



A20-OVA Nanoparticles Inhibit Allergic Asthma in a Murine Model

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Abstract— The skewed T helper (Th) 2 response plays a critical role in the pathogenesis of allergic asthma. Regulatory T (Treg) cells and the regulatory cytokines are required in maintaining the homeostasis in the body. This study aims to determine the effects of a poly(lactic-co-glycolic) acid (PLGA)-ovalbumin (OVA)+A20 (a ubiquitin E3 ligase) nanovaccine on inhibiting allergic asthma in a murine model. In this study, A20 and OVA (a model antigen) were encapsulated into PLGA to be a nanovaccine (PLGA-OVA+A20). An allergic asthma murine model was developed with OVA as the specific antigen to test the role of PLGA-OVA+A20 nanovaccine in maintaining the immune homeostasis in the airway tissues. The results showed that PLGA-OVA+A20 nanovaccine inhibited the asthma responses in mice by suppressing Th2 inflammatory responses, promoting the generation of Treg cells in the airway tissues. We conclude that the PLGA-OVA+A20 nanovaccine has a marked inhibitory effect on the airway allergic response in sensitized mice by significantly promoting the generation of Treg cell and IL-10. The data suggest that PLGA-OVA+A20 has translational potential in the treatment of allergic asthma.

KEY WORDS: allergic asthma; A20; nanovaccine; Th2 response; regulatory T cell.

INTRODUCTION

Allergic asthma is a common respiratory inflammatory disease with high incidence [1]. Its clinical

pathological features include airway hyperresponsiveness (AHR), eosinophil infiltration in the lung, and reversible airway obstruction [2]. The antigen-specific IgE production, the skewed T helper 2 (Th2) polarization, and over-produced Th2 cytokines, along with the reduction of regulatory T (Treg) cell play an important role in the development of allergic asthma [3]. Therefore, to further clarify the mechanism of Th2 polarization and find novel and effective remedies to inhibit allergic asthma is an urgent scientific issue.

Allergen-specific immunotherapy (SIT) is currently the best treatment for allergic diseases. SIT can promote the generation of Treg cells in patients to suppress specific IgE production and inhibit the allergen-specific effector T cell

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activities [4, 5]. It is well known that the increase in Treg cell and IL-10 production is essential for successful immunotherapy of allergic diseases [6–8]. But, the treatment course of SIT takes too long time (more than 3 years), which seriously affects the patient compliance. In addition, traditional SIT still has the risk of inducing anaphylactic shock and exacerbating asthma in patients [9]. Thus, it is necessary to develop a new effective and therapeutic strategies for the treatment of allergic asthma.

Published data indicate that A20, or tumor necrosis factor alpha-induced protein 3 (TNFAIP3), can regulate a variety of immune cell functions and participate in the maintenance of immune homeostasis [10, 11]. A20 can reduce the recruitment of airway inflammatory cells and suppress the production of inflammatory cytokines in bronchoalveolar fluid [12, 13]. Losing of A20 in lung epithelium will abolish the function of immune tolerance and show severe inflammation and tissue damage [14]. Therefore, A20 should be a promising therapeutic agent for the treatment of allergic asthma. However, whether these features of A20 increase the effects of SIT needs to be further investigated.

Poly(lactic-co-glycolic) acid (PLGA) is a biodegradable polymer that has many biological advantages such as biocompatibility, nontoxicity, and metabolic degradation *in vivo*, which make it as an ideal carrier for protein vaccines in allergy immunotherapy as demonstrated by animal model studies [14–16]. Proteins coated with PLGA are slowly released to the environment; it can stably be delivered to specific immune targets and induce the desired immune response in host cells, and thus extending the therapeutic effect [17].

In this study, we used PLGA to coat A20/ovalbumin (OVA) to form the PLGA-OVA+A20 nanovaccine. The immunotherapy effects of PLGA-OVA+A20 nanovaccine on allergic asthma were tested in a mouse asthma model. Our results showed that PLGA-OVA+A20 significantly suppressed the type of Th2 inflammation response and promoted Treg cell generation.

