



Preventive effects of "ovalbumin-conjugated celastrol-loaded nanomicelles" in a mouse model of ovalbumin-induced allergic airway inflammation

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ABSTRACT

Allergies affect a significant proportion of the world's population, and existing vaccination strategies to restrict their adverse pathologies often render side-effects. The aim of this study was to design a new vaccine for allergen-specific immunotherapy (SIT), and to investigate its preventive effects during allergic inflammation. We constructed ovalbumin (OVA)-conjugated celastrol-loaded nanomicelles (OVA-NMs-celastrol), wherein celastrol (a bioactive anti-inflammatory compound) was loaded into carboxyl-functioned polymeric nanomicelles using a thin-film hydration method. OVA was used as a model allergen and conjugated on nanomicelles. The OVA-NMs-celastrol obtained were characterized based on particle size, morphology, drug encapsulation efficiency, and drug loading percentage. Further, the preventive effect of OVA-NMs-celastrol was evaluated in a mouse model of allergic asthma. Our results showed that OVA-NMs-celastrol possessed valuable characteristics such as small particle size (50.72 ± 0.98 nm) and spherical-like shape, with celastrol encapsulation efficiency of $99.89 \pm 0.85\%$ and a drug loading percentage of $4.76 \pm 0.03\%$. Further, in vivo results showed that treatment with OVA-NMs-celastrol could decrease OVA specific IgE and histamine levels, Th2 cytokine (IL-4, IL-5) levels, and inflammatory cell infiltration in the lung tissues. Moreover, it could enhance the OVA specific IgG1 and IgG2a levels and decrease the IgE / IgG2a ratio. These results demonstrate the successful construction of OVA-NMs-celastrol as a potential vaccine candidate for use in SIT for allergic inflammation.

1. Introduction

Allergic diseases, including asthma, rhinitis, and dermatitis are increasing in prevalence, and affect up to 20–30% of the global population (Pawankar et al., 2013). Allergen-specific immunotherapy (SIT) remains the only strategy that can limit the natural course of allergic diseases. The treatment has now been practiced for a century, but the exact mechanisms are not completely understood. Proposed mechanisms involve production of blocking antibody (such as IgG4 and IgG1), generation of regulatory T cells (Tregs) and suppression of effector cells (Akdis and Akdis, 2015). The current SIT methods posit drawbacks such as long treatment duration (3 to 5 years), repeated administration of the allergen, and the risk of local and systemic side-effects (Winther et al., 2006). Thus, the application of SIT in clinical settings is limited. In this front, it becomes necessary to develop novel approaches to improve the safety and efficacy of SIT, including the use of new adjuvants.

In the recent years, nanoparticles (NPs) have been used as adjuvants / vehicles to carry antigens and drugs for vaccines (Zhao et al., 2014). Polymeric micelles are of immense interest for vaccinology due to their biocompatibility and predictable degradation (Smith et al., 2015).

Polymeric micelles have been utilized as vaccine delivery systems to carry hydrophobic drugs, amphiphilic molecules, and hydrophilic substances. Generally, hydrophobic drugs are encapsulated in the hydrophobic core of micelles, while the antigen is covalently conjugated to the hydrophilic surface. Besides, hydrophilic peptides can be transformed into amphiphilic peptides by attaching hydrophobic moieties (i.e. lipids or aliphatic hydrocarbons), and then allowed to self-assemble to form micelles with block copolymers (Jime'nez-Sa'nchez et al., 2014; Trent et al., 2014). The vaccines using micelles as adjuvants / vehicles have shown enhanced T cell priming and antibody responses without the need for adjuvant supplementation.

Celastrol, a quinone methide triterpene, is the pharmacologically active component of Chinese herbal Thunder God Vine, the extracts of which have been used for treating autoimmune and inflammatory diseases in clinical practice. Celastrol was also demonstrated to render broad pharmacological effects and bioactivities, such as anti-cancer, anti-obesity, anti-inflammation, anti-diabetes and neuroprotective (Ng et al., 2019). Recent studies have shown that celastrol exerts immunomodulatory effects and can promote the generation of antigen specific Treg cells (Venkatesha et al., 2016; Zhang et al., 2018).

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However, the low solubility and potential adverse effects on normal organs have hindered the clinical applications of celastrol.

In this study, we aimed to design a novel vaccine for SIT by encapsulating celastrol in the core of polymeric nanomicelles and conjugating model allergen ovalbumin (OVA) on the nanomicelles surface. The constructed OVA-conjugated celastrol-loaded polymeric nanomicelles (OVA-NMs-celastrol) were characterized according to size, morphology, celastrol encapsulation efficiency and drug loading percentage. The vaccines were then subcutaneously administered to the sensitized mice and their preventive effects were evaluated in a mouse model of OVA-induced allergic asthma.

