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Development of a hollow mesoporous silica nanoparticles vaccine to protect against house dust mite induced allergic inflammation

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ABSTRACT

Allergen specific immunotherapy (SIT) is the only specific therapeutic way for house dust mite (HDM) allergy. To improve the efficacy of SIT, hollow mesoporous silica nanoparticles (HMSNs) were used as vehicles for HDM allergen. The HMSNs were prepared and characterized. The major HDM allergen (Der f2) was loaded onto HMSNs, and the drug loading capacity and release profile were determined. Then the Der f2 loaded HMSNs were injected subcutaneously to mouse model of Der f2 induced allergic asthma and the preventive effects were evaluated. Our results showed that HMSNs were spherical (100 nm) with pore diameter of 2.897 nm and successfully loaded with Der f2 protein. The loading capacity is 90 μg Der f2/1 mg HMSNs. The Der f2 loaded on HMSNs released slowly in 72 h. Treatment with Der f2 loaded HMSNs could efficiently decrease Der f2 specific IgE levels, inflammatory cells infiltration in lung tissue, and Th2 cytokine IL4 levels in BALF. In the meanwhile, it could increase the Der f2 specific IgG levels, Th1 cytokine IFN-γ levels, and induce proliferation of splenocytes to Der f2 accompanied by increased IFN-γ levels. These results showed that Der f2 loaded HMSNs were efficient in preventing allergic inflammation, and HMSNs may be potential vehicles for SIT of HDM allergy.

1. Introduction

The allergic diseases are increasing in prevalence recently. About 20–30% of populations in worldwide are suffered by allergic diseases according to World Allergy Organization (WAO) white book on allergy (Pawankar et al., 2013). Allergen is the antigen capable of stimulating allergic diseases such as asthma, rhinitis, and atopic dermatitis in atopic individuals through pathogenic Immunoglobulin E (IgE) responses. House dust mite (HDM) (Dermatophagoides farina and Dermatophsgoides) allergen is one of the most common allergen associated with various allergic diseases, and the major allergens of HDM are classified as group 1 (Der f1 and Der p1) or group 2 (Der f2 and Der p2). The majority of sera from patients allergic to HDM have binding activities to both group 1 and 2 allergens (Takai et al., 1997). Until now, allergen-specific immunotherapy (SIT) represents currently available medical intervention that can limit the natural course of allergic diseases (Akdis and Akdis, 2011). SIT involves repeated administration of the sensitizing allergen (usually by subcutaneous injection or, more recently, by sublingual application) (Akdis and Akdis, 2014). SIT was demonstrated to regulate abnormal immune state in allergic diseases by generation of allergen-specific Treg cells, suppression of allergen-specific effector cells, regulation of antibody isotypes (decreasing specific IgE levels and increasing specific IgG levels), and decrease of effector cells and release of their mediators (Akdis and Akdis, 2014). However, the long treatment duration, occurrence of local side-effects and risk of anaphylaxis, limited the broad application of SIT in clinic (Mellerup et al., 2000). This may be due to the application of aluminum hydroxide ($Al(OH)_3$) as the adjuvant since it has short deposit time for allergen, often induces local reactions at the site of injection, and associates with the induction of Th2 responses (Schöll et al., 2004). Therefore, improved adjuvants are needed to increase the efficacy and safety of immunotherapy. In recent years, researchers have focused on nanoparticle-based allergen delivery systems. Nanoparticles were efficient in improving antigen stability, enhancing antigen processing and immunogenicity, targeted delivery and slow release of antigens, which have become popular vehicles for protein delivery (Ma et al., 2012).

Hollow mesoporous silica nanoparticle (HMSN) is one of the most promising inorganic nanocarriers for antigen delivery with large surface areas, tunable pore sizes, and well-defined surface properties (Sun et al., 2015). Besides, it has properties of good biocompatibility, low cytotoxicity and relatively low cost of production. It has been approved by the Food and Drug Administration as a new biocompatible material (Tang et al., 2012). Different proteins such as bovine serum albumin and lysozyme have been loaded into HMSNs for a variety of applications, and realized prolonged release, improved protein stability and biological function (Mody et al., 2013; Mahony et al., 2013; Mahony

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et al., 2014).

In this paper, we used hollow mesoporous silica nanoparticles (HMSNs) as vehicles/adjuvants to load HDM allergen for SIT. Der f2, one of the main HDM allergens, was chosen as the allergen model, and the Der f2 loaded Hollow mesoporous silica nanoparticles (Der f2-HMSNs) were constructed. The characterization of Der f2-HMSNs was determined and the preventive effect of Der f2-HMSNs was evaluated in mouse model of allergic inflammation. Our results displayed that Der f2-HMSNs could efficiently down-regulate Th2 immune response and prevent allergic inflammation, and showed a higher efficacy than Der f2 adsorbed on $Al(OH)_3$. These results indicated that HMSNs may be potential adjuvants/vehicles for SIT of HDM.

2. Materials and methods

2.1. Materials and animals

The recombinant Der f2 protein (about 20 KD) was expressed in the Escherichia coli strain BL21 under induction with 1 mM IPTG at 37 °C and purified by Ni-NTA gel affinity chromatography according to our previous study (Lin et al., 2012). Mouse IL-4 and IFN-γ ELISA kits (catalog number: BMS613 and BMS606) were purchased from ebioscience (San Diego, California, USA). HRP-conjugated goat anti-mouse IgE monoclonal antibody was obtained from Southern Biotech (Birmingham, AL, USA). HRP-conjugated goat anti-mouse IgG (H + L) antibody (catalog number A28177) was purchased from Thermo Fisher (Waltham, USA). Bicinchoninic Acid (BCA) protein assay kit was purchased from Beyotime (JiangSu, CHN). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Life Technologies (Vienna, Austria). Lymphocyte separation medium (catalog number 0850494X) was purchased from MP Biomedicals (Solon, OH, USA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA).

Specific pathogen-free female BALB/c mice (6–8 weeks old) were obtained from Sion-British Sippr/BK Laboratory. All animal experiments were conducted in the Laboratory Animal Research Center of the Second Military Medical University of Shanghai, and were approved by Chancellor's Animal Research Committee.