METHODS

Reagents

A peroxidase-labeled goat anti-mouse IgE antibodies were obtained from Southern Biotech, USA; tetramethylbenzidine (TMB) was purchased from Solarbio, China; aluminum hydroxide was obtained from Thermo Fisher, USA; and ELISA kits for IL-4, IL-5, IL-10, and IL-13 were

obtained from eBioscience, USA; APC-labeled rat anti-mouse CD4, FITC-rat anti-mouse CD25, and PE rat anti-mouse Foxp3 (BD Biosciences, New Jersey, USA) antibodies and carboxyfluorescein diacetate succinimidyl ester (CFSE) were obtained from eBioscience, USA. The PLGA formulation obtained from Sigma (St. Louis, MO, USA) has a lactide/glycolid molar ratio of 50:50 and viscosity of 0.45–0.60 dL/g.

Preparation of Recombinant A20 Protein and Mutated A20 Protein

A20 genes were constructed into the PAML-C5ET vector, followed by transforming into *Escherichia coli* Top10 cells. Plasmids from positive clones were digested with *KpnI* and *EcoRI*. The target fragment was ligated into PET-32a and then transformed into BL21 for expression. Bacteria were grown in LB broth supplemented with 50 µg/ml ampicillin. After induction using isopropyl-D-thiogalactopyranoside (IPTG), the bacteria were incubated for 4 h at 37 °C and then harvested and resuspended in 50 mM Tris-HCl and 100 mM NaCl (pH 7.5) for sonication. A20 proteins were purified by affinity chromatography. The endotoxin was preliminarily removed using an ion exchange column and further eliminated using the ToxinEraser Endotoxin Removal Kit (GenScript, China). A mutated A20 was also constructed by replacing the CAC with AAA throughout the gene sequence of A20.

Preparation of PLGA-OVA+A20 Nanoparticles

OVA+A20 (0.5 ml, 5 mg) was added to 2 ml dichloromethane and acetone (at a volume ratio of 9:1) containing copolymer PLGA (5% w/v). By probe sonication in an ice bath for 2 min, the mixture was formed to the primary emulsion. One hundred milliliters of aqueous solution containing polyvinyl alcohol (1% w/v) was added to the primary emulsion and sonicated in an ice bath for 1 min to form the secondary emulsion. The organic phase in the secondary emulsion was evaporated after being stirred vigorously overnight, washed twice with distilled water to remove residual polyvinyl alcohol, and centrifuged (20,000 rpm, 15 min, 4 °C) to collect the PLGA-OVA+A20 nanoparticles.

Size of PLGA-OVA+A20 Nanoparticles

PLGA-OVA+A20 (0.2 mg) nanoparticles were diluted in 1 ml distilled water. The nanoparticle size was assessed by the dynamic light scattering (LS230, Coulter, USA).

Protein Loading and Release Efficiency of PLGA-OVA+A20 Nanoparticles

Ten milligrams of freeze-dried nanoparticles was dissolved in 4 ml of aqueous phase with 1% SDS and 0.05 mol/L sodium hydroxide (NaOH) and was shaken for 24 h at 37 °C. Then, protein concentration was determined with a BCA protein quantification kit. Twenty milligrams of PLGA-OVA+A20 nanoparticles was dissolved in 5 ml PBS and loaded in a dialysis bag, and 1 ml suspension was collected followed by centrifugation at 20,000 rpm for 30 min and was replaced with fresh PBS. The collected samples were measured by BCA protein assay to test the fraction of protein released.

Induction of Allergic Airway Inflammation

Female BALB/c mice (6–8 weeks old) were purchased from the Guangdong Experimental Animal Center and maintained in a specific pathogen-free facility at Shenzhen University. All experimental procedures were approved by the Animal Ethics Committee at Shenzhen University. Procedures were performed according to the required guidelines. Mice were sensitized intraperitoneally with a mixture of OVA (100 µg/mouse) in 0.1 ml of 2% aluminum hydroxide on days 0, 3, and 7, respectively. PBS was used as a negative control. The mice were then treated with [nasal drops](#) containing OVA (50 µg/ml), or A20 (50 µg/ml) and OVA (50 µg/ml) + A20 (50 µg/ml), or nanovaccine (50 µg/ml), daily for 7 days. On day 23, the airway resistance was measured *via* the inhalation of incremental doses of methacholine (0 mg/ml, 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, 50 mg/ml, and 100 mg/ml). The mice were euthanized on day 23. The lung tissues were excised and fixed in 4% formalin, then embedded in paraffin wax for histology analysis. Bronchoalveolar lavage fluid (BALF) was collected in 1 ml of PBS; the total cells were stained with Giemsa stain and counted under a light microscope.

