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Original Article

Effect of activation of Toll-like receptor 7 in the inhibition of allergic asthma on a mouse model

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Abstract: Targeting Toll-like receptor 7 (TLR7) is known to have a potential therapeutic effect on experimental allergic asthma, but the exact mechanism is incompletely understood. To investigate the potential therapeutic effect of TLR7 agonist (TLR7a) a new versatile TLR7 agonist conjugated to Der f 1 was synthesized and evaluated *in vivo* here. It was confirmed that the course of airway hyperresponsiveness (AHR) and eosinophilia of the TLR7a vaccine-treated mice were limited. Levels of specific IgG1, IgG2a and IgE antibodies of these mice were changed obviously compared with that of the model mice. The expression of T helper 2 cytokine, interleukin (IL)-4, production in bronchoalveolar lavage fluid (BALF) and splenocytes were significantly decreased, while the levels of IFN-γ, IL-12 and IL-10 were increased after the treatment of TLR7a vaccine. In addition, Muc5 expression and goblet cells were significantly decreased in the lung tissue of asthma model mice treated with TLR7a plus Der f 1. These results suggest that the TLR7a-Der f 1 vaccine exhibits interesting therapeutic potency in suppressing allergic asthma and could be used a new agent in the treatment of allergic diseases.

Keywords: Allergic asthma, dust mite, Der f-1, immunotherapy, TLR7 agonist, adjuvant

Introduction

The prevalence of allergic asthma has significantly increased in the world [1]. Approximately 300 million people suffer from asthma worldwide and more than 80% are sensitized to house dust mites (HDMs) [1, 2]. Patients need to avoid contacting with specific antigens and receive repeat treatments, which impair their health normally [3-5]. Upon inhaling specific allergens, the induction of Th2 type cytokines and recruitment of inflammatory cells into the lungs may be evoked in the airway [6], in which both dendritic cells (DCs) and T cells are involved.

Toll-like receptors (TLRs) play an important role in the innate immunity during the recognition and initiation of adaptive immune responses [7]. The regulation of allergic responses may be induced by activating the innate immune system. DCs are professional antigen-presenting cells that critically link innate and adaptive immunity. The hygiene hypothesis proposes that contacting with microbial components has an effect on the inhibition or/and prevention of allergic diseases [8]. Exposure to microbes or microbial products induces DC maturation through the induction of Th1 response [9, 10].

TLR7 is a kind of important protein which can identify single-stranded RNA virus. TLR7 is mainly expressed on endosome in B cells, plasmacytoid dendritic cells (pDCs) and monocytes [11]. Stimulation of TLR7 in pDCs *in vitro* induce the production of IFN-γ and IL-10 from T cells, proliferation of B-lymphocytes and memory B cells, production of IgG1 and IgG4 antibodies, and inhibit the activation of Th2 effector cells [12-15]. Epicutaneous immunization (EPI) with Bey v 1 (the major brich pollen allergen) plus R848 induced Bet v 1-specific Th1 responses and suppressed asthmatic features *in vivo* [16]. In this study, we synthesized a new versatile TLR7 agonist conjugated to Der f 1 and evaluated the modification of TLR7 signaling on the allergic responses elicited by HDM.

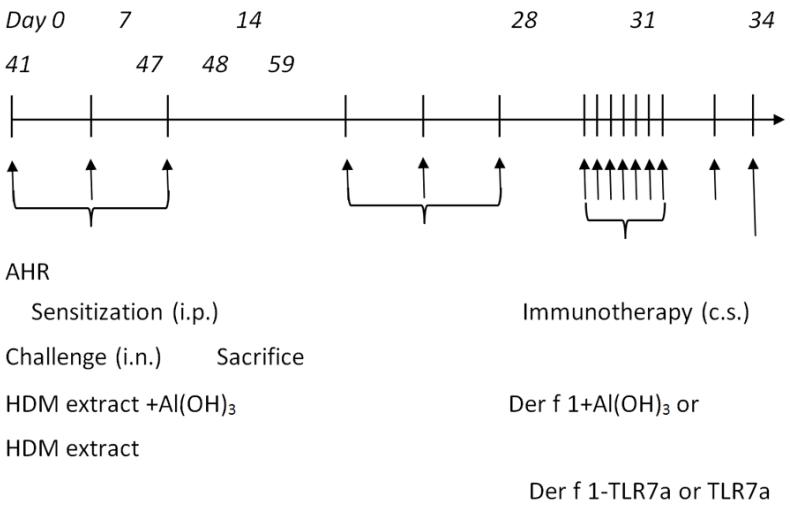

Figure 1. Protocols of allergic asthma sensitization, challenge and treatment. Mice were administered house dust mite (HDM) from day 0 to 14. Treatment was started from day 28 to 34, once every three days, for a total of three times. Epicutaneous applications of Der f 1+Al(OH)3 or Der f 1-TLR7a or TLR7a. After challenge with HDM for a 1-week period, the mice were challenged with Mch for AHR detection, and mice serum and BALF were collected after sacrifice.