2.2. Synthesis of HMSNs

HMSNs were prepared following a minor modification of the protocol described by Hyeon's group (Lee et al., 2010). Firstly, NaOH aqueous solution (2 M, 0.35 mL) was added into water (50 mL) containing cetyltrimethyl ammonium bromide (CTAB) (100 mg), and the resulting solution was heated to 70 °C under stirring. Then tetraethoxysilane (TEOS) (0.5 mL) was introduced drop wise to the reaction mixture. After 1 min, ethyl acetate (0.5 mL) was added and the mixture was stirred for another 30 s, followed by an aging procedure at 70 °C for 2 h. The precipitate was collected after centrifugation and washed with ethanol. Then the collected products were extracted for 6 h with a solution of hydrochloric acid (HCl) in ethanol (10% v/v) at 78 °C by refluxing to remove the template CTAB. Finally, the HMSNs were redispersed in ethanol.

2.3. Characterization of HMSNs

Nitrogen adsorption measurement was done at 77 K using Quadrasorb SI analyser (Quantachrome Corporation, USA). The specific surface areas were calculated by the BET method using adsorption data. The pore size distribution was determined by analyzing the adsorption branch by the BJH method (Wang et al., 2012).

Morphology of HMSNs was analyzed using a JEOL JEM-2010 transmission electron microscope (TEM) (JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 200 kV. One drop of the sample solution (0.1 mg/mL) was placed on a 400 meshes copper grid precoated with a carbon film and the excess droplets were removed with filter paper. The samples were dried overnight and kept in the desiccators until examined with TEM.

Zeta potential of HMSNs was analyzed using a NICOMP 380 ZLS Zeta Potential/Particle Sizer (PSS Nicomp, Santa Barbara, CA, USA).

2.4. Protein adsorption of HMSNs

To load the Der f2 protein into HMSNs, HMSNs solutions were sonicated, and then mixed with 50 μL of Der f2 protein at room temperature for 4 h. Then the solutions were centrifuged at 10,000 rpm for 5 min. The non-loaded Der f2 protein was removed from the solution. The residual Der f2-HMSNs were collected, and dissolved in PBS buffer.

2.5. Drug loading level measurement

To demonstrate the successful load of Derf2 protein in HMSNs, BCA protein assay was used to measure the amount of protein in the supernatant. And the amount of protein adsorbed into the silica was estimated by subtracting the amount of protein unloaded from the protein dissolved in the solution. The protein loaded was further determined by thermogravimetric analysis (TGA) on a TG 209 F1 Iris thermogravimetric analyzer (Netzsch Inc., Selb, Germany) in air at a heating rate of 10 °C/min in a N_2 atmosphere.

2.6. Release kinetics of Der f2 protein from HMSNs

To determine the protein release profile from HMSNs, in vitro release studies were carried out. Der f2-HMSNs (Der f2: 180 μg, HMSNs: 2 mg) were suspended in 1 mL PBS solution (pH = 7.4), and kept in 37 °C. At the 1 h time point, the sample was centrifuged and the supernatant was collected. The particles were resuspended in 1 mL PBS and incubated at 37 °C again. The procedure was repeated at predetermined time intervals (0, 1, 3, 6, 12, 24, 48, 72 h). The supernatant containing Der f2 proteins released by the HMSNs was measured by a BCA protein assay.

2.7. Immunizations of mice with Der f2 loaded HMSNs

As shown in Fig. 1, mice (n = 5 per group) were sensitized with intraperitoneal injections of 20 μg of Der f2 protein adsorbed on 4 mg of $Al(OH)_3$ on days 0, 7 and 14. Two weeks later, mice were injected subcutaneously (s.c.) with 100 μg empty HMSNs, 10 μg Der f2 protein adsorbed on 2 mg $Al(OH)_3$ (Der f2/$Al(OH)_3$), Der f2-HMSNs (10 μg Der f2 with 100 μg HMSNs) every five days for 3 consecutive times respectively as shown in Table 1. Two weeks later, the mice were nebulized with 10 μg/mL Der f2 solutions for 30 min once daily for 5 consecutive days (local challenge) using a nebulizer (Yuwell, Jiangsu, CHN). The control mice were sensitized and challenged with PBS. All mice were euthanized on the following day (day 58) by cervical dislocation.

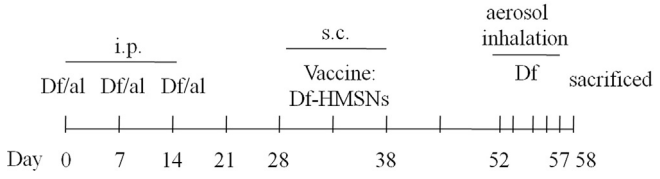

Fig. 1. Experimental design for treatment. Mice were injected intraperitoneally with Der f2 protein mixed with $Al(OH)_3$ on day 0, 7, and 14, and injected subcutaneously with Der f2 loaded HMSNs on day 28, 33, and 38. Two weeks later, mice were challenged with Der f2 protein on 5 consecutive days from day 52. After the final challenge, mice were sacrificed. Note: Df/al, Der f2 adsorbed on $Al(OH)_3$; i.p., intraperitoneal injection; Df-HMSNs, Der f2 loaded HMSNs; s.c., subcutaneous injection.

Table 1
Immunization groups (n = 5) in mice study. All doses were administered by subcutaneous injection.

Treatment group	Group description	Injected dose (150 μL)
1	control	N/A
2	Allergic asthma	N/A
3	Der f2/Al(OH)$_3$	10 μg Der f2 + 2 mg Al(OH)$_3$
4	HMSNs	100 μg HMSNs
5	Der f2-HMSNs	10 μg Der f2 + 100 μg HMSNs

Abbreviations: N/A, non-injected.

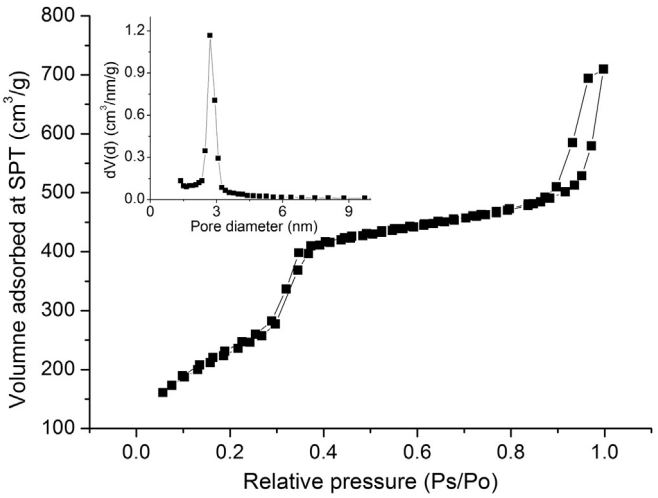

Fig. 2. Nitrogen adsorption/desorption isotherms at 77 K for HMSNs and the pore size distribution obtained from the adsorption branch by the BJH method (inset). STP = standard temperature (273.13 K).