ELISA

Levels of cytokines in culture supernatants and BALF were determined by means of ELISA with commercial reagent kits according to the manufacturer's instruction.

Flow Cytometry

Splenocytes were isolated and cultured for 48 h and then were fixed with 1% formaldehyde containing 0.1% Triton X-100 for 30 min at 4 °C, washed 3 times with 1% bovine serum albumin (BSA) in PBS, and blocked for

30 min at 4 °C with 1% BSA. Cells were incubated with APC-CD4, FITC-CD25, and PE-Foxp3 antibodies or isotype IgG for 1 h at room temperature. After washing with PBS, the cells were analyzed with a flow cytometer (FACS Canto II; BD Biosciences, Franklin Lakes, NJ, USA).

To analyze T cell proliferation, CD4⁺ CD25[−] T cells were isolated from the spleen by flow cytometry cell sorting, labeled with CFSE, and cultured in the presence of OVA (5 mg/ml) or BSA (5 mg/ml), in the presence of dendritic cells (DCs) (T cell:DC = 5:1) for 3 days. Cells were analyzed by flow cytometry.

Statistics

The differences between data (mean ± SD) obtained from two groups were tested by the unpaired two-tailed Student's *t* test. ANOVA followed by Dunnett's test or Bonferroni test was performed in multiple comparisons. A *p* value < 0.05 was set as a significant criterion.

RESULTS

Preparation of A20 Protein

The A20 gene sequence was obtained from the Gene Bank (GenBank: U19463.1), based on which the A20 protein was produced through molecular cloning approaches. The agarose gel shows the amplification recombinant A20 (rA20) gene products after double enzymatic digestion (Fig. 1a). The A20 protein was produced by a prokaryotic expression system (Fig. 1b). The rA20 protein and mutated A20 (mA20) protein were purified by affinity chromatography (Fig. 1c). Figure 1d shows the bioactivities of rA20 and mA20. These results indicate that we successfully cloned A20 gene then produced and purified the A20 protein.

Treatment of Airway Inflammation with A20 or OVA+A20

BALB/c mice were sensitized to OVA to induce a skewed antigen-specific Th2 response in the airway tissues. To test the effects of A20 on regulating allergic airway diseases, we used A20 or OVA+A20 (OVA and A20 were mixed at a ratio of 1:1) or OVA+mA20 to treat sensitized mice. The results showed that, after exposing to OVA (the specific antigen), the immunized mice had asthma-like response, including high airway resistance in response to inhalation of methacholine (Fig. 2a), profound cell infiltration in BALFs (Fig. 2b), high Th2 cytokine

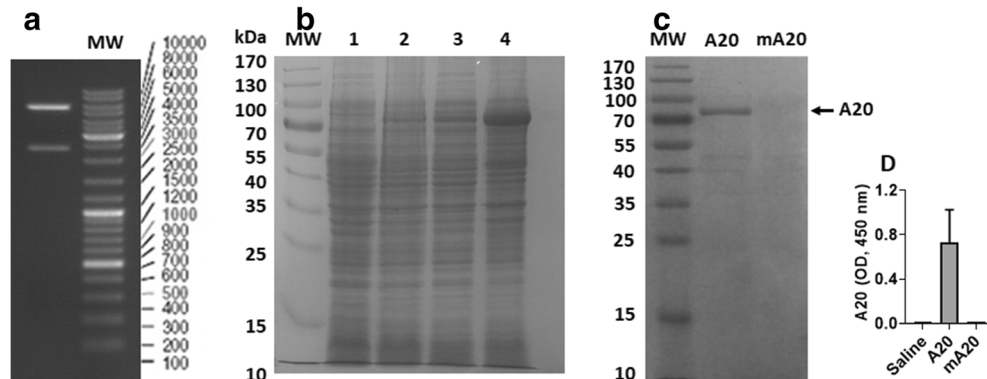


Fig. 1. Cloning, expression, and purification of A20. **a** Restriction enzymatic digestion results of recombinant hyastatin-pET-28a(+) plasmid (A20 DNA). **b** Expressing A20 protein by prokaryotic expression system. The protein band indicates A20 was soluble protein (1: before induction; 2: after induction; 3: supernatant which after ultrasonic broken; 4: precipitation which after ultrasonic broken). **c** Purified A20 and mutated A20 (mA20). MW molecular marker. **d** ELISA results (mean $\pm$ SD) show A20 bio-activities. The data represent 3 independent experiments.

