



CD13-specific ligand facilitates Xanthatin nanomedicine targeting dendritic cells for therapy of refractory allergic rhinitis



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ABSTRACT

Relapse in Allergic Rhinitis (AR) is triggered by various unclear mechanisms. Xanthium strumarium L. as a traditional folk medicine can inhibit inflammatory responses through multiple mechanisms. Xanthatin (XT) is a bioactive compound derived from Xanthium strumarium L. and we developed a polymeric micelle (PM) that is dendritic cells (DCs)-specific targeting delivery system loading XT (NGR-XT-PM) based on a cyclic peptide moiety (NGR) to render DCs maturation-resistant for therapy of refractory AR. A murine model of AR was employed to investigate the *in vivo* therapeutic efficiency and relapse rate compared with the commercial product Budesonide. The results showed intranasal administration of NGR-XT-PM presented significant anti-allergy effect with no recurrence, in contrast, all mice treatment with Budesonide relapsed. NGR-XT-PM could effectively reverse the Th1/Th2 imbalance by depleting the serum inflammatory levels (IgE, histamine and IL-4) and DCs surface costimulatory molecules (CD80, CD86 and I-A/I-E), and promote immune tolerance by up-regulating the level of Treg cells and reducing the levels of Th2, Th9 and Th17 cells. Furthermore, we appealed to virtual screening of inflammatory targets and found XT blocking the COX-2/PGE₂ signaling pathway, which is a key effector in immune responses. These indicated CD13-specific NGR could facilitate XT selectively targeting DCs for efficiently ameliorating refractory rhinitis, and NGR-XT-PM should be a potential anti-AR drug.

1. Introduction

Allergic rhinitis (AR) is a common immune inflammatory disease, affecting billions of people due to its high morbidity and refractory (Greiner et al., 2011; Ricketti et al., 2017). Pharmacotherapy (Rhinocort®, Nasonex® and Flixonase®) and immunotherapy (Alutard®, Novo-Helisen-Depot® and Dermatophagoides Farinae Drops®) are the mainstay of AR management, however, either insensitivity due to non-specific target or serious side effect due to long-term treatment leads to no satisfactory therapeutic effect (Leleux et al., 2015; James and Bernstein, 2017; Meurs et al., 2019). Therefore, developing new anti-

AR agents with multiple therapeutic targets and low side effect is the major clinical challenge for efficiently and safely treating AR.

The imbalance of T-helper type 1/T-helper type 2 (Th1/Th2) cells triggered by excessive Th2 cells differentiation is the most typical feature of AR (Wang et al., 2019; Watts et al., 2019; Yu et al., 2017). Dendritic cells (DCs) have the unique property of differentiating naïve T cells into Th2 cells through the interaction of MHC II complexes bonding to T-cell receptors and B7 costimulatory proteins (CD80 and CD86) bonding to CD28 on the surface of T cells (León and Lund, 2019; Bourque and Hawiger, 2018). On the contrary, inadequate co-stimulatory signals to T cells will induce T-cell anergy or generation of

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CD4⁺CD25⁺ regulatory T (Treg) cells (Luo et al., 2012). Researches revealed that CD40/RelB-silenced DCs could induce antigen-specific hyporesponsiveness (Zheng et al., 2009; Luo et al., 2013). Furthermore, KleinJan and his colleagues demonstrated that elimination of DCs from diseased mice could cure AR, and administration of DCs exacerbated AR (KleinJan et al., 2006). All the above highlighted DC-based immunotherapy should be a promising management in the fight against AR.

Xanthium strumarium L., as a traditional folk medicine with strong anti-inflammatory effect, has been extensively applied for treatment of AR for thousands of years (Peng et al., 2019; Fan et al., 2019), and it has been listed in the Pharmacopoeia of the People's Republic of China 2015, which can be applied to therapy of disperse wind, stuffy nose, rheumatism and nasal congestion, etc. Xanthatin (XT) is a major bioactive compound derived from Xanthium strumarium L., which possesses strong anti-inflammatory activity through multiple mechanisms (Nibret et al., 2011; Sánchez-Lamar et al., 2016). Furthermore, the data of our pilot study *in vitro* has demonstrated XT could render DCs maturation-resistant via functional downregulation of CD80, CD86 and I-A/I-E costimulatory molecules expression, indicating XT as an alternative anti-AR candidate. However, either hydrophobicity or lower effective concentration in the inflammatory region due to non-specific exposure is the major concern during its clinical application. Therefore, realizing XT selectively targeting DCs to enhance uptake may be practical to inhibit AR.

Aminopeptidase N (CD13) was overexpressed on the surface of DCs, which could work as a cell-surface receptor (Garay-Canales et al., 2018; Moffatt and Cristiano, 2006). Furthermore, the data of our pilot study has demonstrated CD13 was overexpressed on the surface of murine bone marrow-derived DCs. NGR (GGCNGRC) is a cyclic peptide Cys-Asn-Gly-Arg-Cys with disulfide bridge constraint, which can specifically recognize and bind to CD13 (Ferreri et al., 2019; Jahanban-Esfahani et al., 2018; Kessler et al., 2018). Therefore, CD13-specific NGR, as the target moiety, may provide an approach to achieve DCs-specific delivery of active agents.

Taken together, we have the following hypothesis that CD13-specific NGR could facilitate XT selectively targeting DCs for efficiently ameliorating refractory rhinitis in a murine model of AR. To test the hypothesis, we fabricated a targeted polymeric micelle (NGR-XT-PM) using poly (ethylene glycol)-block-poly (D, L-lactide) (PEG-PDLLA) copolymers as the carrier, and NGR as the target moiety. We investigated the distribution of NGR-XT-PM at the tissue, the *in vivo* therapeutic efficiency and relapse rate in a murine model of AR compared with the commercial product Budesonide (Rhinocort®), which is the first-line treatment for AR. We appealed to virtual screening of inflammatory targets and found a new molecular pathway of XT to block the activation of the COX-2/PGE₂ pathway, which is essential for the development and regulation of immune responses.

2. Materials and Methods

2.1. Materials

Xanthatin (purity > 99%) was purchased from Huzhou zhanshu biotechnology co., Ltd (Zhejiang, China). Budesonide Nasal Spray was purchased from AstraZeneca AB (Sweden). mPEG₂₀₀₀-PDLLA₂₀₀₀ di-block copolymer, N-hydroxysuccinimidyl-PEG₂₀₀₀-PDLLA₂₀₀₀ (NHS-PEG-PDLLA), the peptide GGCNGRC (NGR) and 1,1'-dioctadecyl-3,3',3'-tetramethyl-indocarbocyanineper-chlorate (DiI) were purchased from Xi'an Ruixi Biological Technology Co., Ltd (Shanxi, China). RPMI-1640 medium was purchased from HyClone (Logan, USA), and fetal bovine serum (FBS) was obtained from Gibco (Australia). Ammonium chloride solution was purchased from Shanghai YuanMu Biological Technology Co. Ltd (Shanghai, China). Recombinant murine IL-4 and granulocyte macrophage colony stimulating factor (GM-CSF) were obtained from PeproTech (USA). Ovalbumin (grade V),

aluminium hydroxide, Lipopolysaccharide and 4'-6-diamidino-2-phenylindole (DAPI) were obtained from Sigma-Aldrich (Saint Louis, USA). 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). ANPEP Polyclonal antibody and Alexa 488-conjugated goat-anti-rabbit antibodies were purchased from ABclonal (Cambridge, MA, USA). Percp/cy5.5-conjugated anti-mouse IFN- γ (Excitation: 488 nm/Emission: 690 nm), FITC-conjugated anti-mouse CD4 (Excitation: 488 nm/Emission: 525 nm), PE-conjugated anti-mouse IL-4 (Excitation: 488 nm/Emission: 578 nm), PE-conjugated anti-mouse Foxp3, APC-conjugated anti-mouse CD25 (Excitation: 633 nm/Emission: 660 nm), Alexa Fluor® 647-conjugated anti-mouse IL-17 α (Excitation: 633 nm/Emission: 668 nm) and APC-conjugated anti-mouse IL-9 were purchased from ABGENT Biotechnology (San Diego, USA). APC-conjugated anti-mouse CD11c, FITC-conjugated anti-mouse CD80, PE-conjugated anti-mouse CD86 and PerCP/Cy5.5-conjugated anti-mouse I-A/I-E, Mouse IL-4 ELISA Kit, Mouse IFN- γ ELISA Kit and Mouse IgE ELISA Kit were purchased from eBioscience (California, USA). Mouse Histamine ELISA Kit was purchased from DRG International Inc. (Marburg, Germany). Mouse Prostaglandin E₂ (PGE₂) ELISA Kit was purchased from Cusabio biotech Co., Ltd (Hubei, China). All other reagents were analytical grade.

Male C57BL/6J mice (6–8 weeks, 20 $\pm$ 2 g), free of murine-specific pathogens and respiratory disease, were obtained from the second Hospital of Shandong University. The animals were housed at temperature of 20–22 °C, relative humidity of 50–60% and 12 h light–dark cycles. Free access to food and water was allowed. All animals would be in quarantine for a week before treatment. All animals care and experimental procedures were conducted according to Institutional Animal Care and Use guidelines.

2.2. Synthesis of targeting copolymer

NGR conjugated copolymer (NGR-PEG-PDLLA) was synthesized according to the previously reported method (Wang et al., 2009). Briefly, NHS-PEG-PDLLA and NGR at 1:2 M ratio were dissolved in borate-buffered saline (BBS, pH = 9.0). The reaction was maintained under stirring at room temperature for 12 h. The product was purified by dialysis (MWCO 3500 Da) against distilled water, lyophilized, and the chemical structure was confirmed by ¹H NMR (in DMSO, 300 MHz). High-performance liquid chromatography (HPLC) was used to quantify the free NGR in the reaction solution. The detection method was detailed in supplemental materials (Supporting Methods, S1).

2.3. Preparation and characterization of NGR-XT-PM

The NGR-XT-PM was prepared using the thin-film hydration method. Generally, 2 mg XT, 35.1 mg mPEG-PDLLA, and 0.9 mg NGR-PEG-PDLLA were co-dissolved in 4 mL dehydrated ethanol. Then, organic solvent was removed under reduced pressure by vacuum rotary evaporator (Laborota 4000; Heidolph Instruments GmbH&Co., KG Germany) at 60 °C. Sequentially, the dried film was hydrated with preheated distilled water to form micelles. Then, the solution was filtered with a 0.22 μ m filter to obtain a clear solution. Finally, the solution was lyophilized to obtain a dried powder. XT-loaded non-targeted polymeric micelles (XT-PM), blank micelles (NGR-PM) and DiI-loaded micelles with different percentages of NGR-PEG-PDLLA/total polymer carrier (N/P = 0%, 2.5%, 5%, 7.5% w/w) were also prepared by the same procedures. Fig. 1 is the schematic illustration of the preparation of NGR-XT-PM micelles.

The drug loading (DL) and encapsulation efficiency (EE) of NGR-XT-PM and XT-PM were quantified as follows: 10 mg lyophilized powder was dissolved in 10 mL acetonitrile and determined by HPLC. The detection method was detailed in supplemental materials (Supporting Methods, S2). The DL and EE of DiI-loaded micelles were quantified by fluorescence spectroscopy with 549 nm excitation and 565 nm emission

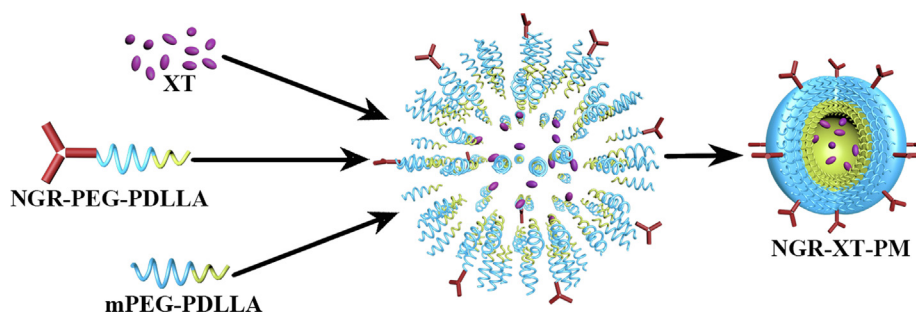


Fig. 1. Schematic representation of preparing NGR-Xt-PM. XT, NGR-PEG-PDLLA and mPEG-PDLLA formed NGR-Xt-PM by self-assembly.

wavelength (PerkinElmer, LS-45).

