveolus. Derp2-mutant-DNA immunization could up-regulate TLR9 in lung tissue. The results indicated that DNA vaccine immunization induced a Th1 immune response and up-regulation of TLR9. However,the function of TLR9 in lung tissue is not clear.

In conclusion, Derp2-mutant- DNA vaccine in which part of amino acid residues were deleted could inhibit airway allergenic inflammation, AHR and specific IgE. The Derp2-mutant-DNA vaccine also activates increased expression of TLR9 in lung. These data indicate that TLR9 plays an important role in the action of the Derp2 DNA vaccine. Our research may lead to the development of a DNA vaccine for use in allergic asthma and provide a new specific immunotherapy for patients with allergic asthma.

Acknowledgements

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