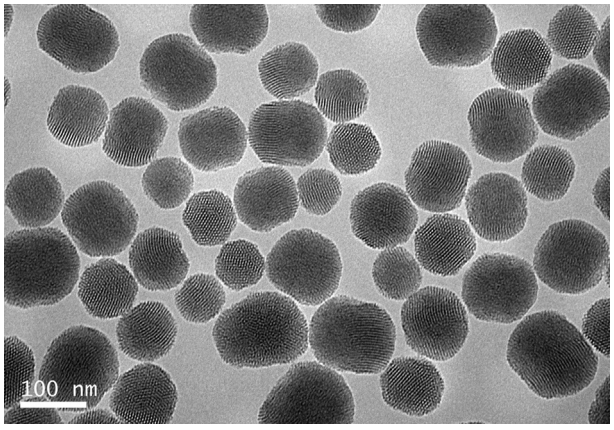

Fig. 3. TEM images of HMSNs. The scale bars represent a size of 100 nm.

2.8. Determination of serum Der f2 specific antibodies

Blood samples were collected immediately after cervical dislocation by retro-orbital bleeding, allowed to clot at room temperature, and then centrifuged at 3000 rpm for 10 min. Serum samples were collected and stored at −80 °C until analyzed for Der f2 specific antibodies.

The levels of Der f2 specific IgE (Der f2 sIgE) and IgG (Der f2 sIgG) in blood samples were determined by ELISA as described previously (Lin et al., 2012). To determine the serum Der f2 sIgE, 96-well microtiter plates (Coster, Corning Inc., NY, USA) were coated with 10 μg/mL of Der f2 overnight at 4 °C. The plate was washed and blocked with PBS containing 1% BSA, and then serum samples (1: 2 diluted) were added to the wells and incubated for 1 h at 37 °C. The plate was washed five times, and incubated with 1 μg/ml HRP-conjugated goat anti-mouse IgE

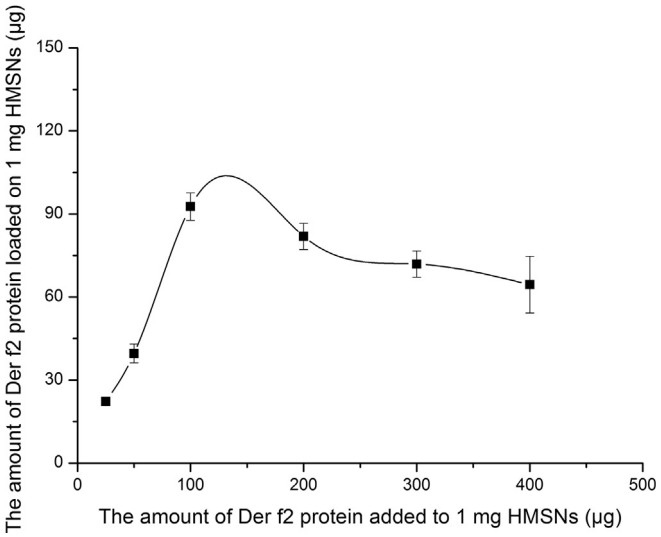

Fig. 4. The adsorption capacity of HMSNs for Der f2 protein. Different amount of Der f2 protein (25, 50, 100, 200, 300, 400 μg) was mixed with 1 mg HMSNs, and the amount of Der f2 protein loaded in HMSNs was determined.

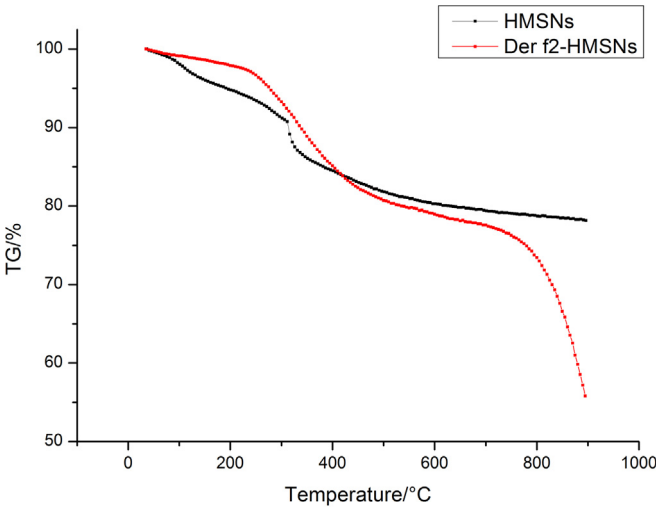

Fig. 5. TGA curves of empty HMSNs and Der f2-HMSNs.

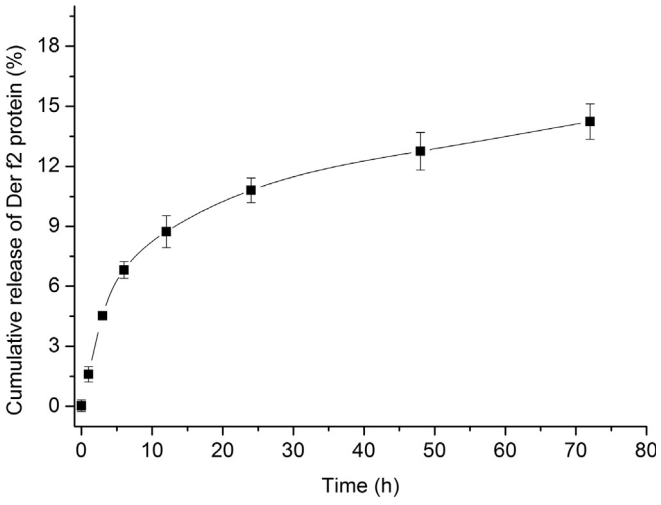

Fig. 6. Release profile of Der f2 protein from HMSNs in PBS.

