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IL-24 Contributes to Neutrophilic Asthma in an IL-17A-Dependent Manner and Is Suppressed by IL-37

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ABSTRACT

Purpose: Neutrophilic asthma is associated with asthma exacerbation, steroid insensitivity, and severe asthma. Interleukin (IL)-24 is overexpressed in asthma and is involved in the pathogenesis of several allergic inflammatory diseases. However, the role and specific mechanism of IL-24 in neutrophilic asthma are unclear. We aimed to elucidate the roles of IL-24 and IL-37 in neutrophilic asthma, the relationships with IL-17A and the mechanisms regulating neutrophilic asthma progression.

Methods: Purified human neutrophils were isolated from healthy volunteers, and a cell coculture system was used to evaluate the function of IL-24 in epithelium-derived IL-17A-dependent neutrophil migration. IL-37 or a small interfering RNA (siRNA) targeting IL-24 was delivered intranasally to verify the effect in a murine model of house dust mite (HDM)/ lipopolysaccharide (LPS)-induced neutrophilic asthma.

Results: IL-24 enhanced IL-17A production in bronchial epithelial cells via the STAT3 and ERK1/2 signaling pathways; this effect was reversed by exogenous IL-37. Anti-IL-17A monoclonal antibodies reduced neutrophil chemotaxis induced by IL-24-treated epithelial cells *in vitro*. Increased IL-24 and IL-17A expression in the airway epithelium was observed in HDM/LPS-induced neutrophilic asthma. IL-37 administration or IL-24 silencing attenuated neutrophilic asthma, reducing IL-17A levels and decreasing neutrophil airway infiltration, airway hyperresponsiveness, and goblet cell metaplasia. Silencing IL-24 inhibited T-helper 17 (Th17) immune responses, but not Th1 or Th2 immune responses, in the lungs of a neutrophilic asthma model.

Conclusions: IL-24 aggravated neutrophilic airway inflammation by increasing epithelium-derived IL-17A production, which could be suppressed by IL-37. Targeting the IL-24/IL-17A signaling axis is a potential strategy, and IL-37 is a potential candidate agent for alleviating neutrophilic airway inflammation in asthma.

Keywords: Asthma; interleukin-24; interleukin-17A; inflammation; neutrophils; epithelial cells

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INTRODUCTION

Asthma is a heterogeneous pulmonary disease and can be classified into various phenotypes and endotypes based on inflammatory cell infiltration.[1] Unlike other asthma phenotypes, the T-helper 17 (Th17)-related neutrophilic asthma (NA) phenotype exhibits more severe asthma or frequent asthma exacerbations.[2] Moreover, patients with a neutrophil-dominated asthma phenotype are generally insensitive to glucocorticoid therapy, and this phenotype currently lacks effective treatments.[3] In this context, it is particularly warranted to explore the pathogenesis of NA. However, the upstream inflammatory factors that drive the activation of neutrophils and their migration into the lungs remain largely unknown.

Interleukin (IL)-17A is a representative neutrophil-attracting cytokine produced by immune cells and resident structural cells.[4] Furthermore, several researchers have pointed out that IL-17A is associated with neutrophil accumulation, steroid resistance, and frequent acute exacerbations, and that knocking out the IL-17A gene in mice fails to induce immune inflammation.[5-8] However, the underlying mechanism of IL-17A production and its interaction with upstream mediators in asthma remain to be completely clarified. In recent years, compelling evidence has indicated that IL-24 is a robust proinflammatory mediator linked to neutrophilic inflammatory responses and is involved in the pathogenesis of various immune and allergic inflammatory diseases.[9,10] IL-24 belongs to the IL-10 family of cytokines and is produced by immune cells such as macrophages, peripheral blood mononuclear cells and Th2 cells.[11,12] It mediates intercellular communication via 2 heterodimeric receptor complexes: IL-20R1/IL-20R2 and IL-22R1/IL-20R2.[13] The 3 receptor subunits of IL-24 are all detected on human lung tissues, suggesting that lung tissue may be one of the important target sites of IL-24.[14] IL-24 is overexpressed in induced sputum and moderately correlated with nasal secretion levels in patients with seasonal asthma, which suggests that IL-24 may be involved in the pathogenesis of asthma exacerbation.[15] A recent study reported that IL-17 induced autocrine production of IL-24 in Th17 cells, indicating that IL-24 and IL-17A can regulate immune responses through a self-feedback loop. Given that chronic inflammatory diseases have a similar pathogenesis, the relationship and mechanism between IL-24 and IL-17A in NA are still unclear.

IL-37 is a potent natural inhibitor with anti-inflammatory and anti-infective properties.[16] The IL-37 level in the serum of asthmatic patients was shown to be significantly lower than that in healthy subjects.[17] Our previous research found that IL-37 suppressed airway inflammation and remodeling by modulating thymic stromal lymphopoietin (TSLP) levels in an house dust mite (HDM)-induced chronic eosinophil-dominated asthma model.[18] To date, the immunological regulation of IL-37 in NA has focused on airway inflammation, and the associated specific mechanisms have not been thoroughly investigated.