The DL and EE of XT-loaded micelles and DiI-loaded micelles were calculated according to Eqs. (1) and (2):

$$DL = \frac{XT}{\text{Amount of XT in feed} + \text{Polymer}} \times 100\% \quad (1)$$

$$EE = \frac{XT}{\text{Amount of XT in feed}} \times 100\% \quad (2)$$

The hydrodynamic particle size and zeta potential of XT-loaded micelles were measured by Malvern Nano-ZS 90 laser particle size analyzer, and the morphological characteristics of the NGR-Xt-PM were examined by transmission electron microscope (TEM, H-6009IV, Hitachi, Japan). Crystallographic assay was performed on the blank carrier materials mixture of NGR-PEG-PDLLA and mPEG-PDLLA, pure XT, NGR-Xt-PM lyophilized powder and physical mixture of XT, NGR-PEG-PDLLA and mPEG-PDLLA using an X-ray diffractometer (XRD, Bruker D8 ADVANCE). The voltage and current used were 40 kV and 40 mA, respectively. The XRD patterns were obtained at 3°/min scan speed from 5 to 50°.

2.4. In vitro release behavior

To determine the release kinetics of NGR-Xt-PM and XT-PM, 1.0 mL NGR-Xt-PM or XT-PM water solution (1.0 mg/mL XT) was placed in dialysis bags (MWCO 3500 Da). The dialysis bags were incubated in 40 mL phosphate buffered solution (PBS, pH = 7.4) containing polysorbate 80 (0.5%, w/v) at 37 °C with gentle shaking (100 rpm). At specific time intervals, the sample solution was drawn and replaced with the equal volume of prewarmed fresh medium. The sample solution was filtered with a 0.45 µm filter and quantified by HPLC. Each batch was analyzed in triplicate.

2.5. Identification of CD13 on the surface of DCs

Murine bone-marrow derived DCs were generated (Supporting Methods, S3) and CD13 overexpressed on the surface of DCs was identified by fluorescent staining. Mice pulmonary microvascular endothelial cells (MPMECs, 1.0×10^5 /well) were seeded on the coverslip named 'a' as negative control, and DCs (1.0×10^5 /well) were seeded on the other two coverslips named 'b', 'c'. The 'a', 'b' cells were treated with PBS, while the 'c' cells were treated with lipopolysaccharide (LPS, 1.0 µg/mL) for 4 h. Cells were rinsed and then fixed with 4% paraformaldehyde for 15 min. The slides were incubated overnight at 4 °C with primary antibodies (ANPEP Polyclonal antibody), and then incubated with secondary antibodies (Alexa 488-conjugated goat-anti-rabbit antibodies) for 60 min at room temperature. The nucleus was stained with DAPI. The cell fluorescence was detected by the fluorescence microscope (Axiovert, Zeiss, Germany), and data were analyzed by software Image J. All the experiments were performed in triplicate.

2.6. Cell viability assay

The viability of cells was determined using the standard MTT assay. DCs were seeded into 96-well plates at a density of 5.0×10^3 cells per well overnight, and then treated with free XT in DMSO solution, NGR-PM, XT-PM and NGR-Xt-PM for 48 h at XT concentrations ranging from 0.0005 to 5.0 µg/mL. After 48 h, the viability of cells was measured using the MTT method. Untreated cells were used as control. Afterward, the UV-Vis absorption of sample wells (A_{sample}) and control wells (A_{control}) was measured using a Microplate reader (Bio-Tek, USA) at 490 nm. Cell viability (%) was equal to $(A_{\text{sample}}/A_{\text{control}}) \times 100$. All the experiments were performed in triplicate.

2.7. Screening and identifying DCs-specific targeting system

Receptor-mediated endocytosis is the primary pathway of the nanoparticle (< 150 nm) for intracellular delivery (Wang et al., 2016). To confirm the specific binding ability of the DCs-specific targeting systems, the delivery systems with different N/P percentages (N/P = 0%, 2.5%, 5% and 7.5%) encapsulating the same concentration of DiI were investigated. Briefly, DCs (1.0×10^5 /well) were incubated with the DiI-loaded micelles with different N/P percentages (3.0 µg/mL DiI) for 4 h. Then, some cells were collected and detected by flow cytometry (BD, FACSaria II, USA). Other cells were fixed with 4% paraformaldehyde, stained with DAPI, and detected by confocal laser scanning microscopy (CLSM, TCS SP8, Leica, Germany).

To elucidate the role of NGR in the cell uptake, competitive inhibition experiment with excess free NGR peptide was performed. DCs (1.0×10^5 /well) were seeded on three coverslips named 'a', 'b', 'c' and cultured for 24 h. Only the 'c' cells were pre-incubated with excessive free NGR (1.0 mg/mL) for 30 min, after that 'a', 'b' and 'c' cells were incubated with DiI-PM, NGR-DiI-PM or NGR-DiI-PM (3.0 µg/mL DiI) for 4 h, respectively. Then, the intracellular fluorescence was determined by CLSM.

To study the intracellular retention time of micelles, DCs were incubated with DiI-PM or NGR-DiI-PM (3.0 µg/mL DiI) for 4 h, respectively. After that, the medium was removed and cells were washed with PBS (designed time as 0 h). Fresh culture medium was added followed by a further incubation for 4 h, 8 h, 16 h and 24 h at 37°C. The intracellular fluorescence intensity was determined by flow cytometry at each time point (FI_T). The fluorescence intensity (%) was calculated according to the formula: $(FI_T/FI_0) \times 100$. All the experiments were performed in triplicate.

2.8. In vitro cell phenotype assay

DCs (1.0×10^5 /well) were seeded in 6-well plates and incubated overnight. Cells treated with PBS or LPS were used as negative or positive controls, respectively. Other cells were pretreated with free XT, XT-PM or NGR-Xt-PM (3.0 µg/mL XT) for 10 min, and then cocultured with LPS (1.0 µg/mL) for 24 h. The cell surface markers were analyzed by flow cytometer after stained with the following anti-mouse mAbs:

Table 1
Superposition quantization scoring.

Score	Nose scratching	Discharging	Sneezing
1	< 4	Reaching the anterior nostril	≤ 3
2	4–10	Over the anterior nostril	4–10
3	Cannot stop	Following all the face	≥ 10

FITC-conjugated anti-CD80, PE-conjugated anti-CD86 and PerCP/cy5.5-conjugated anti I-A/I-E. All the experiments were performed in triplicate.

2.9. In vivo therapeutic efficacy

Animal models of AR were established according to the previously reported methods (Kim et al., 2011; Wang et al., 2008). Briefly, fifty male C57BL/6J mice (6–8 weeks, 20 ± 2 g) were sensitized by intraperitoneal (i.p.) injection of 25 μ g ovalbumin (OVA) and 2 mg aluminum hydroxide in 200 μ L PBS solution on days 0 and 5, followed by intranasal (i.n.) administration once daily from days 12–18 with 6% OVA (20 μ L/nostril) diluted in PBS. Thereafter, prolonged continuous inflammation was maintained by i.n. administration of 6% OVA three times weekly for 8 consecutive weeks. On day 46, the symptoms of AR were scored by recording the secretion, sneezing frequency and the nose-scratching times in 30 min period after the last challenge according to the superimposition quantization scoring (Table 1) (Wang et al., 2017), and the total scores > 5 points would be identified as successful models. In addition, three mice with significant behavioral symptoms (scores > 5) were randomly selected for further histopathological evaluation. Body weight changes were also monitored. Mice were randomly divided into eight groups ($n = 6$ for each group). For the control group, mice were subjected to PBS through i.p. and i.n. challenges. During challenge, mice received corresponding medication via i.n. administration once daily from days 47–60 as follows: group G1 (Normal group) and G2 (AR group) received PBS solution (20 μ L/nostril), group G3 and g3 received XT-PM PBS solution (0.4 mg/Kg XT, 20 μ L/nostril), group G4 and g4 received NGR-XT-PM PBS solution (0.4 mg/Kg XT, 20 μ L/nostril), group G5 and g5 received Budesonide PBS solution (33.28 μ g/Kg, 20 μ L/nostril). The dosage of Budesonide was converted from human-equivalent dose to animal dose. To further investigate the relapse rate, the mice in group g3, g4 and g5 were kept only with 6% OVA challenge from days 61–74.

On day 60 and 74, nasal symptoms were scored in 30 min by three observers blinded to treatment. The serum samples were collected from the mice in treatment groups (G1, G2, G3, G4 and G5) and relapse groups (g3, g4 and g5) by eyeball extirpation and stored at -80°C for biochemical measurements, then the mice were sacrificed on day 60 and 74 through cervical dislocation, respectively. The nasal mucosa and part of spleen tissue were dissected and fixed in 4% paraformaldehyde solution for 24 h. And then, nasal mucosa was decalcified with 10% EDTA solution for 7 days at room temperature. The decalcified nasal mucosa and spleen specimens were further dehydrated and embedded in paraffin, and 4 μ m sections were sectioned. The sections were stained with hematoxylin and eosin (H&E), and examined with a microscope (BX40; Olympus, Hamburg, Germany). Eosinophil counts in randomly selected 4 high power fields (400 $\times$ magnification) were assessed. The rest of spleen tissue was prepared into single cell suspension for determining the percentages of T cell subgroups and DCs phenotype analysis, and the Th1/Th2 ratio was also calculated. In addition, the cervical lymph nodes and blood were also prepared for determining the percentages of T cell subgroups. The T cell subgroups including Th1 ($\text{CD4}^+ \text{IFN-}\gamma^+$), Th2 ($\text{CD4}^+ \text{IL-4}^+$), Th9 ($\text{CD4}^+ \text{IL-9}^+$), Th17 ($\text{CD4}^+ \text{IL-17}^+$) and Treg ($\text{CD4}^+ \text{CD25}^+ \text{Foxp3}^+$) cells were identified using flow cytometry.

2.10. In vivo distribution

To investigate the bio-distribution of such formulations, C57BL/6J mice ($n = 3$ for each group) suffering from OVA-induced AR were administered intranasally with PBS, DiI-PM PBS solution (0.4 mg/Kg DiI) or NGR-DiI-PM PBS solution (0.4 mg/Kg DiI). After 4 h administration, mice were sacrificed. Major organs including nasal mucosa, brain, heart, liver, spleen, lymph node, lung and kidney were excised and fixed in 4% paraformaldehyde, then embedded in paraffin. Sections of 3–5 μ m were stained with DAPI blue-fluorescent dye and observed using CLSM. Data were analyzed by software Image J.

2.11. Virtual screening and confirming new targets for XT

An in-house inflammatory target protein database containing nearly 500 proteins was used in the process of ligand-based reverse targeting. The XT structure was drawn using Chemdraw (version 14.0) and Discovery StudioTM3.5 was used to find new anti-inflammatory targets within the in-house inflammatory target protein database, according to the previously reported method (Yu et al., 2019). All parameters were set as default and the fit values obtained were used to assess the possibility of the receptors binding to XT.

To explore the affinity of new targets to XT, the X-ray crystal structures of new-found receptors were downloaded from the RCSB Protein Database (<http://www.rcsb.org>), and then Ledock (version 1.0) was used to explore the affinity between the new targets and XT. Finally, software PyMOL (<http://www.pymol.org>) was used to analyze and visualize the interaction between ligand and receptor and the 3D configuration, and obtained the optimized interaction pattern.

To verify the new signaling pathway of XT, DCs treated with PBS or LPS were used as negative or positive controls, respectively. Other cells were pretreated with XT-loaded micelles (at the XT dosages of 1.0, 2.0 and 4.0 μ g/mL) or equal amounts of NGR-PM (at the polymer dosages of 18.34, 36.68 and 73.36 μ g/mL) for 10 min, and then cocultured with LPS (1.0 μ g/mL) for 24 h. After treatment, the supernatant was collected and then centrifuged for 10 min at 2000 rpm. The levels of the cytokines were analyzed by ELISA according to the manufacturer's directions.