levels in BALF (Fig. 2c), high serum-specific IgE levels (Fig. 2d), and lung tissue inflammation (Fig. 2e). These mice were then designated asthma mice hereafter. Treatment with A20 alone did not apparently alter the asthma response. Administration of OVA+A20 significantly suppressed the asthma response, which was abolished when OVA+mA20 was administered. The inhibitory effects on serum-specific IgE by OVA-A20 were in a dose-dependent manner (Fig. 2a–e). The results demonstrated that administration of OVA+A20 can suppress asthma response in a murine model.

Characterization of the OVA+A20-Loaded PLGA Nanoparticles

Although the results showed that OVA+A20 protein could improve the efficacy of immunotherapy, the treatment effect of anaphylaxis was not very satisfactory. Thus, we used nanomaterial (PLGA nanoparticles) to carry OVA+A20 protein as a nanovaccine to improve the therapeutic efficacy for allergic asthma. The surface morphology of nanoparticles which we observed by a scanning electron microscope (Fig. 3a) showed that nanoparticles were spherical in shape and their size was 100–250 nm (Fig. 3b). The release rate curve showed that the nanovaccine could gradually release and be released completely within 96 h (Fig. 3c).

Nanovaccine Is more Effective in the Suppression of Allergic Asthma in Mice

To verify if the nanovaccine is more effective than OVA+A20 on the treatment of allergic asthma, we treated

asthma mice with OVA+A20, or nanovaccine, or PLGA alone. We observed that the serum-specific IgE levels, inflammatory cells in BALF, and airway resistance in response to inhalation of methacholine were markedly downregulated in the nanovaccine group compared to the OVA+A20 group (Fig. 4a–c). Similar results were found in the lung tissue histology (Fig. 4d). The results indicate that nanovaccine enhances the therapeutic efficacy in allergic asthma as compared to the OVA+A20 group.

Nanovaccine Promotes Treg Cell Generation in Allergic Asthma Mice

We next observed the effects of nanovaccine on Treg cell generation in the airway tissues. After treatment with the procedures as shown by Fig. 4, the airway tissues were excised; single cells were isolated from the tissues and analyzed by flow cytometry. We found that the nanovaccine markedly increased the IL-10+ Treg cell generation in the airway tissues (Fig. 5a–e). Administration of the OVA-A20 also increased the frequency of Treg cells in the airway tissues, but markedly less than that induced by the nanovaccine. Treating asthma mice with PLGA alone did not alter the Treg cell frequency in the airway tissues. Exposure to OVA even further suppressed the Treg cell frequency in the airway tissues (Fig. 5a–e). In addition, we isolated Treg cells from the airway tissues. The immune-suppressive functions of Treg cells were assessed by CFSE dilution assay (Fig. 5f, g). The results showed that the induced Treg cells had effects on suppressing effector T cell proliferation. The data demonstrate that the nanovaccine can promote the Treg cell generation in the airway