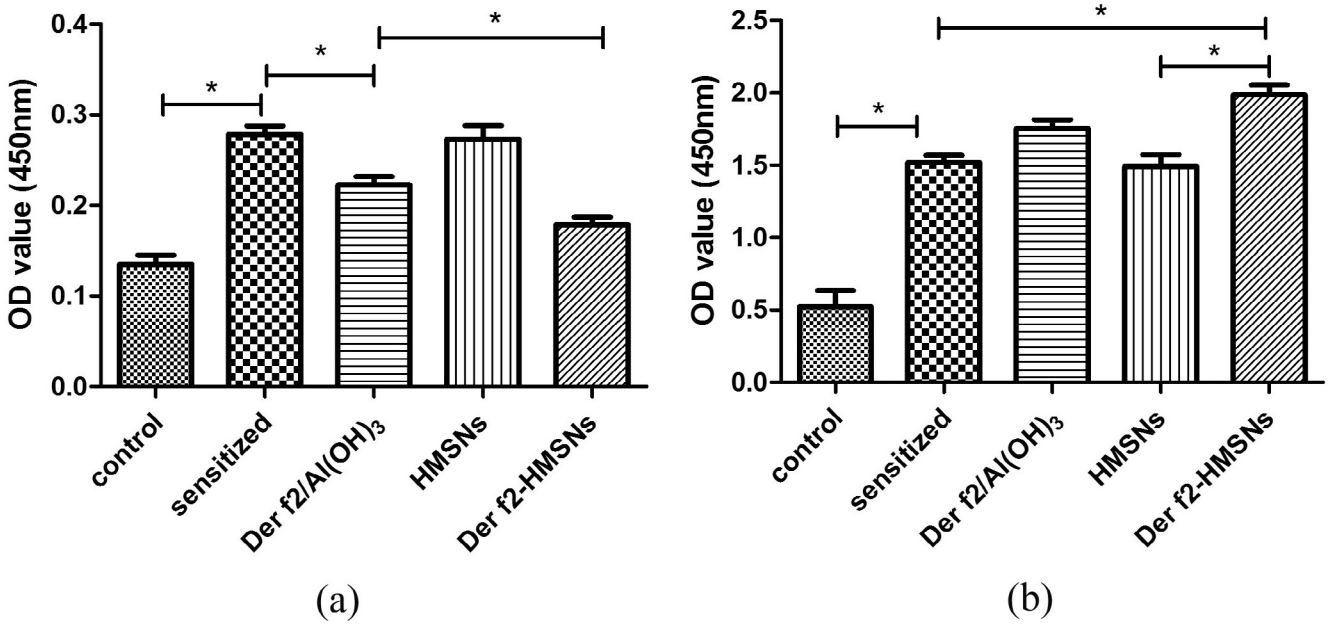

Fig. 7. Effect of different Der f2 formulations on serum Der f2 sIgE (a) and sIgG (b) production in mouse model of allergic asthma. Serum samples were collected from different groups (non-sensitized mice, sensitized mice, sensitized mice treated with Der f2/Al(OH)$_3$, sensitized mice treated with HMSNs, sensitized mice treated with Der f2-HMSNs), and Der f2 sIgE and sIgG were detected using ELSA. Results are reported as mean ± SD (n = 5), $^{*}P < 0.05$.

antibody for 45 min at 37 °C. After washing, 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added, and the absorbance at 450 nm was measured using a microplate reader (Molecular Devices Corp., Menlo Park, CA, USA).

To determine the Der f2 sIgG levels, plate was coated with 5 μg/ml of Der f2 and then incubated with serum samples diluted with 1: 200. For detection, 0.2 μg/mL of HRP-conjugated goat anti-mouse IgG antibody was used.

2.9. Detection of cytokines and inflammatory cells in BALF

Bronchoalveolar lavage fluid (BALF) was collected as previously (Peng et al., 2015). Immediately after euthanization, the lungs were lavaged by flushing 300 μL of cold PBS through the trachea for three times. BALF was centrifuged at 1000 rpm for 3 min at 4 °C. The supernatant and cell sediment of BALF were collected respectively. The supernatants were stored at −80 °C for measuring cytokine levels. Concentrations of interleukin (IL)-4 and interferon (IFN)-γ in BALF were determined by IL-4 Mouse ELISA kit and IFN-γ Mouse ELISA kit. The cell sediment was resuspended with 100 μL PBS and the number of white blood cell (WBC) was counted. A drop of cell suspension was added to the slide, dried, fixed, and stained with Liu's stain. The differential counts of inflammatory cells were conducted.

2.10. Histological examination

The non-lavaged lungs were removed and fixed with 10% neutral-buffered formalin for 48 h. Lung tissues were embedded in paraffin and sectioned at 5 μm. Tissue sections were then stained with hematoxylin and eosin (H&E). Images were obtained under light microscopy at 20× magnification. The pulmonary inflammation was assessed by a blinded observer.

2.11. T-lymphocyte proliferation assay and T-lymphocytes released cytokines measurement

Mice spleens were removed in sterile conditions, grinded and ground through a sterile cuprous mesh (200 meshes). The spleen cells were suspended in RPMI 1640 medium containing 10% FBS (complete medium), gently added to mouse lymphocyte separation medium, and centrifuged at 2400 rpm for 15 min. The lymphocyte layer was aspirated into 15 mL tube, and washed twice with complete medium. 5×10^4 T-lymphocytes were seated in 96-well plate, and co-cultured with Der f2 protein (2 μg/mL). The positive control well was added with concanavalin A (conA), and the negative control well was added with culture medium. After culture for 72 h at 37 °C, 5% CO_2, Cell Counting Kit-8 (CCK-8) regent was added to the wells. After incubation at 37 °C, 5% CO_2 for 3 h, the absorbance at 450 nm was measured, and results were expressed as OD values.

Supernatants of stimulated splenocytes were collected and stored at −80 °C. The cytokines levels were detected as Section 2.9.

2.12. Statistical analysis

Data are presented as mean values ± SD. Statistical analysis was performed using the two-tailed, unpaired Student's *t*-test (two groups) or ANOVA (three or more groups) (software SPSS Version 19.0). P-values < 0.05 were considered statistically significant.

3. Results

3.1. Characterization of HMSNs

The nitrogen adsorption–desorption isotherms are shown in Fig. 2, exhibiting typical type IV features according to the IUPAC classification and indicating the presence of mesopores (Wang et al., 2012). It exhibited a high BET specific surface area (875 $m^2 g^{-1}$) and total pore volume (1.003 $cm^3 g^{-1}$) with pore size of 2.897 nm.

TEM shows that the shape of HMSNs was spherical (Fig. 3), with a diameter of about 99.4 ± 17.6 nm, there is the presence of mesopores in HMSNs.