2.12. Statistical analysis

The results of statistical analysis were presented as mean $\pm$ S.D. Statistical comparison was made by one-way ANOVA analysis following by suitable post-hoc test. Statistical significance is denoted by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results

3.1. Targeting copolymer synthesis and characterization

Fig. 2 presented the synthetic route and the corresponding ^1H NMR spectrum. As shown in Fig. 2A, the NGR-PEG-PDLLA copolymer was prepared via a reaction between the NHS group and the terminal amino group of the NGR. The H specific chemical shift of NHS group on NHS-PEG-PDLLA was at δ 2.75 ppm (Fig. 2B-a). As shown in Fig. 2D, after reaction with NGR, the chemical shift of H on the NHS group disappeared and that of multiplets from NGR (Fig. 2C) were observed at δ 3.81–4.06 ppm and δ 6.95–8.59 ppm. In addition, the ^1H NMR spectrum of the synthesized NGR-PEG-PDLLA also displayed the rest corresponding peaks of PEG ($-\text{CH}_2\text{CH}_2\text{O}-$; $\delta = 3.65$) (Fig. 2D-b) and PDLLA ($-\text{CH}-$; $\delta = 5.1$) (Fig. 2D-c). The HPLC analysis of free NGR was shown in Fig. 3A. The retention time of free NGR was about 5.3 min, and the residual free NGR was detected in the reaction solution at 3 h, while no free NGR was detected in the reaction solution (12 h) after dialysis. These results indicated that the NGR peptide was successfully conjugated to NHS-PEG-PDLLA via an amide bond.

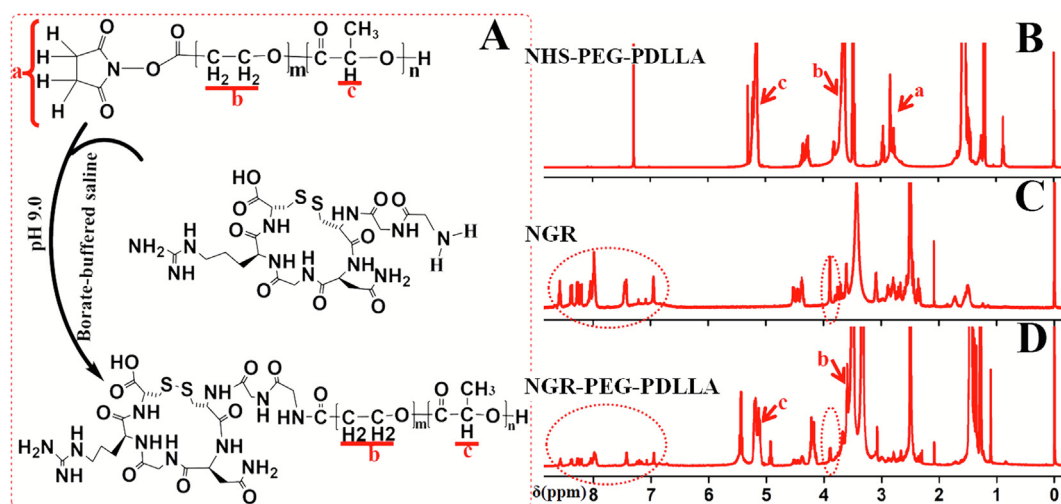


Fig. 2. The synthesis route of NGR-PEG-PDLLA (A), and ^1H NMR spectra of NHS-PEG-PDLLA (B), NGR (C) and NGR-PEG-PDLLA (D). The ellipse represented the peaks of multiple protons of NGR.

3.2. Characterizations of XT formulations

Fig. 3B presented the physical properties of XT-PM and NGR-XT-PM. The EE, zeta potential and particle size of XT-PM and NGR-XT-PM were $98.84 \pm 0.39\%$, -2.69 ± 0.35 mV, 18.10 ± 0.12 nm and $98.89 \pm 1.02\%$, -6.01 ± 0.96 mV, 17.83 ± 0.28 nm, respectively. It was reported that the particle ≤ 500 nm could lead to faster and more efficient uptake by DCs (Foged et al., 2005). Alternatively, the different zeta potential of NGR-XT-PM and XT-PM further confirmed the success of NGR conjugated to the polymer. Fig. 3C showed the TEM image of NGR-XT-PM, which clearly revealed that NGR-XT-PM micelles were well-dispersed with regular spherical shape. Fig. 3D depicted the XRD patterns of the blank carrier materials mixture of NGR-PEG-PDLLA and mPEG-PDLLA (a), pure XT (b), NGR-XT-PM lyophilized powder (c) and physical mixture of XT, NGR-PEG-PDLLA and mPEG-PDLLA (d). The XRD patterns of pure XT (b) and physical mixture (d) presented specific diffraction peaks of XT, which implied the crystalline nature of

XT was not changed in the physical mixture. However, the absence of specific diffraction peaks in diagram of NGR-XT-PM (c) indicated that XT was completely encapsulated within their core-shell structure amorphously. Fig. 3E presented the EE, zeta potential and particle size of DiI-loaded micelles with different N/P percentages, which were $72.63 \pm 6.26\%$, -3.8 ± 0.82 mV, 18.28 ± 0.35 nm (N/P = 0%), $70.77 \pm 6.42\%$, -7.73 ± 0.57 mV, 18.24 ± 0.10 nm (N/P = 2.5%), $68.8 \pm 5.86\%$, -16 ± 4.27 mV, 18.35 ± 0.05 nm (N/P = 5%) and $70.27 \pm 7.72\%$, -17.17 ± 2.06 mV, 18.32 ± 0.21 nm (N/P = 7.5%), respectively. Fig. 3F illustrated the similar release profiles of the XT from XT-PM and NGR-XT-PM. No initial burst effect of both formulations was observed within 0.5 h, only 14.87% XT was released within 1.5 h, and more than 70% XT was released within 20 h.

3.3. Identification of CD13 overexpressed on DCs surface

Fig. 4 showed the expression of CD13 on the surface of MPMECs and

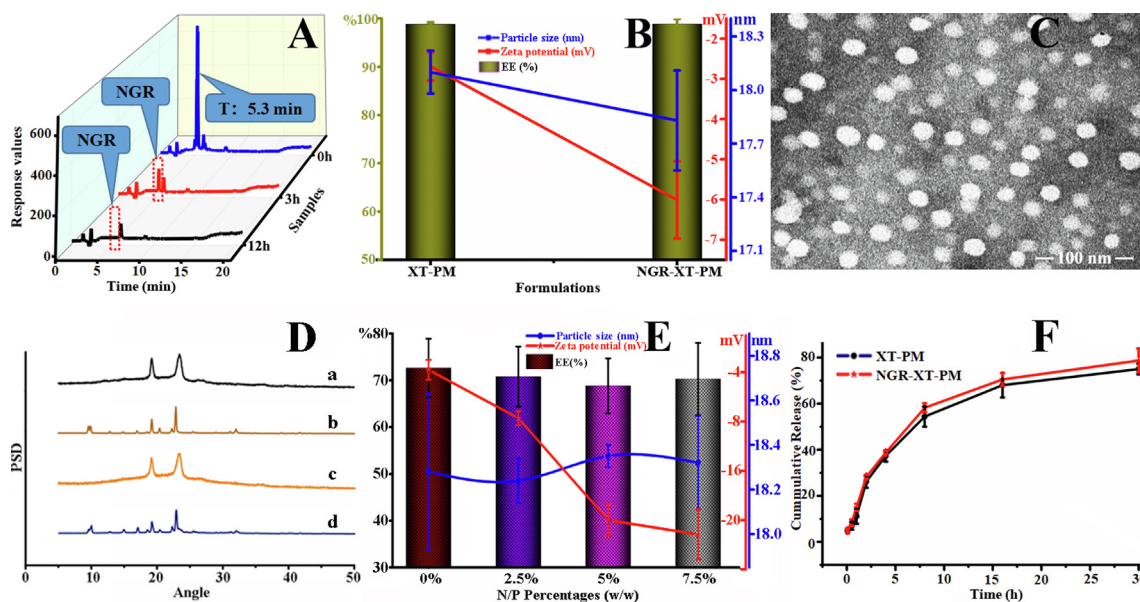


Fig. 3. (A) Qualitative HPLC analysis of NGR in the reaction solution at 0 h, 3 h and 12 h, respectively. (B) The physical properties of XT-PM and NGR-XT-PM. (C) The TEM image of NGR-XT-PM. (D) X-ray diffraction patterns of the mixture of NGR-PEG-PDLLA and mPEG-PDLLA (a), pure XT (b), NGR-XT-PM lyophilized powder (c) and the physical mixture of XT, NGR-PEG-PDLLA and mPEG-PDLLA (d). (E) The physical properties of DiI-loaded micelles with different N/P percentages. (F) In vitro drug release curves of XT-PM and NGR-XT-PM. Results were expressed as mean $\pm$ S.D. (n = 3).

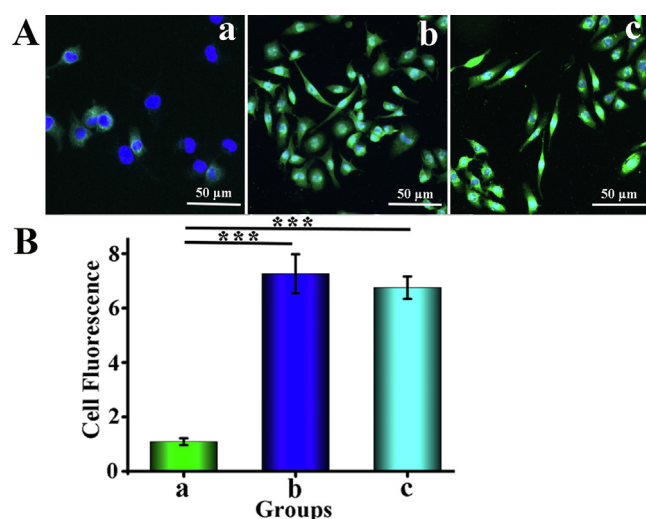


Fig. 4. The identify of CD13 on the surface of MPMECs and DCs. (A) Fluorescent microscopic images (The green fluorescence is the expression of CD13 signal and the blue fluorescence is DAPI signal) and (B) the fluorescence intensity of (a) MPMECs, (b) immature DCs, and (c) mature DCs. Scale bar was 50 μ m. Results were expressed as mean $\pm$ S.D (n = 3). ***p < 0.001 vs. Group a.

DCs. Fig. 4A(b) and Fig. 4A(c) presented a large number of fluorescent signals compared to Fig. 4A(a). Furthermore, the fluorescent intensity of group b and c was approximately 6-fold stronger than that of group a (**p < 0.001) (Fig. 4B). These results indicated CD13 was over-expressed on the surface of DCs whether it was mature or immature, and DCs could be employed as the target cells.

3.4. Cell viability and affinity studies

Fig. 5A presented the viability of DCs incubated with NGR-PM, free XT, XT-PM and NGR-XT-PM solution at different concentrations of XT for 48 h. Results showed the concentrations of XT ranging from 0.0005 to 5.0 μ g/mL had no cytotoxicity on DCs (cell viability > 90%). Fig. 5B showed NGR-DiI-PM (N/P = 2.5%) group displayed the highest

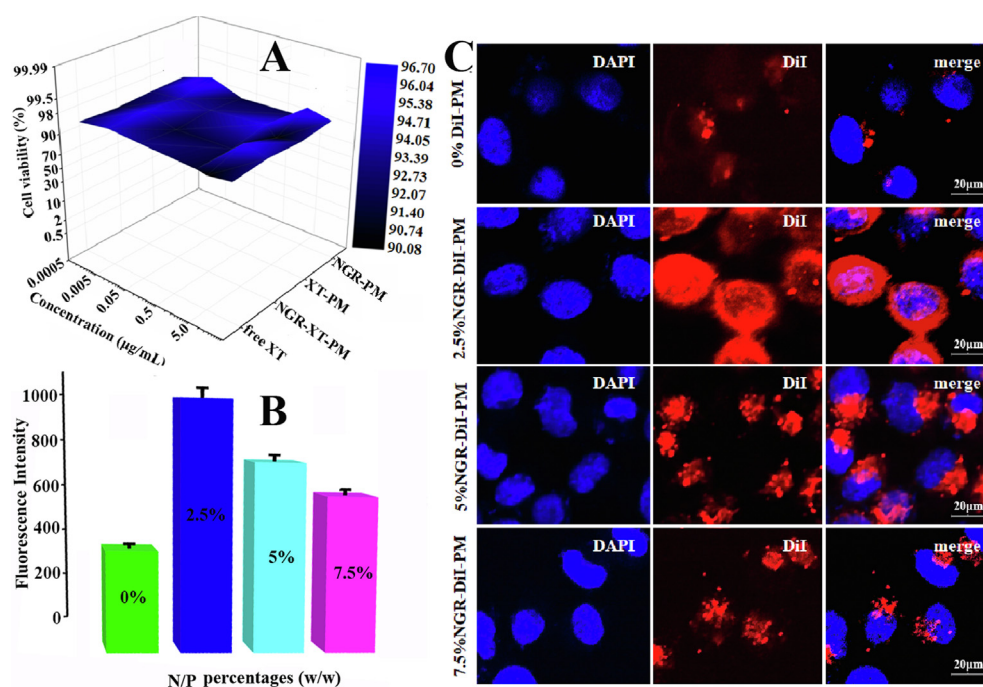


Fig. 5. (A) The viability of DCs incubated with NGR-PM, free XT, XT-PM and NGR-XT-PM solution at different concentrations of XT for 48 h. (B) Quantitative analysis of the fluorescent intensity and (C) Confocal fluorescence images for NGR-DiI-PM with different N/P percentages (N/P = 0%, 2.5%, 5% and 7.5%). Red fluorescence is DiI signal and blue fluorescence is DAPI signal. Scale bar was 20 μ m. Results were expressed as mean $\pm$ S.D (n = 3).

