Aluminum hydroxide nanoparticle adjuvants can reduce the inflammatory response more efficiently in a mouse model of allergic asthma than traditional aluminum hydroxide adjuvants

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Abstract. Traditional aluminum hydroxide is widely used as a vaccine adjuvant. Despite its favorable safety profile, it can cause an inflammatory response at the injection sites. However, multiple studies have shown that aluminum hydroxide nanoparticles have more potent adjuvant activity than their traditional aluminum hydroxide counterparts as antigen carriers; it has also been found that the local inflammation caused by aluminum hydroxide nanoparticle adjuvants is milder than that of other adjuvants. The aim of the present study was to compare the degree of inflammatory response between the aluminum hydroxide nanoparticle adjuvants and the traditional aluminum hydroxide adjuvants in the desensitization treatment of a mouse model of house dust mite (HDM)-induced allergic asthma. Mice were sensitized intraperitoneally with HDM. Subcutaneous desensitization was performed with PBS, traditional aluminum hydroxide adjuvants and aluminum hydroxide nanoparticle adjuvants. The mice were challenged and subsequently euthanized. The skin tissue at the local injection sites was assessed and specific indices were measured, such as the response of specific immunoglobulins, the airway hyper-responsiveness (AHR), and the inflammation in the bronchoalveolar lavage and lung tissues. Early hypersensitivity responses were suppressed in mice treated with subcutaneous immunotherapy (SCIT). Both traditional aluminum hydroxide-SCIT and aluminum hydroxide nanoparticle-SCIT could inhibit AHR. However, aluminum hydroxide nanoparticle-SCIT was able to significantly inhibit the secretion of eosinophils in the lung tissue and the production of type 2 cytokine Interleukin (IL)-5 in blood compared with the corresponding effects noted by traditional aluminum hydroxide adjuvants. Moreover, the aluminum hydroxide nanoparticle group reduced the inflammatory response at the local injection site. Collectively, the data indicated that allergen-specific immunotherapy using aluminum hydroxide nanoparticle adjuvants reduces lung and local inflammation compared with traditional aluminum hydroxide adjuvants.

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Introduction

Allergen-specific immunotherapy (AIT) promotes immune tolerance by repeatedly stimulating the body with small doses of allergens (1). Certain studies have shown that AIT can generate neutralizing antibodies, suppress the number and activity of allergen-specific T helper (Th) 2 and type 2 innate lymphoid cells, and induce the activity of regulatory T cells (2). Current AIT regimens routinely employ subcutaneous immunotherapy (SCIT) or sublingual immunotherapy with crude extracts of allergens for injection, among which the most common allergen is house dust mite (HDM). Therefore, the use of HDM allergens for desensitization treatment can reflect the treatment of this disease in a more efficient way. Aluminum adjuvant is often used in desensitization treatment to enable the slow release of allergens and assist the increase of the immune response.

Aluminum adjuvant induces the production of a specific immune response by the body and reduces the release of antigens and consequently its concentration (3,4). Therefore, certain scientists have employed the application of aluminum adjuvant in desensitization treatment (5,6). However, it has been identified that aluminum adjuvant adsorption of allergens in clinical treatment will produce certain adverse reactions. Ozden *et al* (7) and Mold *et al* (8) demonstrated persistent and pruritus subcutaneous nodular lesions at the injection site in asthmatic patients following desensitization. Smith and Petillo (9) demonstrated that aluminum adjuvants stimulated IgE production.

Therefore, in order to solve the problem of adverse reactions caused by the use of aluminum adjuvants, the development of aluminum hydroxide nanoparticle adjuvants has

been performed. Previous studies have immunized mice with bacterial and viral antigen-adsorbed aluminum hydroxide nanoparticle adjuvants as vaccines and demonstrated that aluminum hydroxide nanoparticle adjuvants induced cell differentiation into Th1 cells and stimulated the early production of antibodies (10-13). Amini *et al* (14) demonstrated that aluminum hydroxide nanoparticle adjuvants could stimulate a potent cellular immune response following immunization of mice with ESXV antigens. Chen *et al* (15) immunized mice with bacterial antigen adsorbed by aluminum hydroxide nanoparticle adjuvants and detected the levels of the cytokine interferon gamma (IFN-γ) by ELISA. The results indicated that aluminum hydroxide nanoparticle adjuvants significantly increased the secretion of IFN-γ and stimulated the immune response of Th1 cells.

Traditional aluminum hydroxide is often widely used as an adjuvant in desensitization therapy; however, a limited number of studies have been performed on the application of aluminum hydroxide nanoparticles in AIT. Aluminum hydroxide nanoparticle adjuvants can produce a rapid and more potent antigen-specific antibody response, inducing the immune response to the stimulation of Th1-type cells in the body. Therefore, in the present study, the application of aluminum hydroxide nanoparticle adjuvants was used to adsorb HDM for specific immunotherapy. This application may cause a more efficient reduction in the inflammatory response than that of the traditional aluminum hydroxide adjuvants.

Materials and methods

Characterization of aluminum hydroxide nanoparticles. The morphology of the aluminum hydroxide nanoparticles was measured via scanning electron microscopy (SEM), (SU8020; Hitachi High-Technologies Corporation). Specimens were prepared by placing a small volume (5 μl) of the aqueous suspension of aluminum hydroxide nanoparticles onto a titanium plate, and after drying, spraying gold (10 sec, three times) for observation.

Synthesis of HDM-adsorbed aluminum hydroxide nanoparticles. The method of adsorbing HDM allergens with aluminum hydroxide nanoparticle hydroxide adjuvant was as follows: i) Aluminum hydroxide nanoparticles were prepared to 1 mg/ml, dispersed ultrasonically for 30 min and stored in a 4˚C refrigerator for later use. ii) A total of 25 mg HDM extract was dissolved in 500-μl sterile PBS to obtain a solution containing 50 μg/μl HDM. Each aliquot was divided into 50 μl and stored at -20˚C for later experimentation. iii) A vortex mixer was utilized to mix HDM allergen and aluminum hydroxide nanoparticle hydroxide adjuvant in a volume ratio of 1:1. Vortex was conducted at 5,000 x g for 30 min and thorough mixing followed to obtain aluminum hydroxide nanoparticle-adsorbed HDM vaccine.