Materials and methods

Animals

Female BALB/c mice (6-8 weeks) were purchased from the Animal Center of Guangdong Province. The mice were maintained in a specific pathogen-free facility at the Experimental Animal Center of Shenzhen University. All animal care and experimental protocols were carried in accordance with the Institutional Guidelines for Animal Care and Use of Laboratory Animals at Shenzhen University.

Preparation of Der f 1 antigen

The pET-28a (+) plasmid containing Der f 1 gene was transformed to the host cell *E. coli* BL21 as previously reported [17]. The protein was purified by Ni affinity chromatography, and used as a specific antigen. SDS-PAGE and Western blot were used to access the purification of the protein.

Conjugation of TLR7 agonist to Der f 1

Succinimidyl 6-hydrazino-nicotinamide acetone hydrazone (SANH) was used as a linker to couple amino groups on proteins. Initially, TLR7a was added to Der f 1 at a ratio of 1:40 and shacking overnight at 15°C. Uncombined agonist was removed by ultrafiltration tubes (10kDa) as previously described [18]. The conjugated TLR7a-Der f 1 was assessed by UVat 280 nm. The secondary structure of TLR7a-Der f 1 was characterized by circular dichroism (CD) [19]. The IgE-binding reactivity was measured by enzyme-linked immunosorbent assay (ELISA).

Immunization protocols

The antigen sensitization and challenge and immunotherapy of the murine model of allergic asthma were performed as previously described [20-22]. Briefly, BALB/c mice received immunization with 50 μg of house dust mite (HDM) extract in 0.2 ml PBS with 2 mg of $Al(OH)_3$ (Sigma, USA) (M) by intraperitoneal injection on day 0, 7 and 14. 14 days after sensitization, then treated with 100 μg Der f 1 adsorbed on 2 mg of $Al(OH)_3$ (D), 100 μg TLR7a-Der f 1 (T-D) or TLR7a (T) in 0.1 ml PBS daily for 3 times. The mice were challenged with 50 μg HDM antigen administered by nasal drop from days 41 to 47. Mice sensitized and challenged with normal saline were used as control group (C). Twenty-four hours after the final challenge, airway hyperreactivity (AHR) was assayed in a Buxco plethys-mograph (Buxco, USA) and next day all the mice were sacrificed (**Figure 1**).

AHR measurement

Airway hyperresponsiveness (AHR) to methacholine (Mch) aerosol was evaluated as an increased pulmonary resistance using unrestrained whole-body plethysmography with a four-chamber system (Buxco Research Systems, Wilmington, NC, USA) [23]. Firstly, mice were put into the chamber and kept steadily respiration for 10 min. The baseline of breathing was monitored for 5 min. The records for Penh values begin with the event that inhaling 0.1 ml NS. Then the mice were subjected to inhale Mch at increasing concentrations (6.25, 12.5, 25, 50 and 100 mg/ml). Each response

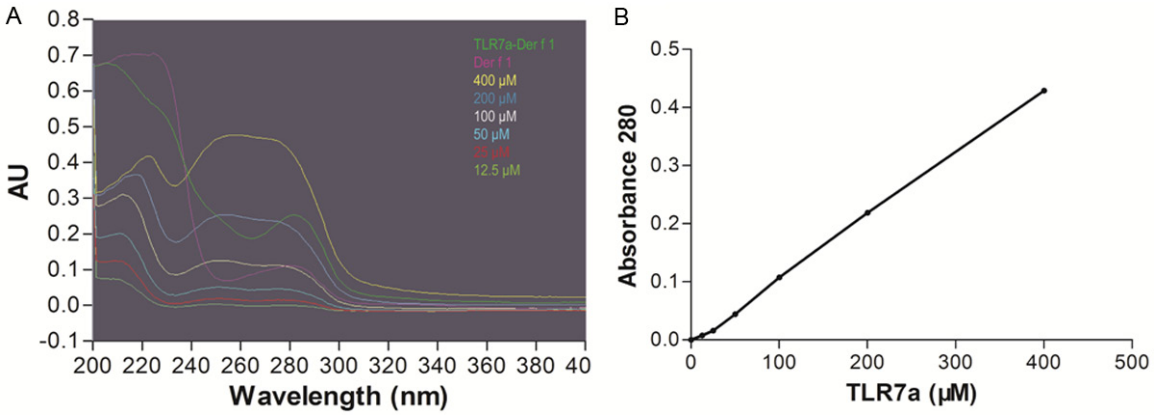

Figure 2. Quantification of TLR7a conjugated to Der f 1. A: Different concentrations of TLR7a, TLR7a-Der f 1 and Der f 1 were analyzed by fluorescence spectroscopy. Conjugation of TLR7a to Der f 1 was indicated by a UV absorption peak at 280 nm. The conjugated TLR7a was determined by subtracting the integrated intensity of TLR7a-Der f 1 for that of Der f 1. B: The readout of TLR7a was used to derive a standard curve. The concentration of TLR7a conjugated to Der f 1 was calculated as (0.25-0.11+0.005)/0.001=145 μM. The concentration of 1 mg/ml Der f 1 was 30.3 μM; therefore, calculation the ratio of TLR7a: Der f 1 was 145/30.3=4.785.

was monitored for 5 min. The tests were performed after some intervals of time to allow the respiration resistance return to the baseline between two different does of Mch.