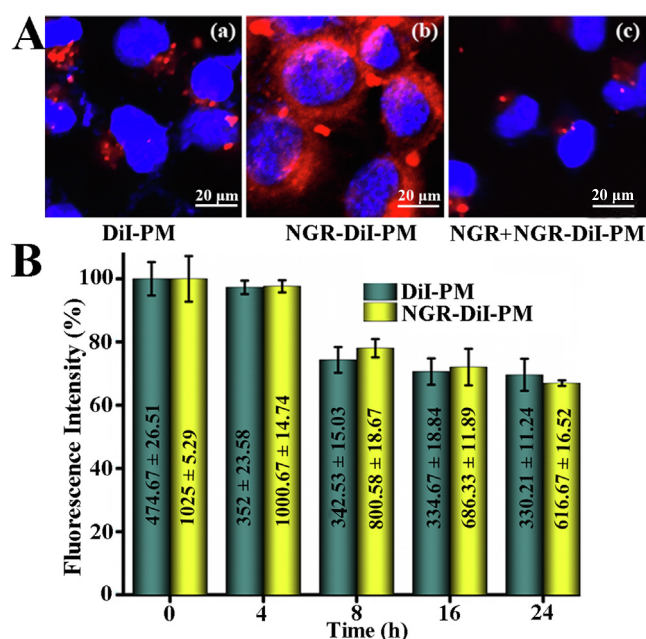


Fig. 6. (A) Confocal images of DCs incubated with (a) DiI-PM, (b) NGR-DiI-PM and (c) NGR-DiI-PM with excess NGR (1 mg/mL) preincubated for 30 min. Red fluorescence is DiI signal and blue fluorescence is DAPI signal. Scale bar was 20 μ m. (B) The fluorescence intensity of DiI-PM and NGR-DiI-PM group in DCs at different time points. The numbers on the column represented the intracellular fluorescence intensity. Results were expressed as mean $\pm$ S.D (n = 3).

fluorescence intensity among four groups, which was approximately 2.5-fold, 1.3-fold and 1.6-fold stronger than that of NGR-DiI-PM (N/P = 0%, 5% and 7.5%) groups, respectively. To visualize intracellular fluorescence, confocal images were presented in Fig. 5C. The NGR-DiI-PM (N/P = 2.5%) group showed the strongest red fluorescence signals, which was consistent with that of flow cytometry. Therefore, NGR-DiI-PM (N/P = 2.5%) was chosen for further study.

Fig. 6A further confirmed the role of NGR-mediated endocytosis. Fig. 6A(a) was the DiI-PM group without DCs-specific targeting moiety

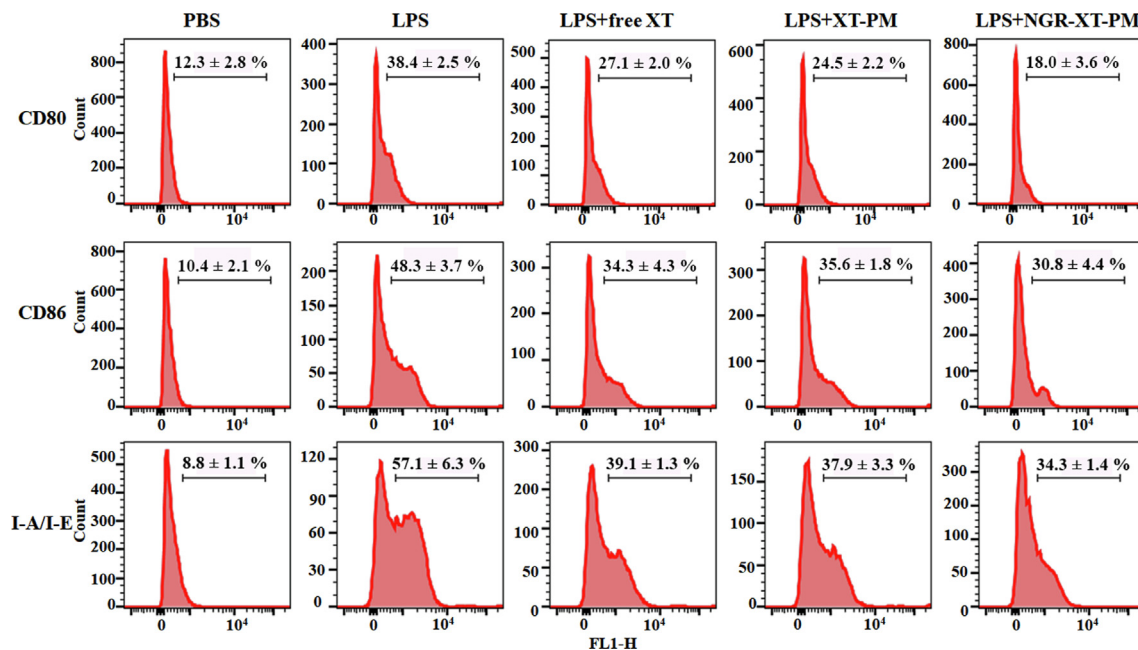


Fig. 7. Phenotypic analysis of DCs in vitro. The expression levels of costimulatory molecules (CD80, CD86 and I-A/I-E) in immature DCs treated with PBS, mature DCs treated with LPS and DCs pretreated with free XT, XT-PM or NGR-XT-PM for 10 min, respectively, and then cocultured with LPS for 24 h ($n = 3$). Results were expressed as mean $\pm$ S.D ($n = 3$).

NGR, and presented weak fluorescence signals. Fig. 6A(b) showed a large number of fluorescent signals compared to Fig. 6A(a) and Fig. 6A(c). The result of Fig. 6A(c) should be ascribed to the pretreatment with excessive NGR, which significantly inhibited the uptake of NGR-Dil-PM. The results of competitive inhibition experiment demonstrated that NGR-mediated endocytosis facilitated the uptake of Dil by cells. In addition, the success of nanocarrier also depends on the retention time of the carrier within the cell without early exocytosis (Kim et al., 2013). As shown in Fig. 6B, the elimination rates of NGR-Dil-PM were comparable to that of Dil-PM, which indicated that NGR showed no significant effect on the elimination of carrier from DCs.

3.5. Phenotype analysis of DCs in vitro

Overexpression of costimulatory molecules MHC II (I-A/I-E), CD80 and CD86 are the most typical features of DCs maturation, and upregulation of CD80 and CD86 is a prerequisite for increasing magnitude of primary and secondary T cells responses (Silva et al., 2013; Pizzurro and Barrio, 2015; Wołkow et al., 2018). Fig. 7 depicted the impact of different formulations on DCs maturation. Compared to mature DCs treated with LPS, lower levels of CD80, CD86 and I-A/I-E molecules were expressed in immature DCs treated with PBS. Furthermore, the levels of CD80, CD86 and I-A/I-E molecules in mature DCs treated with LPS and different XT formulations all were lower than that in mature DCs treated with LPS, among them, the mature DCs treated with LPS and NGR-XT-PM presented the lowest levels of CD80, CD86 and I-A/I-E molecules. These results suggested that NGR-XT-PM could more effectively render DCs resistant to maturation compared to XT-PM and free XT.

3.6. In vivo therapeutic efficacy

3.6.1. Behavioral observation

The experimental procedure was presented in Fig. 8A. The symptom behavior superposition scores were performed and mice (scores > 5) were considered to have successfully suffered from AR with significant pathological changes in nasal mucosa (Supporting Figures, Fig. S1). Moreover, there was no statistically significant difference in body

weight between the AR mice (end point: 20.86 ± 0.92 g) and normal mice (end point: 21.34 ± 0.28 g) ($P > 0.05$).

The nasal symptom scores of each group in 30 min period after the last challenge were presented in Fig. 8B. The nasal symptom scores of group G1 (Normal group), G2 (AR group), G3 (XT-PM group), G4 (NGR-XT-PM group), G5 (Budesonide group), g3 (XT-PM relapse group), g4 (NGR-XT-PM relapse group) and g5 (Budesonide relapse group) were 1.33 ± 0.58 , 7.67 ± 0.58 , 2.67 ± 1.15 , 2.33 ± 0.58 , 2.33 ± 1.15 , 3.33 ± 1.15 , 2.67 ± 0.58 and 6.67 ± 0.58 , respectively. OVA challenge caused a statistically significant increase in the nasal symptom scores in group G2 compared with group G1 ($^{***}p < 0.001$). Notably, the scores of group G3 (scores < 3 , $^{*}p < 0.05$), G4 (scores < 3 , $^{***}p < 0.001$), G5 (scores < 3 , $^{*}p < 0.05$), g3 (scores < 4 , $^{*}p < 0.05$) and g4 (scores < 3 , $^{***}p < 0.001$) appeared to be markedly reduced compared with that of group G2 (scores > 7). Surprisingly, the symptom scores of group g3 ($^{*}p < 0.05$) and g4 ($^{**}p < 0.01$) were significantly lower than that of group g5 (scores > 6), while there was no significant difference in the symptom scores between group G2 and g5. We further evaluated the relapse rate (the symptom scores > 5 of mice would be judged to be relapsed) within 14 days after administrative withdrawal, as shown in Fig. 8C. AR recurrence to 16.7% of mice appeared in the group g3 on the 10th day, while the relapse rate of group g5 up to 50% was noted on the 5th day, 66.7% of mice relapsed on the 8th day, and all mice relapsed on the 11th day. In contrast, no mice in group g4 relapsed. Such results indicated that XT-PM and Budesonide significantly alleviated the nasal symptoms, while DCs-specific targeting nanocarrier loading XT (NGR-XT-PM) not only relieved the nasal symptoms, but also inhibited the recurrence of AR.

3.6.2. Histological evaluation

The light micrographs of spleen and nasal mucosa were presented in Fig. 9A & 9B. Spleen, as a vital organ of the immune and reticuloendothelial system, is often affected by AR (Oh et al., 2011). In group G2 (AR group), we can find large number of hemosiderin-laden macrophages, thicker fibrous capsule and splenic enlargement (hallmark of OVA-induced AR) in spleen, and the histological features of the nasal mucosa also revealed remarkable hyperemia and edema,

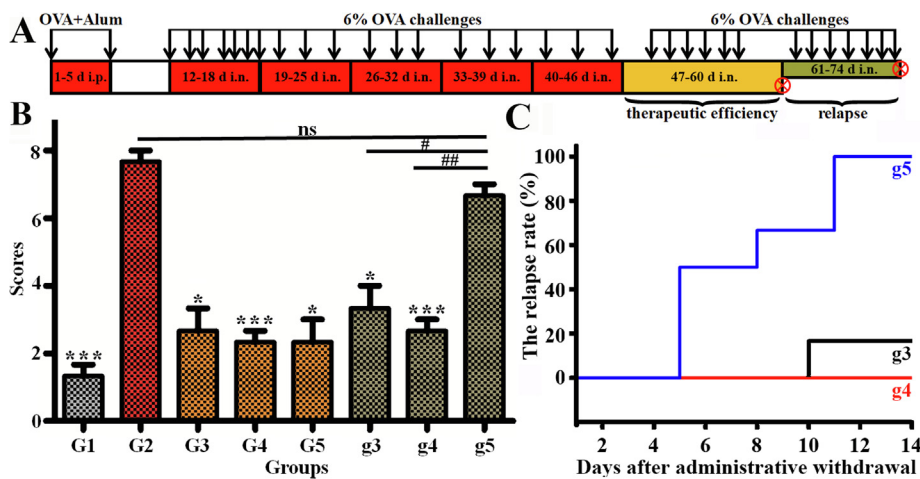


Fig. 8. (A) The experiment procedure. Arrows represented OVA challenges. ⊗ represented that the mice were sacrificed. (B) The nasal symptom scores were calculated in each group after the last challenge. (C) The relapse rate. Results were expressed as mean $\pm$ S.D (n = 6). ns = not significant, *p < 0.05, ***p < 0.001 vs. G2 and #p < 0.05, ##p < 0.01 vs. g5. G1 (Normal group), G2 (AR group), G3 (XT-PM group), G4 (NGR-XT-PM group), G5 (Budesonide group), g3 (XT-PM relapse group), g4 (NGR-XT-PM relapse group) and g5 (Budesonide relapse group).

