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Aerosol inhalation of inflammatory cells-targeted dendrimer-dexamethasone conjugate for efficient allergic asthma therapy

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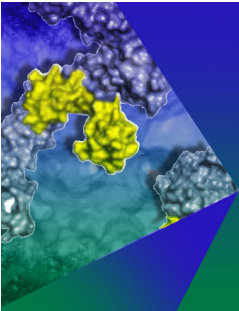
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Aerosol inhalation of inflammatory cells-targeted dendrimer-dexamethasone conjugate for efficient allergic asthma therapy

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ABSTRACT

Allergic asthma (AA) is a common breathing disorder clinically characterized by the high occurrence of acute and continuous inflammation. However, the current treatment options for AA are lacking in effectiveness and diversity. In this study, we determined that the cell membrane receptor of gamma-glutamyl transferase (GGT) was highly overexpressed on the inflammatory cells that infiltrate the pulmonary tissues in AA cases. Therefore, we developed a GGT-specific dendrimer-dexamethasone conjugate (GSHDDC) that could be administered via aerosol inhalation to treat AA in a rapid and sustained manner. The GSHDDC was fabricated by the covalent attachment of 6-hydroxy-hexyl acrylate-modified dexamethasone to polyamidoamine dendrimers via a carbonic ester linkage and the amino Michael addition, followed by the surface modification of the dendrimers with the GGT substrate of glutathione. After aerosol inhalation by the AA mice, the small particle-sized GSHDDC could easily diffuse into pulmonary alveoli and touch with the inflammatory cells via the glutathione ligand/GGT receptor-mediated recognition. The overexpressed GGT on the surface of inflammatory cells then triggers the gamma-glutamyl transfer reactions of glutathione to generate positively charged primary amines, thereby inducing rapid cationization-mediated cellular endocytosis into the inflammatory cells. The dexamethasone was gradually released by the intracellular enzyme hydrolysis, enabling sustained anti-inflammatory effects (e.g., reducing eosinophil infiltration, decreasing the levels of inflammatory factors) in the ovalbumin-induced AA mice. This study demonstrates the effectiveness of an inhalational and active inflammatory cells-targeted dendrimer-dexamethasone conjugate for efficient AA therapy.

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I. INTRODUCTION

Allergic asthma (AA) is a chronic respiratory disease that affects more than 500 million people worldwide, and its prevalence is increasing year over year, particularly in developing countries.^{1,2} Exposure of the respiratory epithelium to inhaled irritants or toxins is considered one of the main drivers of inflammation in AA cases, which is characterized by the hyperinflammation of the pulmonary system, airway hyper-reactivity, and excessive mucus secretion.²⁻⁴ These clinical symptoms are predominantly promoted by the

accumulation of inflammatory cells, including eosinophils, mast cells, and T helper type-2 (Th2) cells, as well as the secretion of proinflammatory mediators, such as interleukin-4 (IL-4), IL-5, and/or IL-13, by these inflammatory cells in the epithelium and lamina propria.⁵⁻⁷ In the past, bronchodilators and corticosteroids were administered to patients with AA to ease bronchoconstriction and inflammation of the airways, respectively, which reduced the hospitalization and mortality of these patients.⁷⁻¹⁰ Despite the seemingly positive clinical outcomes of these treatments, poor compliance

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hinders the effectiveness of these treatment options, and a subset of patients with AA, especially the child patients, are resistant to conventional therapy. Furthermore, molecular disease mediators cannot be specifically targeted to sites of inflammation, and many sufferers of asthma cannot adapt to frequent drug administration.¹ Thus, there is an urgent need for alternative therapeutic approaches that can provide both rapid and sustained efficacy to better control the exacerbations of asthma.

Dexamethasone (DXM) is a glucocorticoid steroid approved for the treatment of inflammatory conditions, including arthritis, allergic reactions, and dermatitis, to name a few.¹¹ Recently, positive results have been achieved with DXM in the treatment of ophthalmic, dermatologic, inflammatory, allergic, and autoimmune diseases.^{12–14} However, as a long-acting corticosteroid, the main limitations of DXM use in AA treatments regard the method of administration (i.e., intravenous injection) and the risk of severe adverse side effects caused by the delivered dosage. As a consequence of the small size, large functional surface area, and high kinetic stability, drug-conjugated nanocarriers are promising vehicles for delivering payloads to specific tissues or regions and enabling the controlled and sustained release of pharmaceuticals *in vivo*; therefore, fewer doses are acquired to achieve the same or even greater effectively compared to the administration of the drug alone.^{15,16} Moreover, the use of nanocarrier drug delivery systems can mitigate the toxicity and off-target effects of drugs as well as reduce the likelihood of multidrug resistance.^{16,17} However, developing a nanocarrier drug delivery system that is specific for a particular tissue or region of the body demands the identification of a stable, membrane-bound receptor that is overexpressed on the surface of the cells within the diseased region that the nanocarriers can recognize and to which they can bind.^{18,19} Gamma-glutamyl transferase (GGT) is an essential membrane protein that functions as an oxidation-reduction enzyme, is involved in protein metabolism, and is implicated in the development and progression of inflammation.²⁰ We previously developed a type of GGT-responsive active accumulating nanomedicine that underwent rapid transcytosis in tumor endothelial cells overexpressing GGT.^{15,21} We here enlighteningly hypothesized that GGT would be overexpressed on the surface of inflammatory cells in AA and sought to select it as the targeting signal of inflammatory cells for AA treatments.