2. Materials and methods

2.1. Materials and animals

Carboxyl-terminated Pluronic P123 copolymer was kindly gifted by Professor Hua Song (Xiamen University, CHN). Celastrol (purity > 99%) was purchased from Nanjing Zelang Pharmaceutical Technology Corporation (Nanjing, Jiangsu, CHN). OVA protein, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and $\text{Al}(\text{OH})_3$ were purchased from Sigma (Shanghai, CHN). Anti-ovalbumin IgE (mouse) ELISA kit (catalog number: 500840) was purchased from Cayman (Michigan, USA). Histamine ELISA kit (catalog number: 93-K4163-100) was obtained from Biovision (California, USA). ProcartaPlex Mouse Th1/Th2 Cytokine Panel (IL-4, IFN- γ , IL-10 and IL-5) (4 plex) was purchased from eBioscience (California, USA). Goat anti-mouse IgG2a (catalog number: 1080-1) and goat anti-mouse IgG1 (catalog number: 1070-01) antibodies were purchased from Southern Biotech (Birmingham, USA). Anti-CD3 antibody (catalog number: ab5690), anti-CD4 antibody (catalog number: 183,685) and anti-CD68 antibody (catalog number: 125,212) were purchased from Abcam (Cambridge, MA, USA).

Female BALB/c mice (6–8 weeks old), generated by Sino-British Sippr/BK Laboratory, were used for animal experiments. All animal experiments were approved by Animal Care and Use Committee of the Second Military Medical University.

2.2. Preparation of OVA-conjugated celastrol-loaded nanomicelles

Carboxyl-terminated Pluronic nanomicelles filled with celastrol were prepared using thin-film hydration method under optimized conditions as described in our previous study (Peng et al., 2012). For conjugation of OVA onto the micelles surface, EDC chemistry was employed (Peng et al., 2015). Briefly, the carboxyl groups on celastrol-loaded nanomicelles were activated with 100 mM EDC for 30 min. Then OVA solutions were allowed to react with the activated nanomicelles for 2 h at room temperature, and the OVA-conjugated celastrol-loaded nanomicelles (OVA-NMs-celastrol) solution was freeze-dried for 24 h in lyophilizer (FD-1A-50, Biocool, Beijing, CHN) at a condenser temperature of -52°C and pressure of less than 0.1 mbar. The freeze-dried samples were stored at -20°C and rehydrated with phosphate buffered saline (PBS) for further experiments.

2.3. Characterization of OVA-NMs-celastrol

The particle size and morphology of nanoparticles were characterized as previously described (Peng et al., 2012). Briefly, the particle size was analyzed by Dynamic light scattering (DLS) analysis using a NI-COMP 380 ZLS Zeta Potential / Particle Sizer (PSS Nicomp, Santa Barbara, CA, USA). The morphology was identified using JEOL JEM-2010 transmission electron microscope (TEM) at an acceleration voltage of 120 kV.

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	OVA/ $\text{Al}(\text{OH})_3$	10 μg OVA + 2 mg $\text{Al}(\text{OH})_3$
4	OVA-NMs	10 μg OVA + 200 μg NMs
5	NMs-celastrol	200 μg NMs + 10 μg celastrol
6	OVA-NMs-celastrol	10 μg OVA + 200 μg NMs + 10 μg celastrol

N/A, non-injected.

2.4. Drug encapsulation efficiency and drug loading percentage

The celastrol concentration was determined by reversed phase high performance liquid chromatography (RP-HPLC) on Agilent 1100 series HPLC system established previously (Peng et al., 2012). The drug encapsulation efficiency and drug loading percentage were calculated according to the celastrol concentration.

2.5. Experimental design of vaccine immunization in OVA-induced mouse model of allergic inflammation

As shown in Table 1, mice were divided into six groups, with each group having five mice. The experimental design is shown in Fig. 1A. On days 0, 7, and 14, mice were sensitized by intraperitoneally injecting 20 μg of OVA protein adsorbed on 4 mg of $\text{Al}(\text{OH})_3$. On days 28, 33 and 38, mice were treated subcutaneously with different OVA vaccines as depicted in Table 1. From day 52 to day 56, mice were challenged with 2% OVA solutions for 30 min every day. The control mice were sensitized and challenged with PBS. On day 57, mice were euthanized by cervical dislocation.

2.6. Determination of serum OVA specific antibodies

Whole blood samples were collected immediately after cervical dislocation by retro-orbital bleeding as described previously (Peng et al., 2018). The blood samples were centrifuged at 3000 rpm for 10 min, and serum was collected and stored at -80°C for analyzing OVA specific antibodies.

The level of OVA specific IgE (OVA sIgE) in serum was determined using mouse anti-OVA-IgE ELISA kit (catalog number: 500840, Cayman Chemical, Michigan, USA) according to the manufacturers' recommendations.

The levels of OVA specific IgG1 (OVA sIgG1) and IgG2a (OVA sIgG2a) were determined using ELISA. Briefly, 100 μL OVA solution (10 $\mu\text{g}/\text{mL}$) was added to 96-well microtiter plates (Costar, Corning Inc., NY, USA). After overnight incubation at 4°C , the plate was washed five times with phosphate buffered saline with Tween 20 (PBST) and then blocked with PBS buffer containing 1% bovine serum albumin (BSA) at 37°C for 1 h. The diluted serum samples (1: 300,000 for IgG1 detection and 1: 3000 for IgG2a detection) were added to the plate wells and incubated for 1 h at 37°C . The plate was washed five times and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 or IgG2a antibody. After incubation for 45 min at 37°C , the plate was washed five times, and the substrate 3,3',5,5'-Tetramethylbenzidine (TMB) was added. The absorbance was measured at 450 nm by a microplate reader (Molecular Devices Corp., Menlo Park, CA, USA).