inflammatory cells infiltration, gland hypertrophy and hyperplasia, especially irregular cell arrangement compared with group G1 (Normal group) with normal architecture of cells. Notably, the inflammatory characteristics in spleen and nasal mucosa of group G3 (XT-PM group), G4 (NGR-XT-PM group), G5 (Budesonide group), g3 (XT-PM relapse group) and g4 (NGR-XT-PM relapse group) were markedly reduced compared with that of group G2. Surprisingly, the inflammatory characteristics in group g3 and g4 were significantly lower than that of group g5 (Budesonide relapse group), while there was no significant difference in the inflammatory characteristics between group G2 and g5. Eosinophils can regulate local immune and inflammatory responses, which is associated with several inflammatory diseases (Fulkerson et al., 2013; Al et al., 2018). As shown in Fig. 9C, the eosinophil count of group G1, G2, G3, G4, G5, g3, g4 and g5 were 4.67 ± 2.52 , 41 ± 4.51 , 16.67 ± 4.51 , 12.33 ± 4.16 , 11.67 ± 4.51 , 25.00 ± 3.61 , 19.33 ± 4.04 , and 37.67 ± 7.51 , respectively. The eosinophils were significantly reduced in group G3 (**p < 0.01), G4

(**p < 0.01), G5 (**p < 0.01), g3 (*p < 0.05) and g4 (*p < 0.05) compared with group G2. Surprisingly, lower levels of eosinophil in group g3 (#p < 0.05) and g4 (#p < 0.05) could be seen compared with that of group g5. However, there was no significant difference in the eosinophil count between group G2 and g5. These results indicated that XT-PM, NGR-XT-PM and Budesonide could significantly inhibit inflammatory pathology and eosinophil infiltration, while XT-PM and NGR-XT-PM presented a long-term inhibitory effect.

3.6.3. Serum inflammation analysis

IgE and histamine are primary mediators involved in the development of allergic responses. Mature DCs can polarize T cells to Th2 cells accompanied by increasing IL-4 secretion and weakening Th1 cells to secrete IFN- γ . As shown in Fig. 10, OVA challenge caused a statistically significant increase of serum IgE, histamine and IL-4 levels in group G2 (AR group) compared with group G1 (Normal group, ***p < 0.001). The levels of IgE, histamine and IL-4 were significantly reduced in

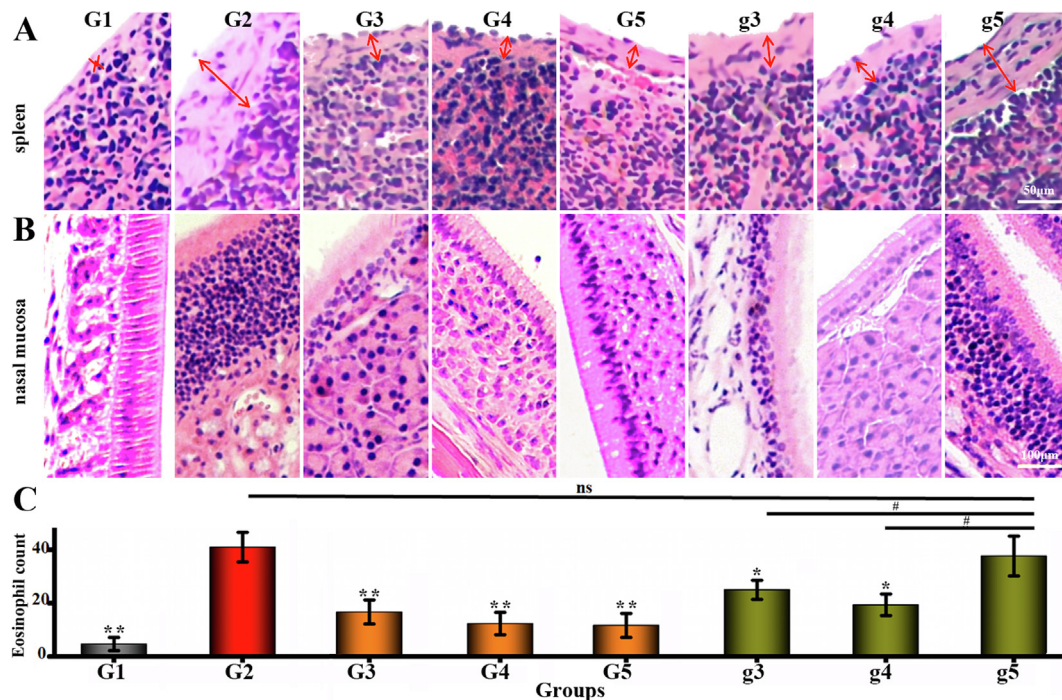


Fig. 9. Histological analysis of (A) spleen and (B) nasal mucosa ($\times 100$). Red arrows represented the thickness of the fibrous capsule. (C) Eosinophil count on the nasal mucosa. Results were expressed as mean $\pm$ S.D (n = 6). ns = not significant, *p < 0.05, **p < 0.01 vs. G2 and #p < 0.05 vs. g5. G1 (Normal group), G2 (AR group), G3 (XT-PM group), G4 (NGR-XT-PM group), G5 (Budesonide group), g3 (XT-PM relapse group), g4 (NGR-XT-PM relapse group) and g5 (Budesonide relapse group).

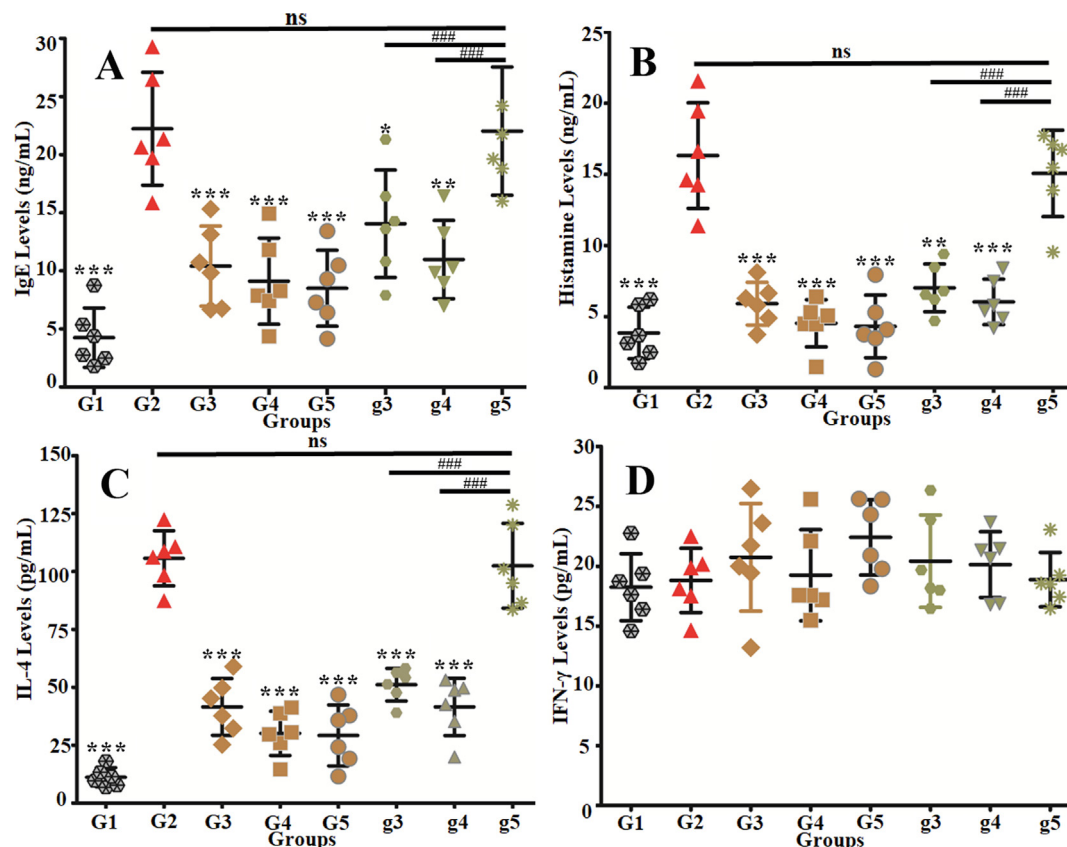


Fig. 10. The analysis of serum mediators (A) IgE, (B) Histamine and cytokines (C) IL-4, (D) IFN- γ . Results were expressed as mean $\pm$ S.D (n = 6). ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001 vs. G2 and ###p < 0.001 vs. g5. G1 (Normal group), G2 (AR group), G3 (XT-PM group), G4 (NGR-XT-PM group), G5 (Budesonide group), g3 (XT-PM relapse group), g4 (NGR-XT-PM relapse group) and g5 (Budesonide relapse group).

group G3 (XT-PM group, ***p < 0.001), G4 (NGR-XT-PM group, ***p < 0.001), G5 (Budesonide group, **p < 0.001), g3 (XT-PM relapse group, *p < 0.05) and g4 (NGR-XT-PM relapse group, **p < 0.01) compared with group G2. Surprisingly, lower levels of IgE, histamine and IL-4 in group g3 (###p < 0.001) and g4 (###p < 0.001) could be seen compared with that of group g5 (Budesonide relapse group). However, the levels of IgE, histamine and IL-4 in group g5 were similar to those in group G2. In contrast, there was no significant difference in the IFN- γ levels among all groups. These results indicated that XT-PM, NGR-XT-PM and Budesonide could significantly suppress the production of inflammation mediators and cytokines (IgE, histamine and IL-4), but had no influence on IFN- γ .

3.6.4. Phenotype analysis of DCs in spleen

DC population isolated from mice spleen were defined by CD11c⁺, and the costimulatory molecules (CD80, CD86 and I-A/I-E) on this gated population were analyzed by flow cytometry (Fig. 11A). Fig. 11B & 11C & 11D presented the levels of CD80, CD86 and I-A/I-E, which were significantly decreased in group G3 (XT-PM group, **p < 0.01), G4 (NGR-XT-PM group, ***p < 0.001), G5 (Budesonide group, *p < 0.05), g3 (XT-PM relapse group, *p < 0.05) and g4 (NGR-XT-PM relapse group, **p < 0.01) compared with group G2 (AR group). In contrast, the levels of CD80, CD86 and I-A/I-E in group g3 and g4 were lower than that of group g5, and there was statistical difference between group g4 and g5 at the levels of CD80 (#p < 0.05), CD86 (##p < 0.01) and I-A/I-E (#p < 0.05). However, the levels of CD80, CD86 and I-A/I-E in group g5 (Budesonide relapse group) were comparable to that of group G2. These results further confirmed that NGR-XT-PM could more effectively render DCs resistant to maturation by reducing the levels of CD80, CD86 and I-A/I-E than Budesonide.