Given the above, we, therefore, designed a GGT-responsive cationized dendrimer-DXM conjugate (GSHDDC) fabricated by the covalent attachment of 6-hydroxyhexyl acrylate-modified dexamethasone to polyamidoamine dendrimers via a carbonic ester linkage and the amino Michael addition, followed by the surface modification of the dendrimers with the GGT substrate of glutathione. In this strategy, we hypothesized that the GSHDDC nanocarriers could easily and directly diffuse into the pulmonary alveoli via aerosol inhalation and specifically be internalized into inflammatory cells in the pulmonary tissues via glutathione ligand/GGT receptor-mediated endocytosis, which can avoid the limitations of biological barriers and improve the sensitivity and tissue accumulation, even at lower doses compared to those currently needed for DXM administration alone. The GGT on the surface of the inflammatory cells triggered the transfer of the gamma-glutamyl moiety of glutathione, generating positively charged primary amines that induced the rapid internalization of the conjugates into the

inflammatory cells. We anticipated that DXM would then be gradually released via the enzyme hydrolysis of carbonic ester, which would enable sustained anti-inflammatory effects.

II. EXPERIMENT

A. Materials

All chemical reagents other than indicated were purchased from Sigma-Aldrich Inc. or Aladdin Reagent Inc. RPMI 1640 medium, fetal bovine serum, and 0.25% trypsin solution were purchased from GIBCO (USA). The eosinophilic isolation kit was purchased from STEMCELL Technologies Inc. The enhanced BCA protein assay kit was purchased from Beyotime Biotechnology (Nanjing, China). *N*-hydroxysuccinimide ester-Cyanine 5 (Cy5-NHS) was purchased from Xi'an Ruixi Biological Technology Co. Ltd. (Xi'an, China). The GGT antibody was purchased from Proteintech Group (USA). PEG succinimidyl propionate (Mw: 550 Da) was purchased from Shanghai Yanyi Biotechnology Corporation. Boc-gamma-Glu(otbu)-Cys(Trt)-Gly (pro-GSH) and Boc-gamma-Glu(otbu)-Gly-Gly (pro-EGG) were purchased from Qiang Yao Biotechnology (Shanghai, China).

B. Cell culture and animal

Eosinophils were isolated and purified from the rat's whole blood according to the manufacturer product description (Eosinophilic Isolation Kit, STEMCELL Technologies Inc.) and maintained in the full RPMI 1640 medium.

Male C57BL/6 mice (5–6 weeks of age) were supplied by the Laboratory Animal Center of Zhejiang Chinese Medical University and kept at 25 °C and 45% relative humidity and were exposed to 12 h light/12 h dark cycles. All animal experiments (License No. 20211101-13) were performed in accordance with the guidelines for the care and use of animals and approved by the Institutional Animal Ethics Committee of Zhejiang Chinese Medical University.

C. Synthesis and characterization of dendrimer-dexamethasone conjugate

The 6-hydroxyhexyl acrylate-modified DXM prodrug (PDXM) was first synthesized. 6-Hydroxyhexyl acrylate (1.1 g, 6.0 mmol) and dimethyl aminopyridine (2.2 g, 18.0 mmol) were suspended in dry dichloromethane (DCM; 50 ml) under an argon atmosphere. Triphosgene (0.6 g, 2.0 mmol) was added, and the mixture was stirred for 30 min at room temperature. DXM (2.4 g, 6.0 mmol, in 30 ml dry tetrahydrofuran) was added dropwise via a constant pressure funnel. The reaction mixture was stirred for 8 h during which a white precipitate was formed. After filtration and evaporating all the solvents, the residues were diluted with diethyl acetate and washed once with water and twice with brine. The organic layer was dried over anhydrous MgSO₄, filtered, and concentrated on a rotary evaporator. The crude product was purified by column chromatography using ethyl acetate as eluent (100% EtOAc) to give PDXM as a white solid powder (1.8 g, yield: 51%) and as determined by ¹H-NMR (400 MHz, CDCl₃): δ 7.23 (s, 1.0H), 6.38 (s, 2.1H), 6.11 (s, 2.0H), 5.84 (s, 1.1H), 4.94 (s, 2.1H), 4.37 (s, 1.0H), 4.16 (s, 4.0H), 3.10 (s, 1.0H), 2.62 ~ 2.26 (d, J = 91.9 Hz, 5.3H), 2.10 (s, 2.0H), 1.71 ~ 1.41 (15.0H), 1.23 (s, 1.0H), 1.06 (s, 3.0H), 0.91 (s, 3.0H).

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The dendrimer-DXM conjugates were then synthesized and characterized according to our previous work.¹⁵ Briefly, PDXM (77 mg, 130 μ mol) and PAMAM (288.3 mg, equivalent $-\text{NH}_2$ moiety 1.3 mmol) were dissolved in dimethylformamide (DMF; 20 ml) and stirred at 45 °C. The reaction was monitored by high performance liquid chromatography (HPLC). Above 95% of PDXM was conjugated with PAMAM at 24 h, and then, the solution was purified by dialysis (Mw cutoff 10 kDa) in de-ionized water (volume ratio of sample to dialysis buffer was 1/100) for 24 h by replacing the fresh water every 6 h. After freeze drying, the dendrimer-dexamethasone conjugate (DDC) was obtained (ca. 300 mg, 82% yield) with about 10% conjugation as determined by ^1H -NMR, indicating 12 drug molecules per dendrimer.