2.7. Measurement of histamine, cytokines and eosinophils in BALF

Bronchoalveolar lavage fluid (BALF) was collected as described previously (Peng et al., 2018). Immediately after euthanization, the left

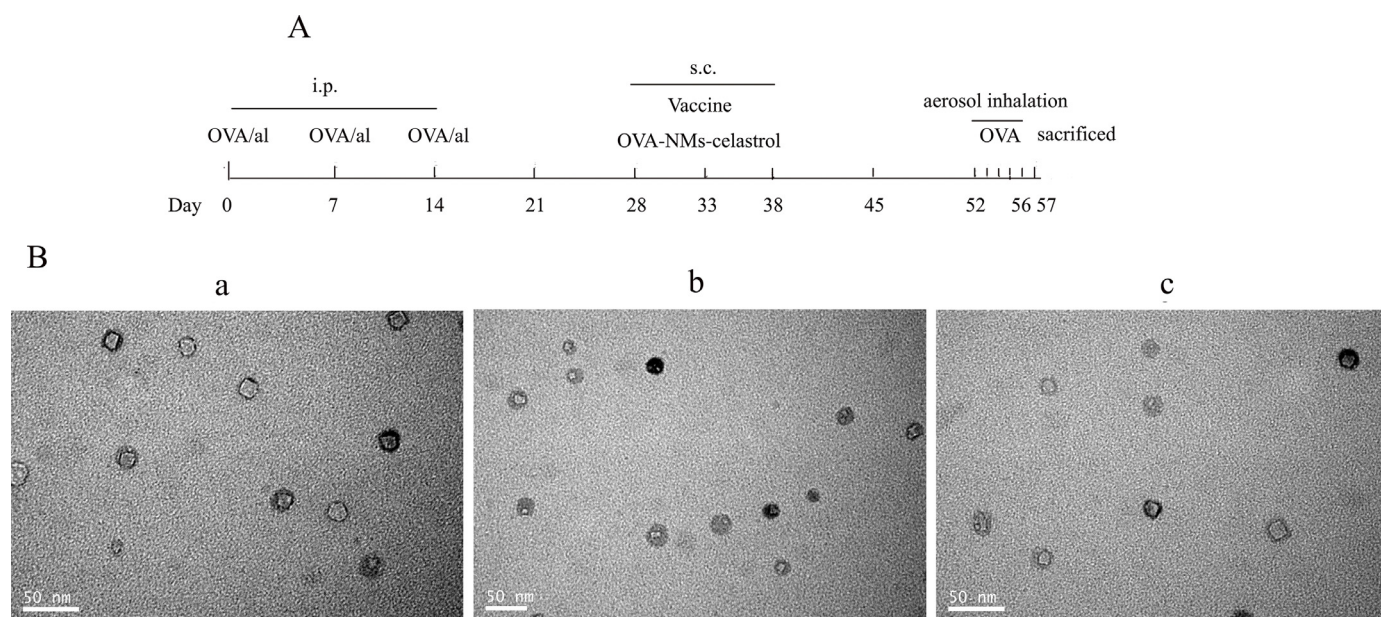


Fig. 1. (A) Experimental design for SIT of allergic inflammation. Mice were injected intraperitoneally with OVA protein mixed with $\text{Al}(\text{OH})_3$ on days 0, 7 and 14, and injected subcutaneously with OVA-NMs-celastrol on days 28, 33 and 38. Two weeks later, mice were challenged with OVA for 5 consecutive days from day 52 onwards. After the final challenge, mice were sacrificed. OVA/al, OVA adsorbed on $\text{Al}(\text{OH})_3$; i.p., intraperitoneal injection; s.c., subcutaneous injection. (B) TEM images of OVA-NMs (a), NMs-celastrol (b) and OVA-NMs-celastrol (c). Scale bar: 50 nm.

lungs were washed by passing 300 μL cold PBS through trachea to collect BALF. BALF was centrifuged at 1000 rpm for 3 min at 4 °C. The supernatants were collected and kept at -80 °C. Histamine level was determined using Histamine ELISA kit (Biovision, San Francisco, USA). Concentration of interleukin (IL)-4, interleukin (IL)-5, interleukin (IL)-10, and interferon (IFN)- γ were measured by ProcartaPlex Mouse Th1/Th2 Cytokine Panel (eBioscience, California, USA).

The cell sediments from BALF were resuspended in 100 μL PBS and stained with Liu's stain to obtain the differential counts of inflammatory cells as observed under microscope (CX31, Olympus, Tokyo, Japan).

2.8. Histological examination

For histopathological study, the non-washed right lungs were fixed with 10% neutral-buffered formalin and embedded in paraffin. 5 μm tissue sections were prepared and stained with hematoxylin and eosin (H and E). Inflammatory cell infiltration was detected under light microscope (DM6000B, Leica, Wetzlar, Germany) at 200 $\times$ magnification.

IHC was performed as described by Ye et al. (2007). Briefly, paraffin-embedded lung tissue sections were deparaffinized and immersed in 3% hydrogen peroxide to eliminate endogenous peroxidase activity. Antigen retrieval was performed by heating the tissue sections at 96 °C in 0.01 mol/L citrate buffer (pH 6.0) for 10 min, 10 min and 12 min, respectively. The sections were blocked with 5% BSA and incubated with primary monoclonal antibodies against the following antigens: CD3 (1:200), CD4 (1:600) and CD68 (1:200). Immunodetection was performed using Dako Real™ EnVision™ system, Peroxidase/DAB+, Rabbit/Mouse (Code: K5007, Dako, Glostrup, Denmark). DAB was

added to the tissue sections for 3 min and cells were counterstained with hematoxylin. Images were scanned and analyzed using an Aperio Imagescope (Leica Biosystems Inc., Wetzlar, Germany).

