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•Original article•

Exposure to ephedrine attenuates Th1/Th2 imbalance underlying OVA-induced asthma through airway epithelial cell-derived exosomal lnc-TRPM2-AS

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[ABSTRACT] Although various anti-inflammatory medications, such as ephedrine, are employed to manage cough-variant asthma, their underlying mechanisms are yet to be fully understood. Recent studies suggest that exosomes derived from airway epithelial cells (AECs) contain components like messenger RNAs (mRNAs), micro-RNAs (miRNAs), and long noncoding RNA (lncRNA), which play roles in the occurrence and progression of airway inflammation. This study investigates the influence of AEC-derived exosomes on the efficacy of ephedrine in treating cough-variant asthma. We established a mouse model of asthma and measured airway resistance and serum inflammatory cell levels. Real-time polymerase chain reaction (RT-qPCR), Western blotting, and enzyme-linked immunosorbent assay (ELISA) analyses were used to assess gene and protein expression levels. Exosomes were isolated and characterized. RNA immunoprecipitation (RIP) and RNA pull-down assays were conducted to examine the interaction between hnRNPA2B1 and lnc-TRPM2-AS1. In the ovalbumin (OVA)-challenged mouse model, ephedrine treatment reduced inflammatory responses, airway resistance, and Th1/Th2 cell imbalance. Exosomes from OVA-treated AECs showed elevated levels of lnc-TRPM2-AS1, which were diminished following ephedrine treatment. The exosomal lnc-TRPM2-AS1 mediated the Th1/Th2 imbalance in $CD4^+$ T cells, with its packaging into exosomes being facilitated by hnRNPA2B1. This study unveils a novel mechanism by which ephedrine ameliorates OVA-induced $CD4^+$ T cell imbalance by suppressing AEC-derived exosomal lnc-TRPM2-AS1. These findings could provide a theoretical framework for using ephedrine in asthma treatment.

[KEY WORDS] Asthma; Ephedrine; Exosomal lnc-TRPM2-AS1; Th1/Th2 imbalance; HnRNPA2B1; Airway epithelial cells
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Introduction

Asthma is a prevalent chronic airway condition marked by pulmonary inflammation, airway remodeling, and shedding of airway epithelial cells [1]. It is triggered by various factors, including allergen exposure, genetic predispositions, and microbial infections [2]. Asthma manifests in several forms, such as allergic asthma, cough-variant asthma, and exercise-induced asthma [3]. Among these, cough-variant asthma is commonly managed with anti-inflammatory medications, including β2 receptor agonists, leukotriene receptor antagonists, and corticosteroids; these are often used in combination with inhaled corticosteroids and ephedrine [4]. However, the use of ephedrine in treating asthma is restricted in many patients due to varying efficacies.

Ephedrine, a sympathomimetic drug, functions as both an α- and β-adrenergic agonist, stimulating the central nervous system, raising blood pressure and heart rate, and dilating bronchial tubes [5]. Historically, ephedrine has been

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employed in the management of asthma [6]. Previous research has shown that combining ephedrine and Riparin II improves inflammation and airway remodeling in asthmatic rats [7]. Additionally, due to its anti-allergic properties, ephedrine is a component of various cough syrups and traditional medicines, like Kracie, used to treat allergies, including the common cold and cough-variant asthma [4]. Nevertheless, the specific mechanisms by which ephedrine mitigates cough-variant asthma remain poorly understood. This study aims to elucidate the regulatory mechanisms of ephedrine on asthma, providing more precise insights into its therapeutic potential.

The process of airway hypersensitivity and inflammation in asthma involves immune cells and airway epithelial cells (AECs), which are pivotal in mediating this response [8]. When stimulated by allergens, AECs can influence immune effector cells by secreting cytokines such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), thereby participating in allergic airway inflammation [9]. Recent research has further demonstrated that exosomes derived from AECs, containing various components, including mRNA and cytokines, play a significant role in the progression and onset of airway inflammation [10]. For instance, AEC-derived exosomes have been shown to activate macrophage-regulated allergic inflammation [10]. Additionally, a study by ZHANG *et al.* revealed that exosomal contactin-1 from AECs could activate monocyte-derived dendritic cells, inducing allergic responses in asthma [11]. A crucial aspect of asthma pathophysiology is the imbalance between $CD4^+$ T helper 1 (Th1) and Th2 cells, which drives the immune response [12]. In asthma patients, $CD4^+$ T cells differentiate into Th1 cells under IL-12 stimulation, whereas IL-4 activates the signal transducer and activator of transcription 6 (STAT6)/GATA3 pathway, promoting their differentiation into Th2 cells [13]. The heightened Th1 and Th2 responses, particularly from Th2 cells, lead to the secretion of proinflammatory cytokines such as interleukin (IL)-4, IL-5, IL-9, and IL-13. These cytokines contribute to airway inflammation, mucosal hyperplasia, methacholine responsiveness, and airway remodeling [14]. However, the potential regulatory role of AEC-derived exosomes on $CD4^+$ T cell differentiation and their involvement in the mechanisms by which ephedrine mediates asthma treatment remain unclear.

In this study, we discovered that ephedrine mitigates the effects of ovalbumin (OVA)-induced exosomal long noncoding RNA (lnc)-TRPM2-AS1 from AECs in asthma, thereby restoring the Th1/Th2 imbalance in $CD4^+$ T cells. This finding provides a theoretical basis for employing ephedrine in asthma treatment.

Materials and Methods

Animal studies

All animal experiments conducted in this study received approval from the Ethical Committee of Hunan University of Chinese Medicine （License No. SCXK [Jing] 2016-0006）. Female balb/c mice (approximately six weeks old, weighing 20–25 g, sourced from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were housed in a controlled animal facility. A total of 15 mice were randomly allocated into three groups: (1) the control group, (2) the OVA group, and (3) the OVA + ephedrine group. For the control group, mice were administered an intraperitoneal injection of sterile 0.9% saline on days 0 and 14 and subjected to inhalation of 0.9% saline for 30 min, three times per week from weeks 3 to 10. In the OVA-induced asthma model group, mice were sensitized with OVA (20 μg combined with 2 mg Inject Alum Adjuvant in 200 μL of sterilized 0.9% saline, Grade V, Sigma-Aldrich, St Louis, MO, USA) on days 0 and 7. These mice were then exposed to a 5% aerosolized OVA in saline for 30 min, three times per week from weeks 1 to 3. In the OVA + ephedrine group, following the same sensitization and challenge protocol as the OVA group, the mice received oral forced feeding of 0.2 $mmol \cdot kg^{-1}$ ephedrine (Sigma-Aldrich, St. Louis, MO, USA) on days 21, 23, 25, 27, and 29. Subsequent analyses were performed on these mice.

Assessment of airway resistance

Twenty-four hours following the treatment, mice were anesthetized using a combination of ketamine and xylazine. They were then ventilated with a volume-cycled ventilator (FlexiVent, SCIREQ Scientific, Montreal, QC, Canada) set to maintain a positive-end expiratory pressure of 2 cm H_2O. To evaluate airway hyperresponsiveness, the animals were exposed to aerosolized methacholine at increasing concentrations (0, 3.125, 6.25, 12.5, 25, 50, and 100 $mg \cdot mL^{-1}$). Airway resistance was measured using the "snapshot" protocol *via* FlexiVent software, alongside Pulmodyn data-acquisition software (Hugo Sachs Electronic). Following the inhalation of each methacholine dose, airway resistance for each mouse was recorded. Lung resistance values were calculated using the single-compartment model, from which dose-response curves were derived to assess the airway's responsiveness to methacholine.