Boc- γ -Glu(otbu)-Cys(Trt)-Gly (176.3 mg, 250 μ mol), DDC (34 mg, equivalent to $-\text{NH}_2$ moiety 118 μ mol), PyBop (260 mg, 0.5 mmol), HoBt (68 mg, 0.5 mmol), and 80 μ l diisopropylethylamine were dissolved in DMF (5 ml) and stirred at room temperature for 12 h. The solution was first purified by dialysis (Mw cutoff 10 kDa) in DMF for 12 h by replacing the fresh dialysis buffer every 3 h at 4 °C. Then, the solution was dropped to tenfold volume of DCM/TFA (1/1) mixture solution and stirred for 4 h. Subsequently, the mixture was vacuumized on a rotary evaporator by using a vacuum oil pump to remove the solution. After precipitating twice in freezing ether and drying in freeze drying, the yellow solid of GSHDDC was obtained (ca. 50 mg, 73% yield). GSHDDC was kept at -20°C and dissolved in water when it was used. EGGDDC was synthesized by using Boc- γ -Glu(otbu)-Gly-Gly as the same method. PEG succinimidyl propionate (100 mg, 180 μ mol) and DDC (34 mg, equivalent to $-\text{NH}_2$ moiety 118 μ mol) were dissolved in DMF (10 ml) and stirred at room temperature for 12 h. The solution was purified by dialysis (Mw cutoff 10 kDa) in de-ionized water for 12 h by replacing the fresh dialysis buffer every 3 h at 4 °C. After dried in freeze drying, the yellow solid of PEGDDC was obtained (about 80 mg, 83% yield).

D. GGT-responsive charge-switching

GGT (10 U/ml) was added into a solution of GSHDDC or EGGDDC (equivalent to 10 mM γ -glutamyl moiety) and PEGDDC and shaken using an orbital shaker at 100 rpm and 37 °C. After incubation for a timed interval, every sample (100 μ l) was removed and transferred into 900 μ l PBS solution. The corresponding zeta potential values were measured using Zetasizer Nano (Malvern Instrument Ltd. Inc., UK).

E. In vitro cellular uptake

In 12-well plates, fluorescent molecular Cy5-labeled GSHDDC, EGGDDC, and PEGDDC (equivalent to 20 μ M DXM) were added to the cell culture of eosinophils (1×10^5 cells per well in 2 ml medium) and incubated for 0.08, 0.16, 0.32, 0.5, 1, 2, 4, and 6 h. The cells from each well were collected, washed twice with PBS, and centrifuged at 1000 rpm for 3 min. The precipitate was retained and added to 0.5 ml PBS, and a total of 2×10^4 cells were analyzed by flow cytometry (FACS Aria III Becton, Dickinson Company) to determine the uptake ratio of the nanocarriers by the eosinophils based on the differences in the mean fluorescence intensity of each sample ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 640/670 \text{ nm}$).

F. Intracellular drug releasing

GSHDDC and DXM (equivalent to 20 μ M DXM) were incubated with eosinophils for 3 h, and then, the cell culture was replaced with a fresh medium. At timed intervals, the cells were sampled, dissociated, extracted, and subjected to HPLC (Agilent 1200 series). The HPLC system consisted of a 1525 binary pump, a 2475 multi- λ -fluorescence detector, a 2998 photodiode array detector, and a SunfireTM C₁₈ ($4.6 \times 250 \text{ mm}$, 5 μ m) column. The mobile phase used was acetonitrile/water = 40/60 (v/v) at a flow rate of 1.5 ml/min at 25 °C.

G. Western-blotting analysis and immunohistochemical analysis

The lung tissues were dissected separately and lysed using the protein extraction reagent RIPA (Invitrogen, Carlsbad, CA) supplemented with phenylmethanesulfonyl fluoride (Roche). After grinding, the tissue lysate was centrifuged to obtain its supernatant. Equivalent amounts of proteins (30 μ g) from each sample were electrophoresed on a 4%–20% SDS-polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane, blocked in 5% bovine serum albumin solution for 2 h at room temperature, and incubated with the specific primary GGT antibody. The β -actin protein was used as a control. After incubation with the primary antibody, the polyvinylidene difluoride (PVDF) membrane was washed with Tris buffered saline with Tween-20 buffer and then incubated with horseradish peroxidase-linked goat antirabbit IgG secondary antibody. Prestained Color Protein Standard Marker (10–250 kDa, Thermo Fisher Scientific) was used as a molecular marker. A hypersensitivity ECL chemiluminescence kit (Beyotime Biotechnology, China) was used to detect specific bands, and the PVDF membrane was analyzed using the Azure c600 Imager (Azure Biosystem, USA). At the same time, a second dissection of the lung tissue from AA was conducted to perform immunohistochemical analysis. The tissue was dissected separately and fixed in 4% paraformaldehyde at room temperature. The tissue samples were stained with GGT antibody and analyzed by pathological examination to confirm the expression of GGT.

H. Pharmacodynamics in ovalbumin (OVA)-induced AA model

The OVA-induced AA model was established according to the reported study using male C57BL/6-mice.^{12,14} The mice were treated with a single intraperitoneal injection of a solution of OVA (80 μ g) in sterile PBS (0.1 ml) and a gel suspension of alum (4 mg) in sterile PBS (0.1 ml) three times with 7-day intervals from days 0 to 14. Then, the mice were randomly divided into six groups ($n = 7$ mice per group): healthy control (HC), AA model control (AAC), DXM, PEGDDC, EGGDDC, and GSHDDC (equivalent to 0.05 mg/kg DXM). From days 21 to 27, the mice were treated daily with either PEGDDC, EGGDDC, or GSHDDC (0.05 mg/kg) via aerosol inhalation using an aerosol micropipettor. The two control groups received sterilized PBS instead of the drugs, while the mice in the positive group received an intraperitoneal injection of DXM (3 mg/kg). On day 24, all AA model mice were given 5% OVA in sterile PBS via aerosol inhalation once daily for 40 min over four consecutive days. Mice in the HC group were given PBS at the

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same time interval. On day 28, all the mice were euthanized, and the bronchoalveolar lavage fluid (BALF) and lung tissues were collected from the mice and used for cell classification, RNA analysis, and histological examination.

I. Biodistribution

PEGDDC, EGGDDC, and GSHDDC (equivalent to 0.05 mg/kg DXM) were administrated to mice via aerosol inhalation. At the time points of 1 and 48 h, the organs lung, heart, liver, spleen, kidney, intestines, and stomach were dissected, and the fluorescence intensity in each tissue was measured using a living IMAGING SYSTEM. The wavelength of excitation and emission was set at 640 and 670 nm, respectively.