2.9. Statistical analysis

Data were analyzed using the SPSS statistical software (version 19.0). Two-tailed, unpaired Student's *t*-test and analysis of variance (ANOVA) were used for statistical significance analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of OVA-NMs-celastrol

According to DLS measurements, OVA-NMs, NMs-celastrol and OVA-NMs-celastrol exhibited small particles size (38.75 ± 1.76 , 46.73 ± 1.44 , and 50.72 ± 0.98 nm, respectively) with a relatively narrow size distribution (0.306 ± 0.05 , 0.335 ± 0.04 , and 0.24 ± 0.04) (Table 2). TEM analysis showed that OVA-NMs, NMs-celastrol and OVA-NMs-celastrol were majorly spherical, with sizes approximately 20 nm smaller than that obtained from DLS results (Fig. 1B). The OVA-NMs-celastrol had a drug encapsulation efficiency of $99.89 \pm 0.85\%$ and drug loading percentage of $4.76 \pm 0.03\%$ (Table 2).

Table 2

Characteristics of OVA-NMs, NMs-celastrol and OVA-NMs-celastrol. Particle size, polydispersity index (PDI), encapsulation efficiency (EE) and drug loading percentage (DLP) were determined.

Group	Size (nm)	PDI	Drug encapsulation efficiency (%)	Drug loading percentage (%)
OVA-NMs	38.75 ± 1.76	0.306 ± 0.05	–	–
NMs-celastrol	46.73 ± 1.44	0.335 ± 0.04	98.7 ± 1.56	4.7 ± 0.07
OVA-NMs-celastrol	50.72 ± 0.98	0.24 ± 0.04	99.89 ± 0.85	4.76 ± 0.03

*Data were expressed as mean $\pm$ SD ($n = 3$).

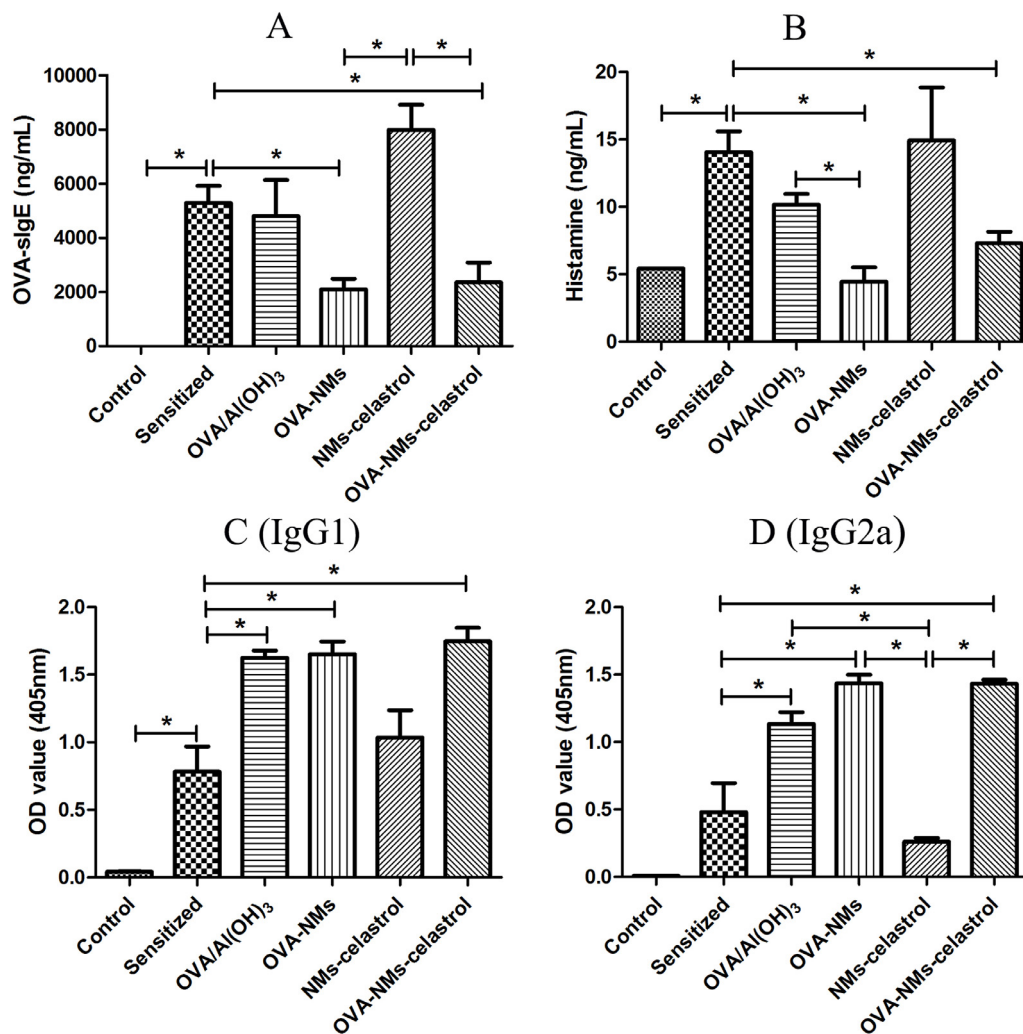


Fig. 2. Effect of OVA vaccines on histamine in BALF, serum OVA sIgE and sIgG production in a mouse model of allergic asthma. BALF and serum samples were collected from different groups (non-sensitized mice; sensitized mice; sensitized mice treated with OVA/Al(OH)₃ or OVA-NMs or NMs-celastrol or OVA-NMs-celastrol), and OVA sIgE (A), histamine (B), OVA sIgG1 (C) and OVA sIgG2a (D) were detected using ELSA. Results are reported as mean $\pm$ SD ($n = 5$), * $P < 0.05$.