Inflammatory cell counting of bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was obtained from each mouse after sacrifice by means of trachea cannulation with three times repeat of 0.8 ml PBS lavage from left lung. The supernatant was collected for cytokine level determination. The cells in the pellets were resuspended with 500 μl of PBS. Total cells in BALF were counted with 10 μl suspension with hemocytometer, and 300 μl suspension was centrifuged in a cytocentrifuge (Spring scientific, USA). Differential cell counting was performed manually by Liu's stain. Eosinophils and lymphocytes were classified in accordance with morphologic features.

Enzyme-linked immunosorbent assay (ELISA)

Twenty-four hours after the final aerosol challenge, the mouse serum was collected from angular vein. Der f-specific IgE, IgG1 and IgG2a levels were measured with ELISA. The levels of IL-4, IL-10, IL-12 and INF-γ in BALF and spleen cell culture supernatant were determined by ELISA according to the instructions of reagent kits.

Lung histology

The fresh right lungs were removed immediately and fixed in 4% neutral-buffered formalin for 24 h and subjected to graded alcohol dehydration. The lung tissues were embedded in paraffin, and 5-μm thick sections were stained with hematoxylin and eosin or periodic acid-Schiff (PAS). The histological analysis was observed under an optical microscope.

Immunohistochemistry

After deparaffinization and rehydration, the lung tissue paraffin sections were boiled with citrate buffer (PH6.0) for antigen retrieval. The sections were incubated with mucin 5 antibody at 4°C overnight. Following washing, they were immersed in 3% H_2O_2 in methanol to inhibit endogenous peroxidase. The secondary antibody (HRP-conjugated goat anti-rabbit antibody) was added and incubated for 40 minutes, with 3,3'-diaminobenzidine (DAB) color development. The expression of mucin 5 was observed with a light microscope.

Statistical analyses

Data were expressed as mean ± standard error. The SPSS 13.0 software was used for statistical analysis. Differences between two groups were determined using. A *P*-value <0.05 was considered statistically significant.