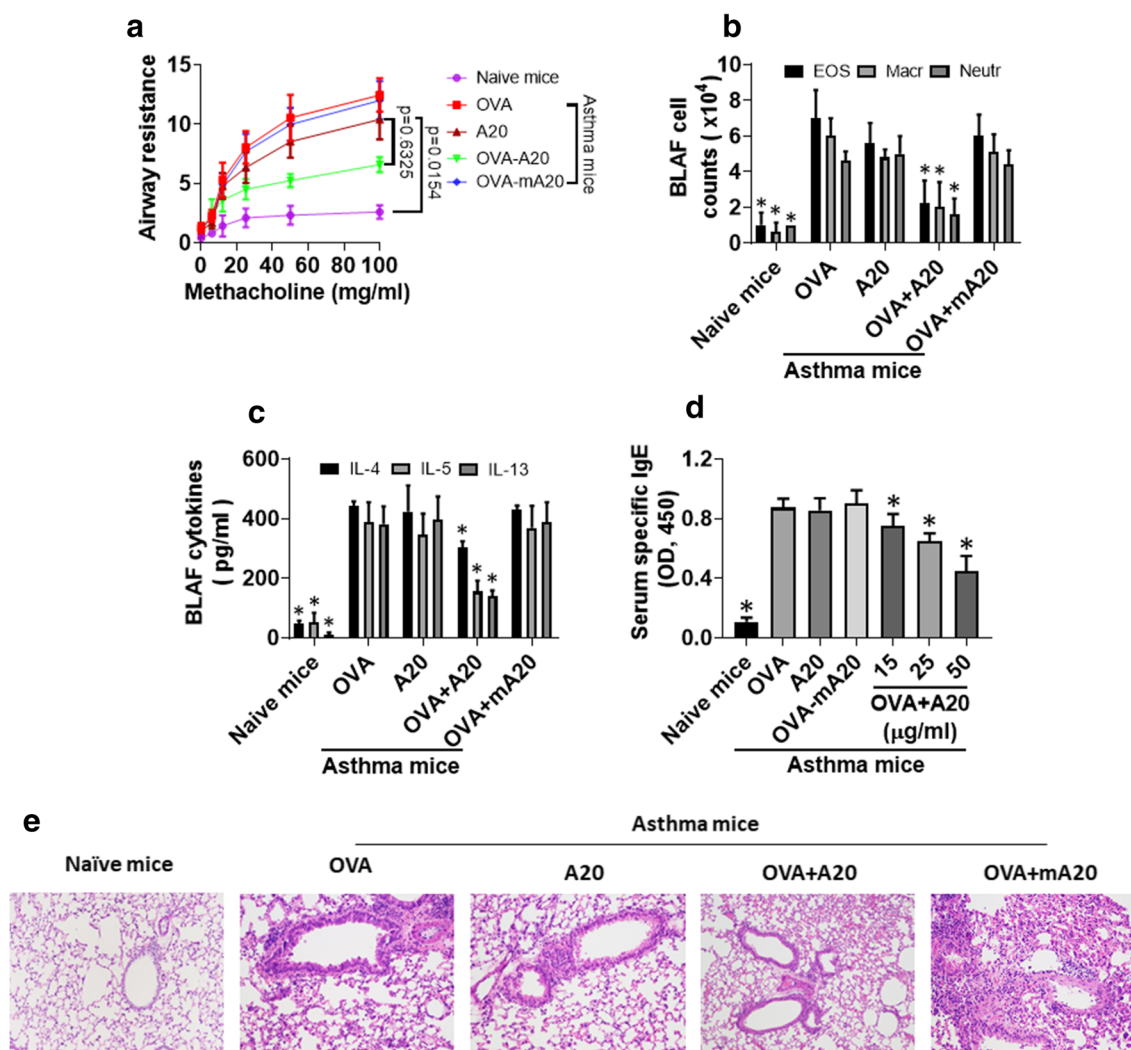


Fig. 2. OVA+A20 reduces asthma symptoms in mice. Asthma mice (6 mice per group) were treated with the procedures on the X-axis of bar graphs. **a** Airway resistance records of mice in response to inhalation of methacholine. **b** Inflammation cell counts in bronchoalveolar lavage fluids (BALFs). **c** Th2 cytokine levels in BALF. **d** Serum OVA-specific IgE levels. **e** Representative lung histology images (magnification $\times 200$). The data are presented as mean $\pm$ SD. Statistics: ANOVA + Dunnett's test; $*p < 0.001$, compared with the OVA group.