Animal vaccination using HDM-adsorbed aluminum hydroxide nanoparticles. When mice were sensitized with HDM allergens, they became hypersensitive. When the mice were challenged again with HDM allergens, the inflammatory factors produced in the mice remained highly expressed. Both female and male mice developed the aforementioned allergic reactions. However, male mice living in groups are prone to fights. After being injured in a fight, the skin surface will be red and swollen, and the mouse's organism will produce inflammatory factors. This would make it difficult to determine whether the production of inflammatory factors was due to injury from fighting between male mice or due to sensitization to the allergen. Therefore, female mice (32 mice; 6 to 8 weeks old; weight, 18 to 22 g) were selected for experiments in the present study. Female BALB/c mice used in the present study were obtained from Curegenix Corporation [permit no. SCXK (YUE)2022-0063]. The mice were grouphoused (2-4 per cage) and maintained with a 12/12-h light/dark cycle (light at 6 a.m.) under a specific temperature range (19-25˚C) and humidity (55-65%) controlled environment. They were provided free access to food and water and acclimated for at least 7 days to adapt to the environment. All procedures involving animals complied with the Guiding Principles for the Care and Use of Vertebrate Animals in Research and Training. The experiments were approved (approval no. YSDW2023020-04) by the animal Ethics and Welfare Committee of Curegenix Corporation (Guangzhou, China). Crude extract of HDM was purchased, its information being as follows: Allergen, *Dermatophagoides Pteronyssinus*, (cat. no. XPB82D3A2.5; Greer Laboratories). The crude extract of HDM was dissolved in sterile PBS for subsequent experimentation. The specific operation was as follows: A total of 25 mg HDM extract was dissolved in 500-μl sterile PBS to get a solution containing 50 μg/μl HDM; this stock was aliquoted in 50 μl portions and stored at -20˚C. All experiments in the present study used the same batch of HDM allergen.

The experiments were performed blinded. The experimental mice were divided into 4 groups (eight mice in each group): i) Negative control (NC), PBS challenged; ii) Positive control (PC), HDM challenged; iii) HDM + Al, traditional aluminum adjuvant adsorbing HDM allergen control group; and iv) HDM + NP, aluminum hydroxide nanoparticle adjuvant adsorbing HDM allergen control group. For studying immunogenicity, mice (n=8) were sensitized on days 0, 7, and 14 (on the abdomen) with 50 μg HDM (cat. no. XPB82D3A2.5; Stallergenes Greer) extract in PBS containing 2.25 mg Alum (cat. no. 77161; Thermo Fisher Scientific, Inc.) by intraperitoneal injection. For studying the therapeutic efficacy, the mice (n=8) were immunized on days 29, 31, 33 (on the back) by subcutaneous injection of 100 μg HDM extract adsorbed (100 μl) aluminum hydroxide nanoparticles (Chongqing University) or 2.25 mg aluminum. The adsorption of HDM on aluminum hydroxide nanoparticles was carried out by mixing the aluminum nanoparticles with an equal volume of the HDM suspensions, followed by gently rotating at 4˚C for 12 h to assist 20 conjugations (15). Following the last treatment used for induction of lung inflammation, the mice were challenged on days 45, 47 and 49 by intranasal instillation with native HDM extract (50 μl PBS containing 50 μg native HDM extract). Mice were anesthetized using 3% isoflurane and then euthanized by cervical dislocation. The spines and brains of mice were severed. It was observed that the pain response of mice disappeared and there was no response when the toes were pressed with hands; the heartbeat and breathing were observed to stop, thus confirming the death of the mouse. The blood samples were obtained on day 51

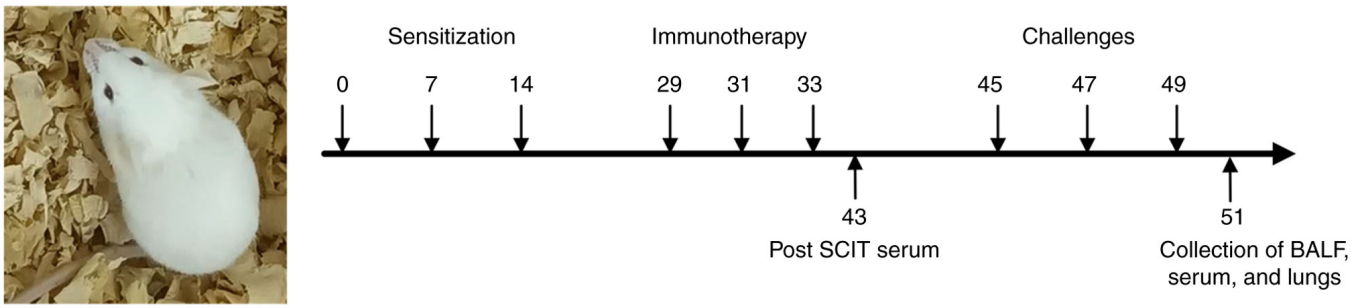

Figure 1. A controlled experimental method is used in the present study. The experimental mice were divided into 4 groups. NC, Negative control, PBS challenged; PC, Positive control, HDM challenged; HDM + Al, Traditional aluminum adjuvant adsorbing HDM allergen control group; HDM + NP, Aluminum hydroxide nanoparticle adjuvant adsorbing HDM allergen control group. BALB/c mice of PC, HDM + Al and HDM + NP were sensitized i.p. on days 0, 7 and 14, and HDM adsorbed aluminum hydroxide adjuvant and injected i.p.; On days 29, 31 and 33,the NC group was given PBS desensitization treatment, the PC group was given PBS desensitization treatment; the HDM + NP and HDM + AI groups were administered neck desensitization therapy by subcutaneous injection (100 μg HDM adsorbed to 100 μl of aluminum hydroxide nanoparticles or 2.25 mg Alum). Mice were challenged by nasal drip on days 45, 47 and 49. The mice were anesthetized using 3% isoflurane and then euthanized by cervical dislocation 51 days after the last HDM stimulation for blood collection, alveolar lavage, lung tissue and other immune indexes analysis. HDM, house dust mite; Al, Aluminum; NP, nanoparticle; i.p., intraperitoneally.

(therapy) by cardiac puncture and the sera were stored at -20˚C until further analysis (Fig. 1).