Measurement of inflammatory cell counts and cytokine detection via enzyme-linked immunosorbent assay (ELISA)

Bronchoalveolar lavage fluid (BALF) samples were collected from the right lungs of mice from each experimental group. This collection was facilitated by flushing with phosphate-buffered saline (PBS) using a 1 mL syringe connected to a cannula, performed under anesthesia one day post-treatment, and repeated three times. After centrifugation of these samples, the supernatant was reserved for cytokine analysis *via* ELISA, and the cell pellet was resuspended in PBS for cellular analysis. Total leukocytes, lymphocytes, eosinophils, and neutrophils were counted using microscopic evaluation, with distinct cell types identified based on their morphological characteristics. The concentrations of inflammatory cytokines in the BALF supernatants were quantified using specific ELISA kits: murine IL-12 (M1270, R&D Systems, NE Minneapolis, MN, USA), mouse γ-IFN (KMC4021, Thermo Fisher Scientific, Waltham, MA, USA), mouse IL-4 (BMS613, Thermo Fisher Scientific), and mouse IL-13

(BMS6015, Thermo Fisher Scientific). These assays were conducted following the manufacturer's recommended protocols.

Histological examinations (hematoxylin-eosin (HE), Masson's trichrome (Masson), and Periodic Acid Schiff (PAS))

Airway tissues from mice were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned into 5 μm slices for histological analysis. The sections underwent hematoxylin and eosin staining as follows: sections were incubated with hematoxylin (Sigma-Aldrich) for 3 min and then rinsed for 1 min. Subsequently, they were stained with eosin (Sigma-Aldrich) for 45 s. After dehydration and mounting, the sections were examined under a microscope. For Masson's trichrome staining, we used the Trichrome Stain Kit (Sigma-Aldrich) according to the manufacturer's instructions. The stained sections were then photographed using a light microscope. PAS staining was performed using a PAS staining kit (Beijing Solarbio Life Sciences Co., Ltd., Beijing, China), adhering to the provided manual. The resulting stained sections were visualized under a microscope to assess mucopolysaccharide accumulation in the airway tissues.

Serum OVA-specific IgE and total IgE

Two days following the final OVA challenge, blood samples were collected from the mice *via* the inferior vena cava. The serum was then assessed for anti-OVA-specific antibodies using an ELISA protocol. Initially, microtiter plates (Nunc, Roskilde, Denmark) were coated with 2 μg·mL^{-1} of OVA dissolved in 50 mmol·L^{-1} carbonate buffer and incubated overnight at 4 °C. After blocking the plates with 2% bovine serum albumin (BSA, Gibco, Grand Island, NC, USA), the samples were incubated with horseradish peroxidase-labeled goat anti-mouse IgE (Pharmingen, San Diego, USA) at 20 °C for 1 h. The reaction was developed, and the optical density was subsequently measured at 490 nm to quantify the level of OVA-specific IgE.

Total serum IgE levels were determined using the IgE ELISA kit (555248, BD Biosciences, Erembodegem, Belgium) following the manufacturer's instructions. This assay allowed for the accurate measurement of overall IgE concentrations in the serum, providing insights into the systemic immune response of the mice.

Flow cytometry

CD4$^+$ T cells were isolated from airway tissue and washed twice with phosphate-buffered saline (PBS, Gibco). The cells were then seeded in 24-well plates and allowed to adhere for approximately 24 hours. For surface marker staining, the cells were treated with anti-mouse FITC-CD4 Antibody (11-0042-82, Thermo Fisher Scientific) and incubated for 15 min at room temperature. Subsequent intracellular staining involved PE anti-mouse IFN-γ Antibody (505807, Biolegend, San Diego, CA, USA) and or PE anti-mouse IL-4 antibody (504103, Biolegend), each for more than 1 hour at 4 °C. AECs were stained with PE anti-mouse IL-13 antibody (159403, Biolegend) for more than 1 hour at 4 °C and APC anti-mouse CD326 antibody (118213, Biolegend) for 15 min at room temperature. After staining, the cells were analyzed using FACS cytometry (BD Biosciences) to assess the expression of these markers, providing insights into the immune response modulation in the airway tissue.

Separation of airway epithelial cells (AECs)

The trachea was harvested from mice, and mucosal debris was removed from the surface. The trachea was then placed in PBS containing 0.5 mg·mL^{-1} protease and incubated overnight at overnight 4 °C. Following this, it was centrifuged for 5 min at 180 × g and 4 °C. The supernatant was discarded, and the pellet was washed twice with PBS. The airway epithelial cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 5% fetal bovine serum and cultured in a 100 mL dish.

Exosome isolation and identification

Exosomes were isolated from the airway epithelial cells using a total exosome isolation reagent (Cat# 4478359, Thermo Fisher Scientific) based on methods described in previous research [4]. Initially, the airway epithelial cells were cultured in plates until they reached approximately 70% confluence. The cells were then washed with PBS and incubated for 24 h in DMEM supplemented with 10% bovine exosome-depleted FBS (Gibco). After incubation, the cell culture medium was collected and centrifuged at 2000 × g for 30 min. The supernatant was subsequently filtered through a 0.22-μm filter (Sigma-Aldrich). Exosome isolation reagent (Invitrogen, Waltham, MA, USA) was added to the filtered culture medium, followed by overnight incubation at 4 °C. The mixture was then centrifuged at 10 000 × g at 4 °C for 1 h. The resulting exosome pellet was resuspended in 150 μL PBS. For characterization, the exosomes were diluted in PBS (1 ∶ 100) and analyzed using dynamic light scattering, transmission electron microscopy (TEM), and Western blotting techniques.

RNA immunoprecipitation (RIP) assay

AECs were collected, and a RIP assay was conducted using the EZ-Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Merck Millipore, Billerica, MA, USA). Cells were lysed using RIP lysis buffer, followed by sonication for 5s and subsequent centrifugation. The supernatant was collected and pre-washed with magnetic beads. Either the mouse anti-hnRNPA2B1 antibody (1 ∶ 500, 14813-1-AP, Proteintech, Rosemont, IL, USA) or a nonspecific anti-IgG antibody (1 ∶ 50, 30 000-0-AP, Proteintech) was added and the mixture incubated at 4 °C overnight. Post-incubation, the samples were treated with proteinase K buffer and centrifuged five times to prepare for analysis *via* Western blotting assay.

RNA pull-down assay

Cell lysates from AECs were incubated with streptavidin-coated magnetic beads to isolate the biotin-coupled RNA complex, following the manufacturer's instructions (Invitrogen). The capability of lnc-TRPM2-AS to enrich hnRNRA2B1 was evaluated by qRT-PCR. Proteins bound to the complex were eluted and analyzed by SDS-PAGE and

Western blotting assay.