Based on this, the purpose of this project was to explore the pathophysiological features of IL-24 in an HDM/lipopolysaccharide (LPS)-induced NA model and the mechanisms underlying the interaction with IL-17A signaling in airway epithelium. In addition, we further expanded our earlier findings by determining the role of IL-37 in a HDM/LPS-induced NA model and the mechanisms underlying the interaction with IL-24 signaling.

MATERIALS AND METHODS

Cell culture and stimulation

Human bronchial epithelial cells (16-HBE) were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Walthan, MA, USA) supplemented with 10% fetal bovine serum (Gibco), 100 IU/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco) at 37°C in a 5% CO_2 atmosphere. The cells were exposed to various concentrations of human recombinant IL-24 (rhIL-24, cat# 200-35-20; PeproTech, Rocky Hill, NJ, USA) for 24 hours (reverse transcription-quantitative polymerase chain reaction [RT-qPCR]) or 48 hours (western blotting) in the presence or absence of an 8 hours pretreatment with 100 ng/mL (cat# 200-39-25; PeproTech).

Neutrophil chemotaxis assays

Peripheral blood neutrophils were isolated from healthy control subjects using a neutrophil isolation kit (Tianjin Haoyang Biotechnology, Tianjin, China) according to the manufacturer's instructions. The morphology of neutrophils was examined by Diff-Quik staining. The viability of neutrophils was determined using flow cytometry. Anti-CD15 and anti-CD66b antibodies were used to analyze the purity of neutrophils by flow cytometry. For neutrophil migration assays, neutrophils (1.0×10^5 cells per well in 1640 medium) were seeded on the upper chambers of 24-well Transwell plates (8 μm pore, Corning), and the lower chambers were filled with 600 μL 16-HBE cell suspensions (1.0×10^5/mL in 1640 medium) supplemented with rhIL-24 (100 ng/mL), anti-IL-17A neutralizing antibodies (20 μg/mL, RD Biotech, Besançon, France) or immunoglobulin G isotype control antibody (20 μg/mL, RD Biotech). After incubation for 2 hours at 37°C, the suspensions of the upper chamber were removed, and the migrated membrane cells were stained with Diff-Quik solution and counted under a light microscope.

Asthma animal models

Wild-type (WT) BALB/c mice (female, 7–8 weeks old) were purchased from Yancheng Biotechnology Co., Ltd. (Guangzhou, China; license number: SCXK (Liao) 2020-0001) and housed in a specific pathogen-free environment with a 12-hour light-dark cycle and free access to food and water. Animal experiments were carried out in compliance with institutional guidelines, and were approved by the Ethics Committee of Animal Experiments of the Third Affiliated Hospital of Sun Yat-sen University (No. [2019]02-148-01).

Mice were randomly divided into 6 groups (n = 8 each group) as follows: 1) the Control (phosphate-buffered saline [PBS]) group, 2) the HDM group; 3) the HDM plus LPS group, 4) the HDM plus LPS plus small interfering interleukin (si-IL)-24 group, 5) the HDM plus LPS plus si-negative control (si-NC) group, and 6) the HDM plus LPS plus IL-37 group. The present asthma model was developed according to previous methods.[19,20] Briefly, mice were sensitized intranasally (i.n.) using HDM (25 μg/mouse, cat# XPB82D3A25; Greer, London, USA) with or without LPS (10 μg/mouse, *E. coli* serotype O111: B5, cat# L2880, Sigma-Aldrich, St. Louis, MO, USA) dissolved in 20 μL of PBS once daily on days 0, 1, and 2; then, challenged with HDM (25 μg/mouse) alone i.n. once daily for 9 consecutive days (days 7–15). In some groups, si-IL-24 (1 nmol/mouse; Ruibo Biotechnology, Guangzhou, China) or si-NC as an isotype control in 10 μL of PBS was given i.n. 1 hour preceding allergen exposure once every 2 days from day 7. Some mice received rhIL-37 i.n. prior to allergen instillation at a dose of 1 μg/mouse (dissolved in 10 μL PBS) 1 hour before allergen challenge. For budesonide (BUD) inhalation suspension treatment, mice were i.n. administered 20 μL BUD (0.5 mg/

kg) 1 hour before allergen exposure.[21] The mice were administered an equivalent volume of vehicle (PBS) as a negative control. The animal experimental protocols are depicted in **Supplementary Fig. S1A and B**. The small interfering RNA (siRNA) sequence of si-IL-24 was F-CCGCAGAGCAUUCAAACAGUUtt, R-AACUGUUUGAAUGCUCUGCGGtt.