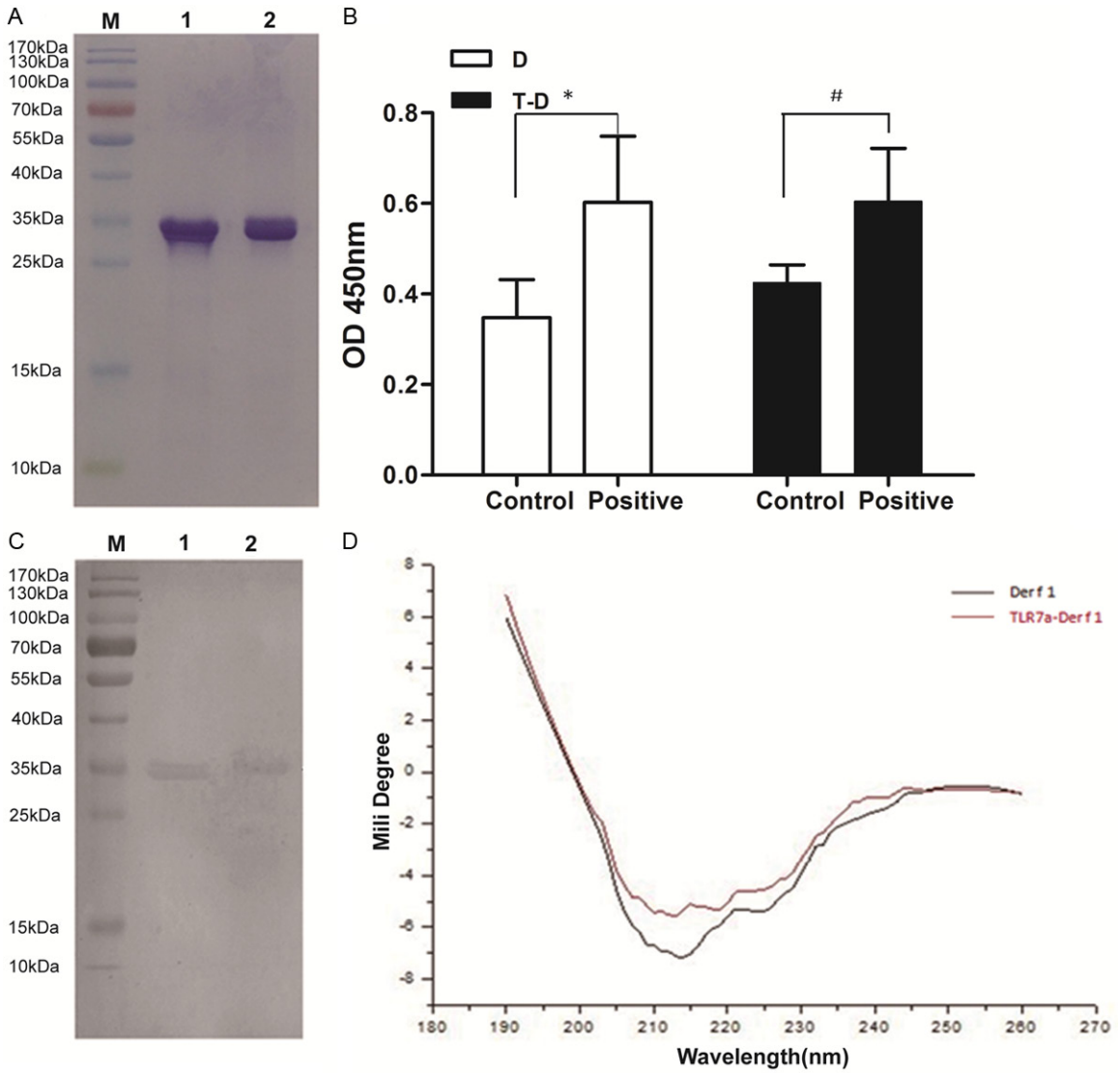

Figure 3. Immunology and structural characteristics of TLR7a-Der f 1 conjugate. A: SDS-PAGE analysis of TLR7a-Der f 1 (M: marker, 1: Der f 1, 2: TLR7a-Der f 1). The MW of TLR7a-Der f 1 was a litter bigger than Der f 1. B: ELISA detection of the antigenicity of TLR7a-Der f 1. *P<0.05 positive serum vs control group of Der f 1, #P<0.05 positive serum vs control group of TLR7a-Der f 1. C: Western blotting identification of the ability of TLR7a-Der f 1 binding to specific IgE antibody in positive serum (M: marker, 1: Der f 1, 2: TLR7a-Der f 1). D: CD spectra of TLR7a-Der f 1 and Der f 1.

Result

Conjugation of TLR7 agonist to Der f 1

TLR7a was conjugated to Der f 1 protein through an amide linkage and was identified by UV at 280 nm (**Figure 2**). Quantification of TLR7a molecules conjugated per Der f 1 protein was calculated based on a standard curve of different concentrations of TLR7a (12.5 μM, 25 μM, 50 μM, 100 μM, 200 μM, 400 μM). The TLR7a-Der f 1 was turned out to be conjugated at a ratio of 4.785:1.

Characterization of TLR7a-Der f 1

SDS-PAGE was preformed to detect the conjugates of TLR7a/Der f 1. The bands of TLR7a-Der f 1 and Der f 1 were identified (**Figure 3A**). The result indicated that TLR7a and Der f 1 were stably combined. The antigenicity of TLR7a-Der f 1 was detected by ELISA. The obvious increase of OD values stated that TLR7a-Der f 1 can recognize and bind to IgE antibodies specifically (**Figure 3B**). Furthermore, the bands in Western blotting indicated that TLR7a-Der f 1 bind to specific IgE in the serum (**Figure 3C**).

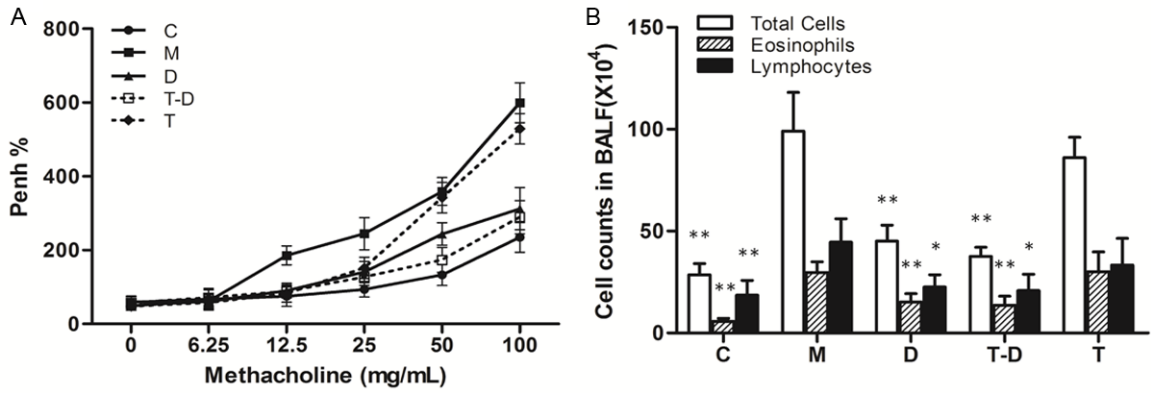

Figure 4. Effect of immunization with TLR7a-Der f 1 on lung inflammation. A: Airways hyperresponsiveness assay from control group and groups of animals sensitized and treated with Der f 1 and TLR7a-Der f 1. Results are expressed as mean ± SEM. B: Differential inflammation cell counts in BALF including total cells, eosinophils and lymphocytes. *P<0.05 others vs control group, **P<0.01 others vs control group.