Separation of CD4$^+$ T cells

Approximately 5 mL of peripheral blood was collected from mice and treated with ethylene diamine tetraacetic acid-K2. Peripheral blood mononuclear cells (PBMCs) were isolated using a lymphocyte separation medium (Sangon Biotech, Shanghai, China) and washed with PBS. The PBMCs were then resuspended in a mixture of 20 mL·L^{-1} FBS, 2 mmol·L^{-1} EDTA, PBS, and CD4 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) solution and incubated for 20 min in the dark. Following incubation, the samples were centrifuged at 1000 r·min^{-1} for 10 min. The supernatant was discarded, and the cell pellets were resuspended in 2 mL of buffer. CD4$^+$ T cells were then separated using a magnetic-activated cell sorting system (Miltenyi Biotech).

Cell transfection and treatment

Cell transfections were performed based on previously published literature [2]. Airway epithelial cells (AECs) were seeded at a density of 5 × 10^6 cells per well in 24-well plates, 24 h prior to transfection. Short hairpin RNAs targeting lnc-TRPM2-AS or a non-targeting control shRNA (sh-NC) were designed by Genesee Biotech and cloned into GV102 vectors by GenePharma (Shanghai, China). Short interference RNAs for hnRNPA2B1 (si-hnRNPA2B1) and a control siRNA (siNC) were obtained from GenePharma. Additionally, an overexpressing plasmid was created by amplifying the full-length hnRNPA2B1 sequence *via* PCR and cloning it into the pcDNA3.1 vector (Promega, Madison, WI, USA), resulting in constructs named p-hnRNPA2B1 and p-vector. These constructs were transfected into AECs using Lipofectamine 2 000 (Invitrogen, CA, USA) in a final volume of 20 μL lipofectamine per well. 6 h later, the culture medium was changed to the normal medium. The culture medium was replaced with fresh medium 6 hours post-transfection. Cells were utilized for *in vitro* experiments 48 h after transfection.

Primary AECs were also seeded in 24-well plates at 5 × 10^6 cells per well and treated either with 1 μg·mL^{-1} RNase alone or in combination with 0.1% Triton X-100 for 30 min. Subsequently, the cells were analyzed using RT-qPCR. In a separate set of experiments, AECs were treated with or without GW4869 (20 μmol·L^{-1}, Sigma-Aldrich) for 2 h followed by exposure to lipopolysaccharide (LPS, 10 μg·μL^{-1}, Sigma-Aldrich) for an additional 24 hours.

AECs were also cultured in 15-cm dishes (Corning Inc. NY, USA) at a density of 5 × 10^5 cells/mL. After treatments, as mentioned, cells were washed with PBS and replenished with 10 mL DMEM medium. Two days later, the conditioned medium was collected. This conditioned medium was then used to culture CD4$^+$ T cells for 24 h after removing their original medium and washing the cells with PBS.

Real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the cells using the Trizol reagent (Invitrogen) according to the manufacturer's protocol. One microgram of RNA was reverse-transcribed into cDNA using the SYBR Premix Ex Taq (RR82WR, Takara, Osaka, Japan), following the instructions provided by the manufacturer. Quantitative polymerase chain reaction (qPCR) was then performed using the Taqman® Universal PCR Master Mix (Thermo Fisher Scientific). PPrimer sequences for the target genes were obtained from Origen Biotech (Wuxi, Jiangsu, China). The following primers were used: lnc-TRPM2-AS forward primer: 5′-GCGGTTACG AGGGCAAATA-3′, reverse primer: 5′-CACGTAG GCTGAGTGACGAG-3′; hnRNPA2B1 forward primer: 5′-CGGTGGCAATTTTGGACCAGGA-3′, reverse primer: 5′-CCATAACCAGGGCTACCTCCAA-3′; IL-4 forward primer: 5′-ATCATCGGCATTTTGAACGAGGTC-3′, reverse primer: 5′-ACCTTGGAAGCCCTACAGACGA-3′; IL-10 forward primer: 5′-CGGGAAGACAATAACTGCACCC-3′, reverse primer: 5′-CGGTTAGCAGTATGTTGTCCAGC-3′; GATA3 forward primer: 5′-CCTCTGGAGGAG-GAACGCTAAT-3′, reverse primer: 5′-GTTTCGGGTC-TGGATGCCTTCT-3′; IFN-γ forward primer: 5′-CAGCAA-CAGCAAGGCGAAAAAGG-3′,reverseprimer:5′-TTTCCG-CTTCCTGAGGCTGGAT-3′; IL-2 forward primer: 5′-GCG-GCATGTTCTGGATTTGACTC-3′,reverseprimer5′-CCACC-ACAGTTGCTGACTCATC-3′; T-bet forward primer: 5′-CCACCTGTTGTGGTCCAAGTTC -3′, reverse primer: 5′-CCACAAACATCCTGTAATGGCTTG -3′; U6 forward primer: 5′- GCTTCGGCAGCACATATACTAAAAT-3′, reverse primer: 5′-CGCTTCACGAATTTGCGTGTCAT-3′; GAPDH forward primer: 5′-CATCACTGCCACCCAGAA-GACTG-3′,reverse primer: 5′- ATGCCAGTGAGCTTCCCG-TTCAG-3′. The relative mRNA levels were quantified using the $2^{-\triangle\triangle Ct}$ method and normalized to U6/GPDAH.

Western blotting assay

Proteins were extracted from cells using radio-immunoprecipitation assay (RIPA) buffer (Beyotime Inc., Hiamen, Jiangsu, China), supplemented with protease inhibitors, and incubated at 4 ℃ for 30 min. Concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). For electrophoresis, 30 μg of protein from each sample was separated by SDS-PAGE and then moved to polyvinylidene fluoride membranes (Merck Millipore). The membranes were blocked and washed with PBS prior to overnight incubation with primary antibodies: TSG101 (ab125011, 1 : 1000, Abcam, Cambridge, UK), CD63 (ab217345, 1 : 1000, Abcam), GRP94 (ab238126, 1 : 1000, Abcam), and GAPDH (ab8245, 1 : 500, Abcam) as a loading control. After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (A-11001 and 31402, both 1 : 2000, Invitrogen). Finally, an ECL kit (Merck Millipore) was used to visualize the protein bands.

Statistical analysis

Data were analyzed using GraphPad Prism version 5.0. Results, derived from at least three independent experiments, are expressed as mean ± standard deviation (SD). Statistical significance was assessed using an unpaired Student's *t*-test for comparisons between two groups or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple group comparisons. A *P*-value of less than 0.05 was considered statistically significant.