3.6.5. Analysis of lymphoid immune response

To evaluate the lymphocyte immune responses, the percentages of T-helper cell subsets and Treg cells in spleen (Fig. 12), blood (Supporting Figures, Fig. S2) and lymph nodes (Supporting Figures, Fig. S3) were investigated. As shown in Fig. 12A, the percentages of Th1 cells were not significantly different among all groups. The percentages of Th2, Th9 and Th17 cells were significantly decreased in group G3 (XT-PM group, *p < 0.05), G4 (NGR-XT-PM group, **p < 0.01), G5 (Budesonide group, **p < 0.01), g3 (XT-PM relapse group, **p < 0.01) and g4 (NGR-XT-PM relapse group, **p < 0.01) compared with those in group G2 (AR group). However, the percentages of Th2, Th9 and Th17 cells in group g3 and g4 were lower than that of group g5 (Budesonide relapse group). Oppositely, the percentages of Treg cells in group G3 (*p < 0.01), G4 (***p < 0.001), G5 (***p < 0.001), g3 (**p < 0.01) and g4 (**p < 0.01) were significantly increased compared with group G2, whereas the percentages of Treg cells in group g3 (##p < 0.01) and g4 (##p < 0.01) were higher than that of group g5. Furthermore, the percentages of T-helper cell subsets and Treg cells were kept in similar abundance between group G2 and g5. Fig. 12B further presented the Th1/Th2 ratio in spleen. The Th1/Th2 ratios of group G1, G2, G3, G4, G5, g3, g4 and g5 were $65.41 \pm 5.85\%$, $15.45 \pm 6.97\%$, $44.85 \pm 10.34\%$, $53.30 \pm 6.34\%$, $57.31 \pm 5.52\%$, $31.98 \pm 3.71\%$, $39.63 \pm 3.80\%$ and $17.39 \pm 7.35\%$, respectively. The Th1/Th2 ratios of group G3 (*p < 0.05), G4 (**p < 0.01), G5 (**p < 0.01), g3 (*p < 0.05) and g4 (*p < 0.05) were significantly increased compared with group G2. Surprisingly, the Th1/Th2 ratios of group g3 and g4 were significantly higher than group g5, although there was no statistical difference between group g3 and g5. Furthermore, no significant difference was observed in the Th1/Th2 ratio between group G2 and g5. The results of lymphocyte immune responses in blood and lymph nodes were similar

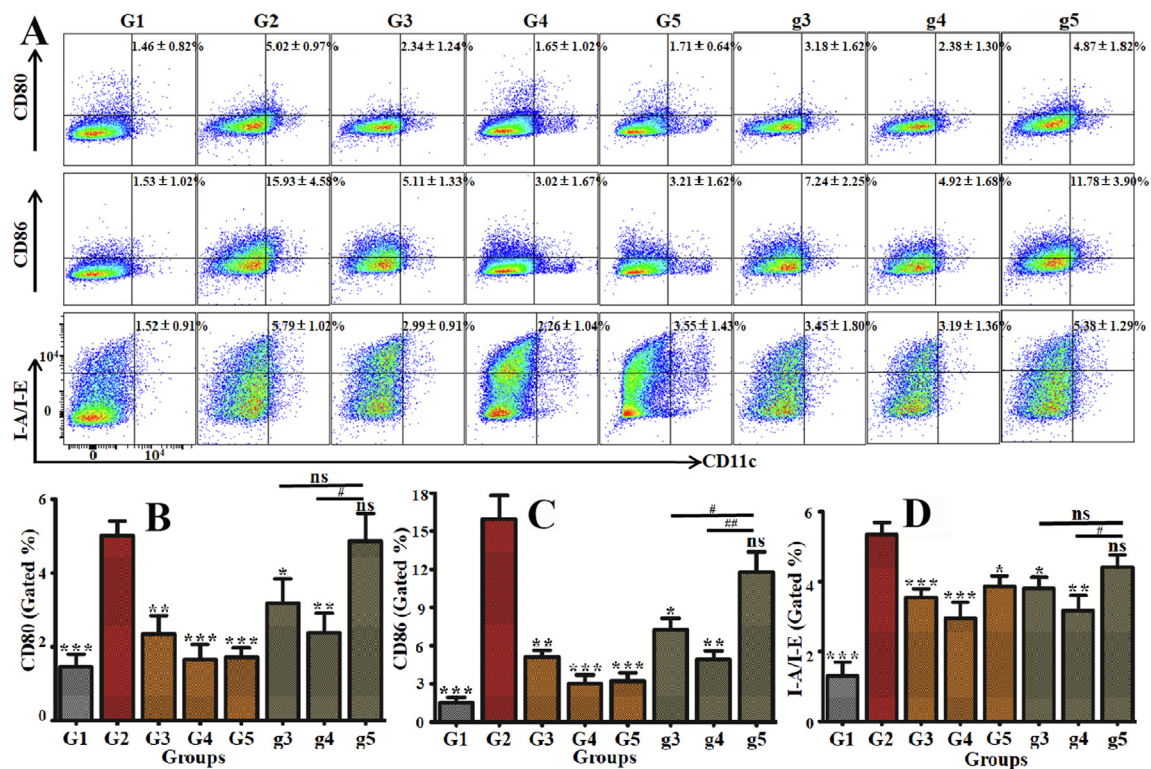


Fig. 11. Phenotype analysis of DCs in spleen. (A) Representative flow cytometry plots of CD80, CD86 and I-A/I-E. The histogram analysis of CD80 (B), CD86 (C) and I-A/I-E (D). Results were expressed as mean $\pm$ S.D (n = 6). ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001 vs. G2 and #p < 0.05, ##p < 0.01 vs. G5. G1 (Normal group), G2 (AR group), G3 (XT-PM group), G4 (NGR-XT-PM group), G5 (Budesonide group), g3 (XT-PM relapse group), g4 (NGR-XT-PM relapse group) and g5 (Budesonide relapse group).

to that of spleen. These results indicated that XT-PM, NGR-XT-PM and Budesonide possessed strong ability to restore Th1/Th2 balance and improved immune tolerance, and the mice administrated with XT-PM and NGR-XT-PM presented lower rebound of lymphocyte immune responses than that administrated with Budesonide.

3.7. In vivo distribution study

The biodistribution of such formulations were analyzed using CLSM after 4 h administration. As displayed in Fig. 13A, there was no obvious fluorescence in heart, lung and kidney, and few red fluorescent spots could be observed in brain and liver, while clear fluorescence signals were presented in the nasal mucosa, spleen and lymph node. In addition, the fluorescence intensity of DiI-PM group in nasal mucosa, spleen and lymph node was relatively weaker than those of NGR-DiI-PM group. Quantitative fluorescence results of tissue sections were shown in Fig. 13B. There was no statistical difference in the fluorescence intensity of brain, heart, liver, lung and kidney among all groups. Although there was no statistically significant difference, the fluorescence intensity of NGR-DiI-PM was approximately 1.9-fold stronger than that of DiI-PM in nasal mucosa, which may be due to drug retention and antigen-presenting DCs targeted by NGR-based delivery system. Furthermore, the fluorescence intensity of NGR-DiI-PM in spleen and lymph node was approximately 1.7-fold and 2.3-fold stronger than that of DiI-PM (##p < 0.01), which should be ascribed to the NGR-mediated delivery system targeting CD13 receptors enriched in the spleen and lymph node, especially in spleen. These results indicated that NGR-mediated delivery system loaded active agents could penetrate the nasal mucosa into the blood system and then targeted DCs in spleen and lymph node.

3.8. In silico new-found action pathway

We appealed to the work of ligand-based reverse targeting, and found cholesterol oxidase (PDB ID: 1coy, fit value = 0.97293), prostaglandin-endoperoxide synthase 2 (PDB ID: 3ntb, fit value = 0.936673) and androgen receptor (PDB ID: 1coy, fit value = 0.914947) with higher fit values. Ledock was then used to evaluate the affinity of cholesterol oxidase, prostaglandin-endoperoxide synthase 2 (COX-2) and androgen receptor to XT. Surprisingly, the grid scores were -5.63, -5.24, -4.40, respectively, which suggested XT could be a promising inhibitor of them. The optimized interaction modes were presented in Fig. 14A & 14B & 14C, respectively. Among them, COX-2 is the primary source of prostaglandin E2 (PGE₂) production, which promoted inflammation production, DCs maturation, antigens uptake and lymphocyte homing. Then, we further tested the influence of XT on the PGE₂ production by ELISA experiment *in vitro*. Fig. 14D presented the levels of PGE₂ in immature DCs treated with PBS (5.02 $\pm$ 0.48 pg/mL), mature DCs treated with LPS (16.91 $\pm$ 0.60 pg/mL), mature DCs treated with NGR-PM at the polymer dosages of 18.34 μ g/mL (16.62 $\pm$ 0.60 pg/mL), 36.68 μ g/mL (16.28 $\pm$ 0.18 pg/mL) and 73.36 μ g/mL (16.92 $\pm$ 0.46 pg/mL), and mature DCs treated with XT-PM or NGR-XT-PM at the XT dosages of 1.0 μ g/mL (16.15 $\pm$ 0.25 or 15.94 $\pm$ 0.46 pg/mL), 2.0 μ g/mL (15.63 $\pm$ 0.53 or 14.92 $\pm$ 0.68 pg/mL) and 4.0 μ g/mL (14.85 $\pm$ 0.20 or 13.23 $\pm$ 0.48 pg/mL). Mature DCs treated with LPS produced high levels of PGE₂ compared to immature DCs treated with PBS (***p < 0.001). Mature DCs treated with NGR-PM had no significant effect on the levels of PGE₂ compared to mature DCs treated with LPS. However, the levels of PGE₂ were significantly reduced in mature DCs treated with XT-PM or NGR-XT-PM compared to mature DCs treated with LPS, especially for NGR-XT-PM, which could be attributed to the fact that NGR-mediated endocytosis enhanced XT uptake. Based on the preliminary verification results, we derived the new

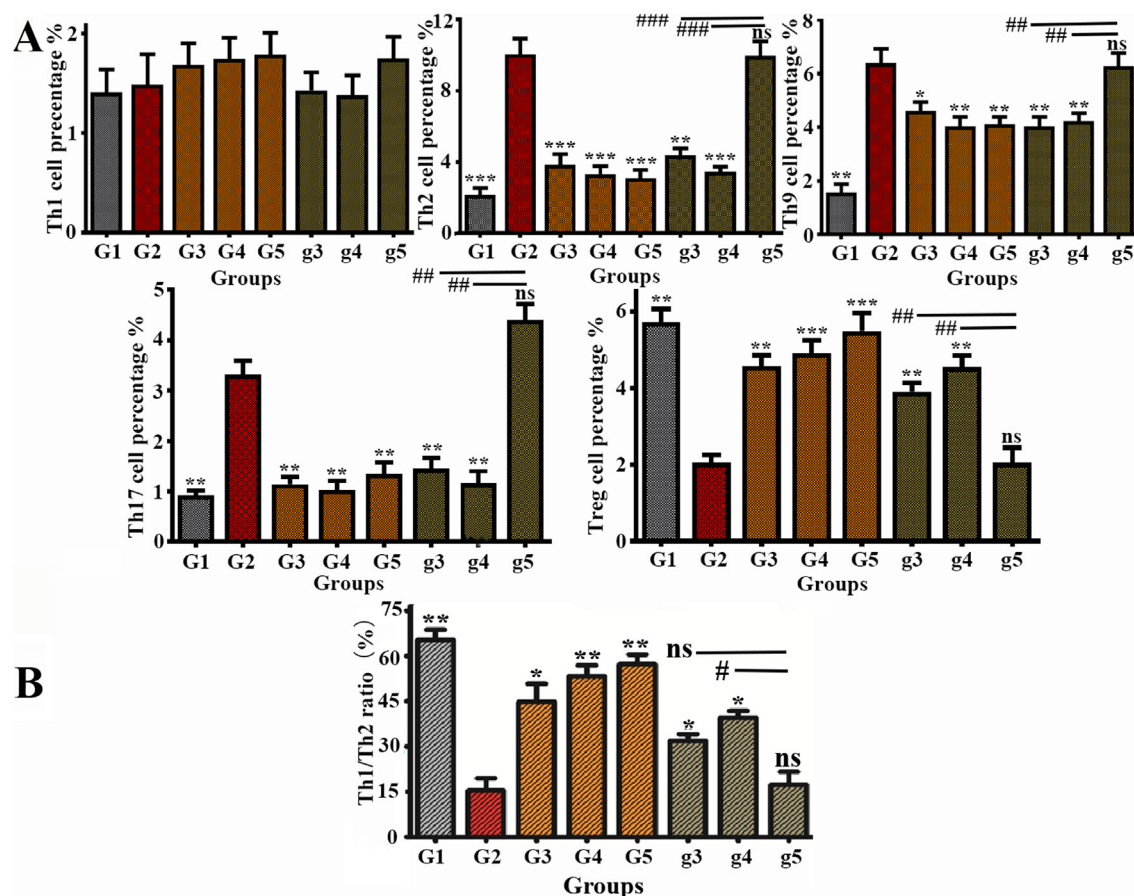


Fig. 12. (A) The percentages of Th1, Th2, Th9, Th17 and Treg cells in spleen, analyzed by flow cytometry. (B) The Th1/Th2 ratio in spleen. Results were expressed as mean $\pm$ S.D (n = 6). ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001 vs. G2 and #p < 0.05, ##p < 0.01, ###p < 0.001 vs. g5. G1 (Normal group), G2 (AR group), G3 (XT-PM group), G4 (NGR-XT-PM group), G5 (Budesonide group), g3 (XT-PM relapse group), g4 (NGR-XT-PM relapse group) and g5 (Budesonide relapse group).