Collection and processing of bronchoalveolar lavage fluid (BALF). In the present study, the accumulation of leukocytes was evaluated by studying the phenotype of cells collected from the BALF samples of vaccinated mice. These mice were intranasally exposed with HDM from day 45 to day 49 to induce an allergic airway inflammation model (16). The mice were anesthetized with 3% isoflurane by inhalation on day 51 (Cyprane Fluotec Vaporizer). BALF was collected and processed as previously described by Debeuf *et al* (17) with minor modifications. Briefly, the trachea was exposed and BALF was extracted using 1 ml PBS. BALF was centrifuged at 800 x g for 5 min at 4˚C, and the supernatant was stored at -20˚C for the determination of cytokine levels. The cells in the pellet were resuspended in PBS for the determination of the total cell counts. A total of 300 cells per/slide were counted to detect the number of macrophages, eosinophils and neutrophils under an light microscope (Olympus Corporation), followed by staining with hematoxylin-eosin (H&E) according to the manufacturer's procedure protocols (Beijing Solarbio Science & Technology Co., Ltd.).

Analysis of inflammatory cytokine infiltration in BALF. The mice were sacrificed on day 51, BALF was centrifuged at 800 x g for 5 min at 4˚C, and the concentration levels of interleukin (IL)-4 (QuantiCyto® Mouse IL-4 ELISA kit; cat. no. EMC003.96.5), IL-5 (QuantiCyto® Mouse IL-5 ELISA kit; cat. no. EMC108.96.2), IL-10 (QuantiCyto® Mouse IL-10 ELISA kit; cat. no. EMC005.96.2) and IFN-γ (QuantiCyto® Mouse IFN-γ ELISA kit; cat. no. EMC101g.96) were determined by ELISA according to the manufacturer's instructions (Neobioscience Technology Co., Ltd.).

Histological analysis. The lung tissues (mouse lung tissue thickness, 5 mm; cross-sectional lung tissue thickness, 6-8 μm) were fixed in 10% formalin at 4˚C for 24 h and subsequently embedded in paraffin wax following dehydration in alcohol. The lungs were removed and processed for H&E staining. Following the final challenge, the mice from each group were sacrificed for skin histological examination. The skin tissues were fixed in 10% formalin at 4˚C for 24 h, paraffin-embedded, and stained with H&E. The skin tissues were evaluated for allergic airway inflammation to assess the presence of inflammatory cell infiltrates, and the degree of perivascular and peribronchiolar inflammation.

Evaluation of airway hyper-responsiveness (AHR). AHR was assessed on day 51 (24 h following the final intranasal instillation of HDM). The mice were anesthetized by inhalation of 3% isoflurane. The animals were subsequently connected to a small animal ventilator (BUXCO Finepointe-NAM; Data Sciences International; Harvard Bioscience, Inc.) set at a frequency of 150 breaths/min, a tidal volume of 10 ml/kg, and a positive end-expiratory pressure of 2-3 cm H_2O. The mice were challenged with increasing concentrations (3.125, 6.25, 12.5 and 50 mg/ml) of methacholine chloride (cat. no. A2251-25G; Sigma-Aldrich; Merck KGaA) aerosol that were administered for 10 sec with an in-line nebulizer. Airway resistance was measured using a 'snapshot' protocol each 20 sec for 5 min, ensuring that the measured parameters were stabilized.

Statistical analysis. The data were analyzed using the statistical and graphing software SPSS 24.0 (IBM Corp.) and GraphPad Prism 8.0 (Dotmatics). Statistical analyses were performed by Oneway ANOVA followed by Tukey's post-hoc test. The data are presented as mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Physicochemical characterization of aluminum hydroxide nanoparticles. The ultimate morphologies and size of the as-synthesized aluminum hydroxide nanoparticles were characterized with microscopic techniques. As demonstrated in Fig. S1A, SEM images revealed that the obtained products have a uniform pellet morphology with a mean diameter of 200±80 nm.

Adsorption of HDM antigens on aluminum hydroxide nanoparticles. HDMs and aluminum hydroxide nanoparticle adjuvant

were emulsified (volume ratio was 1:1), and then combined at a certain temperature and rotation speed. The surface charge of the adjuvant depends on its pH value; a previous study (18) determined that the surface of aluminum hydroxide nanoparticle adjuvants is positively charged at neutral pH. The surface of HDM allergens had a negative charge at neutral pH (19), and the adsorption of the adjuvant to the antigen occurs through static electricity. Primary adsorption mechanisms for aluminum adjuvants: When the antigen and the adjuvant have opposite charges, electrostatic interaction occurs, which illustrates the adsorption of the antigen to the adjuvant. The particle size of the aluminum hydroxide nanoparticle adjuvant becomes notably larger after adsorption of HDMs. The mean diameters of the HDM-adsorbed aluminum hydroxide nanoparticles were 600±80 nm; a representative SEM image clearly demonstrated the adsorption effect of whole HDM onto aluminum hydroxide nanoparticles (Fig. S1B).

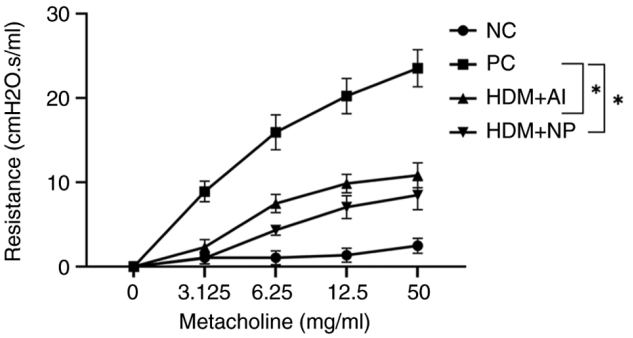

Figure 2. Clinical manifestations after SCIT treatment. An ED of Methacholine when the airway resistance reaches 3 cm H_2O.s/ml (ED3). Absolute values are expressed as the mean ± standard deviation (n=8). *P<0.05 compared with PC. SCIT, subcutaneous immunotherapy; ED, effective dose; SEM, scanning electron microscopy; PC, positive control NC; negative control; HDM + Al, house dust mite traditional aluminum adjuvants; HDM + NP, house dust mite nanoparticle.

Nanoparticle-HDM (NP-HDM)- and aluminum-HDM (Al-HDM)-SCIT suppress AHR. To evaluate whether SCIT protects against asthma, the effects of SCIT on lung function were assessed following HDM challenge. Acetylcholine was used to stimulate the mice to produce AHR, and it was measured that the PC group was in a state of high airway resistance, which was specifically manifested as a significant upward trend in airway resistance values. Compared with the PC group, NP-HDM and Al-HDM mice showed a significant decrease in airway resistance (6.25, 12.5 and 50 mg/ml) after desensitization treatment (Fig. 2).