Results

Ephedrine relieves inflammatory response and airway resistance in asthmatic mice

To evaluate the impact of ephedrine on asthma, we developed a murine model by sensitizing mice to either OVA alone or OVA combined with ephedrine. The experimental protocol for creating the OVA-induced asthma mouse model is depicted in Fig. 1A. As shown in Fig. 1B, mice treated with OVA alone displayed increased inflammatory infiltrates and mucus secretion in airway tissues. Additionally, Masson

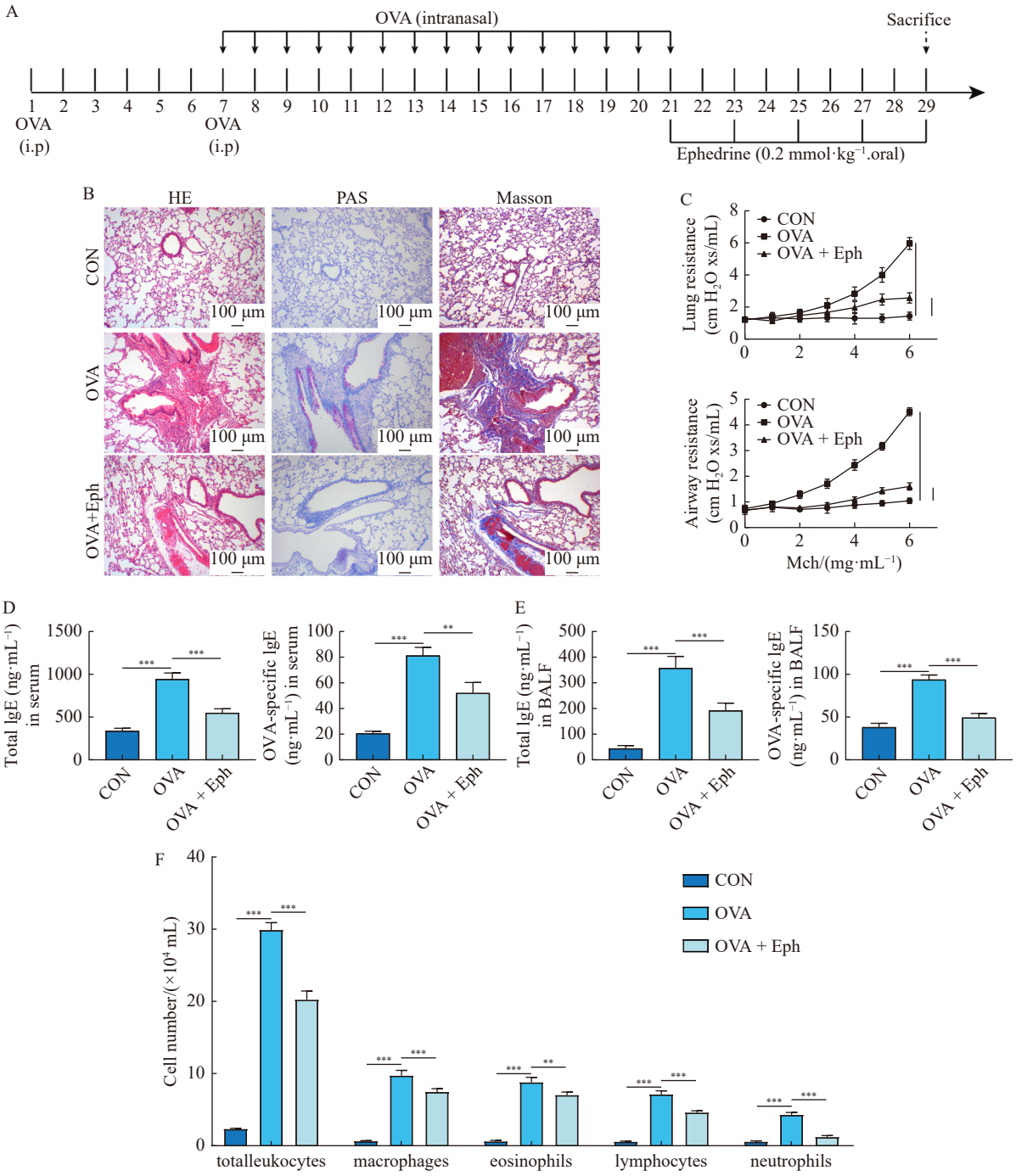

Fig. 1 Ephedrine relieves inflammatory response and airway resistance in asthmatic mice (n = 5/group) were treated with saline (control group, CON), sensitized and challenged with OVA, or sensitized and challenged with OVA and treated with ephedrine (OVA + Eph). (A) Experimental protocol for the establishment of OVA-induced asthma mouse model. (B) Representative HE, PAS, and Masson staining images of lung tissue were obtained from the mice. (C) Airway resistance and lung resistance were assessed. (D) The serum OVA-specific IgE level in mice from different groups. (E) Total serum IgE level in BALF from mice. (F)The count of inflammatory cells in BALF from mice. $^{**}P < 0.01$; $^{***}P < 0.001$ *vs* CON.

staining indicated that OVA sensitization resulted in thickened airway collagen deposition, which was mitigated by ephedrine treatment. Furthermore, OVA exposure significantly increased airway resistance to escalating doses of methacholine, an effect that was reduced by ephedrine. OVA also resulted in enhanced airway resistance to increased concentrations of methacholine in mice (Fig. 1C). Th2 cytokines, known to stimulate IgE production, were also examined. Serum levels of OVA-specific IgE and total IgE in the BALF were significantly higher in the OVA-induced asthma group compared to controls. However, these levels markedly decreased following ephedrine treatment (Figs. 1D–Fig. 1E). To assess the influence of ephedrine on inflammatory cell accumulation in the airways, we analyzed BALF. The total leukocyte count, along with macrophages, lymphocytes, eosinophils, and neutrophils, was significantly increased in OVA-induced asthmatic mice. Conversely, ephedrine treatment led to a significant reduction in these cell counts (Fig. 1F, Table 1). In summary, ephedrine significantly attenuates the inflammatory response and airway resistance induced by OVA in asthmatic mice, demonstrating its potential as a therapeutic agent in asthma management.

Table 1 Analysis of the complete cell count in the blood of mice (n = 5/group). $^{***}P < 0.001$ *vs* CON; $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* OVA.

	Total leukocytes (10^4/mL)	Macrophages (10^4/mL)	Eosinophils (10^4/mL)	Lymphocytes(10^4/mL)	Neutrophils(10^4/mL)
Control	2.33 ± 0.10	0.62 ± 0.10	0.60 ± 0.15	0.54 ± 0.11	0.56 ± 0.12
OVA	29.87 ± 1.05***	9.71 ± 0.75***	8.78 ± 0.70***	7.11 ± 0.52***	4.27 ± 0.35***
OVA + Eph	20.22 ± 1.24***	7.47 ± 0.44***	7.00 ± 0.46**	4.57 ± 0.28***	1.18 ± 0.22***

$^{**}P$ <0.01; $^{***}P$ < 0.001

Ephedrine restores the Th1/Th2 balance in asthmatic mice

Further analysis was conducted to assess the impact of ephedrine on the Th1/Th2 imbalance, a critical factor in the pathophysiology of asthma [15]. $CD4^+$ T cells were isolated from different groups of mice. As shown in Figs. 2A–2B, in OVA-treated mice, the proportion of Th1-positive cells was reduced while the proportion of Th2-positive cells was elevated, indicating a shift towards a Th2-dominant immune response. Additionally, the levels of IL-12 and γ-IFN in the BALF from OVA-treated mice were decreased. However, ephedrine treatment restored the levels of these cytokines (Fig. 2C). Furthermore, OVA sensitization led to significantly increased levels of IL-4 and IL-13 in the BALF, cytokines typically associated with Th2 responses. Remarkably, these elevated levels were reversed when ephedrine was coadministered with OVA (Fig. 2C). In conclusion, ephedrine effectively counteracted the OVA-induced Th1/Th2 imbalance in asthmatic mice, highlighting its potential therapeutic value in rebalancing immune responses in asthma.