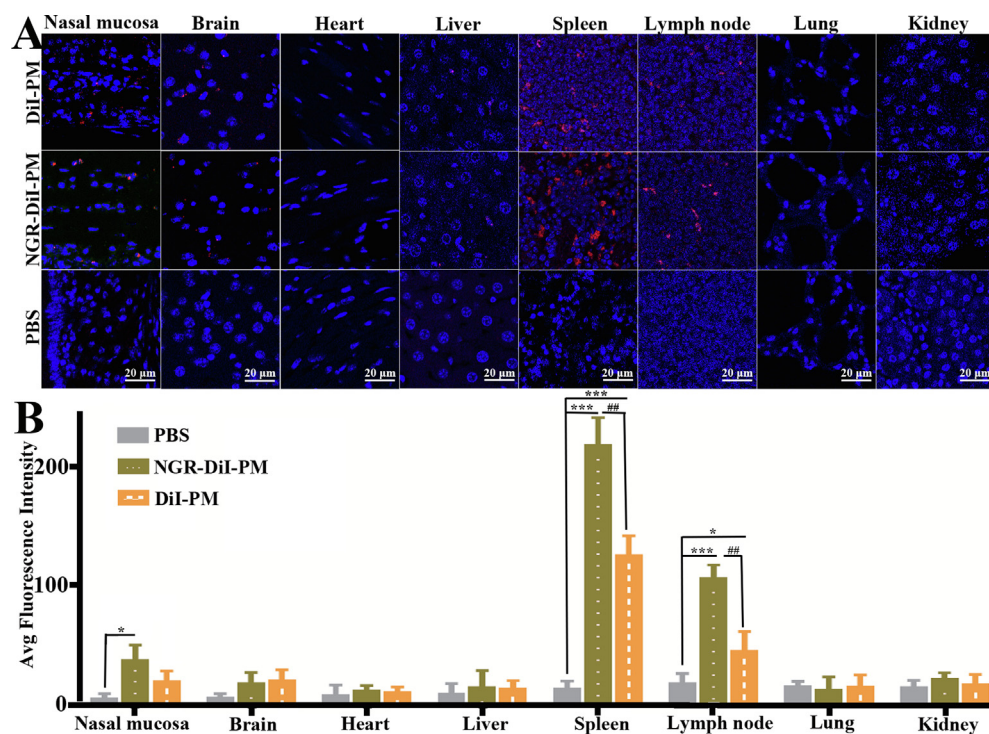


Fig. 13. Tissue distribution of DiI-PM and NGR-DiI-PM *in vivo*. (A) Representative confocal images and (B) the quantitative analysis of tissue sections including nasal mucosa, brain, heart, liver, spleen, lymph node, lung and kidney. Red fluorescence is DiI signal and blue fluorescence is DAPI signal. Scale bar was 20 μ m. Results were expressed as mean $\pm$ S.D (n = 6). *p < 0.05, ***p < 0.001 vs. PBS group and ##p < 0.01 vs. NGR-DiI-PM group.

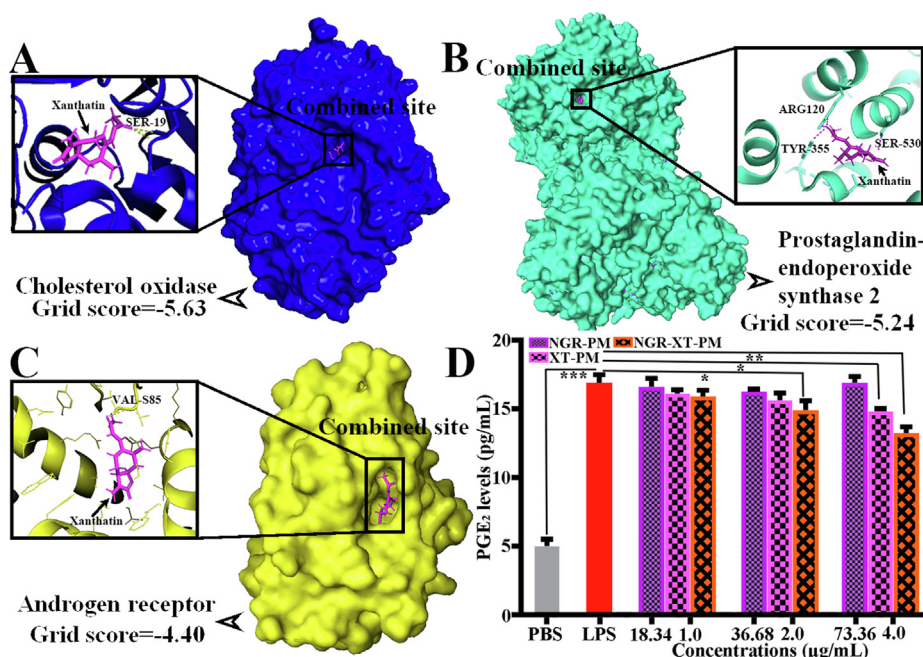


Fig. 14. Interaction modes of (A) XT and cholesterol oxidase (Grid score = -5.63), (B) XT and prostaglandin-endoperoxide synthase 2 (Grid score = -5.24), and (C) XT and androgen receptor (Grid score = -4.40), in silico. (D) The levels of PGE₂ in the culture supernatant of immature DCs treated with PBS, mature DCs treated with LPS, and mature DCs treated with NGR-PM (at the polymer dosages of 18.34, 36.68 and 73.36 µg/mL) or XT-loaded micelles (at the XT dosages of 1.0, 2.0 and 4.0 µg/mL). Results were expressed as mean ± S.D (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 vs. mature DCs treated with LPS.

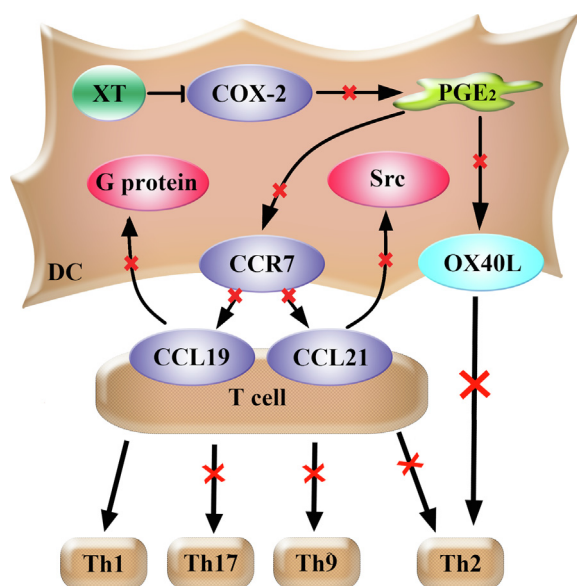


Fig. 15. Schematic illustration of immunotherapy pathways mediated by XT. XT inhibited the activation of the COX-2/PGE₂ pathway by combining with COX-2, thereby reducing the production of PGE₂ and further blocking the two downstream signaling pathways, including inhibiting CCR7-driven migration and down-regulating the expression of OX40L. 'x' represented the 'inhibition' signal.

signaling pathway of XT blocking the COX-2/PGE₂. In addition, the results of ligand-based reverse targeting also displayed high affinity to cholesterol oxidase, which can affect cell growth and metabolism, and high affinity to androgen receptor, which plays a pivotal role in the development and progression of prostate cancer, however, further research should be very interesting.

4. Discussion

AR has a complex inflammatory microenvironment involving a variety of inflammatory cytokines and inflammatory cells. Ongoing efforts have been made to eradicate AR, but the results are discouraging

and high relapse remains insurmountable. DCs play a pivotal role in the inception of allergic sensitization, modulation of T cells responses, and balancing tolerance versus inflammatory responses to allergens (Shen et al., 2014). DC-based management such as DC vaccines and interfering RNA has been developed for therapy of allergic diseases (Suzuki et al., 2018; Li et al., 2016; Sirvent et al., 2016), however, either lower gene-silencing efficiency due to non-specificity or lower cellular uptake due to macromolecules leads to limited therapeutic effects.

To address such hurdles, we constructed NGR-XT-PM nanomedicine with DCs-specific targeting function and excellent particle size to facilitate cellular uptake. In addition, long-term intracellular retention of NGR-XT-PM also prevented early exocytosis as a prerequisite for drug effectiveness. XT enhanced the resistance of DCs maturation by down-regulating the expression of the costimulatory molecules CD80, CD86 and I-A/I-E, which blocked the necessary signals for Th2 cells activation provided by DCs. All these were beneficial to dampen subsequent immune responses.

Topical challenge with OVA is reported to induce symptoms similar to human AR (Aswar et al., 2015). In the present study, OVA challenge resulted in the increase of nasal symptoms (scratching, discharging and sneezing) and serum inflammatory levels (IgE, histamine and IL-4), as well as pathological changes (spleen and nasal mucosa) in AR mice, which were in line with reported characteristics signs of AR in mice (Oh et al., 2011; Yao et al., 2017). NGR-XT-PM showed significant prevention of allergy-related changes compared with Budesonide. Th1, Th2, Th9, Th17 and Treg cells are important components of lymphoid immunity (Yao et al., 2017). After administration of NGR-XT-PM, Th2, Th9 and Th17 cells were markedly down-regulated, but there was no apparent effect on Th1 cells. However, Treg cells were significantly upregulated in mice administrated with NGR-XT-PM, which are a class of immunosuppressive T cells. Though Budesonide showed an inhibitory effect on the imbalance of Th1/Th2, the dramatic rebound after withdrawal indicated that the remission should be temporary. There was no significant difference in the therapeutic efficacy between NGR-XT-PM and XT-PM *in vivo*, but NGR-XT-PM significantly inhibited AR recurrence with 0% relapse rate compared to XT-PM with 16.7% relapse rate. In contrast, NGR-XT-PM presented significant anti-allergy effect with no recurrence. These results suggested that NGR-XT-PM should be a promising anti-inflammatory drug.

Herein, we found a new target COX-2 for XT to inhibit the

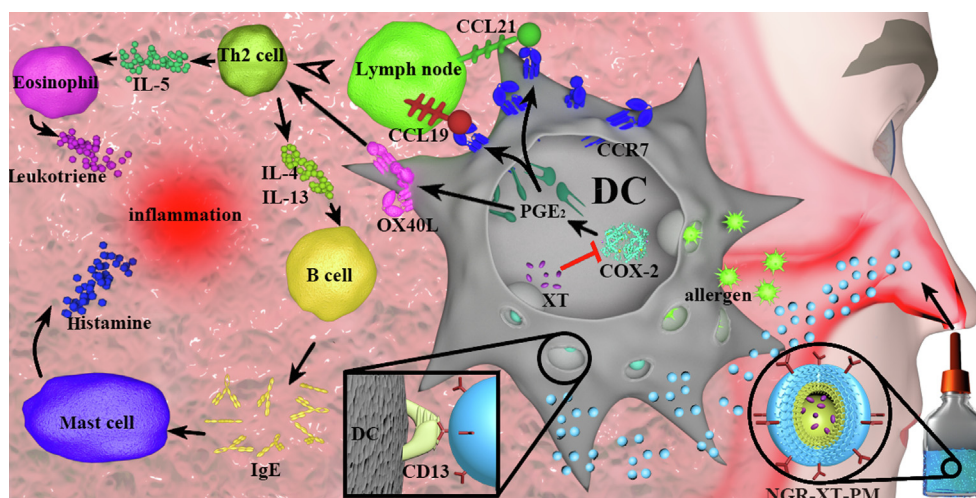


Fig. 16. Schematic illustration of the anti-inflammatory pathway of NGR-XT-PM. During allergic sensitization, DCs captured, processed, and presented allergens to lymph node, promoting excessive differentiation of Th2 cells and further triggering the inflammatory responses. After intranasal administration, NGR-XT-PM was absorbed by DCs via NGR-mediated endocytosis, and then released XT, which could combine with COX-2 to inhibit PGE₂ synthesis, thereby blocking the downstream signaling pathways and finally depleting excessive differentiation of Th2 cells and subsequent inflammatory responses.

expression of PGE₂, which is closely associated with allergic severity (Ebrahim et al., 2019). Mature DCs migration into the draining lymph nodes depended on the oligomerization of the chemokine receptor CCR7, which is stringently dependent on the presence of the proinflammatory mediator PGE₂ (Sokol et al., 2018). Furthermore, CCR7 oligomerization established a signaling hub to elicit distinct signaling pathways including G protein-dependent and Src-dependent signaling pathways controlling cell migration toward the lymph node-derived chemokines CCL19 (EBI1 ligand chemokine/macrophage inflammatory protein-3β) and CCL21 (secondary lymphoid tissue chemokine [SLC]/6CKine) (Hua et al., 2016; Hauser et al., 2016; Hauser and Legler, 2016; Guak et al., 2018). After DCs migrated to the draining lymph nodes, it interacted with T cells and directly promoted the differentiation of T cells into Th2, Th9 and Th17 cells, mainly in Th2 cells. Alternatively, PGE₂ was perceived by DCs itself, resulting in the expression of OX40 ligand (OX40L), which enabled DCs to facilitate Th2 proliferation at later phases (Kaisar et al., 2018; Krause et al., 2009). Fig. 15 was the anti-inflammatory pathway of XT, which blocked the activation of the COX-2/PGE₂ pathway to suppress inflammatory responses.