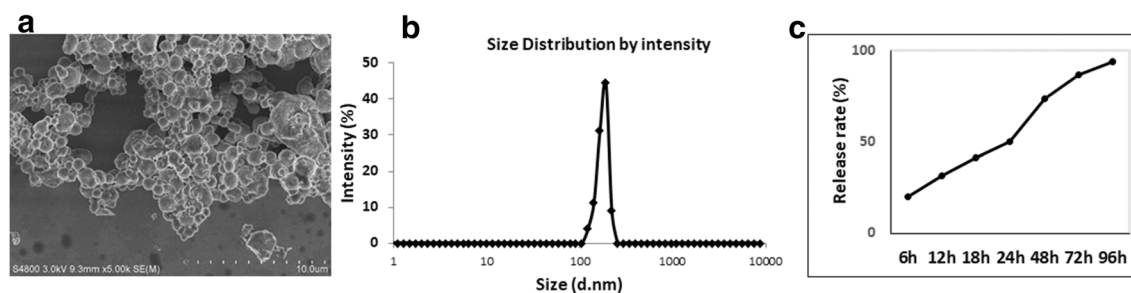


Fig. 3. Characterization of nanoparticles. **a** Field emission scanning electron microscopic image. **b** Dynamic light scattering spectra. **c** *In vitro* cumulative protein release of OVA+A20-loaded PLGA nanoparticles.

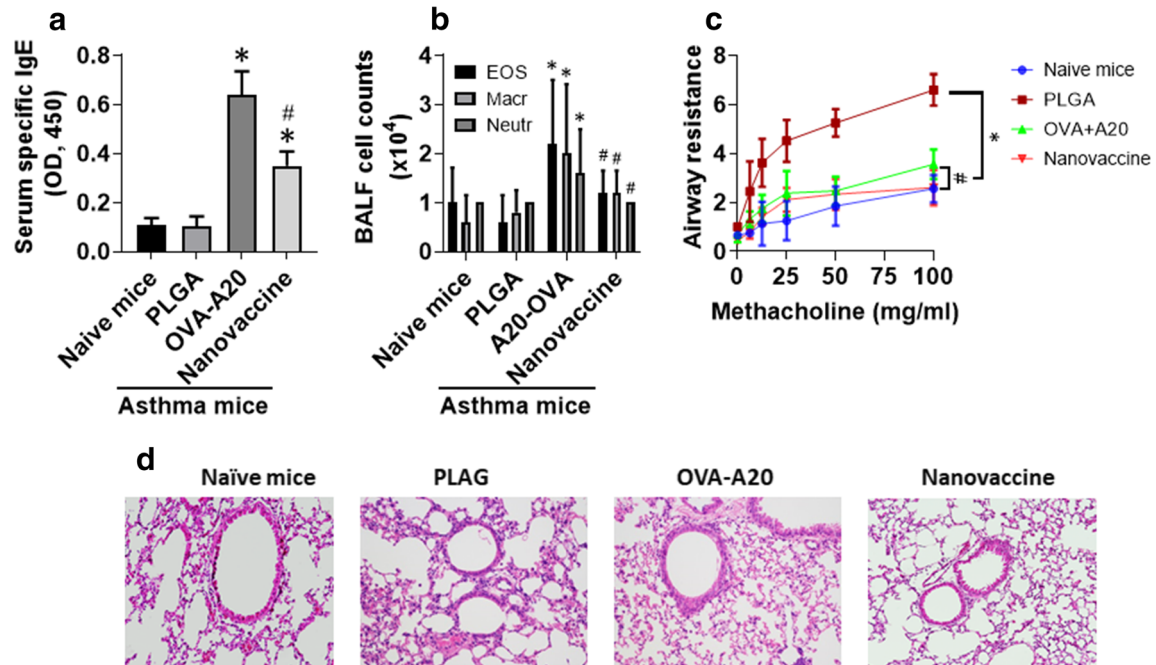


Fig. 4. Nanovaccine enhances the therapeutic efficacy in allergic asthma mice. Mice were treated with the procedures as denoted on the X-axis of bar graphs. **a** OVA-specific IgE levels in the serum (by ELISA). **b** Inflammatory cell counts in BALF. **c** Airway resistance in response to inhalation of methacholine. **d** Representative lung tissue histology images (magnification $\times 200$). The data of bars are presented as mean $\pm$ SD. Statistics: ANOVA + Dunnett's test. * $p < 0.01$, compared to the naïve mouse group. # $p < 0.05$, compared to the OVA+A20 group. The data represent 6 independent experiments.

tissues; these Treg cells have immune-suppressive functions.