OVA exposure induces airway epithelial cells to yield TRPM2-AS-containing exosomes, which is reversed by ephedrine treatment

To elucidate the protective mechanism of ephedrine in OVA-induced asthma, we first isolated AECs from a mouse model. Using flow cytometry, we confirmed successful isolation, as indicated by the cells testing positive for CD326, an epithelial cell marker (Fig. 3A). Subsequent experiments focused on examining the expression of the lncRNA TRPM2-AS in AECs. Using RT-qPCR, we assessed lnc-TRPM2-AS levels after treating cells with 1 μg·mL^{-1} RNase, both alone and in combination with 0.1% Triton X-100 for 30 min. The results showed that RNase treatment alone did not significantly alter lnc-TRPM2-AS levels, whereas the combination of RNase and Triton X-100 significantly reduced its expression. This suggests that lnc-TRPM2-AS is compartmentalized within membrane-bound structures, likely exosomes, which are disrupted by detergent treatment (Fig. 3B). We then isolated exosomes from the AEC supernatant to analyze lnc-TRPM2-AS expression further. Comparative analysis revealed no significant differences in lnc-TRPM2-AS levels between the culture medium and the exosomes derived from it, indicating a consistent presence of lnc-TRPM2-AS across conditions (Fig. 3C). Characterization of these exosomes was performed using TEM, dynamic light scattering, and Western blotting assay, confirming typical exosomal markers (TSG101 and CD63) and size (64 to 150 nm in diameter). These findings demonstrate that the exosomes maintain a consistent morphology and protein composition across different treatment groups (Figs. 3D–3E). Interestingly, exosomes from OVA-challenged AECs exhibited higher levels of lnc-TRPM2-AS compared to those from control groups (Fig. 3F). This increase was mitigated by ephedrine treatment, suggesting that ephedrine may interfere with the exosomal packaging or secretion of lnc-TRPM2-AS (Figs. 3G–3H). These findings suggest a novel aspect of asthma pathophysiology where ephedrine may exert its anti-inflammatory effects by modulating exosomal lncRNA content. The attenuation of lnc-TRPM2-AS expression in AEC-derived exosomes could be a key mechanism through which ephedrine alleviates the symptoms of OVA-induced asthma (Figs. 3G–3H). This study highlights the potential of targeting exosomal pathways in the development of therapeutic strategies for asthma.

Taken together, ephedrine treatment diminished the OVA-treatment-induced increase of TRPM2-AS expression in OAEs.

HnRNPA2B1 mediates the packaging of lnc-TRPM2-AS into exosomes

To elucidate the mechanistic role of hnRNPA2B1 in reg-

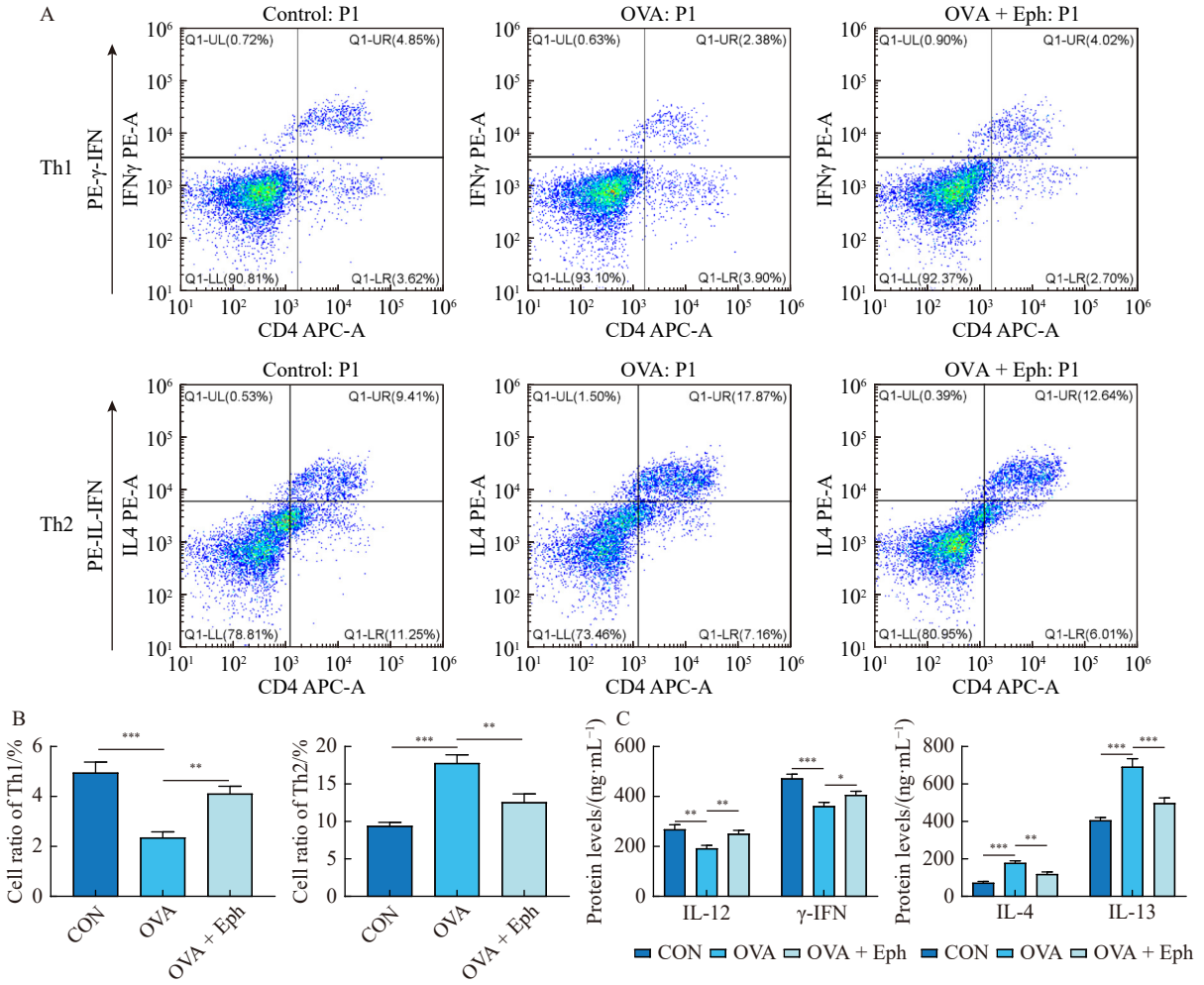

Fig. 2 Ephedrine restores the Th1/Th2 balance in asthmatic mice. (A) Flow cytometry was used to examine the quantity of IL-4 and CD4-positive cells. (n=5/group). (B) The quantitative results of (A) showed the ratio of Th1 and Th2 cells. (n=5/group). (C) ELISA detected the protein levels of IL-12, γ-IFN, IL-4 and IL-13. (*n* = 5/group). $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* CON, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* OVA .