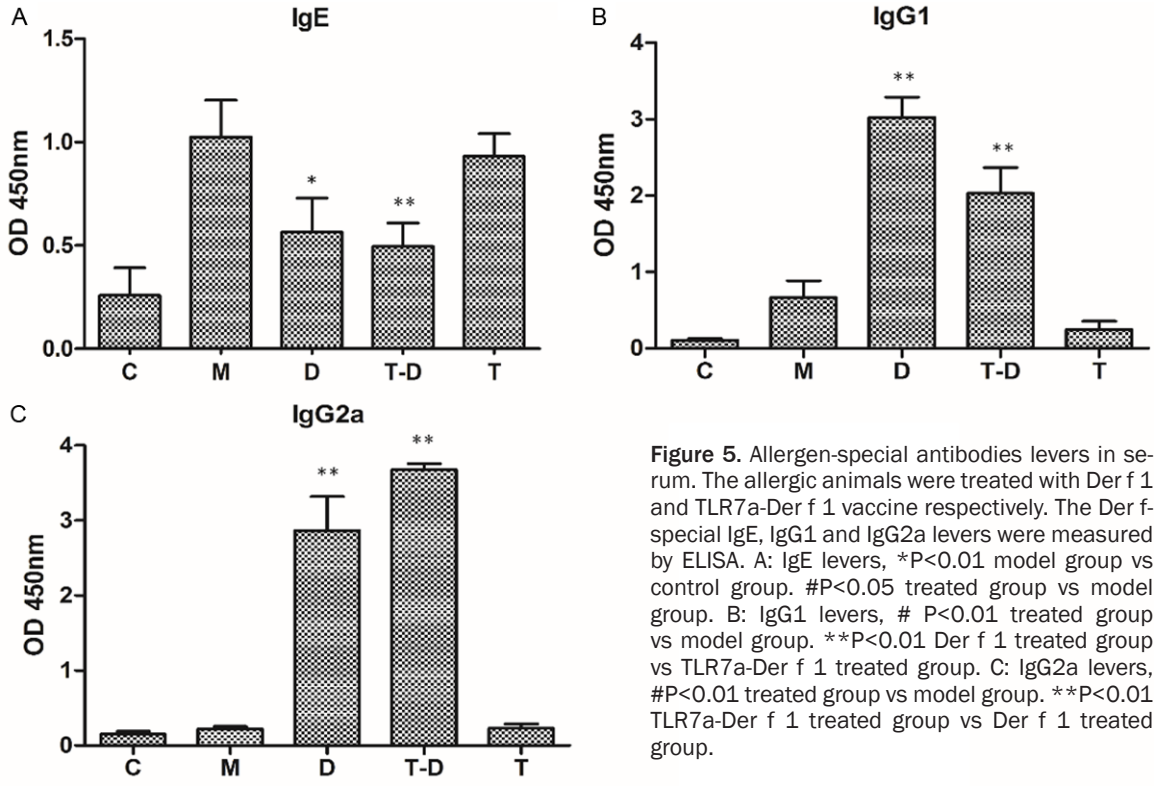

Figure 5. Allergen-special antibodies levers in serum. The allergic animals were treated with Der f 1 and TLR7a-Der f 1 vaccine respectively. The Der f-special IgE, IgG1 and IgG2a levers were measured by ELISA. A: IgE levers, *P<0.01 model group vs control group. #P<0.05 treated group vs model group. B: IgG1 levers, # P<0.01 treated group vs model group. **P<0.01 Der f 1 treated group vs TLR7a-Der f 1 treated group. C: IgG2a levers, #P<0.01 treated group vs model group. **P<0.01 TLR7a-Der f 1 treated group vs Der f 1 treated group.

To confirm the conjugating of TLR7a to Der f 1, the CD was utilized. As shown in **Figure 3D**, CD spectra of the samples exhibited negative bands in UV region at 218 and 224 nm, which were characteristic α-helical structures of protein [24]. CD spectra of TLR7a-Der f 1 exhibited lower absolute θ values than those of Der f 1 from 218 to 224 nm. It could be seen the formation of some α-helical structures in TLR7a-Der f 1. This result suggested that conjugation between TLR7a and Der f 1 can cause slight spatial conformational changes of proteins.