Zeta potential of HMSNs and Der f2-HMSNs was −32.7 mV and −19.7 mV respectively.

3.2. Protein level loaded on HMSNs

To determine the protein loading capacity of HMSNs, 1 mg HMSNs was mixed with different amount of Der f2 protein, and the protein

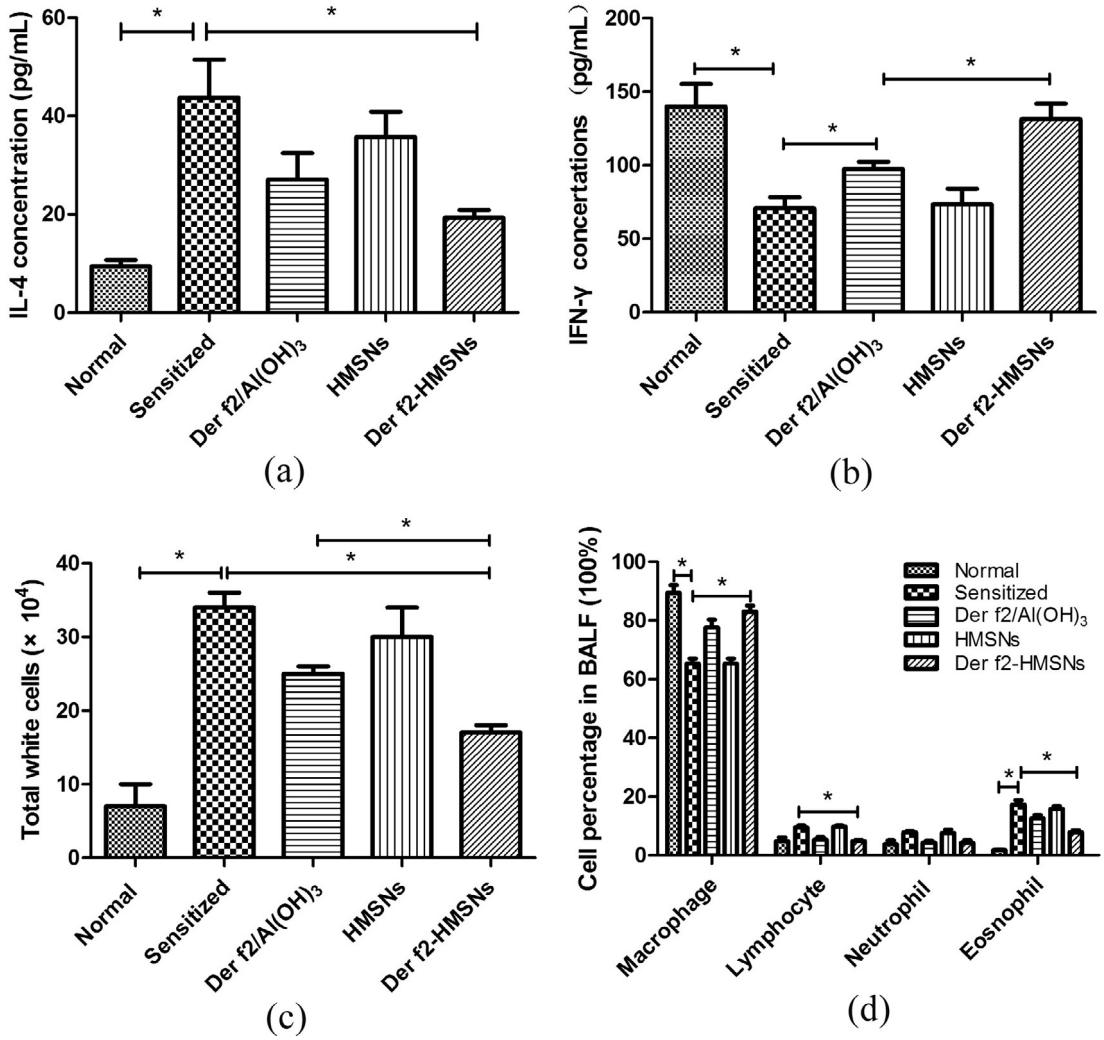

Fig. 8. Effect of different Der f2 formulations on cytokine production and inflammatory cell infiltration in BALF in a mouse model of allergic asthma. BALF samples were collected from different groups (non-sensitized mice, sensitized mice, sensitized mice treated with Der f2/Al(OH)$_3$, sensitized mice treated with HMSNs, sensitized mice treated with Der f2-HMSNs). Cytokines IL-4 (a) and IFN-γ (b) were detected using ELISA. Total white cells were counted (c). Cells were stained with Liu' staining and counted differently (d). Results are reported as mean ± SD (n = 5), $^{*}P < 0.05$.

loading level was determined by detecting the concentrations of protein in the supernatant before and after loading with Der f2 protein by BCA. According to Fig. 4, the loaded Der f2 amount was dependent on the amount of Der f2 added into HMSNs solutions. The highest loading Der f2 protein in the HMSNs is obtained when 0.1 mg Der f2 protein was added. The maximum loading capacity is about 90 μg per 1.0 mg of HMSNs. When the protein added exceeded 0.1 mg, the loading capacity of HMSNs decreased slightly.

Besides, the protein loading level was also determined by TGA. TGA curves of HMSNs and Der f2 loaded HMSNs are shown in Fig. 5. The results showed that the weight loss from 150 to 800 °C was 25.18% in Der f2-loaded HMSNs, while that in HMSNs was 17.29%. The increased weight loss (7.89%) of Der f2-loaded HMSNs indicated that Der f2 protein was successfully loaded onto HMSNs.

3.3. Release profile of Der f2 protein from HMSNs

The release of Der f2 protein (180 μg/mL) from HMSNs (2 mg/mL) at room temperature was conducted in PBS. Fig. 6 showed the release kinetics of the Der f2 from HMSNs. Our results showed that during the first 6 h the Der f2 protein released slightly fast from HMSNs, and about 6.81% of the protein was released. Then it showed a much slower release from the particles. After 24 h and 72 h, only 10.8% and 14.5% Der f2 on HMSNs was released respectively.

3.4. The efficacy of Der f2 loaded HMSNs in allergic inflammation

The preventive effect of Der f2-HMSNs in allergic inflammation was evaluated in mouse model of allergic asthma. Mice were divided into five groups and subcutaneously injected with different Der f2 formulations as Fig. 1 and Table 1.