J. Bronchoalveolar lavage and staining

The mice were anesthetized by receiving an intraperitoneal injection of 4% pentobarbital sodium. PBS (0.4 ml) was injected into the left lung from the weasand three times to obtain the BALF in 24 h. Aqueous acetic acid (20 μ l of a 2% solution) was diluted with 20 μ l of the BALF to lyse erythrocyte to count the number of total inflammatory cells in the BALF. Then, the supernatant was collected by centrifuging the lysate at 4 $^{\circ}$ C and 6000 rpm for 8 min and then stored at -80° C. The residual cell sediment was resuspended in PBS and spun onto a microscope slide. Subsequently, the cells were classified and counted by electron light microscope under a 100 $\times$ oil lens with at least 200 total cells per slide after staining with Wright-Giemsa stain (Baso, BA4017).

K. Lung histological analysis

After collecting the bronchoalveolar lavage fluids, paraformaldehyde solution (0.3 ml, 4%) was injected into the left lung, and the trachea was laced up with string as tight as possible. The left lung tissue from all groups was then removed, fixed in 4% paraformaldehyde overnight, embedded in paraffin wax, and processed to be cut into 4 μ m slices. The sections stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS) were evaluated by NanoZoomer S60 (Hamamatsu, Japan). The residual lungs were isolated by dissection and divided into three lobes stored at -80° C for the analysis of mRNA expression and enzyme linked immunosorbent assay (ELISA).

L. RNA extraction and quantitative real-time polymerase chain reaction (RT-PCR)

Lysis solution (0.5 ml) was added to the middle lobe of lung tissues, which was ground into a homogenate using a high-speed, low-temperature tissue grinding machine, and the supernatant was removed following centrifugation at 10 000 rpm for 10 min. Total RNA was isolated using the SteadyPure universal RNA extraction kit, according to the manufacturer's recommendations to prepare the complementary DNA using a reverse transcription system (Accurate Biology). The relative expressions of the mRNA genes of interest were quantified using QuantStudio5 RT-qPCR system (Thermo Fisher Scientific) and SYBR[®] Green dye (Accurate Biology); β -actin was used as an internal reference. The following primer sequences (mouse) were used for PCR: β -actin

(forward)-GGCTGTATCCCCCTCCATCG, β -actin (reverse)-CCAGTTGGTAACAATGCCATGT; IL-25 (forward)-TATGAGTTGGACAGGGACTTGA; IL-25 (reverse)-TGGTAAAGTGGGACG GAGTTG; IL-33 (forward)ATTTCCCCGGCAAAGTTCAG; IL-33 (reverse)-AACGGAGTCTCATGCAGTAGA; MUC5AC (forward)-CTGTGACATTATCCCATAAGCCC; MUC5AC (reverse)-AAGGG GTATAGCTGGCCTGA; TSLP (forward)-ACGGATGGGGCTAAC TTACAA; TSLP (reverse)-AGTCCTCGATTGCTCGAACT. In addition to the quantification of the mRNA expression levels of IL25, IL33, MUC5AC, and TSLP, the protein expression levels of IL-4, IL-5, and IL-13 were determined using cytokine-specific ELISA assay kits (Thermo Fisher Scientific) and standardized to the total protein content following the manufacturer's recommendations.

M. Statistical analysis

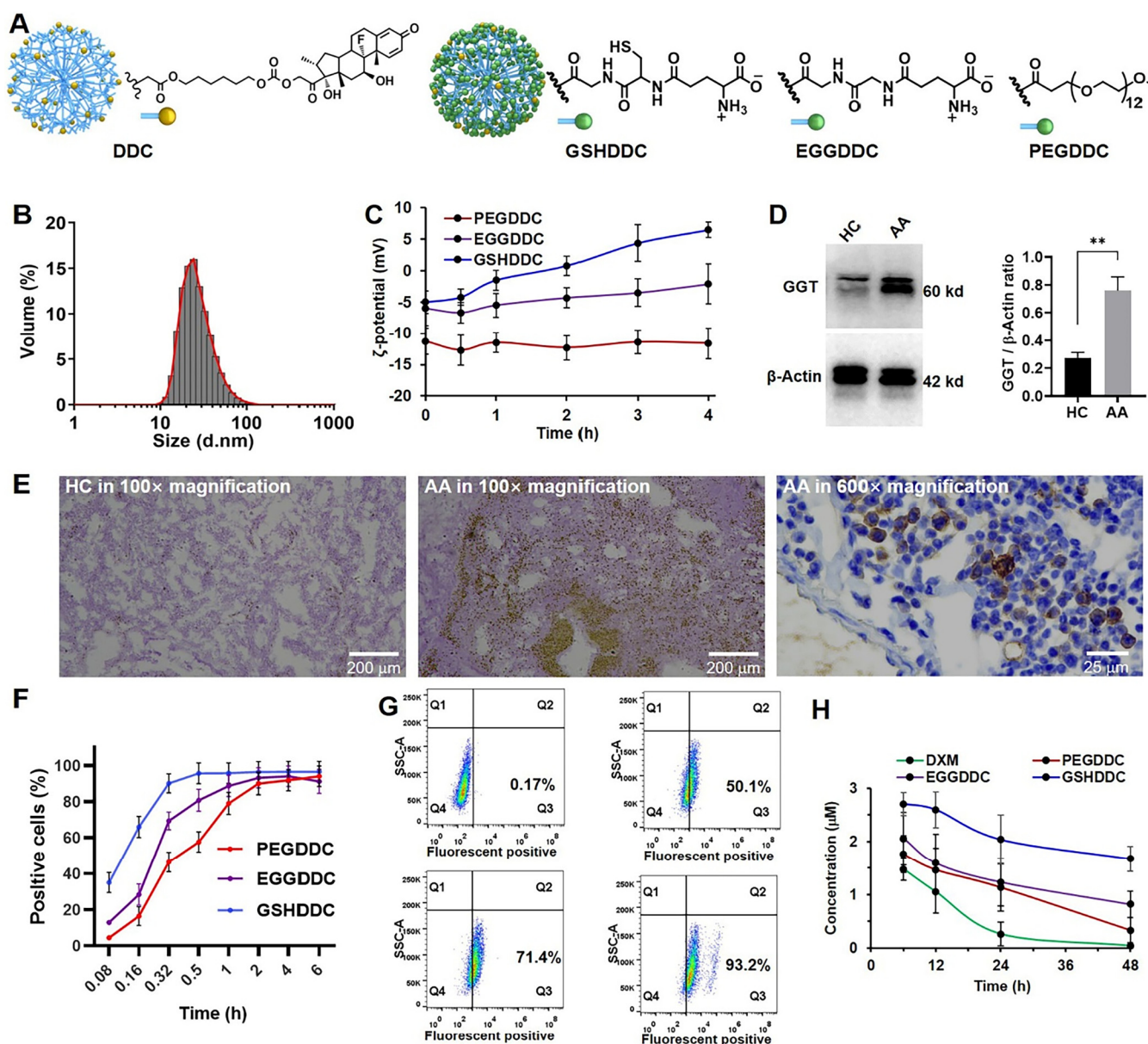
All statistical data involved in this study were collected with GraphPad Prism statistical software 8.0.1 and expressed as means $\pm$ standard deviation (SD). The cell counts and lung RT-PCR data were assessed by ordinary one-way ANOVA with Tukey's multiple comparisons test to compare the differences among the groups. In each case, the results of PEGDDC, EGGDDC, and GSHDDC groups were compared with HC or AAC mice, respectively. The significance was classified by the value of P, * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001.