ulating the packaging of lnc-TRPM2-AS into exosomes, several molecular techniques were employed. Initial investigations using an RIP assay demonstrated that hnRNPA2B1 selectively enriches lnc-TRPM2-AS in AECs (Fig. 4A). This interaction was further confirmed by an RNA pull-down assay, providing additional evidence of the specific binding between lnc-TRPM2-AS and hnRNPA2B1 (Fig. 4B). To directly assess how hnRNPA2B1 influences the incorporation of lncTRPM2-AS into exosomes, we manipulated the expression levels of hnRNPA2B1 in AECs. Cells were transfected with si-hnRNPA2B1 to reduce its expression and with a plasmid (p-hnRNPA2B1) to enhance its expression. The transfection efficiency was verified through RT-qPCR and Western blotting analyses, confirming the effective modulation of hnRNPA2B1 levels (Figs. 4C–4D). Analysis following these modifications revealed that depletion of hnRNPA2B1 led to a decrease in the level of lnc-TRPM2-AS in exosomes derived from AECs. Conversely, overexpression of hnRNPA2B1 resulted in increased levels of lnc-TRPM2-AS in these exosomes (Fig. 4E). Moreover, the expression of hnRNPA2B1 itself was observed to increase in AECs following OVA exposure, but significantly decreased under ephedrine treatment, suggesting a potential mechanism through which ephedrine mitigates asthma symptoms (Fig. 4F). These findings highlight the critical role of hnRNPA2B1 in the packaging of lnc-TRPM2-AS into exosomes.

Airway epithelial cell-derived exosomes (OAEs) carrying lnc-TRPM2-AS are crucial for imbalancing CD4$^+$ T cells

The primary AECs were treated with LPS or LPS plus GW4869, which is an exosome inhibitor, to confirm the role of lnc-TRPM2-AS carrying exosomes on asthma. LPS exposure induced a higher lnc-TRPM2-AS expression in AECs compared to the control group (Fig. 5A). This upregulation was also observed in the conditioned medium from LPS-treated AECs, whereas GW4869 treatment reversed this increase, suggesting the involvement of exosomes in lnc-TRPM2-AS transport (Fig. 5B). Conditioned medium from both LPS-stimulated and LPS plus GW4869-stimulated AECs was used to treat CD4$^+$ T cells exposed to the conditioned medium from LPS-treated AECs showed significant

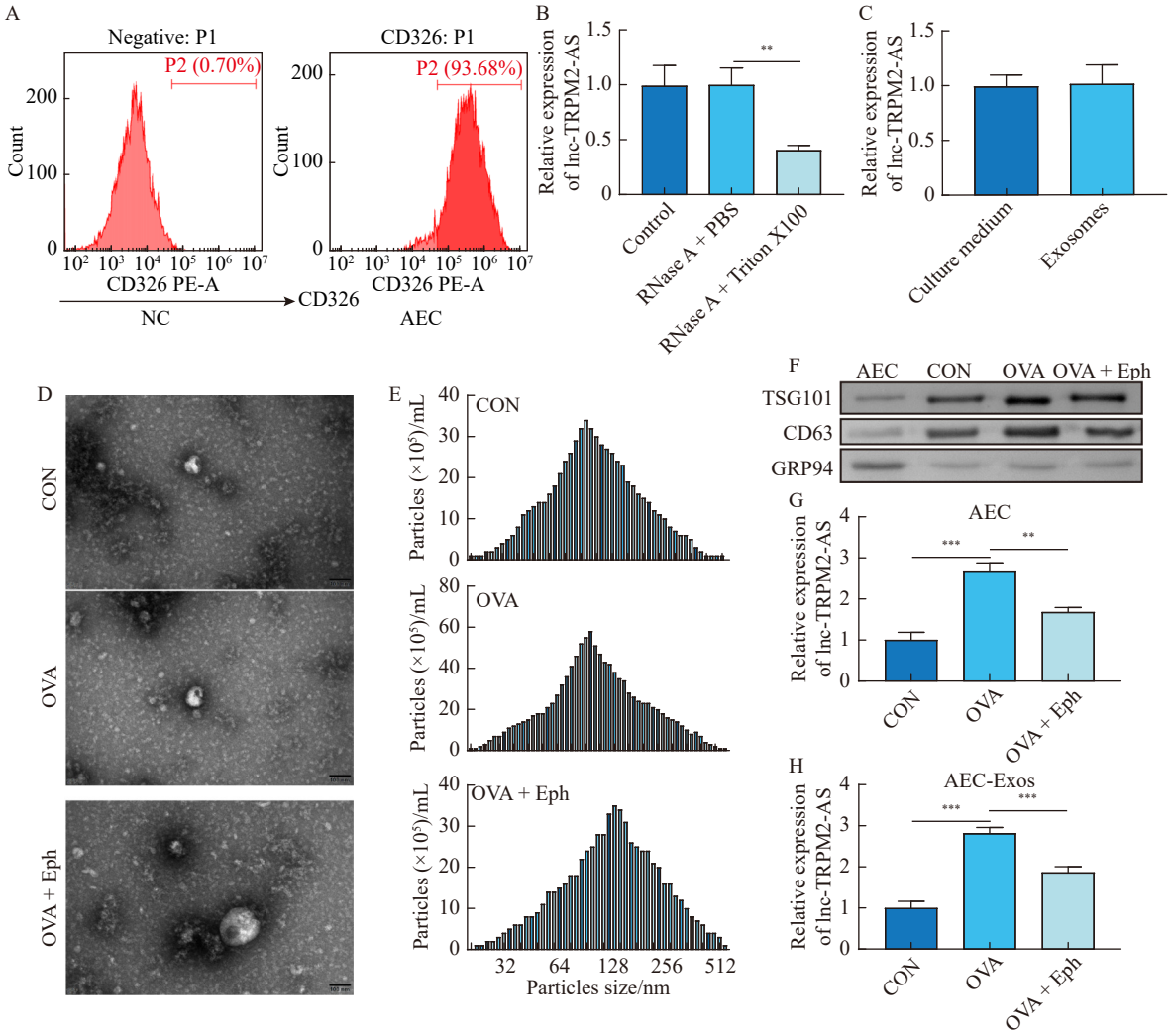

Fig. 3 OVA exposure induces airway epithelial cells to yield TRPM2-AS-containing exosomes, which is reversed by ephedrine treatment. (A) Primary AECs were isolated from mice. Flow cytometry assessed the CD326 surface marker expression in the isolated cells. (B) RT-qPCR was performed to detect the expression level of lnc-TRPM2-AS after treatment with 1 μg·mL^{-1} RNase alone or combined with 0.1% Triton X-100. (C) RT-qPCR analysis of lnc-TRPM2-AS expression in exosomes and whole culture medium from AECs. (D) TEM, (E) dynamic light scattering, and (F) Western blotting investigated the exosomes derived from AECs. RT-qPCR assay for determining the expression levels of lnc-TRPM2-AS in AECs (G) and exosomes isolated from AECs (H) within different indicated treatments. (n = 3/group). $^{**}P < 0.01$ *vs* RNase A + PBS; $^{***}P < 0.001$ *vs* CON; $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* OVA.

increases in IL-4, IL-10, and GATA3 expression, along with decreases in IFN-γ, IL-2, and T-bet levels, indicative of a shift towards a Th2-dominant response (Fig. 5C). The influence of LPS on these cytokine levels was mitigated when the conditioned medium was treated with GW4869, highlighting the critical role of exosomes in mediating these effects (Fig. 5C). The silencing of lnc-TRPM2-AS significantly reduced its expression in both LPS-treated AECs and their derived exosomes (Figs. 5D–5E). Importantly, exosomes from LPS-treated AECs significantly increased the proportion of IL-13 positive CD4$^+$ T cells, an effect that was reduced when lnc-TRPM2-AS was knocked down in the AECs (Fig. 5F). However, the promotive effect of exosomes was diminished after knocking down of lnc-TRPM2-AS in AECs (Fig. 5F). These findings collectively indicate that exosomal lnc-TRPM2-AS derived from AECs plays a pivotal role in shifting the Th1/Th2 balance towards Th2 in CD4$^+$ T cells, thereby contributing to the pathophysiology of asthma.