DISCUSSION

Allergic asthma is a chronic airway inflammatory disease involving eosinophils, lymphocytes, and other cells. The classic pathogenesis of allergic asthma is the imbalance of the immune system caused by the polarization of Th2 cells [18]. Th2 cells play a key role in allergic reactions such as allergic rhinitis and allergic asthma. The secreted Th2 cytokines IL-4, IL-5, and IL-13 can promote the migration of eosinophils to inflammatory sites and induce the release of antigen-specific IgE antibodies in the body [19–21]. Therefore, the purpose of this study is to regulate the proportion of Th2 cells in the immune system and the release of inflammatory factors, which is an important aim of allergic asthma treatment.

In this study, we treated asthma mice with A20 mixed with allergen OVA in nasal drops. The results showed that OVA+A20 could reduce the number of inflammatory cells in BALF as well as reduce serum-specific IgE and Th2

inflammatory cytokine levels. In addition, OVA+A20 can also promote the IL-10 expression in spleen cell culture supernatant. The airway resistance of the murine model was also significantly improved compared with that of the OVA group. The results of pathological section also showed that OVA+A20 protein had a certain inhibitory effect on the Th2 response of asthma mice.

PLGA can promote drug stability by preventing drug degradation, which has been approved by the FDA to be used in the disease treatment in the USA [22]. Studies on human immunodeficiency virus (HIV) and foot-and-mouth disease virus have confirmed that PLGA particles can significantly enhance the therapeutic efficacy of vaccination [23, 24]. Vaccines prepared with PLGA and protein antigens can effectively improve systemic and mucosal immunity [25, 26]. In addition, the size of PLGA-coated protein affects its passage through the pore of endothelial cells in the inflammatory tissues, and appropriate nanoparticles can improve the therapeutic efficacy [27]. In order to improve the inhibitory effects of OVA+A20 on the inflammatory response of allergic asthma, we used PLGA-coated OVA+A20 nanoparticles to treat allergic asthma animal model by nasal drops. By comparing with