III. RESULTS AND DISCUSSION

A. Design and characterization of dendrimer-DXM conjugates

To obtain the dendrimer-DXM conjugate, the DXM was first conjugated with 6-hydroxyhexyl acrylate via a carbonic ester linkage, which can not only guarantee a fully Michael reaction between the terminal alkene bond with the primary ammonia bond but also be capable of long-time drug releasing via enzymolysis of carbonic ester. The 6-hydroxyhexyl acrylate-modified DXM was then conjugated to PAMAM dendrimer by Amino Michael Addition between amines and acrylates to obtain DXM with $\sim$ 10% conjugation. The remaining amines were fully modified by GSH precursor (pro-GSH, Boc- γ -Glu(OtBu)-Cys(Trt)-Gly) and followed by deprotection to give the GGT-responsive dendrimer-DXM conjugate, GSHDDC. The GGT nonresponsive dendrimer-DXM conjugates, EGGDDC and PEGDDC, were synthesized by surface modification with a GSH-similar precursor (EGG, γ -Glu-Gly-Gly) or a classical PEG chain (Mw = 550 Da) [Fig. 1(a)]. These dendrimer-DXM conjugates formed well-dispersed nanoparticles in aqueous solution with a diameter of around 20 nm and polydispersity index around 0.1 as measured using dynamic light scattering [Fig. 1(b)]. Moreover, these dendrimer-DXM conjugates have negative charge in PBS buffer (pH 7.4), but that was significantly changed after incubation in GGT solution (10 U/ml, pH 7.4). The surface charge of GSHDDC changed from -5.9 to $+11$ mV within 3 h while those of EGGDDC and PEGDDC had no significant change [Fig. 1(c)]. This GSH-modified dendrimer-DXM conjugate exhibited much faster and more specific GGT-triggered charge-reversal property. The efficient and specific GGT-responsive

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FIG. 1. *In vitro* characterization of the dendrimer-DXM conjugates. (a) Chemical structure of dendrimer-DXM conjugates: DDC, GSHDDC, EGGDDC, and PEGDDC. (b) Size distribution determined by dynamic light scattering. (c) Changes in the zeta potential of the conjugates after incubation with 10 U/ml GGT within 4 h. (d) Western-blotting results of the expression of GGT in the lung tissues of the AA model. (e) Immunohistochemical staining of GGT in the lung tissues of AA model. (f) Cellular uptake of the cy5-labeled conjugates in eosinophils. (g) Positive ratio of the conjugates-internalized cells at 20 min. (h) Concentration of free DXM in eosinophils within 48 h after one-time incubation with the DXM and the conjugates.

charge-reversal of GSHDDC would make it more effective to induce cationization-initiated endocytosis in GGT-overexpressed cells.

B. GGT protein expression in lung tissues of AA model

Inflammatory cells are central to the pathology of body's natural defense response to injury or disease including infectious

diseases, allergy, cancer, and autoimmunity. However, their immunomodulation is always a two-edged sword, as both hypersensitive and hyposensitive of immunoreactions induce disorders of immunologic functions. Targeting the inflammatory cells at sites of inflammation is the best effective strategy for disease treatment. Therefore, there is an urgent need to screen the targeting signal of