Discussion

Cough variant asthma, characterized by chronic cough, airway remodeling, and inflammatory cell infiltration, is a specific form of asthma that predominantly affects children [16]. Patients with cough variant asthma are commonly children [17]. The standard treatment for cough variant asthma involves anti-inflammatory medications, including ephedrine [18]. This study explored the mechanisms through which ephedrine regulates asthma and identified its protective effects in an OVA-induced murine asthma model. We demonstrated that ephedrine mitigated the inflammatory response,

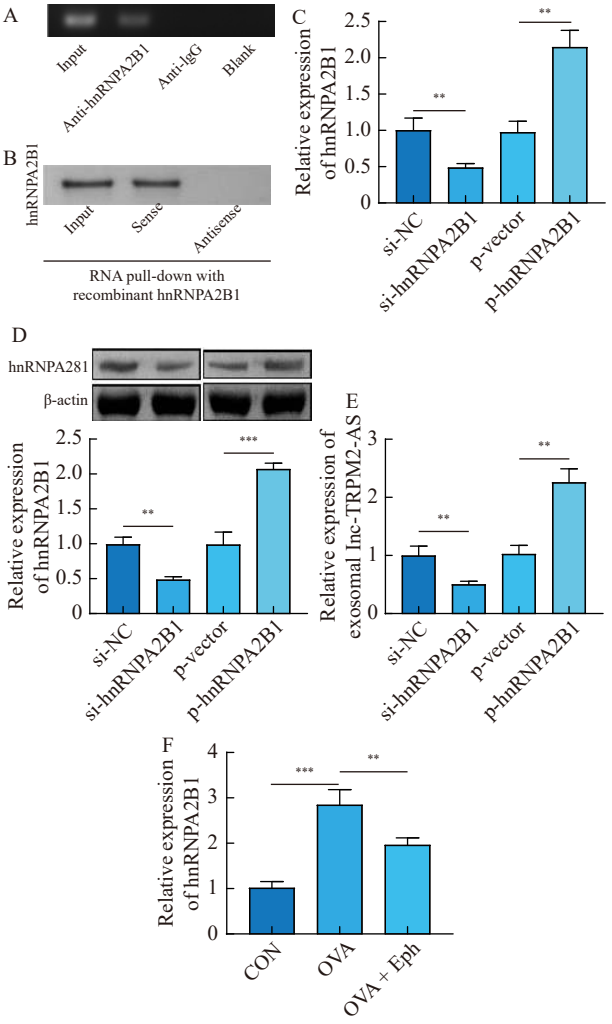

Fig. 4 hnRNPA2B1 mediates the packaging of lnc-TRPM2-AS into exosomes. (A) The RIP assay revealed that lnc-TRPM2-AS was enriched by hnRNPA2B1 in AECs. (B) RNA pull-down demonstrated the interaction of lnc-TRPM2-AS and hnRNPA2B1. (C–D) The relative gene and protein expression of hnRNPA2B1 in AECs transfected with si-hnRNPA2B1 or si-NC was examined using RT-qPCR and Western blotting assays, respectively. (E) RT-qPCR detected the exosomal lnc-TRPM2-AS expression in AECs. (F) The expression of hnRNPA2B1 in AECs isolated from different mouse groups was assessed using RT-qPCR assay. (*n* = 3/group). $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* si-NC or CON; $^{**}P < 0.01$ and $^{***}P < 0.001$ vs p-vector or OVA.

airway resistance, and Th1/Th2 imbalance in asthmatic mice. Our findings revealed that exposure to OVA increased the production of TRPM2-AS-containing exosomes from AECs, which significantly influenced the Th1/Th2 imbalance. Importantly, this effect was substantially reduced by ephedrine treatment. Further investigations showed that hnRNPA2B1 plays a critical role in packaging lnc-TRPM2-AS into these exosomes.

Ephedrine acts as an α- and β-adrenergic agonist, which has been shown to control symptoms of cough and asthma effectively. Previous studies, such as the combination of Ripariin II and ephedrine, have demonstrated protection against inflammation and airway remodeling in asthma models [7]. Consistent with these findings, our study confirmed that ephedrine not only reduced inflammation and airway resistance but also restored the Th1/Th2 balance in OVA-induced asthmatic mice.

AECs are pivotal in the innate immune functions of the lungs [19]. They play a crucial role in trapping pathogens under inflammatory conditions [20]. However, the excessive activation of this mechanism can lead to the overproduction of epithelium-derived cytokines such as IL-25, IL-33, and TSLP. These cytokines are known to activate dendritic cells, thereby enhancing the Th2 immune response [21], which is commonly associated with allergic reactions and asthma. Previous research has shown that treatment with IL-25 in the lungs of mice induces eosinophilic inflammation, airway hyperresponsiveness, and Th2 cytokine expression [22]. Soumelis and colleagues highlighted that TSLP released by AECs can activate dendritic cells and promote the production of Th2-attracting chemokines [23]. Moreover, exosomes, which contain various transcripts, such as messenger RNA (mRNAs), lncRNAs, and micro-RNAs (miRNAs), serve as crucial mediators for intracellular communication among innate immune cells. For instance, IL-13-challenged AEC-derived exosomes have been shown to induce chemotaxis and proliferation of lung macrophages under inflammatory asthmatic conditions [24]. In this study, we observed that conditioned medium from LPS-treated AECs induced a Th1/Th2 imbalance in $CD4^+$ T cells. Notably, this promotive effect of LPS treatment was significantly reduced following the administration of the exosome inhibitor GW4869. This finding underscores the critical role of AEC-derived exosomes in modulating the Th1/Th2 balance.

LncRNAs, characterized by lengths exceeding 200 nucleotides, have been increasingly recognized for their roles in regulating various lung diseases, including asthma, lung cancers, and idiopathic pulmonary fibrosis. LncRNA metastasis-associated lung Specifically, lncRNAs have been shown to influence immune cell functions and inflammatory responses, critical factors in these conditions. In asthma, the lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was found to be overexpressed in patients and crucially impacted the Th1/Th2 balance within $CD4^+$ T cells, promoting airway inflammation [25]. Another study by WEI et al. described how the activation of the lncRNA PVT1-miR-15a-5p/miR-29c-3p-PI3K-Akt-mTOR axis contributed to an Ozone-induced Th1/Th2 imbalance in $CD4^+$ T cells, further implicating lncRNAs in asthma development [26]. Additionally, lnc-TRPM2-AS has been demonstrated to inhibit autophagy-induced macrophage apoptosis in asthma [27]. Our findings extend the understanding of lncRNA functions by showing that OVA exposure increases the presence of lnc-TRPM2-AS-containing exosomes derived from airway epithelial cells (AECs), an effect that is mitigated by ephedrine treatment. LPS stimulation also induced lnc-TRPM2-AS expression in