3.2. Effect of OVA-NMs-celastrol on antibody production and histamine release

Allergen-induced airway inflammation is considered to be a Th2-driven disease (Kearley et al., 2007). Generation of antigen-specific IgE antibody is one of the characteristics in allergic responses, while histamine release is usually used as an indicator of mast cell activation. Our results showed that OVA sensitization / challenge induced a substantial increase in OVA sIgE production (5288 ± 634.7 ng/mL) and histamine release (14.05 ± 1.56 ng/mL) compared to that in the control group (OVA sIgE: 0.001 ± 0.0003 ng/mL, histamine: 5.43 ± 0.02 ng/mL) ($P < 0.05$). Treatment with OVA-conjugated nanoparticles, including OVA-NMs and OVA-NMs-celastrol, significantly decreased OVA sIgE (2098 ± 388.8 ng/mL, 2353 ± 733.2 ng/mL, respectively) and histamine (4.46 ± 1.07 ng/mL, 7.31 ± 0.85 ng/mL, respectively) levels (Fig. 2A and B).

Production of IgG blocking antibodies is an important aspect of SIT. The level of OVA sIgG1 was significantly elevated after OVA sensitization / challenge compared to that in the control group, while no apparent difference in OVA sIgG2a level was observed between sensitized and control groups. Treatment with all the OVA vaccines (OVA/Al(OH)₃, OVA-NMs and OVA-NMs-celastrol) could elevate OVA sIgG1 and OVA sIgG2a levels compared to that in the sensitized mice ($P < 0.05$). However, there was no difference in OVA sIgG1 and OVA sIgG2a levels

among groups treated with different vaccines. Nevertheless, there was a rising trend of OVA sIgG2a levels in OVA-NMs and OVA-NMs-celastrol treated mice compared to that in the OVA/Al(OH)₃ treated group (Fig. 2C and D).

3.3. Effect of OVA-NMs-celastrol on cytokines levels in BALF

Th1 / Th2 cytokines in BALF were also evaluated. Th2 cytokines (IL-4, IL-5 and IL-10) in OVA sensitized/challenged mice were significantly increased compared to those in the control group (Fig. 3B). OVA-NMs-celastrol treatment dramatically decreased IL-4 and IL-5 levels. However, none of the OVA vaccine formulations could decrease IL-10 level. Surprisingly, the OVA-NMs treated group showed a rising trend of Th2 cytokine levels. Besides, IFN- γ level was not affected by sensitization / challenge or vaccine treatment.

3.4. Effect of OVA-NMs-celastrol on inflammatory cell infiltration in lung tissue

Infiltration of inflammatory cells is another important feature of allergic inflammation. Fig. 3A shows an increase in the total cell, macrophage, lymphocyte and eosinophil numbers in BALF upon OVA sensitization / challenge compared to that in the control group. A significant decrease in total cell and macrophage numbers was observed in