Antigen-mediated allergic responses are known to induce Th2-specific Ig production. The clinical effect of specific immunotherapy treatment is mainly attributed to the production of IgG blocking antibodies and/or the reduction of IgE-reaginic antibodies (Nakagawa et al., 1987). Thus, we examined whether Der f2-HMSNs affected Der f2 sIgE and sIgG concentrations. Our results showed that Der f2 sIgE and sIgG levels increased after Der f2 challenge. Der f2 sIgE level was decreased after treated with Der f2/Al(OH)$_3$ and Der f2-HMSNs. However, in comparison with Der f2/Al(OH)$_3$ treated group (OD value 0.222 ± 0.009), Der f2-HMSNs treated group showed a significantly lower Der f2 sIgE levels (OD value 0.178 ± 0.008) ($P < 0.05$) (Fig. 7a). In addition, Der f2-HMSNs treated group could significantly increase the Der f2 sIgG level compared with sensitized group (OD value 1.987 ± 0.066 VS 1.520 ± 0.048) ($P < 0.05$) (Fig. 7b). However, the Der f2 sIgG level between Der f2/Al(OH)$_3$ treatment and

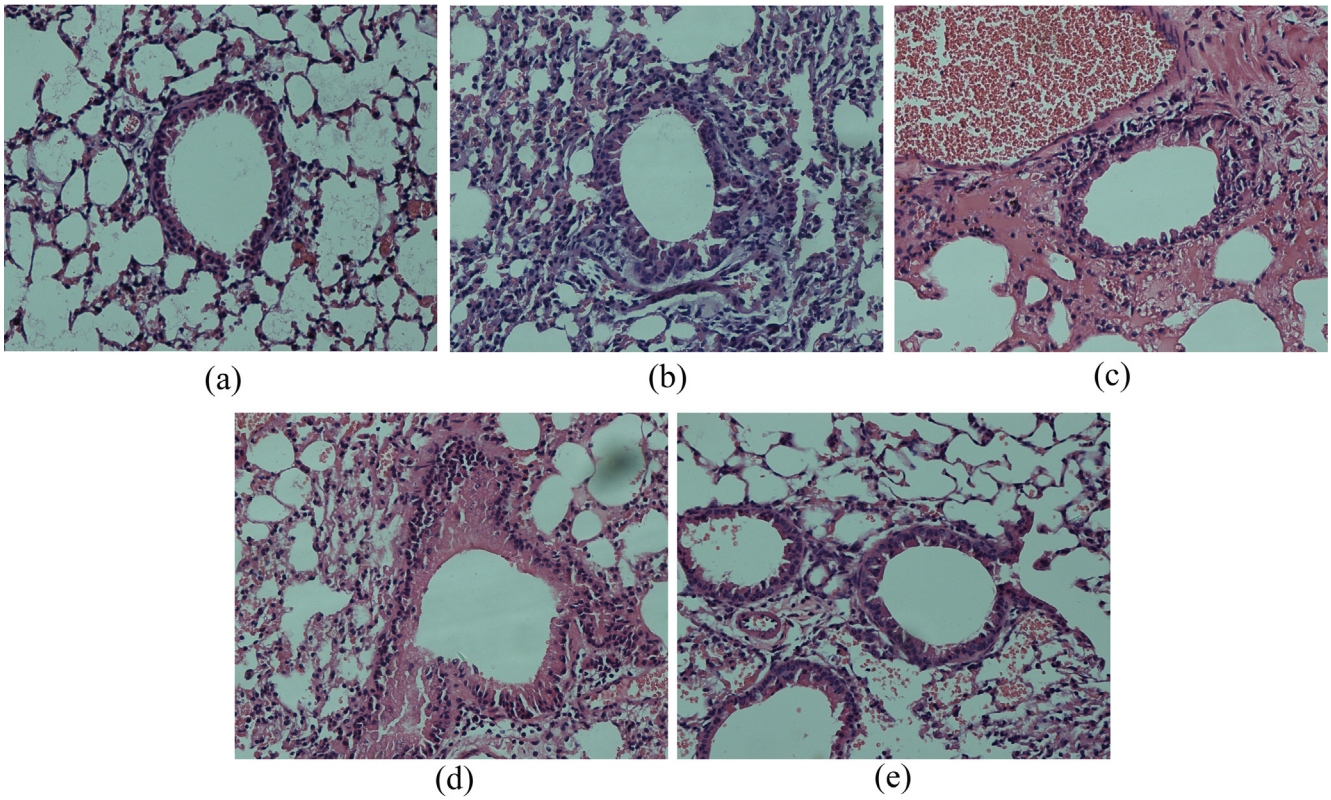

Fig. 9. Histopathology changes in the lung tissues. HE staining of the lung tissues from (a) non-sensitized mice, (b) sensitized mice, (c) sensitized mice treated with Der f2/Al(OH)$_3$, (d) sensitized mice treated with empty HMSNs, and (e) sensitized mice treated with Der f2-HMSNs. All images were captured at 20× magnification.

sensitized group has no difference (OD value 1.755 ± 0.059 VS 1.520 ± 0.048).

In allergic status, Th1/Th2 cytokines imbalance plays an important effect in early phase asthma. Therefore, we detected the cytokine levels in BALF. Der f2 challenge increased levels of Th2 cytokine IL-4 and decreased the level of Th1 cytokine IFN-γ. Der f2-HMSNs treatment could significantly reduce the IL-4 levels compared with the sensitized group (19.33 ± 1.52 pg/mL VS 43.79 ± 7.70 pg/mL), while the IL-4 levels between Der f2/Al(OH)$_3$ treated (27.09 ± 5.34 pg/mL) and sensitized group (43.79 ± 7.70 pg/mL) have no difference in statistics (Fig. 8a). Besides, both Der f2/Al(OH)$_3$ (97.47 ± 4.97 pg/mL) and Der f2-HMSNs treatment (131.50 ± 10.37 pg/mL) could increase the IFN-γ levels compared with the sensitized group (70.72 ± 7.45 pg/mL) ($P < 0.5$). However, Der f2-HMSNs treated group had a significantly higher IFN-γ concentration than Der f2/Al(OH)$_3$ treated group ($P < 0.5$) (Fig. 8b).