TLR7a-Der f 1 reduce AHR and airway inflammation

Non-specific airway hyperreactivity is typical symptom of allergic asthma. The Mch is widely used to study AHR. One day after the last HDM extract challenge, the mice were challenged with increasing doses of Mch. Exposures to HDM antigen induced a significant increase in airway hyper-reactivity in model mice compared with that in control group (P<0.05, **Figure 4A**). penh values from TLR7a-Der f 1 and Der f 1

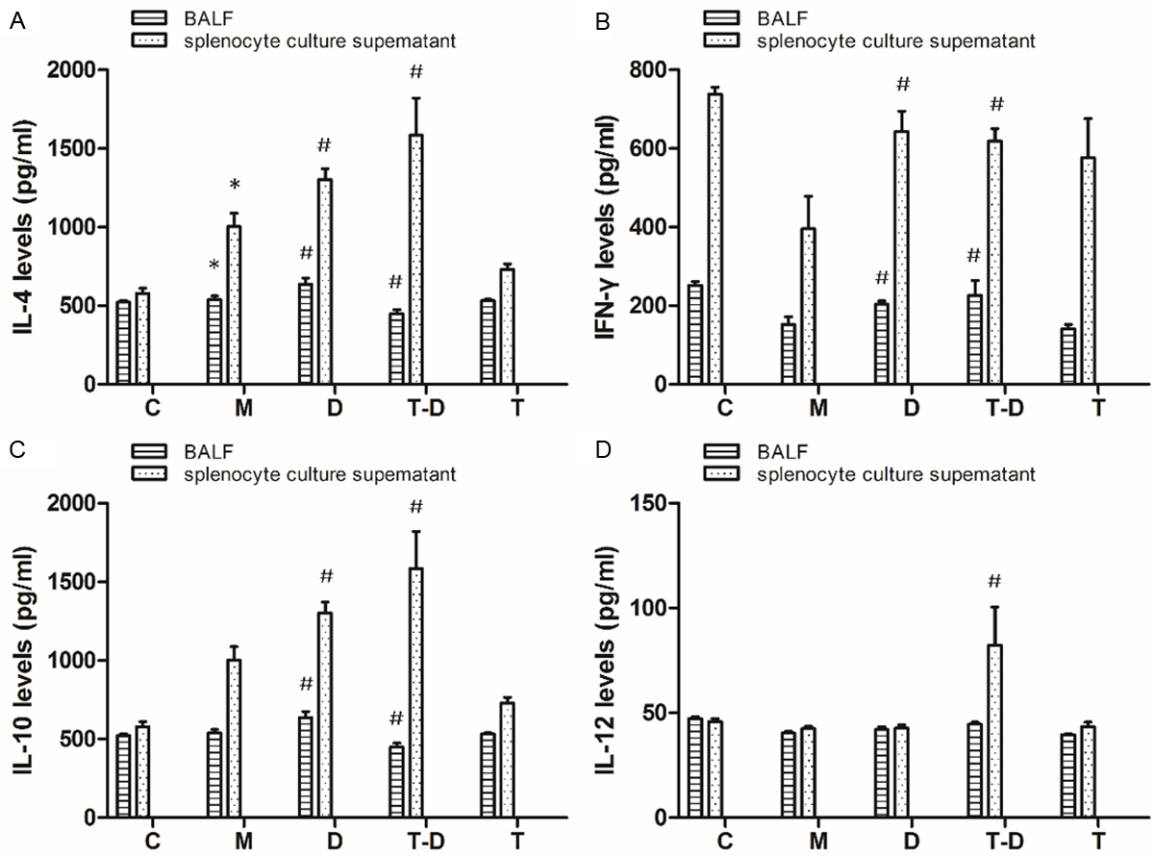

Figure 6. Cytokine production in BALF and splenocyte culture supematant. A-D: Levels of IL-4, IFN-γ, IL-10 and IL-12 were shown. Results are expressed as mean ± SEM. Data are representative for three independent experiments. *P<0.05 model group vs control group. #P<0.05 treated group vs model group.

treated groups were both significantly decrease compared with that of un-treated asthma group (P<0.05). Airway responses to Mch challenge in TLR7a-Der f 1 group induced lower penh values than in Der f 1 group.

We also observed that the allergen sensitization and challenge resulted in severe inflammation in the airways. HDM antigen induced a marked increase in BALF total cell, eosinophil and lymphocyte counts in treated group compared with in control (P<0.05, **Figure 4B**). The results indicate that treatment with TLR7a-Der f 1 and Der f 1 mitigates allergic airway inflammation in mice.

TLR7a-Der f 1 attenuates IgE and increases IgG1 and IgG2a antibodies

As shown in **Figure 5**, sensitization to HDM and aerosol challenges induced higher serum specific IgE levels in model group than in the normal control group (P<0.01). The levels of IgE was significantly reduced in mice treated with Der f 1 and TLR7a-Der f 1 (P<0.05). There was no difference significantly between two treatment groups. But specific IgG1 and IgG2a levels were significantly increased in Der f 1 or TLR7a-Der f 1 vaccine groups compared with that in model group (P<0.01). After Der f 1 conjugated with TLR7a-Der f 1, the expression of IgG2a antibody could be up-regulated, while Der f 1 could induce more obvious up-regulation of IgG1 antibody than TLR7a- Der f 1 group do.