NP-HDM- and Al-HDM-SCIT suppress eosinophilic airway inflammation and cytokine levels. AIT suppressed the Th2 cell activity and reduced airway inflammation following treatment. Therefore, the number of inflammatory cells and the expression levels of the Th1/2 cytokines were assessed in BALF samples. As expected, the HDM challenge in the PC group induced a pronounced eosinophilic and neutrophil airway inflammation. Both NP-HDM- and Al-HDM-SCIT groups resulted in a significant decrease in the eosinophil and neutrophil numbers in BALF samples compared with controls (Fig. 3B and D, respectively). Moreover, the BALF total cell count was significantly reduced in the HDM + Al and the HDM + NP groups compared with the PC group (Fig. 3A); whereas the number of macrophages was increased in in the HDM + Al and the HDM + NP groups compared with the PC group (Fig. 3C). To evaluate the activity of the Th1/2 cells, the expression levels of IL-5 and IL-10 were assessed in BALF samples and a significant reduction was only observed in the IL-5 levels of NP-HDM- and Al-HDM-SCIT-treated mice compared with those observed in PC (Fig. 4A); however, IL-10 levels were unaffected by either treatment (Fig. 4B).

NP-HDM- and Al-HDM-SCIT suppress type 2 responses. Given the results of the type 2 cytokines in BALF, the expression levels of the signature type 1/2 inflammatory cytokines were analyzed in the blood samples of the NP + HDM and Al + HDM groups and in the corresponding samples derived from sham-SCIT-treated mice. In the present study, it was revealed that the HDM challenge in sham-treated mice induced a strong IL-5 and IL-4 response compared with that noted in the NC (Fig. 5A and C, respectively). SCIT with NP + HDM and Al + HDM was able to suppress IL-4 and IL-5 levels. It was found that NP + HDM used for vaccination could reduce the IL-5 response compared with Al + HDM (Fig. 5A). In addition, NP + HDM- and Al + HDM-SCIT induced an increase in the levels of IL-10 in blood compared with the PC group, but no statistical significance was identified (Fig. 5D). IFN-γ levels were also assessed to control for any effect on Th1 cell activity, however IFN-γ levels were increased by either treatment compared with the PC group, but no statistical significance was identified (Fig. 5B).

NP-HDM- and Al-HDM-SCIT reduce IgE responses and enhance HDM-IgG2a levels. The majority of the PCs induced a sufficiently high IgE response, which was visualized in the total IgE levels plotted in Fig. 6A. In addition, the expression levels of IgE were significantly decreased following treatment with NP + HDM- and Al + HDM-SCIT compared with the levels of the PC group. The results indicated increased HDM-IgG2a levels following treatment with NP + HDM-and Al + HDM-SCIT (Fig. 6B). However, IgE levels did not differ between the NP + HDM and Al + HDM experimental groups.

NP + HDM-SCIT reduces the inflammatory response. Allergic diseases can cause infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues; the latter were examined by histological analysis. Compared with the NC group, the PC group induced more accumulation of inflammatory factors such as neutrophils and eosinophils (Fig. 7A and B). The PC group indicated the most severe perivascular cuffing in the lung tissue, which was reduced in mice vaccinated with NP + HDM and Al + HDM (Figs. 7C and D). The numbers of the inflammatory cells were also analyzed in the skin tissues of NP + HDM, Al + HDM and in sham-SCIT-treated mice. The present study demonstrated that HDM challenge in Al-HDM-treated mice induced a potent inflammatory response compared with that of the NC group (Fig. 8A and C). By contrast, the data indicated that NP-HDM-SCIT did not induce a more potent increase in the inflammatory response levels in the skin tissues compared