The pulmonary infiltration of inflammatory cells is also an important feature of allergic inflammation. Der f2 challenge could significantly increase the WBC number and the percentage of eosinophils in BALF (Fig. 8c and d). Our results showed that only Der f2-HMSNs treatment could decrease the WBC number and eosinophils percentage in BALF (Fig. 8c and d), and Der f2-HMSNs treated group has a significantly lower WBC number than Der f2/Al(OH)$_3$ treated group (Fig. 8c). Histological analysis of lung tissue sections revealed that Der f2 challenge induced severe pulmonary infiltration of inflammatory cells around the bronchial and perivascular areas, which was obviously alleviated in mice treated with Der f2-HMSNs, and slightly improved in Der f2/Al(OH)$_3$ treated mice (Fig. 9).

3.5. Lymphocytes proliferation assay

To evaluate the cellular immunologic state, T lymphocyte proliferation and cytokine release responded to Der f2 stimulation were determined.

In T cell proliferative experiment, single-cell suspensions from the spleens of control mice, allergic mice, and Der f2 sensitized mice treated with Der f2/Al(OH)$_3$, empty HMSNs, or Der f2-HMSNs were stimulated in vitro with purified Der f2 protein, con A or medium. As shown in Fig. 10a, the proliferative capacity of the splenocytes is significantly higher in Der f2-HMSNs treated group compared with that of sensitized group. Although Der f2/Al(OH)$_3$ treated group had a tendency of increased proliferative reaction.

T cell proliferation was accompanied by production of anti-inflammatory cytokines. Our results showed that the spleen lymphocytes from sensitized mice treated with Der f2-HMSNs showed higher levels of IFN-γ, but lower levels of IL-4 (Fig. 10b and c) compared with sensitized group ($P < 0.5$).

4. Discussion

The design of more powerful adjuvants/vehicles is of crucial interest to improve SIT effect. It was reported that HMSNs as vaccine carriers could induce stronger humoral and T-lymphocyte mediated immune responses, but not induce any local tissue damage or granuloma formation (Mody et al., 2013). Thus, in this study, we used HMSNs as carrier of HDM allergen for SIT, and recombinant Der f2 (a major allergen of house dust mite) was used as the model allergen to be loaded on HMSNs. The Nitrogen adsorption experiment showed that HMSN had a large surface area, thereby enhancing their capacity for protein adsorption. TEM showed that the Der f2 loaded HMSNs were spherical morphology with diameter of about 100 nm. It is reported that the spherical nanoparticles were more effective in inducing humoral immunity than other shapes (Marrack et al., 2009). Zeta potential results showed that the charges of HMSNs changed after Der f2 adsorption. It is reported that the zeta potential measurements are sensitive mainly to the charging at the outer surface of the particles and are not sensitive to

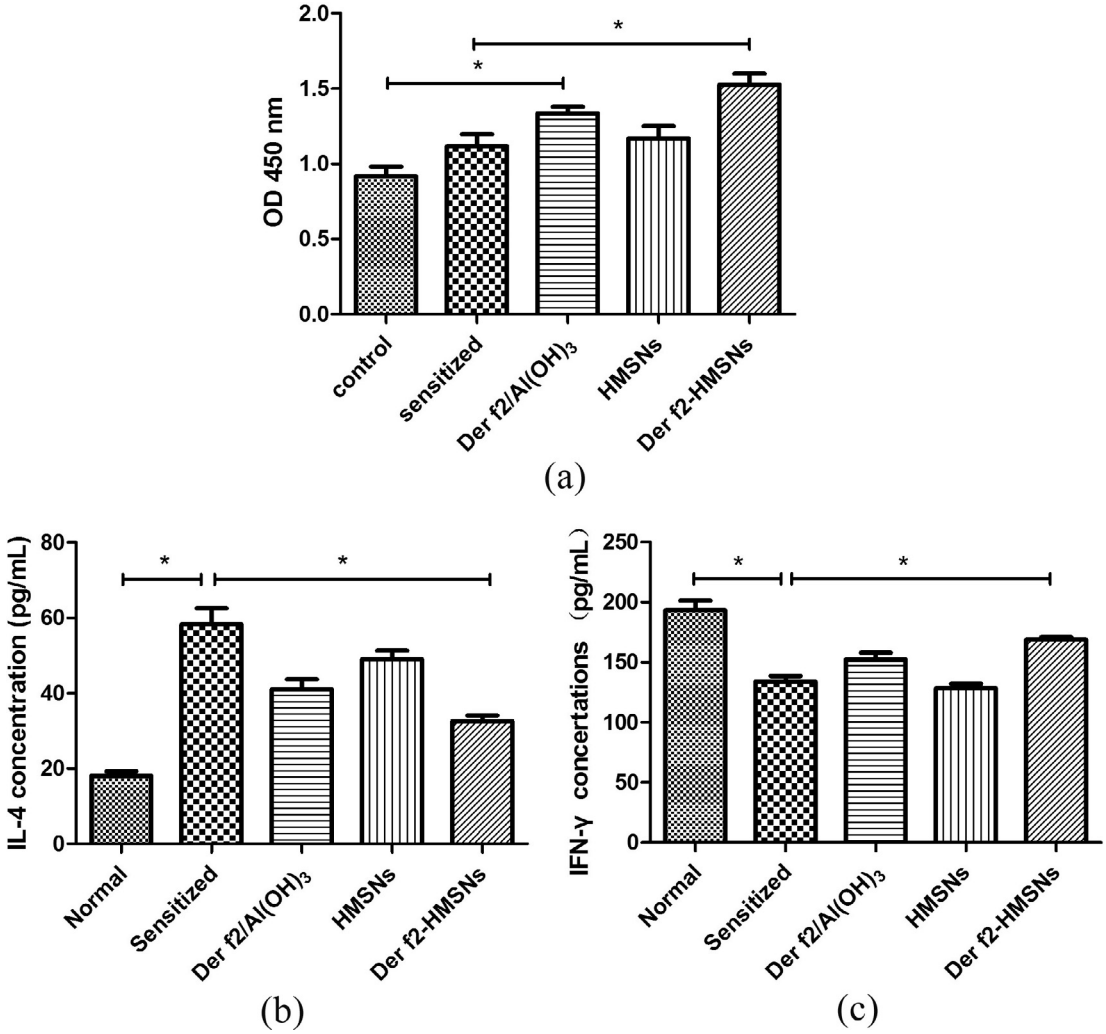

Fig. 10. Der f2 specific lymphocyte proliferation and cytokine release after treated with different Der f2 formulations. (a) Spleen cells were harvested and stimulated in vitro with Der f2 protein for 72 h, and then the CCK-8 regent was added to detect the lymphocytes proliferation. Results showed mean ± SD (OD 450 nm) of triplicate wells in each condition. (b) Before added with CCK-8, supernatant from lymphocyte cultures was reserved and cytokines IL-4 (b) and IFN-γ (c) were analyzed by ELISA. Results are reported as mean ± SD (n = 5), $^{*}P < 0.05$.