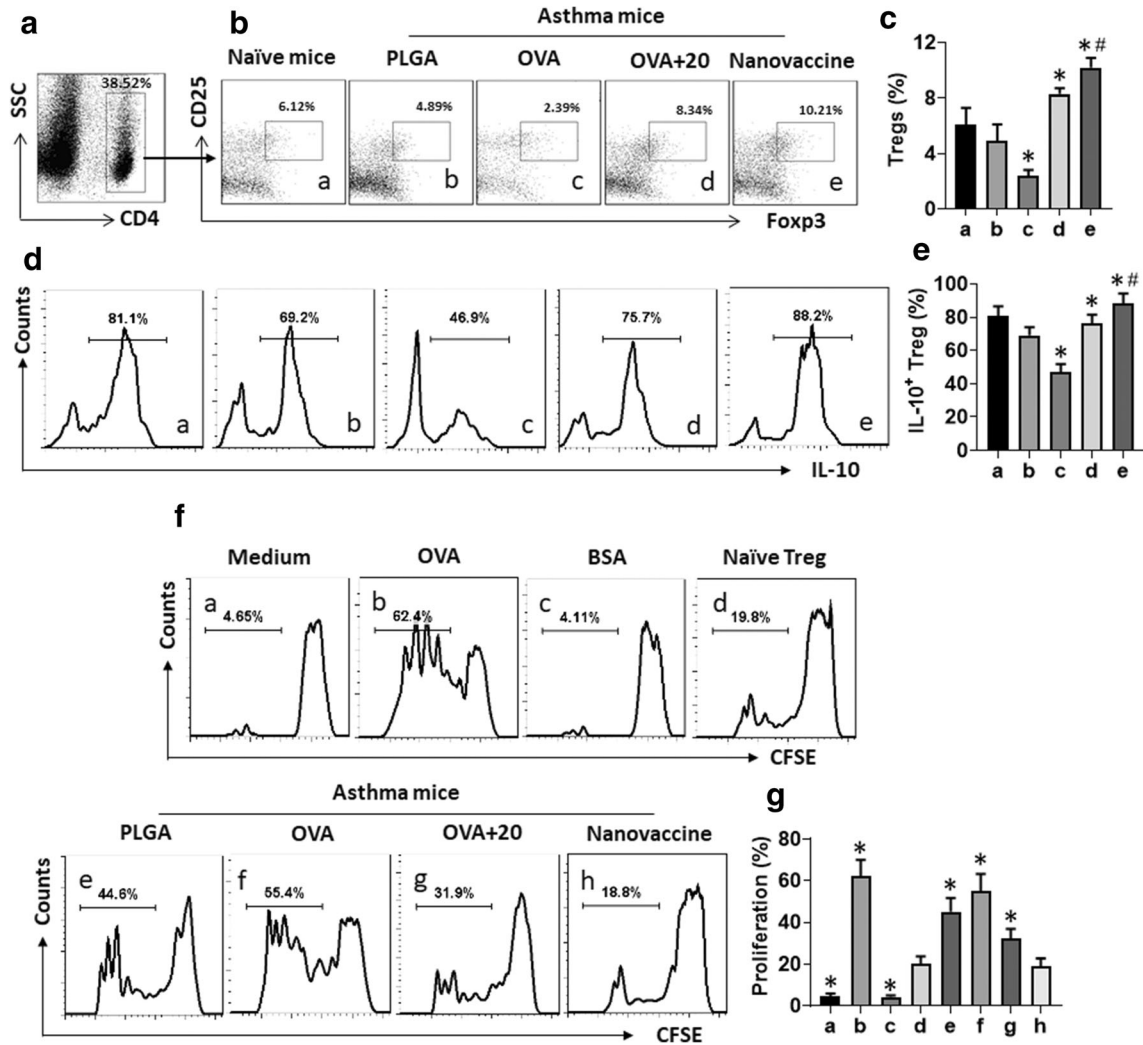


Fig. 5. Assessment of airway mucosal Treg cell number and their immune regulatory function. **a–e** Single cells were prepared from the airway tissues of naïve mice and asthma mice. The cells were analyzed by flow cytometry. **a** Gated dot plots are CD4+ T cells. **b** Gated dot plots show frequency of Treg cells. **c** Bars show summarized Treg cell numbers in the airway tissues. **d** Gated histograms show IL-10+ T cells in the gated cells of panel **b**. **e** Bars show summarized IL-10+ Treg cells in panel **d**. **f, g** By FCSS, CD4+ CD25+ CD127– Treg cells were isolated from airway tissue single cells; CD4+ CD25– effector T cells and DCs were isolated from the asthma mouse spleen. Effector T cells were labeled with CFSE and cocultured with Treg cells (sources of Treg cell are presented above each subpanel) at a ratio of $5 \times 10^3:1 \times 10^3$ per well in the presence of OVA (or BSA) and DCs. Three days later, the cells were analyzed by flow cytometry. **f** Gated histograms show proliferating effector cell frequency. **g** Bars show proliferating effector cell frequency. The data of bars are presented as mean $\pm$ SD. Statistics: ANOVA + Dunnett's test. * $p < 0.01$, compared with group "a" (**b** and **e**) or group "d" (**g**). The data represent 6 independent experiments.

OVA+A20, we observed PLGA-OVA+A20 nanovaccine had better effects on inhibiting inflammation of allergic asthma mice.

The particle size of PLGA-OVA+A20 prepared in this study ranges from 100 to 250 nm, which is the optimal size range for cell community nanoparticles. From the release rate

of nanovaccine *in vitro*, the release rate of nanovaccine at 24 h is as high as 50%. Such release is beneficial for vaccine delivery because it provides primary immune effects. Moreover, due to the constant release of OVA+A20 driven by diffusion through the rigid PLGA core, the nanovaccine was fully released after 96 h as shown by the present data.

When the body's immune regulatory system is normal, it inhibits the aberrant immune response. The components of the immune regulatory system include Treg cells and the regulatory cytokines [28, 29]. Our results show that PLGA-OVA+A20 nanovaccine can inhibit Th2-type inflammation and increase the number of Treg cells and the release of IL-10 in a murine model of allergic asthma.

In summary, our results show that PLGA-OVA+A20 administration was able to alleviate allergic asthma in mice by promoting the production of Treg cells better than OVA+A20. The results suggest that PLGA-OVA+A20 vaccine may provide a novel therapeutic vaccine for allergic asthma immunotherapy.

AUTHOR CONTRIBUTIONS

XQL, JWZ, SYQ, LQY, ZYL, MZ, and FXZ performed the experiments, analyzed the data, and reviewed the manuscript. LHM, DBL, and PCY organized and supervised the experiments. LHM and DBL designed the project and prepared the manuscript. PCY reviewed and edited the manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no competing interests.

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