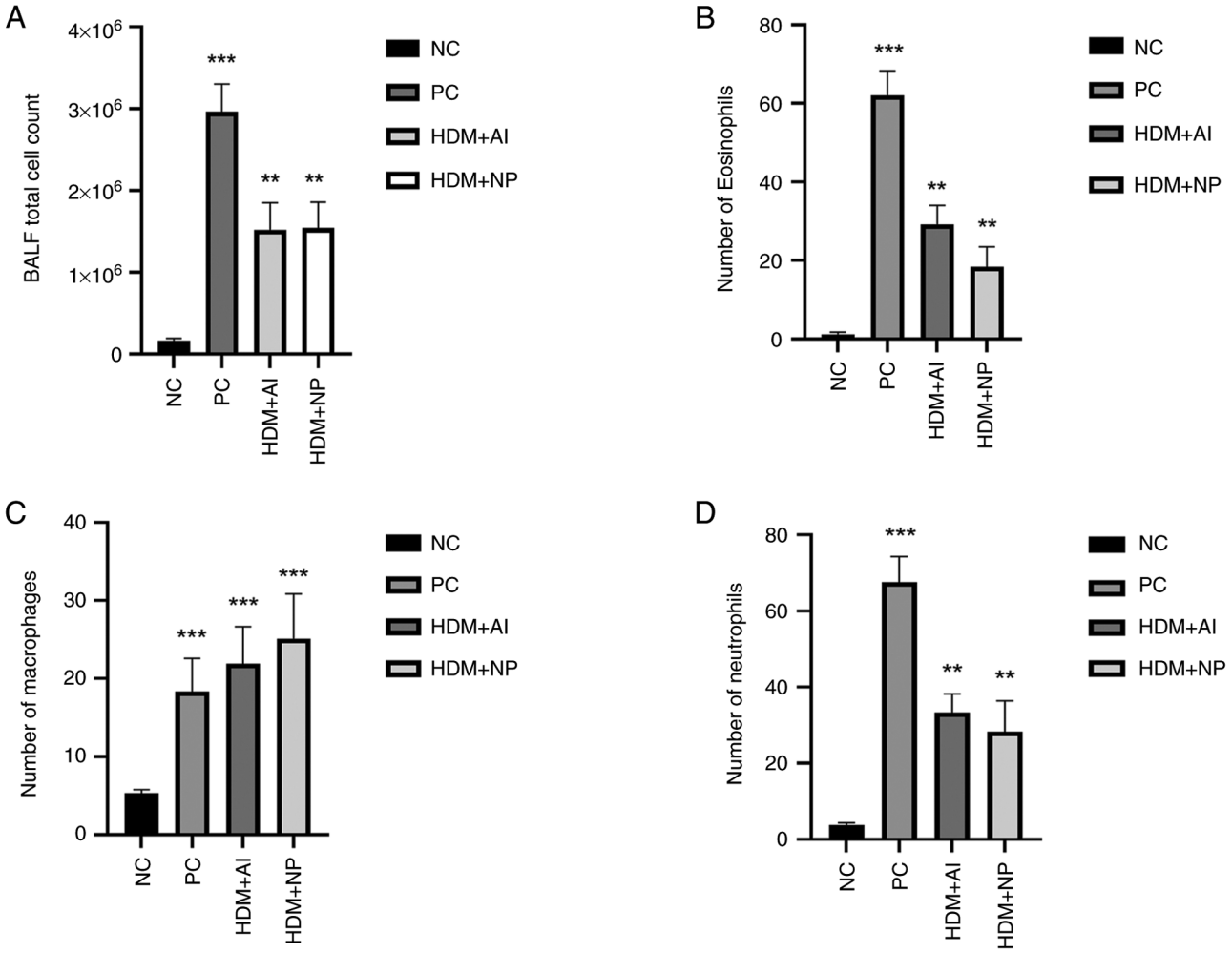

Figure 3. Airway inflammation response after subcutaneous immunotherapy treatment. (A) Total cell count in BALF. (B) Number of eosinophils. (C) Number of macrophages. (D) Number of neutrophils. Values are expressed as the mean ± standard deviation (n=8). **P<0.01 and ***P<0.001 compared with PC. BALF, bronchoalveolar lavage fluid; PC, positive control; NC; negative control; HDM + Al, house dust mite traditional aluminum adjuvants; HDM+NP, house dust mite nanoparticle.

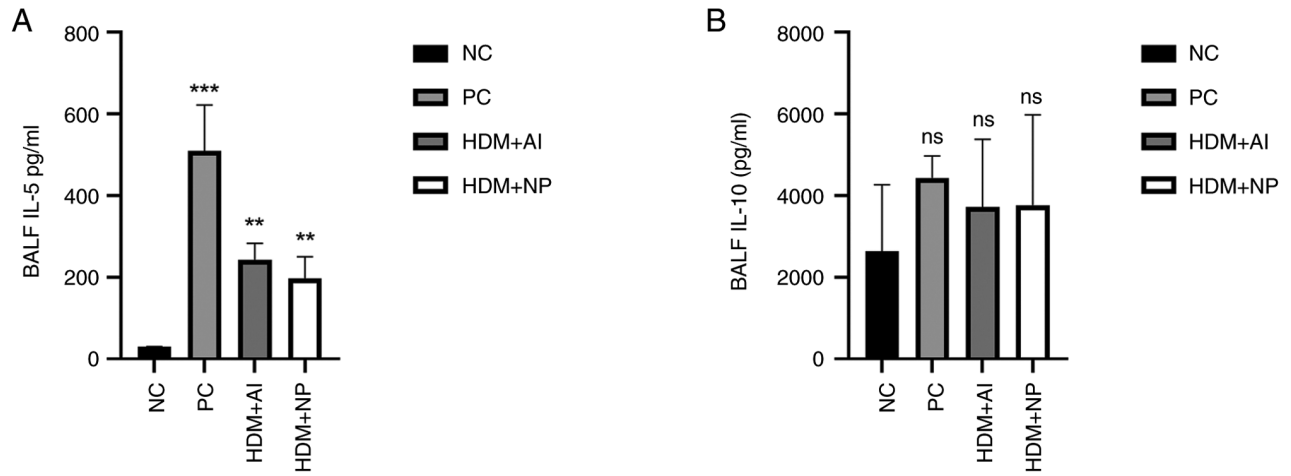

Figure 4. Cytokine response after subcutaneous immunotherapy treatment. (A) Levels of IL-5 and (B) levels of IL-10 measured in re-stimulated lung single cell suspensions. Values are expressed as the mean ± standard deviation (n=8). **P<0.01 and ***P<0.001 compared with PC. IL, interleukin; PC, positive control; NC; negative control; HDM + Al, house dust mite traditional aluminum adjuvants; HDM + NP, house dust mite nanoparticle; BALF, bronchoalveolar lavage fluid.

with that of the PC group (Fig. 8B and D). Overall, the findings indicated that the application of NP-HDM for vaccination can reduce the perivascular cuffing in skin and lung tissues compared with Al-HDM.