the surface potential inside the porous particles (Kecht et al., 2008). Thus, we speculated that the changed charge of HMSNs after Der f2 adsorption should be due to the effect of protein binding on the outer surface of HMSNs. The loading capacity for Der f2 proteins in HMSNs was at ratio of 90 μg (Derf2)/mg (HMSNs), which approached to the protein loading percentage reported by Guo HC and Mahony D (Guo et al., 2012; Mahony et al., 2014). The relatively high protein loading capacity could help decrease the dosage of HMSNs in the HMSN/protein mixture and eliminate potential adverse effects resulting from high concentrations of HMSNs. Besides, our results also showed that the protein loading capacity decreased slightly when the Der f2 protein added exceeded 0.1 mg (per 1 mg HMSNs). The reason may be that the protein and nanoparticles may slightly form aggregation (Kayitmazer et al., 2013). The Der f2 protein loaded on HMSNs was released relatively slowly in 72 h. The slow in vitro release of Der f2 from HMSNs is useful as both an adjuvant and a delivery vehicle with bound protein remaining stable and biologically functional. It is reported that the slow release rate of proteins could enhance the humoral immune response and improve the antibody titers when used in immunization studies (Wang et al., 2012). In this way, HMSNs could overcome several disadvantages of traditional adjuvants, and may offer an opportunity to reduce the necessary shots in SIT, thereby improving patients' compliance.

The allergic immune response is characterized by the activation of allergen-specific Th2 cells. Allergen presentation by activated antigen presenting cell (APC) to naïve CD4+ T cells induces the activation of naïve CD4+ T cells, which will then differentiate to Th2 cells. Secondary exposure to allergen leads to Th2 cell activation and release of IL-4, IL-5 and IL-13. These cytokines stimulate B cell activation and IgE secretion and activate cells in the immune system (eosinophils, mast cell, and so on), causing the release of vasoactive, pro-inflammatory mediators, mucus hypersecretion, and so on (Bosnjak et al., 2011). An important aspect of SIT in humans should be based on the decrease of the Th2 response by enhancing the Th1 response. Thus, we evaluated the effect of Der f2 formulations in mouse model of allergic asthma. After sensitization with Der f2, mice were treated with different Der f2 formulations (Der f2/Al(OH)$_3$ and Der f2-HMSNs), followed by challenge with Der f2 to provoke airway inflammation.

Our results showed that levels of Der f2 sIgE, a pathogenic antibody isotype for allergy, was reduced both in mice treated with Der f2-HMSNs and Der f2/Al(OH)$_3$. However, Der f2-HMSNs treated group had a lower level of Der f2 sIgE than Der f2/Al(OH)$_3$ treated group. The role of IgG in allergy development has been controversial because of their binding affinity to different Fcγ receptors which may lead to different immune response outcomes (Bruhns, 2012; Williams et al., 2012). In SIT, IgG can block the allergen binding to IgE on the mast

cell/basophil surface and thereby inhibit the allergic responses (Strait et al., 2006). Our results showed that only Der f2 sIgG level in mice treated with Der f2-HMSNs was significantly higher than sensitized mice. In our future study we should determine the subtypes of Der f2 sIgG, and make certain whether there is any shift between the Th2-associated IgG1 production and Th1-associated IgG2a following the Der f2 challenge and Der f2-HMSNs treatment. However, the decreased ratio in Der f2 sIgE to Der f2 sIgG in Der f2-HMSNs treated mice may also help indicate a shift of the Th2 to the Th1 response by the vaccine.

IL-4 level is an indicator for Th2 immune responses, and IFN-γ is an important cytokine in Th1 immune response, which could inhibit Th2 cytokines (Geginat et al., 2014). Our results showed that Der f2-HMSNs could decrease IL-4 level, and increase IFN-γ level in the BALF of allergic mice. Furthermore, splenocytes from Der f2-HMSNs treated mice had an increased proliferative ability in response to Der f2, accompanied by increased IFN-γ level and decreased IL-4 level. These cytokine profiles thus also showed a somewhat downregulation of Th2 response in Der f2-HMSNs-treated mice.

Another important characteristic of allergic airway inflammation is the increased pulmonary recruitment of inflammatory cells, especially eosinophils (Ge et al., 2016). Our results showed that challenge with Der f2 induced an apparent airway inflammation in Der f2-sensitized mice with increased inflammatory cells (especially eosinophils) in lung tissue according to BALF cell count and lung histopathology, and the inflammatory cells infiltration was decreased in Der f2-HMSNs treated mice. It is reported that Th2 cells appear to be the critical IL-4/IL-13-expressing cell type for the induction of allergic airway inflammation. Recruitment of effector cells (eosinophils and basophils) to the lung during Th2 immune responses is largely dependent on IL-4- or IL-13-induced activation of endothelial cells and secretion of the chemokines Ccl11 (eotaxin-1) and Ccl17 (TARC) (Oeser et al., 2015). In this way, we speculated that the reduction of the Th2 cytokine release might be a cause of decreased inflammatory cells infiltration in lung tissue of Der f2-HMSNs treated mice.

Above all, our results showed that Der f2-HMSNs exhibited a relatively higher effect in preventing allergic inflammation than Der f2/Al$(OH)_3$. Such increased efficacy of Der f2-HMSNs could be explained by the relatively good characterization (including relatively small particle size, spherical shape and slow release profile), which could help induce strong humoral and cellular immune response. These characteristic of Der f2-HMSNs may further help reduce the injection during treatment, thus decreasing treatment costs and enhancing patients' compliance.

Through these results, we found that Der f2-HMSNs could efficiently prevent the pathological Th2-response to Der f2, which indicate that HMSNs may be potential adjuvants for Der f2 protein in SIT of house dust mite allergy.

5. Conclusions

In this study, we successfully constructed Der f2 loaded HMSNs. The Der f2-HMSNs showed relatively good characterization with appropriate particle size, relatively high drug loading percentage and slow release profile. In vivo, it exhibited good efficacy in preventing allergic inflammation by shifting Th2 immune response to Th1 immune response. The Der f2-HMSNs may be potential vaccine for SIT of dust mite allergy.

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Conflicts of interest

The authors declare no conflicts of interest in this work.

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