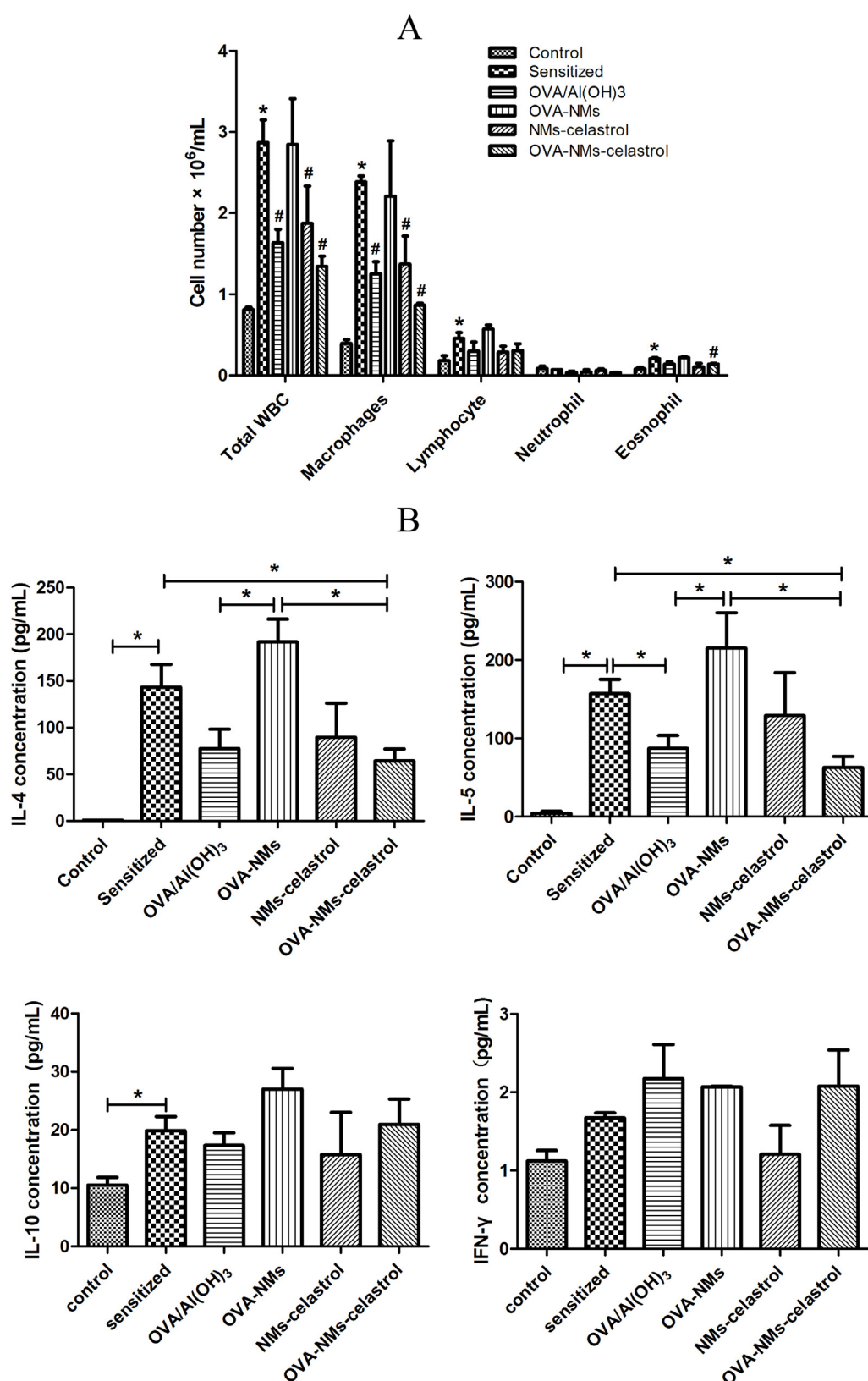


Fig. 3. Effect of different OVA vaccines on inflammatory cell infiltration and cytokine production in a mouse model of allergic asthma. BALF samples were collected from different groups (non-sensitized mice, sensitized mice, sensitized mice treated with OVA/Al(OH)₃ or OVA-NMs or NMs-celastrol or OVA-NMs-celastrol). (A) Cells were stained with Liu's stain. Total and differential white blood cell number were counted. Results are reported as mean $\pm$ SD ($n = 5$). *Significant differences ($P < 0.05$) between sensitized and control group. #Significant differences ($P < 0.05$) between sensitized group and treatment groups. (B) Cytokines were detected using Luminex xMAP technology. Results are reported as mean $\pm$ SD ($n = 5$), * $P < 0.05$.

OVA/Al(OH)₃, NMs-celastrol and OVA-NMs-celastrol treated conditions. Among all the treatment groups, only OVA-NMs-celastrol treated group showed decreased eosinophil number. A decreasing trend in eosinophil numbers was observed in OVA/Al(OH)₃ and NMs-celastrol treated groups. H and E staining showed severe pulmonary inflammation with infiltration of inflammatory cells around the bronchial and perivascular

areas in OVA sensitized / challenged mice (Fig. 4), while IHC exhibited a significantly increased number of lung tissue T cell, CD4+ cell and macrophage based on staining for CD3, a T cell-specific marker, CD4, and CD68, a macrophage-specific marker (Fig. 5). The inflammatory cell infiltration was ameliorated by treatment with OVA/Al(OH)₃ and NMs-celastrol, and almost suppressed by OVA-NMs-celastrol.

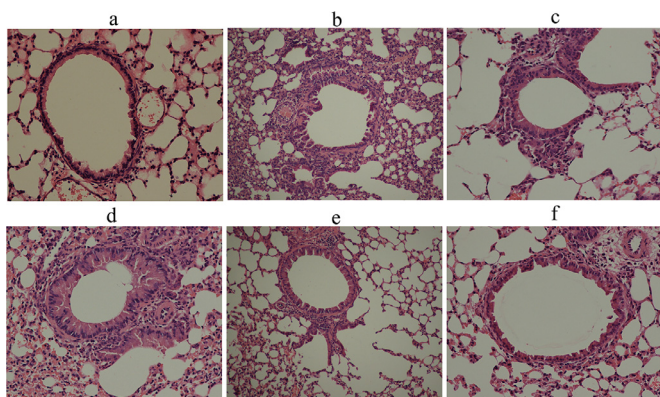


Fig. 4. H and E staining of the lung tissues from (a) non-sensitized mice, (b) sensitized mice, (c) sensitized mice treated with OVA/Al(OH)₃, (d) sensitized mice treated with OVA-NMs, (e) sensitized mice treated with NMs-celastrol, and (f) sensitized mice treated with OVA-NMs-celastrol. All images were captured at 200 × magnification.

4. Discussion

Improvement of safety and efficiency is of crucial importance in SIT. In this study, a novel vaccine for SIT of allergic diseases was designed. The vaccine is polymeric nanomicelles encapsulating the traditional Chinese medicine (celastrol) and conjugated with the model antigen OVA on the surface. The study aimed at assessing the safety and efficacy of the constructed OVA-NMs-celastrol in inducing a strong and long-term immune response.