In addition, nasal administration offered lucrative way for NGR-XT-PM of both topical and systemic therapies. The high permeability, high vasculature and low enzymatic environment of nasal cavity are well suitable for NGR-XT-PM via nose. Pathological analysis of nasal mucosa and spleen all showed significant improvement in mice treated with NGR-XT-PM. In vivo distribution further demonstrated that NGR-mediated delivery system was highly selectively accumulated in the DCs-populated region of the spleen and lymph node through penetrating the vascularity of the nasal mucosa. The schematic illustration of anti-AR pathway of NGR-XT-PM was showed in Fig. 16 and video.

5. Conclusions

In summary, we successfully constructed the DCs-specific targeting nanomedicine (NGR-XT-PM) with significant immunosuppressive and anti-inflammatory effect, confirmed a new molecular pathway of XT blocking the activation of the COX-2/PGE₂, and found that intranasal administration of NGR-XT-PM presented superior anti-allergy effect than the commercial product Budesonide for therapy of refractory AR. The results can be as a reference that researchers can modify the XT to construct molecular targeted drugs, such as synthesizing XT-NGR conjugate for selectively targeting DCs to confirm its efficacy, this is also what we will do in future.

CRediT authorship contribution statement

Xue Zheng: Conceptualization, Methodology, Formal analysis,

Investigation, Data curation, Writing - original draft. **Chao Sun:** Conceptualization, Methodology, Formal analysis, Investigation, Resources. **Rilei Yu:** Software, Formal analysis. **Xin Chu:** Software, Formal analysis. **Jiahao Xu:** Software, Formal analysis. **Chengcheng Liu:** Resources, Software. **Miaoqing Zhao:** Data curation. **Xuelian Xu:** Methodology. **Ming Xia:** Resources, Data curation, Supervision, Project administration. **Cheng Wang:** Conceptualization, Methodology, Data curation, Writing - original draft, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpharm.2020.119034>.

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Supporting information

CD13-specific Ligand Facilitates Xanthatin Nanomedicine Targeting Dendritic Cells for

Therapy of Refractory Allergic Rhinitis

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1 **Supporting Methods**

2 ***S1. Determination of free NGR by HPLC***

3 Free NGR in reaction solution was determined by HPLC equipped with C18 column
4 (Diamonsil). The detection wavelength was 214 nm, 0.1% trifluoroacetic acid as mobile
5 phase A and acetonitrile-water (1:99, v/v) as phase B. The gradient program was as follows:

Time (min)	A%	B%
0	95	5
25	70	30
25.01	0	100
30	0	100

6 The column temperature was 35 °C and the flow rate was 1.0 mL/min.

7 ***S2. Determination of free XT by HPLC***

8 Free XT in the supernatant was determined by HPLC, using C18 column (Xterra®),
9 acetonitrile as mobile phase A and water as phase B. The gradient program was as follows:

Time (min)	A%	B%
0	20	80
10	20	80
40	60	40
45	60	40

10 The column temperature was 25 °C. The flow rate was 1.0 mL/min and the detected
11 wavelength was 280 nm.

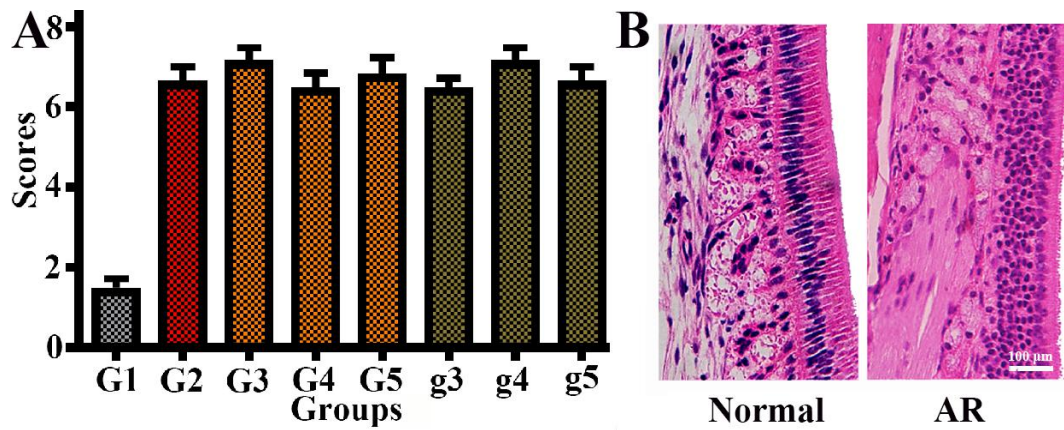
12 ***S3. Generation of murine bone-marrow derived DCs***

13 Murine bone-marrow derived DCs were generated from bone marrow of C57BL/6J mice,
14 as previously described ([Zhang C et al., 2017](#)). Red blood cells were lysed with an

15 ammonium chloride solution. The remaining cells were washed two times and re-suspended
 16 in the complete RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 µg/mL
 17 streptomycin, 100 IU/mL penicillin, 10 ng/mL recombinant GM-CSF and IL-4 at 37 °C with
 18 5% CO₂. Every 2 days, non-adherent cells were removed, and adherent cells were cultured in
 19 fresh media supplemented with 10 ng/mL GM-CSF. On the 6th day, non-adherent and loosely
 20 adherent cells were harvested.

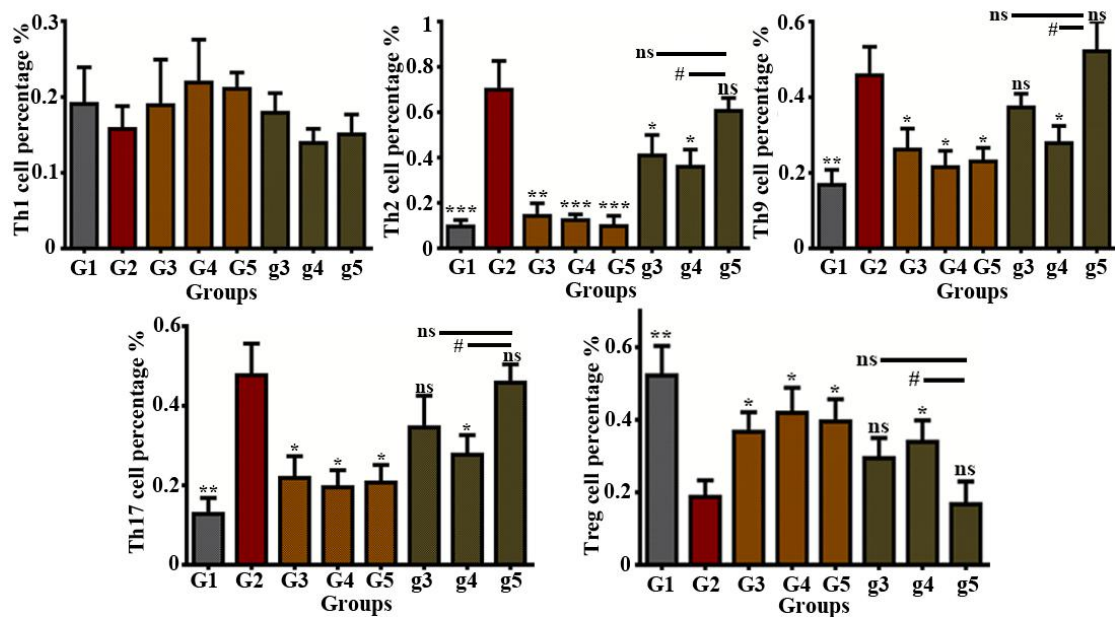
21

22 Supporting Figures



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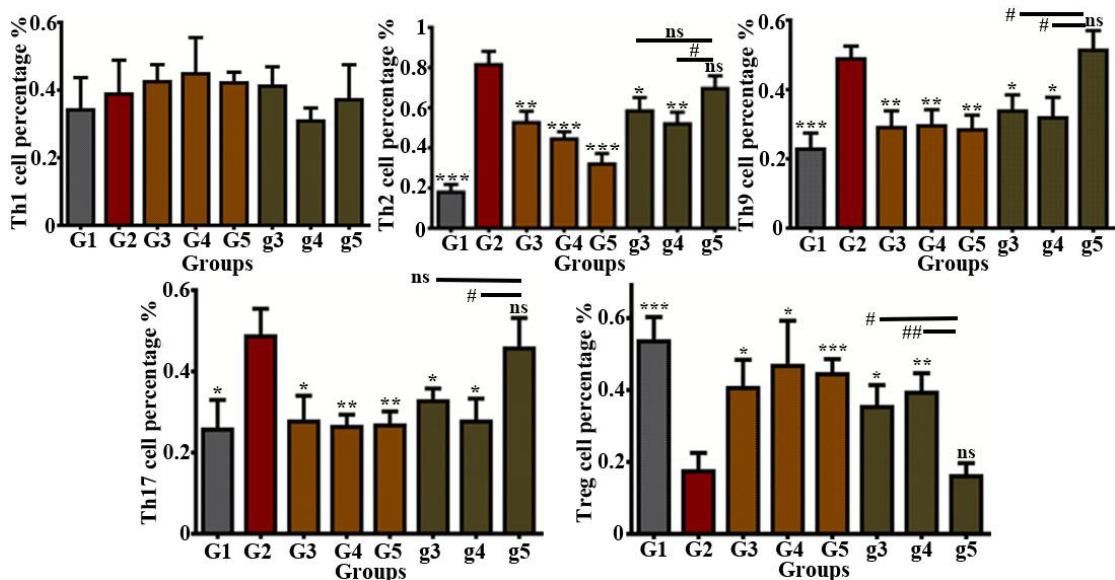
24 **Fig. S1.** Results of OVA sensitized AR mice. (A) Nasal symptom scores. Results were
 25 expressed as mean $\pm$ S.D (n = 6). G1 (Normal group), G2 (AR group), G3 (XT-PM group),
 26 G4 (NGR-XT-PM group), G5 (budesonide group), g3 (XT-PM relapse group), g4
 27 (NGR-XT-PM relapse group) and g5 (Budesonide relapse group). (B) Histological analysis of
 28 nasal mucosa ($\times 100$).



30

Fig. S2. The percentages of Th1, Th2, Th9, Th17 and Treg cells in blood, analyzed by flow cytometry. Results were expressed as mean $\pm$ S.D (n = 6). ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001 vs. G2 and #p < 0.05 vs. g5. G1 (Normal group), G2 (AR group), G3 (XT-PM group), G4 (NGR-XT-PM group), G5 (Budesonide group), g3 (XT-PM relapse group), g4 (NGR-XT-PM relapse group) and g5 (Budesonide relapse group).

36



37

Fig. S3. The percentages of Th1, Th2, Th9, Th17 and Treg cells in lymph nodes, analyzed by flow cytometry. Results were expressed as mean $\pm$ S.D (n = 6). ns = not significant, *p < 0.05, **p < 0.01, ***p < 0.001 vs. G2 and #p < 0.05, ##p < 0.01 vs. g5. G1 (Normal group), G2 (AR group), G3 (XT-PM group), G4 (NGR-XT-PM group), G5 (Budesonide group), g3 (XT-PM relapse group), g4 (NGR-XT-PM relapse group) and g5 (Budesonide relapse group).

43

44 **Reference**

45 Zhang C, Shi G, Zhang J, et al., 2017. Targeted antigen delivery to dendritic cell via functionalized
46 alginate nanoparticles for cancer immunotherapy. *Journal of controlled release*. 256, 170-181.

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