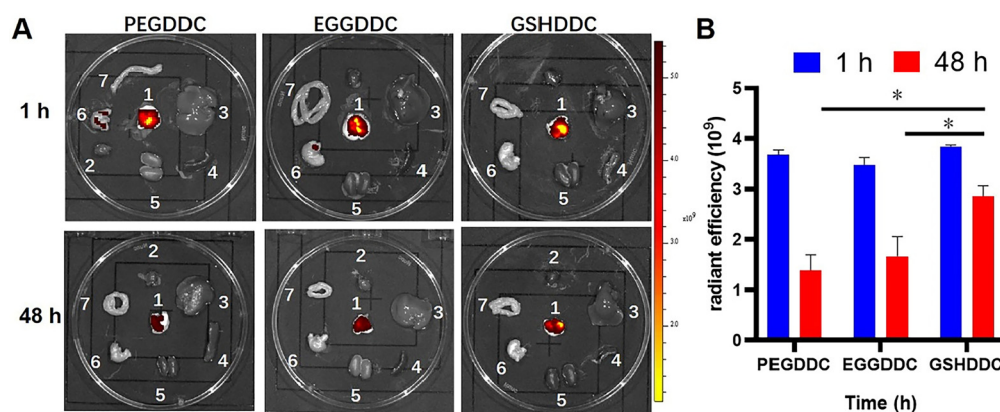


FIG. 2. Biodistribution of the dendrimer-DXM conjugates in AA mice after aerosol inhalation. (a) Fluorescent images of the cy5-labeled conjugates at 1 and 48 h in AA mice. 1: lung, 2: heart, 3: liver, 4: spleen, 5: kidney, 6: stomach, 7: intestine. (b) Fluorescence intensity of lung tissue retention was quantitatively analyzed by LIVING IMAGE-4.5 software. The radiant efficiency was presented as means $\pm$ SD, * $P < 0.05$.

inflammatory cells for AA treatments. Western-blotting assay was primarily performed to investigate the expression of GGT protein in lung tissue of the AA model. The relative-expression of GGT in lung tissue of AA model is approximately twofold higher than that of health mice, [Fig. 1(d)], indicating the GGT would be a good potential receptor for AA-targeting therapy. In addition, immunohistochemical analysis was further performed to investigate the distribution of the GGT protein expression. In the immunohistochemical staining images, the expression of GGT protein has significant increase in the AA model and mostly distributed in the inflammatory cells, including neutrophils, eosinophils, macrophages, and mast cells [Fig. 1(e)], confirming the GGT would be a good potential receptor for inflammatory cells-targeting in AA therapy.

C. Cellular uptake and release profile of DXM in inflammatory cells

The cellular uptake of the PEGDDC, EGGDDC, and GSHDDC was evaluated using eosinophils to determine the functional properties of conjugates. The results show that with the prolongation of time, the proportion of cells engulfing conjugates gradually increased. The 100% cellular uptake of GSHDDC, EGGDDC, and PEGDDC, respectively, tends to be finished at about 20 min, 1 h, and 2 h [Fig. 1(f)], indicating the fast internalization of GSHDDC. Moreover, the results of flow cytometry analysis show the GSHDDC can achieve 93.2% cellular uptake at 20 min, which is significantly higher than that of PEGDDC (50.1%) and EGGDDC (71.4%) [Fig. 1(g)], proving that the internalization of GSHDDC occurs via GGT-triggered cationization-mediated cellular endocytosis into the inflammatory cells. The intracellular drug release was then investigated using the HPLC. As shown in Fig. 1(h), after one-time incubation with equal drug dose, the GSHDDC not only has enough drug internalization but also has long time of drug release and retention. The free DXM concentration in GSHDDC-treated cells was 2–10-fold higher than that of EGGDDC, PEGDDC and DXM solution at 48 h post-treatments.

The DXM can be released after enzymolysis of carbonic ester, suggesting a prolonged pharmaceutical effect.

D. Biodistribution of the conjugates

A biodistribution study was conducted in the AA mice model to assess the *in vivo* delivery of the conjugates to the lung and the ability of the conjugates to be retained in the lung after aerosol inhalation.^{22,23} All of the three conjugates were obviously distributed into lung tissue after one hour administration, and nearly negligible conjugates were found in the other tissues (Fig. 2), indicating remarkable lung specificity distribution. Delivering precise dosages of inflammatory modulatory drugs with spatiotemporal control to the target tissues and cells, while avoiding unwanted off-target distribution, is indispensable for ensuring a safe and effective anti-inflammatory response. As time going on, the fluorescent intensity of GSHDDC still kept strong, while that of EGGDDC and PEGDDC degrade obviously at 48 h, indicating the long retention ability of GSHDDC in lung. Given that GSHDDC could be rapidly internalized into inflammatory cells, the GSHDDC would keep a long-time controlled-release of drug in the lung. Most of the nanodrugs are administrated via intravenous injection and oral administration, which are always hindered by a series of biological barriers of blood circulation, tissues accumulation and penetration, cellular internalization, and drug release, and resulting in low delivery efficiency. In contrast, aerosol inhalation can make the nanodrugs easily diffuse into pulmonary alveoli and directly touch with the inflammatory cells with a higher delivery efficiency, which avoids the biological barriers in blood circulation, tissues accumulation, and penetration.

E. Pharmacodynamics of conjugates on AA mice model

The therapeutical effects of the conjugates were then investigated in the OVA-induced C57BL/6 AA mice, whose phenotype