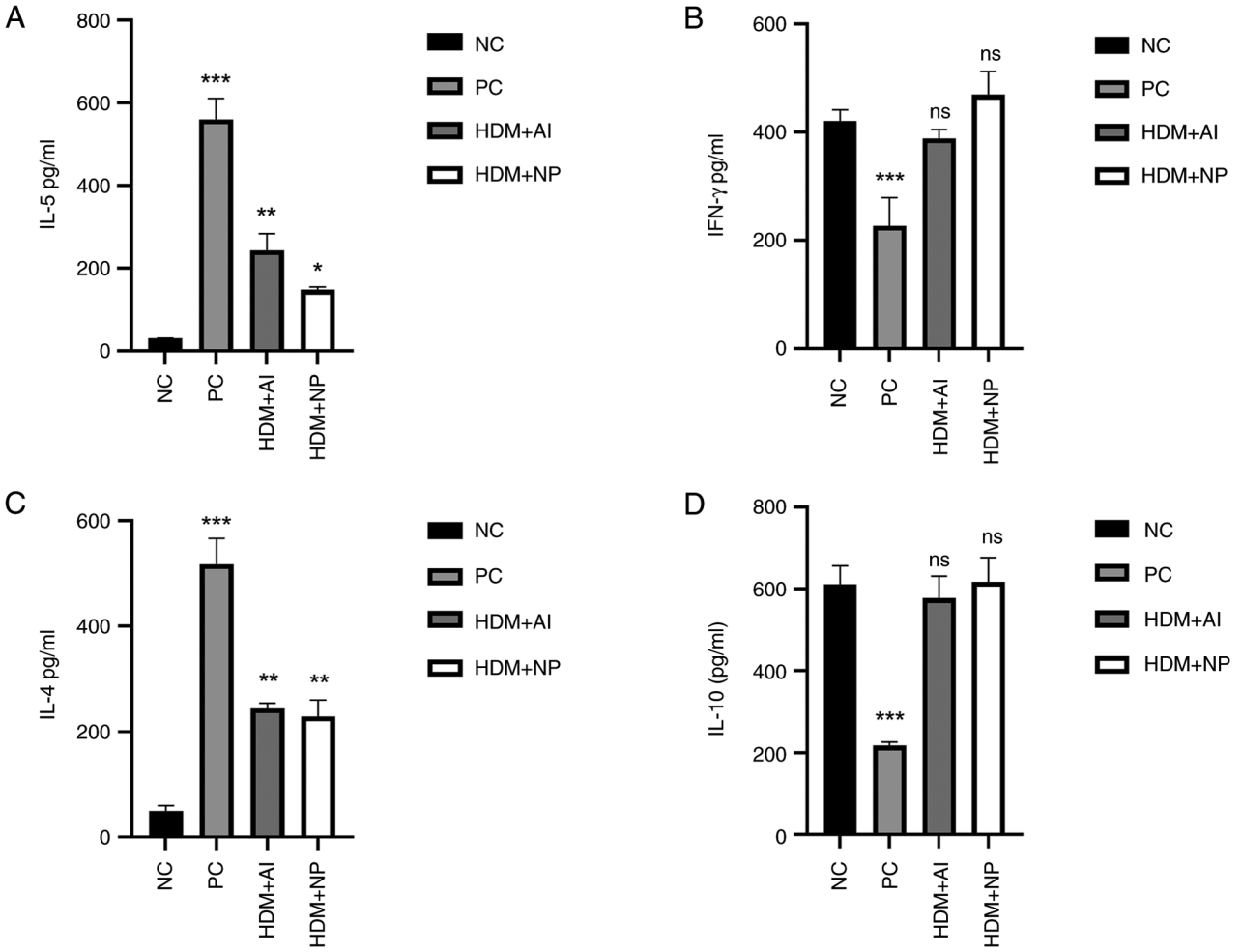

Figure 5. Overview of cytokine profile after SCIT treatments, measured in blood. (A) Type-2 inflammatory cytokines IL-5 (pg/mg), (B) IFN-γ levels (pg/mg), (C) IL-4 levels (pg/mg) and (D) IL-10 levels (pg/mg) in blood of HDM + Al, HDM + NP and sham-SCIT treated mice. Concentrations (pg/mg) are expressed as mean ± standard deviation (n=8). *P<0.05, **P<0.01 and ***P<0.001 compared with PC. SCIT, subcutaneous immunotherapy; IL, interleukin; IFN-γ, interferon gamma; HDM + Al, house dust mite traditional aluminum adjuvants; HDM + NP, house dust mite nanoparticle; NC, negative control; PC, positive control.

Discussion

The aim of the present study was to compare the degree of inflammatory response between the aluminum hydroxide nanoparticle and the traditional aluminum hydroxide adjuvants in the desensitization treatment of a mouse model of HDM-induced allergic asthma.

The results indicated that in the mouse model of allergic asthma immunotherapy with aluminum hydroxide nanoparticle adjuvant-adsorbed HDM, the AHR of the aluminum hydroxide nanoparticle adjuvant group was decreased compared with that of the sensitization group; however, the difference was not statistically significant when this parameter was compared with the AHR of the aluminum hydroxide adjuvant (Fig. 2). However, no significant difference was observed in the total number of alveolar lavage fluid cells, eosinophils, neutrophils and macrophages compared with the traditional aluminum hydroxide adjuvant group (Fig. 3A-D). The aforementioned results indicated that the aluminum hydroxide nanoparticle adjuvants adsorbing HDM could effectively reduce AHR and total cells when used as immunotherapeutic agents for allergic asthma. In the present study, it was revealed that aluminum hydroxide nanoparticle adjuvants were shown to induce Th1-type immune responses. The decrease in the number of eosinophils and neutrophils may be related to the Th1-type immune responses induced by aluminum hydroxide nanoparticle adjuvants.

The concentration levels of the cytokine IL-5 in the alveolar lavage fluid and the levels of the Th2-type cytokine IL-5 in serum were significantly reduced following immunotherapy with the aluminum hydroxide nanoparticle adjuvants (Figs. 4A and 5A); however, the levels of the Th1 cytokine IFN-γ in serum were increased (Fig. 5B). The aluminum hydroxide nanoparticle adjuvants adsorbing HDM immunotherapy could inhibit the increase in the levels of the Th2-cytokine IL-5. When the organism is exposed to the allergen again, the allergen binds to the mast cells present in the organism, causing them to release inflammatory mediators, including histamine and cytokines. Among these inflammatory factors, IL-4 and IL-5 are Th2-type cytokines, which mainly participate in the inflammatory response and promote the secretion of the Th1-type cytokine IFN-γ and IL-10. IFN-γ can promote antigen presentation, enhance macrophage lysosomal activity, and induce regulatory T cell immune response. The mechanism of aluminum hydroxide nanoparticle adjuvant desensitization therapy may be related to the reduction of the activation state of the innate immune