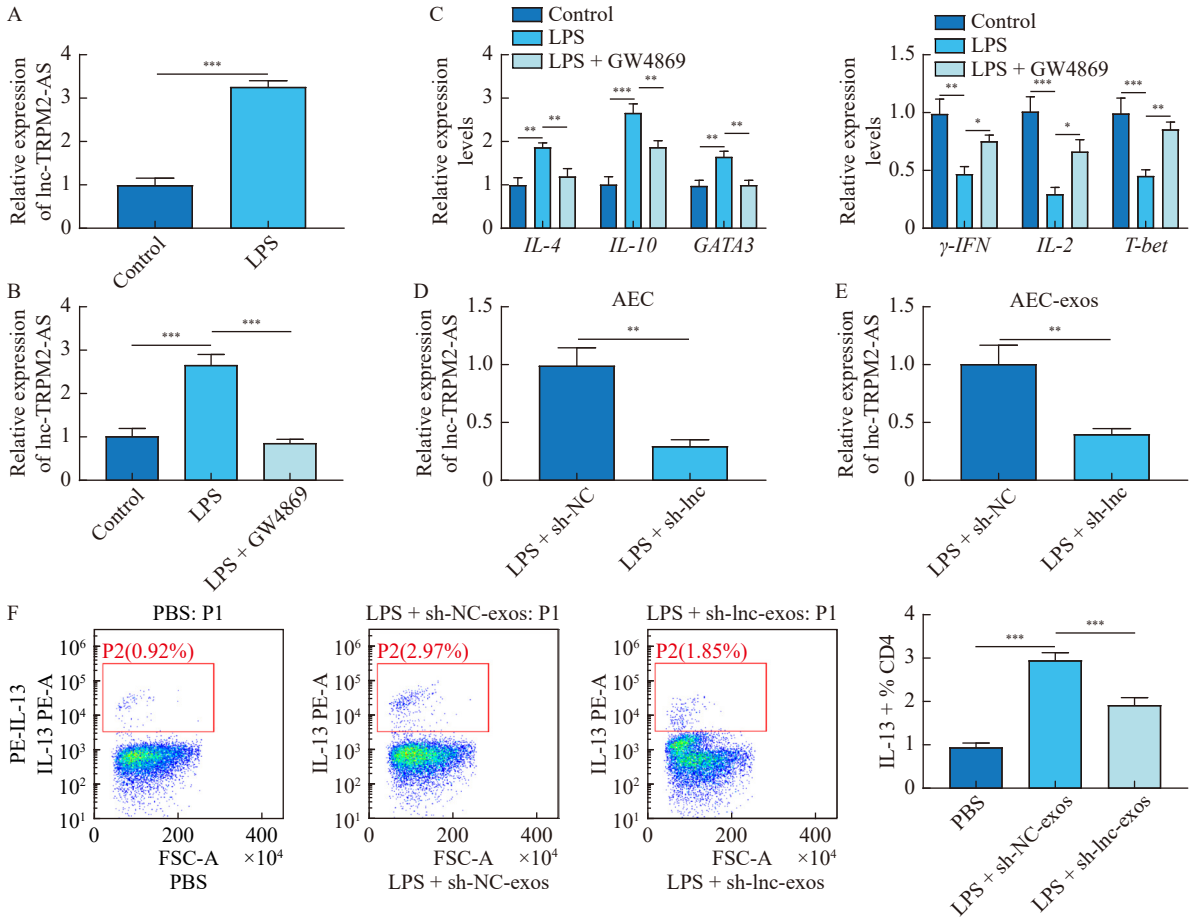

Fig. 5 Airway epithelial cell-derived exosomes (OAEs) carrying lnc-TRPM2-AS are crucial for imbalancing CD4$^+$ T cells. Primary AECs were isolated from mice and treated with LPS or LPS plus GW4869. RT-qPCR detected the expression level of lnc-TRPM2-AS in (A) AECs and (B) conditioned medium from AECs. The conditioned medium was collected from AECs treated with LPS or LPS plus GW4869. Afterward, CD4$^+$ T cells were treated with the collected conditioned medium. (C) The levels of cytokines relevant to Th1/Th2 imbalance were determined within CD4$^+$ T cells using RT-pPCR. The AECs were exposed to LPS and then transfected with sh-lnc-TRPM2-AS or sh-NC; the expression of sh-lnc-TRPM2-AS in (D) AECs and (E) exosomes isolated from AECs were detected using RT-qPCR. The exosomes isolated from different groups of AECs were used to treat CD4$^+$ T cells; (F) The quantity of IL-13 positive CD4$^+$ T cells was examined by flow cytometry. (n = 3/group). $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* Control; $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* LPS; $^{**}P < 0.01$ *vs* LPS + sh-NC; $^{***}P < 0.001$ *vs* PBS; $^{***}P < 0.001$ *vs* LPS + sh-NC-exos.

conditioned mediums from AECs. Importantly, exosomes carrying lnc-TRPM2-AS derived from LPS-stimulated AECs were found to disrupt the Th1/Th2 balance within CD4$^+$ T cells. However, this effect was attenuated after knocking down lnc-TRPM2-AS in AECs, highlighting the pivotal role of AEC-derived exosomal lnc-TRPM2-AS in modulating the Th1/Th2 balance in asthma. Further investigations revealed that hnRNPA2B1, a known RNA-binding protein, is responsible for packaging lnc-TRPM2-AS into AEC-derived exosomes. HnRNPA2B1's role in exosomal RNA transport is supported by its ability to interact with cytoskeletal components [28]. This mechanism is not unique to asthma, as similar RNA packaging processes have been observed in non-small cell lung cancer, where lncRNA H19 is packaged into exosomes [29].

This study presents several limitations that warrant acknowledgment and consideration for future research. Firstly, the focus primarily on the lnc-TRPM2-AS pathway might not capture the entirety of the signaling mechanisms through which ephedrine mitigates asthma symptoms. A broader examination of the potential signaling pathways involved could enrich our understanding of ephedrine's pharmacological effects. Another significant limitation is the absence of a separate experimental group solely treated with ephedrine (Eph treatment). This decision was influenced by the commitment to adhere to the 3R principle (Reduce, Refine, Replace) aimed at minimizing the use of experimental animals and the constraints imposed by limited research funding. Consequently, the study could not comprehensively assess the independent effects of ephedrine, potentially overlooking some of its impacts as a standalone treatment. Moreover, while the study highlighted the general influence of ephedrine on white blood cell counts, it did not delve into the effects on specific types of cells within this heterogeneous group. Future studies

should focus on dissecting the impacts of ephedrine on individual cell types to provide a more detailed understanding of its immunomodulatory actions.

In summary, despite these limitations, this study has demonstrated that exosomes secreted from OVA-induced AECs containing lnc-TRPM2-AS contribute to the differentiation of $CD4^+$ T cells into Th2-like cells, thereby promoting the progression of asthma. The reversal of this effect by ephedrine highlights the potential therapeutic value of targeting AEC-derived exosomal lnc-TRPM2-AS in asthma treatment. Further investigations into this pathway and broader signaling mechanisms may enhance the strategic use of ephedrine and potentially other treatments for asthma.

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