TLR7a-Der f 1 regulates the levels of Th2/Th1 cytokines

The evaluation of IL-4, IL-10, IL-12 and IFN-γ in the splenocyte culture supernatant and bronchoalveolar lavage fluid indicated that the Th1 immune responses were promoted after treatment. IL-4 level was higher in sensitized group that that in control group (**Figure 6A**). The IFN-γ levels in sensitized mice were lower compared

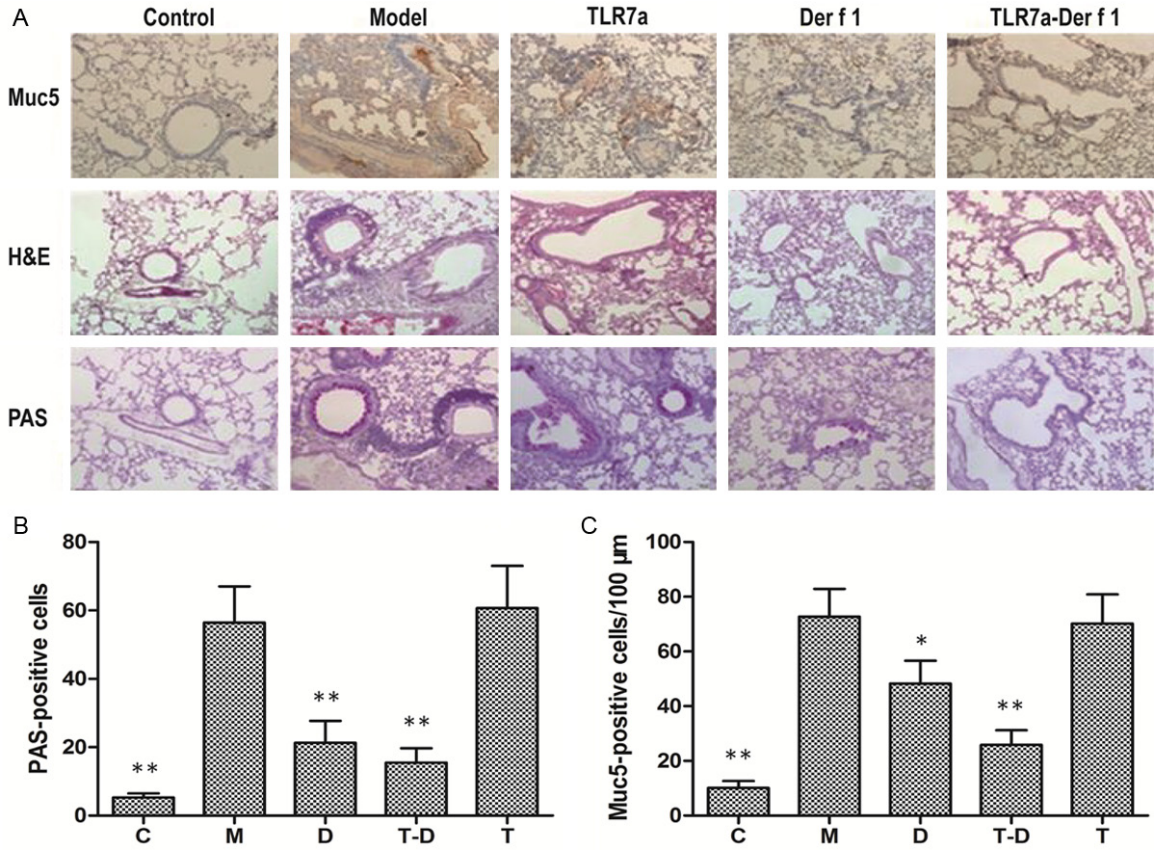

Figure 7. Effects of treatment on lung tissue pathology. A: Muc5 expression was analyzed by immunohistochemical staining. Representative histologic sections of lobar bronchi stained with routine H&E and PAS. Magnification, 200×. B: The number of MUC5AC-positive cells per 100 μm was presented. C: The number of PAS-positive cells per 100 μm of basement membrane was visualized graphically. *P<0.05 compared with model group. **P<0.01 compared with model group. The data are representative for three independent experiments.

with that in the normal control mice (**Figure 6B**). The results indicated that a Th2 immune response has been induced in the mice after exposing to HMD. Treatment with Der f 1 or TLR7a-Der f 1 induced the decrease of IL-4 levels for 41% or 45% respectively; the increasing of IFN-γ level indicated the treatment has induced a shift toward a Th1 response. IL-10 level was elevated by 0.38 folds in TLR7a-Der f 1 group, suggesting that the activation of TLR7 induce a tolerogenic response (**Figure 6C**). In addition, treating with TLR7a-Der f 1 induced a significant increase in the levels of IL-12, while treating with Der f 1 only induced a slight increase in IL-12 (**Figure 6D**).