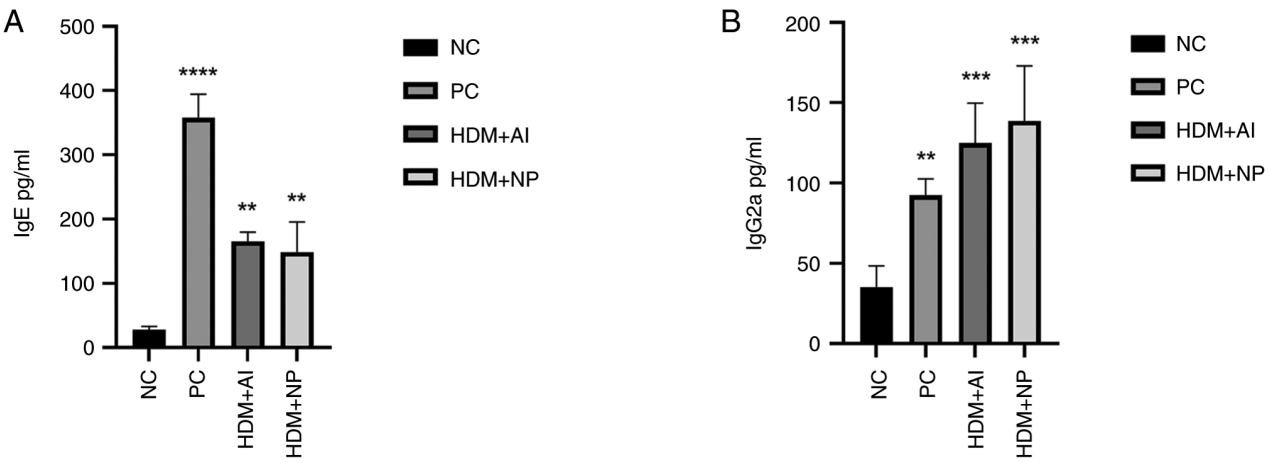

Figure 6. Overview of immunoglobulin response after subcutaneous immunotherapy treatment. (A) HDM + Al and HDM + NP IgE levels measured in sera received after treatments [pg/ml, post- treatments). (B) HDM + Al, HDM + NP IgG2a levels measured in sera received after treatments (Arbitrary Units (AU)/ml, Post challenges). Values are expressed as the mean ± standard deviation (n=8). **P<0.01,***P<0.001 and ****P<0.0001 compared with PC. HDM + Al, house dust mite traditional aluminum; HDM + NP, house dust mite nanoparticle; NC, negative control; PC, positive control.

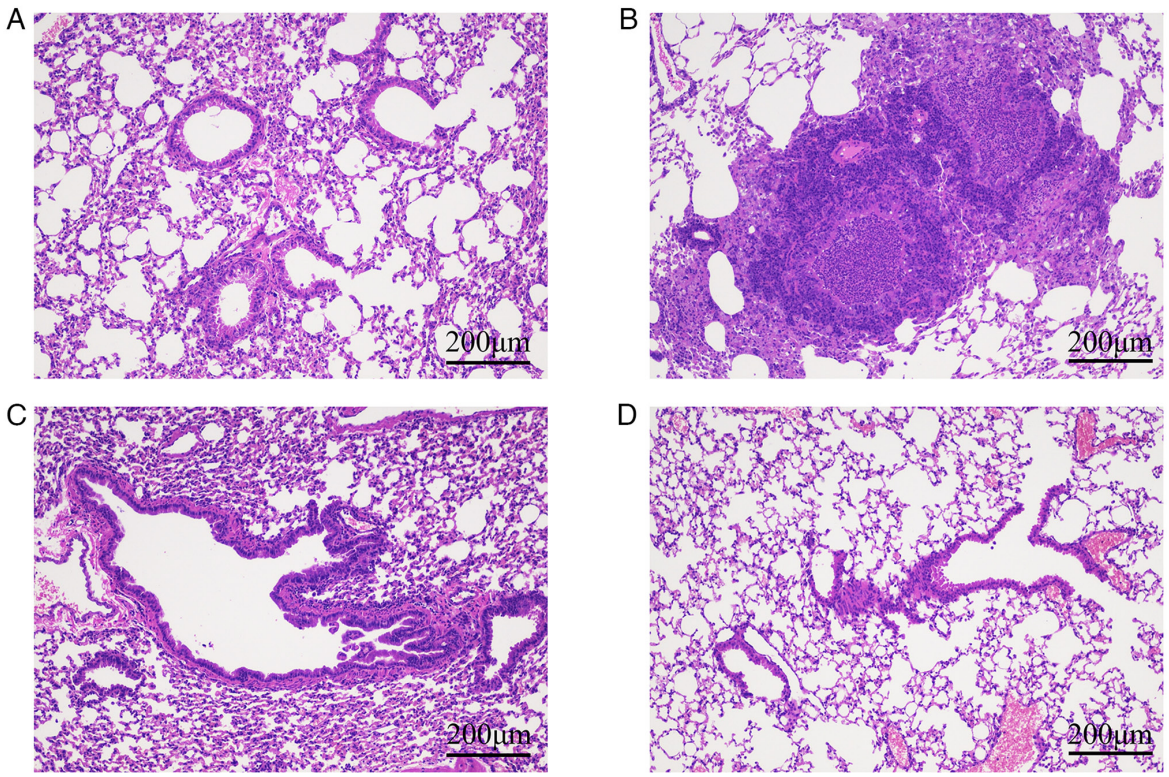

Figure 7. Representative H&E-stained lung sections of mice vaccinated with HDM coated on NP; (A) PBS, (B) shams, (C) HDM + Al and (D) HDM + NP. Lung sections were collected on day 51. Boxes indicate critical areas of pathology that are almost exclusively infiltrated by perivascular inflammatory cells consisting of macrophages, eosinophils and neutrophils, these are highlighted in the high-resolution image insert. Scale bar, 200 μm. HDM + NP, house dust mite nanoparticle; HDM + Al, house dust mite traditional aluminum adjuvants; H&E, hematoxylin and eosin.

system (20-23). However, in the present study, BALF IL-10 exhibited no statistical significance, which may be related to the number of mice used, the individual differences, and the experimental errors. A previous study have demonstrated that aluminum hydroxide nanoparticle adjuvant adsorption of HDM immunotherapy can promote the immune response of Th1 cells, which is consistent with the study of Hesse *et al* (24).

Following immunotherapy of the aluminum hydroxide nanoparticle adjuvant group, the levels of IgE were decreased and those of IgG2a were increased; however, no statistical significance was observed compared with the traditional aluminum hydroxide adjuvant (Fig. 6A and B). Following aluminum hydroxide nanoparticle adjuvant immunotherapy, the number of inflammatory cells was significantly reduced, as determined by lung histopathological analysis compared with that noted following traditional aluminum hydroxide adjuvant immunotherapy (Fig. 7C and D). Certain studies have reported that the combination of ovalbumin (12) and aluminum