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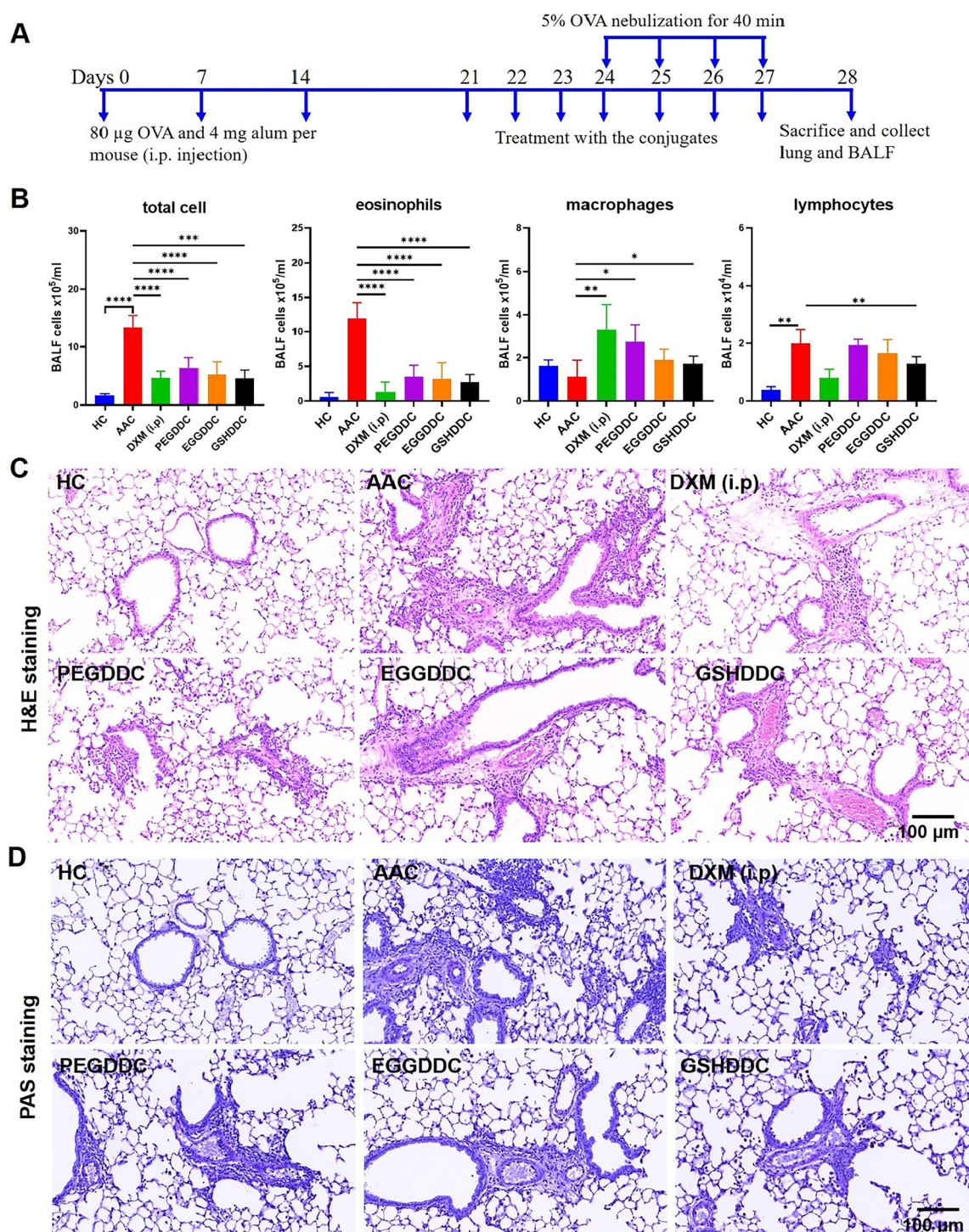


FIG. 3. Pharmacodynamics of the dendrimer-DXM conjugates in AA mice after aerosol inhalation. (a) Schematic design of the experimental procedures. (b) Number of total leukocyte and inflammatory cells in the BALF obtained from the mice in the different treatment groups. Histological analysis of the lung sections in the different treatment groups after H&E staining (c) and PAS staining (d). Scale bar = 100 µm. In each case, the results of PEGDDC, EGGDDC, and GSHDDC groups were compared with the HC or AAC groups. The significance was classified by the P value: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

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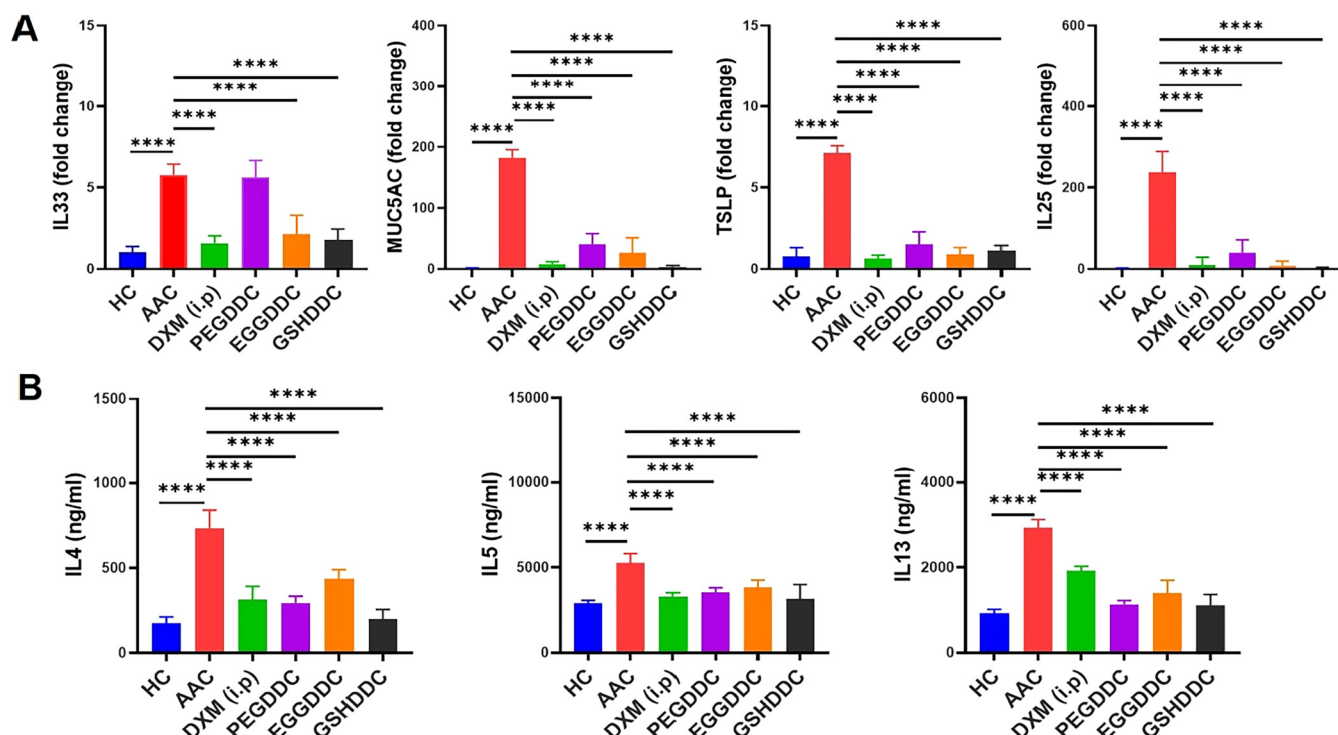