Lung histologic changes

The damage in the lung and goblet cell hyperplasia were observed by H&E and PAS staining as shown in **Figure 7A**. The results in model group showed that the lung was severely injured and edema, the peribronchial smooth muscle and blood vessels were infiltrated by inflammatory cells and the organization of bronchial mucosal cilia was in a mess condition. Eosinophils have been considered to play a key role in the pathogenesis of airway pathology in allergic asthma. The TLR7a-Der f 1 and Der f 1 treated animals exhibited a consistent reduction in the numbers of perivascular and peribronchiolar eosinophils. The tissue structure in TLR7a-Der f 1 group was close to the normal group. Expression of Muc5 protein in sensitized mice was elevated compared to that in normal mice revealed by immunohistochemical staining. However, Muc5 expression was significantly inhibited in TLR7a-Der f 1-treated mice, compared to that in HDM-sensitized mice and treated mice with Der f 1 along. The goblet cell hyperplasia was observed by lung tissue PAS staining. Treatment with Der f 1 and TLR7a-Der f 1 reduced the number of PAS-positive goblet

cells significantly and suppressed the lung inflammation.

Discussion

In the present study, administration of TLR7 agonist-Der f 1 protein conjugates protected animals from anaphylaxis on challenge. This protection was associated with the shift toward a Th1 response consistent with previous allergen-based immunotherapies that promote tolerance by reshaping T-cell responses [25, 26]. The TLR7a-Der f 1 vaccine was confirmed to have good capacity to bind IgE antibody specifically in allergic serum in our research. Our study demonstrated that the TLR7a-Der f 1 can be directly delivered to the respiratory system to modulate the immune responses of host cells in the airway mucosa. TLR7a-Der f 1 may be avidly captured by pDCs expressing TLR7 [27]. Published data indicate that there is an increased migratory potency of circulating pDCs in patients with allergic asthma [28, 29]. These changes are accompanied by a reduced TLR7-mediated cytokine response [30]. Therefore, the vaccine of TLR7a-Der f 1 may mitigate allergic diseases that have been associated with TLR7-reduction. Thus, The TLR7a-Der f 1 may provide an approach for a selective delivery of the immunotherapeutic to lung tissue and to certain DCs subset.

$CD4^+$ T cells play a key role in the pathological changes in the lung tissues. IL-4 is an important Th2 pattern inflammatory mediator, and is important for inducing and maintaining Th2 response. TLR7a-Der f 1 vaccine significantly reduced IL-4 levels compared with Der f 1, indicating that the targeting TLR7 effectively suppresses Th2 type immune response, thereby reducing the lung tissue and airway inflammation.

The present data indicate that activation of TLR7 suppresses the Th2 polarization response by increasing Th1 response. IL-12 has been shown to play a role in the prevention and treatment of experimental asthma [31]. In this study we found that the activation of TLR7-mediating immune response produced IFN-γ and IL-12, suggesting that the Th1 response is attributed to the activation of TLR7. In addition, IL-10 is an important immune regulatory molecule, which can inhibit allergic airway responses, improve the body's immune tolerance to antigenic stimulation and enhance the function of regulatory T cells [32]. The present results also showed that IL-10 levels were elevated after treatment with the TLR7a-Der f 1. Our data are in line with the reports by Kawamura et al [12] that virus-stimulated pDCs are capable of inducing naïve CD4+ T cells to differentiate into IL-10-producing regulatory T cells in vitro. TLR-7 activation through TLR ligands has preventive and suppressive effects on experimental asthma which is mediated by the additive effects of IL-12 and IL-10.

It is reported that stimulation of B cells with intracellular TLR7 induces an activation cascade leading to memory B-cell generation and particularly IgG1 [13]. The TLR7 immunotherapy increases the serum specific antibodies of IgG1 and IgG2a levels over 10-folds as we observed in the present study. Previous studies have shown that high levels of IgG antibodies are capable of competing with IgE antibodies to bind to allergens [33]. Antibody phenotype changes may reflect in T cell immune response changes. IFN-γ is an important transforming factor in mediating IgG2a antibody [34]. These data suggest that TLR7 activation may induce the production of protective antibody by B cells.

In conclusion, the present study demonstrates for the first time that TLR7a-Der f 1 vaccine has a protective and suppressive potential in allergic inflammation against dust mite allergic asthma. The activation of TLR7 modifies the T cell response by suppressing Th2 cytokine production and promoting Th1 cytokine production. The TLR7a-Der f 1 vaccine is expected to become a new approach in the treatment of allergic diseases.

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Disclosure of conflict of interest

None.

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