DLS and TEM results showed that OVA-NMs-celastrol exhibited spherical morphology with small particle size. Spherical nanoparticles have been reported to be efficient in inducing strong humoral immune reaction (Bachmann and Jennings, 2010). Besides, smaller nanoparticles have been demonstrated to penetrate tissue barriers and traffic to the draining lymph nodes more rapidly than larger nanoparticles (greater than 100 nm), thus generating stronger adaptive immune responses (Smith et al., 2013). In our study, particle size detected by DLS was found to be slightly larger than that detected by TEM (20 nm difference). The reason may be that the samples used in these two methods are of different forms. DLS determines size of hydrodynamic micelle, while TEM determines that in dried state (Xie et al., 2017).

We further investigated the *in vivo* effects of micelles in a mouse model of allergic asthma. Typical Th2 type allergic inflammation was successfully induced by OVA sensitization / challenge with heightened production of OVA sIgE and histamine, increased release of Th2 cytokines (IL-4, IL-5, and IL-10) and infiltration of inflammatory cells.

Interestingly, we found that OVA sIgE levels are reduced both in mice treated with OVA-NMs and OVA-NMs-celastrol, but not in OVA/Al(OH)₃ treated mice. Usually, traditional SIT does not induce decreased allergen-specific IgE levels during the first 6 months, probably because memory B cells produce allergen-specific IgE after administration of allergens independent of T cells (Winther et al., 2006; Smarr et al., 2016). Consistent with the OVA sIgE results, the histamine level was also not decreased in mice treated with OVA/Al(OH)₃, however it was significantly reduced in OVA-NMs and OVA-NMs-celastrol treated groups. The decreased histamine level in the indicated groups could probably be attributed to the process of ECD coupling antigen to carboxylated nanoparticles, which could obscure conformational epitopes recognized by Fcε receptor (FcεR)-bound OVA sIgE, thus reducing the activation of sensitized effector cells (mast cells and basophils) and histamine release (Smarr et al., 2016). It is known that the long-term maintenance of antigen-specific antibody titers is achieved from long-lived plasmacytes (PCs) and memory B cells activated by allergens (Dullaers et al., 2012; Gould and Ramadani, 2015). The reason for decreased OVA-sIgE production may be due to the lack of recognition of

OVA-nanoparticle conjugates by memory B cells.

The detection of IgG subtypes may indicate the basis for Th bias. Usually, production of IgG2a is induced by Th1 cytokines such as IFN-γ, while generation of IgG1 and IgE antibodies is mediated by Th2 cytokines such as IL-4 and IL-5 (Joshi et al., 2014). Our results showed that OVA/Al(OH)₃, OVA-NMs and OVA-NMs-celastrol treatment could significantly increase OVA sIgG1 and OVA sIgG2a levels as compared to the sensitized mice. However, no difference among these three treatment groups was observed. It was reported earlier that the response curve for allergen specific IgG during immunization and challenge is different among different vaccines (Schöll et al., 2004). Long period observation may be needed to evaluate the OVA sIgG levels. Nevertheless, OVA-NMs-celastrol treated mice showed a significantly lower OVA sIgE / OVA sIgG2a ratio compared to OVA/Al(OH)₃ and OVA-NMs treated mice, indicating an inhibition of Th2 response.

The cytokine results indicate that OVA-NMs-celastrol can significantly decrease Th2 cytokine (IL-4 and IL-5) levels. However, the OVA-NMs treated group exhibited a reverse trend of increasing IL-4 and IL-5 levels. These results show that the inhibitory effect of OVA-NMs-celastrol on Th2 cytokines is partly due to celastrol, which had previously been demonstrated to have anti-inflammatory and immunoregulatory effects. (Astry et al., 2015; Kim et al., 2009). Besides, the low dose of conjugated OVA could help induce immune tolerance, which may further reduce Th2 cytokine (IL-4 and IL-5) release. It was reported that IL-4 and IL-5 are mainly released by memory Th2 cells upon re-exposure to allergens during allergic inflammation (Wang et al., 2006). The increasing trend of Th2 cytokines by OVA-NMs treatment may be due to the exposure of surface OVA, which promotes the antigen recognition by antigen presenting cells (APCs), thereby activating memory Th2 cells. The cytokine results also show that none of the vaccines could decrease IL-10 level. This may be attributed to the broad source of IL-10, which was reported to be produced not only by Th2 cell, but also by Treg cells and non-T cells (Saraiva and O'Garra, 2010). SIT will lead to decreased IL-10 release from Th2 cells, but increased IL-10 production from Treg cells (Palomares et al., 2014). This may be a possible explanation for the unaltered IL-10 levels in vaccine treated groups compared to the sensitized group.

Consistent with the IL-4 and IL-5 levels, OVA-NMs-celastrol treated mice also showed reduced inflammatory cell infiltration in lung tissue than sensitized mice, and NMs-celastrol treated mice exhibited a decreasing trend of inflammatory cell infiltration.

Taken together, OVA-NMs-celastrol show higher efficiency in preventing allergic inflammation compared to OVA/Al(OH)₃ and OVA-NMs. This may be attributed to: (1) the relatively better morphological characteristics (spherical shape and small particle size) of OVA-NMs-celastrol, which help induce strong humoral immune responses; (2) the hypoallergenic feature of OVA-NMs-celastrol, which would inhibit OVA-sIgE generation and histamine release; and (3) the anti-inflammatory and immunomodulatory function of the loaded celastrol. The hypoallergenic property of OVA-NMs-celastrol may also decrease local and systemic side-effect induced by allergen administration, which may assist in increasing the therapeutic dose and improve SIT efficacy.