FIG. 4. Cytokine analysis after treatment with the dendrimer-DXM conjugates in AA mice. (a) mRNA expression levels of IL25, IL33, MUC5AC, and TSLP in the lung tissue homogenates determined by RT-qPCR. (b) Protein expression levels of IL-4, IL-5, and IL-13 in the lung tissues homogenates determined by ELISAs.

was characterized by excessive airway inflammation and infiltration of eosinophils.^{12,14} According to the protocol in Fig. 3(a), OVA and alum were administered to the mice were administered for 14 days to establish the AA mouse model, after which the mice were randomly divided into six groups ($n = 7$ mice in each group): healthy control (HC, PBS); AA model control (AAC, PBS); DXM (3 mg/kg); and PEGDDC, EGGDDC, and GSHDDC experimental (equivalent to 0.05 mg/kg DXM) groups. From days 21 to 27, DXM was daily administrated to the mice via intraperitoneal injection, while the conjugates and sterile PBS were administrated daily via aerosol inhalation. On day 24, the AA model mice received 5% OVA daily for 40 min for four consecutive days. Finally, on day 28, all mice were euthanized, and the BALF was collected from the lungs of the mice to conduct cell classification, mRNA analysis, and histological examination.

To assess the effects of OVA and alum on the extent of airway inflammation in the AA model mice, the quantities of total leukocytes and inflammatory cells in the airways of the mice were determined. The mice in the AAC group had fourfold higher amounts of leukocytes than the mice in the HC group, and thousands of eosinophilic granulocytes were observed in the airway of the mice in the AAC group [Fig. 3(b)]. These results indicated that the OVA-induced AA mouse model was successfully established. After treatment of the mice with DXM and the three conjugates, the number of total leukocyte cells and eosinophilic granulocytes

dramatically decreased compared with the mice in the AAC group. The changes in the number of lymphocytes present in the airway in each group were consistent with the number of eosinophils, but the opposite trend was observed with the number of macrophages in the BLAF. The therapeutic efficacy of GSHDDC (0.05 mg/kg) in reducing airway inflammation was similar to DXM (3 mg/kg), despite the 60-fold lower dose of the GSHDDC compared to DXM, indicating the high efficacy on anti-inflammation of the GSHDDC. H&E staining images showed that airway inflammation had extended to the lung in the AA model mice, which was characterized by eosinophil infiltration, goblet cell proliferation, and mucosal hyperemia. Encouragingly, a sharp decrease in inflammation cell infiltration was observed in the lung tissue of the mice treated with the conjugates and DXM [Fig. 3(c)]. PAS staining images showed the excessive production of mucus in the lung tissues of mice in the AAC group. After treatment with GSHDDC, the mucus production significantly decreased and to a greater extent than in the mice treated with DXM, PEGDDC, and EGGDDC [Fig. 3(d)]. The histopathologic data of lung tissues demonstrated that aerosol inhalation of GSHDDC could effectively reduce the number of leukocytes, especially eosinophils, and mucus production, even at a lower dose, compared to DXM.

Cytokines always regulated the activation, aggregation, and degranulation of eosinophils.^{5–7} To further evaluate the ability of the conjugates to attenuate the level of cytokines, the mRNA

expression levels of the inflammation-related factors IL25, IL33, MUC5AC, and TSLP in the lungs were measured by RT-qPCR [Fig. 4(a)], while the protein expression levels of IL4, IL5, and IL13 in the lungs were measured using ELISAs [Fig. 4(b)]. Compared to the AAC group, the expression levels of IL4, IL5, IL13, IL25, IL33, MUC5AC, and TSLP were all significantly reduced in the mice treated with PEGDDC, EGGDDC, and GSHDDC. Moreover, some of the inflammation-related factors (e.g., IL33, MUC5AC, and IL25) in the mice after treatment with GSHDDC even recovered to levels similar to those of the mice in the HC group, confirming the potent anti-inflammatory effects of GSHDDC in AA therapy.

IV. CONCLUSIONS

In summary, we determined that GGT was overexpressed in inflammatory cells in the lung tissues of AA mice, and we developed a GGT-selective, dendrimer-DXM conjugate (GSHDDC) to treat the inflammation associated with AA. Following aerosol inhalation, the GSHDDC could easily and rapidly diffuse into the pulmonary alveoli and be taken up by inflammatory cells in the pulmonary tissue via the glutathione ligand/GGT receptor-mediated endocytosis. Furthermore, DXM was gradually released from the GSHDDC via the intracellular enzymatic hydrolysis, enabling sustained anti-inflammatory effects in the pulmonary tissue of the AA mice. This study provided a promising strategy for the treatment of AA, especially for the populations of people who either do not respond to or who are poorly compliant with current treatment regimens and options.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Ethics Approval

All animal experiments (License No. 20211101-13) were performed in accordance with the guidelines for the care and use of animals, and approved by the Institutional Animal Ethics Committee of Zhejiang Chinese Medical University.

Author Contributions

Danfei Chen: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Validation (equal); Visualization (equal); Writing – original draft (equal). **Xiaobo Xuan:** Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Validation (equal). **Yuyan Chen:** Data curation (equal); Investigation (equal); Methodology (equal); Validation (equal).

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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