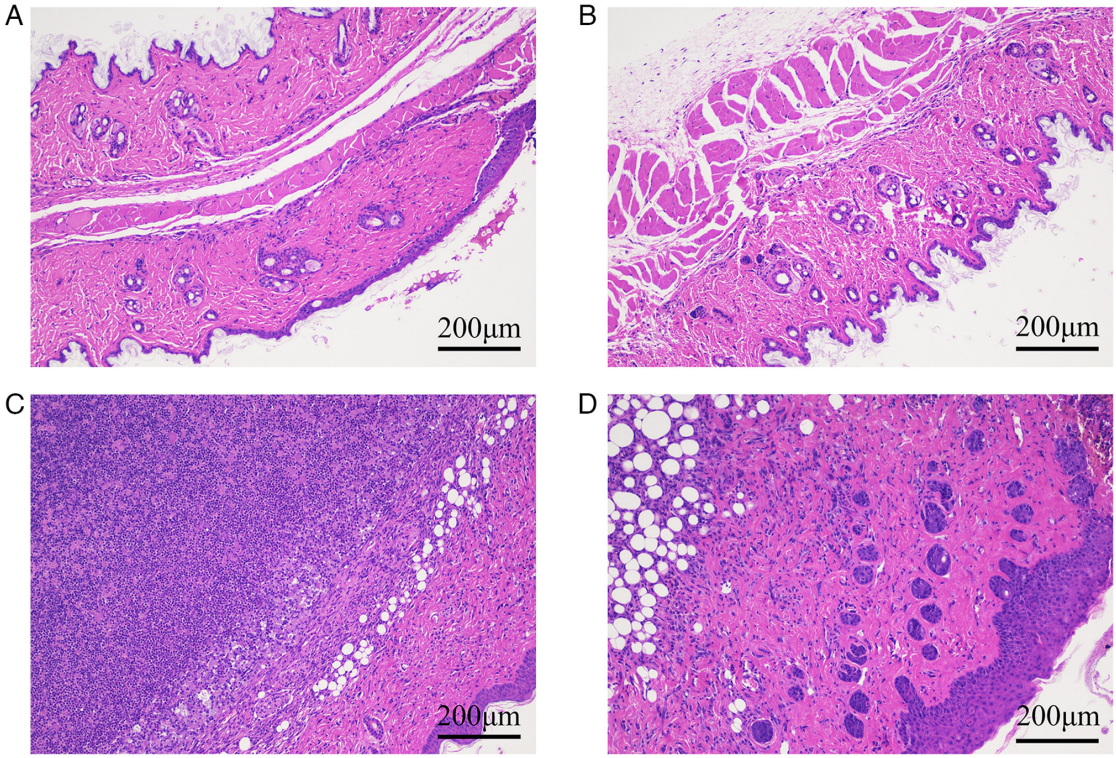

Figure 8. Representative H&E-stained subcutaneous sections of mice skin samples in the subcutaneous injection sites. Samples were received from mice 51 days after injection. (A) Negative control. (B) Positive control. (C) HDM-Al. (D) HDM-NP. Scale bar, 200 μm. HDM-Al, house dust mite traditional aluminum adjuvants; HDM-NP, house dust mite nanoparticle; H&E, hematoxylin and eosin.

hydroxide nanoparticles in animal models can enhance the organism's antibody response and increase the IgG2a:IgG1 ratio, indicating that aluminum hydroxide nanoparticles can enhance the organism's auxiliary activity and reduce inflammation (13), which is consistent with the experimental results of the present study.

The present study demonstrated that the aluminum hydroxide nanoparticle adjuvant adsorption HDM and the traditional aluminum hydroxide adjuvant adsorption as HDM immunotherapies caused local inflammatory reactions at the injection site. However, compared with the traditional aluminum hydroxide adjuvant adsorption HDM immunotherapy, the local inflammatory reaction at the injection site caused by the aluminum hydroxide nanoparticle adjuvant adsorption HDM immunotherapy was milder as determined by the subcutaneous tissue biopsy results (Fig. 8C and D). Therefore, the local inflammatory reactions induced by the aluminum hydroxide nanoparticle adjuvants were less severe than those induced by traditional aluminum hydroxide adjuvants, which is consistent with the results of Chen *et al* (15).

In summary, aluminum hydroxide nanoparticle adjuvant adsorption of HDM-specific immunotherapy is improved compared with the traditional aluminum hydroxide adjuvant adsorption of HDM treatment. This development can be summarized as follows: Firstly, aluminum hydroxide nanoparticle adjuvant adsorption of HDM-specific immunotherapy can reduce the number of total cells; secondly, it can reduce the levels of the Th2 cytokine IL-5; thirdly, it can increase the levels of the Th1 cytokine IFN-γ and IL-10; fourthly, it can reduce skin inflammation at the injection site.

It was hypothesized that the reason as to why the inflammatory response in mice is relatively small when adsorbing allergens with aluminum hydroxide nanoparticle adjuvants for immunotherapy is that aluminum hydroxide nanoparticle adjuvants have stronger adjuvant activity than traditional aluminum hydroxide adjuvants. Compared with traditional aluminum hydroxide adjuvants, aluminum hydroxide nanoparticle adjuvants stimulate antigen-presenting cells (APCs) after adsorbing allergens, and APCs present allergens to macrophages faster, thereby reducing inflammation at the local injection site and enhancing antigen-specific immune responses. This is also reflected in the study by Chen *et al* (15).

Regarding the research content of the present study, there are a number of directions that require further in-depth research in the future. First, the authors will continue to experimentally explore the mechanism by which adsorption of HDM allergens by aluminum hydroxide nanoparticles can reduce skin validation reactions during immunotherapy. Secondly, in the immunotherapy of HDMs adsorbed by aluminum hydroxide nanoparticle adjuvant, it has not yet been clearly studied which specific substances act on cells and which pathways inhibit cells from producing inflammatory factors. The authors will continue their research on the relevant immune mechanisms through cell model experiments in subsequent studies. Thirdly, the application of aluminum hydroxide nanoparticle adjuvants in desensitization requires further research. In future research, the metabolism and distribution of aluminum hydroxide nanoparticle adjuvants in the organism will be further analyzed in order to create an experimental theoretical foundation for the application of aluminum hydroxide nanoparticle adjuvants in immunotherapy. Finally, the present experimentation was

conducted in mice. After repeated exploration in the patient studies, when it can be used as a mature technology to human benefit, it will be possible to conduct clinical stage experiments.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to being used under license for the current study, but are available from the corresponding author on reasonable request.

Authors' contributions

WZ and YZ designed the study, performed the experiments and revised the manuscript. WZ analyzed the data. YZ interpreted the data and drafted the manuscript. Both authors have read and approved the final version of the manuscript. WZ and YZ confirm the authenticity of all raw data.

Ethics approval and consent to participate

The experiments described in the present study were performed at Curegenix Corporation (Guangzhou, China). The present study was approved by the Ethics Committee of Curegenix Corporation (approval no. YSDW2023020-04). All methods were performed in accordance with the relevant guidelines and regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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