5. Conclusions

In this study, we constructed OVA-conjugated celastrol-loaded polymeric micelles as new vaccines for SIT. The constructed OVA-NMs-celastrol vaccine had an appropriate particle size and spherical shape. *In vivo*, it exhibited appreciable efficacy in limiting allergic inflammation in a mouse model of allergic asthma. Thus, the allergen conjugated celastrol loaded nanomicelles may provide a valuable platform for advancing vaccine design strategies for SIT of allergic diseases.

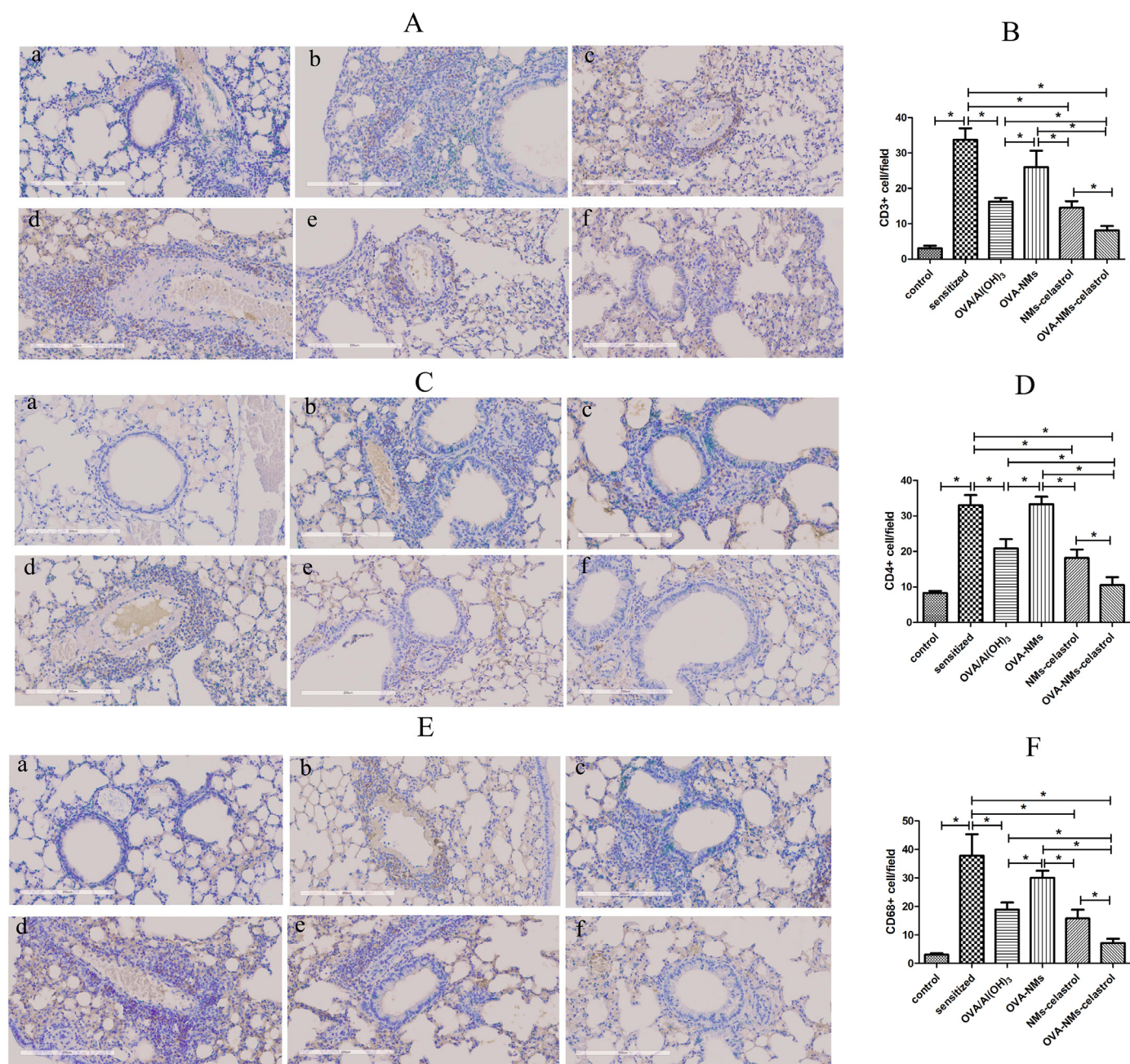


Fig. 5. CD3+, CD4+, and CD8+ cells were stained using immunohistochemistry staining in the lung of (a) non-sensitized mice, (b) sensitized mice, (c) sensitized mice treated with OVA/Al(OH)₃, (d) sensitized mice treated with OVA-NMs, (e) sensitized mice treated with NMs-celastrol, and (f) sensitized mice treated with OVA-NMs-celastrol. Images for CD3+ lung cells (A), CD4+ lung cells (C) and CD68+ lung cells (E) were captured at 20 × magnification by ImageScope. The CD3+ lung cell (B), CD4+ lung cell (D) and CD68+ lung cell (F) numbers were counted, and results are reported as mean ± SD, * $P < 0.05$.

Declaration of Competing Interest

None.

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