Airway hyperresponsiveness (AHR)

Invasive lung function of mice was assessed using a Buxco® FinePointe™ RC system (DSI, St. Paul, MN, USA). Briefly, mice were anesthetized with 1% pentobarbital sodium (90 mg/kg, intraperitoneally) 24 hours after the last allergen exposure. The mice were annulated intratracheally with a 24-gauge cannula and connected to a ventilator at a frequency of 120 breaths per minute. Then, airway resistance (R_L, cm $H_2O \cdot s/mL$) was recorded in response to 200 μL of nebulized PBS or incremental concentrations of aerosolized methacholine (Mch, 6.25, 12.5, 25, and 50 mg/mL; Sigma-Aldrich) during a 3-minute period at each dose, at 5-minute resting intervals.

Bronchoalveolar lavage fluid (BALF)

The BALF of mice was collected immediately after lung function measurement. Briefly, the trachea was cannulated and flushed 3 times with 1 mL of PBS using a syringe while withdrawing as much fluid as possible. After centrifugation at 3,000 rpm/min for 5 minutes, the supernatants were frozen at -80℃ for subsequent enzyme-linked immunosorbent assay (ELISA) analysis. The cell pellets of BALF were resuspended in PBS and stained with appropriate antibodies for flow cytometry analysis of the population of neutrophils, eosinophils, and CD4+ T lymphocytes after splitting red cells. **Supplementary Table S1** contains a list of fluorescence-activated cell sorting antibodies.

ELISA

The concentrations of IL-24 and IL-17A in murine BALF and serum were measured using ELISA kits (Multi Sciences, Hangzhou, China) according to the manufacturers' instructions.

Flow cytometry analysis

The detailed steps of flow cytometry are depicted in the **Supplementary Data S1**. More details of the antibodies used for flow cytometric analysis are listed in **Supplementary Table S1**.

Gene mRNA analysis

The detailed steps of RT-qPCR are depicted in the **Supplementary Data S1**. The sequences of different primers for RT-qPCR are listed in **Supplementary Table S2**.

Western blotting

The detailed steps of western blotting are depicted in the **Supplementary Data S1**. The antibodies used in the western blot assay are described in **Supplementary Table S3**.

Immunofluorescent (IF) staining

The detailed IF steps are depicted in the **Supplementary Data S1**. The antibodies used in IF are described in **Supplementary Table S3**.

Supplementary methods

More details of the methods can be seen in the **Supplementary Data S1**.

Statistical analysis

All data were processed and analyzed using GraphPad Prism Software (version 9.0, GraphPad, San Diego, CA, USA) and are presented as the mean ± standard deviation. The significant differences among different groups were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) between 2 or more groups. Bonferroni's multiple comparison tests were used to adjust *P* values following one-way ANOVA. In all cases, values of $P < 0.05$ were considered significant.

RESULTS

HDM exposure increases IL-24 expression in 16-HBE cells via the nuclear factor-κB (NF-κB) pathway

Given the complicated pathogenesis of asthma, exposure to various allergens in the airways can cause the release of inflammatory mediators. We treated 16-HBE cells with HDM with or without LPS to evaluate the expression of IL-24 and its receptors. The results showed that HDM and LPS coexposure enhanced the protein levels of IL-24, IL-20R1, IL-20R2, and IL-22R1 (**Supplementary Fig. S2A**). IL-25, IL-33 and TSLP are well-known key epithelial-derived inflammatory factors linked to the pathogenesis of asthma. We also observed that HDM and LPS treatment significantly increased the levels of IL-25 and IL-33, with a slight upregulation of TSLP (**Supplementary Fig. S2B**). Next, our findings revealed that HDM and LPS treatment caused a significant phosphorylation of NF-κB in a time-dependent manner (**Supplementary Fig. S2C**) and that blocking the NF-κB pathway with the specific inhibitor BAY 11-7082 led to an inhibition of IL-24 production (**Supplementary Fig. S2D**). Overall, these results suggested that HDM and LPS coexposure upregulated the production of epithelial-derived IL-24 via the NF-κB pathway.

IL-24 enhances IL-17A production in 16-HBE cells via the STAT3 and ERK1/2 signaling pathways

Previous studies have shown that IL-24 receptors can be detected in human bronchial epithelial cells, so we attempted to define the biological function of IL-24 using 16-HBE cells.[14,22] We initially determined the expression pattern of IL-24 receptors on the 16-HBE cell surface. The results of agarose gel electrophoresis revealed that all IL-24 receptor subunits (IL-20R1, IL-20R2 and IL-22R1) could be detected in 16-HBE cells (**Fig. 1A**). To explore whether IL-24 can modulate IL-17A secretion, 16-HBE cells were treated with different concentrations of rhIL-24, and then IL-17A levels were evaluated by RT-qPCR, western blotting, and immunofluorescence. As shown in **Fig. 1B-E**, IL-24 gradually promoted IL-17A mRNA and protein expression in a dose-dependent manner in 16-HBE cells. After that, to ascertain which specific pathways are responsible for IL-24-induced IL-17A secretion, we used western blotting analysis to confirm the effects of IL-24 on the activation of the NF-κB, STAT3, p38-MAPK, ERK1/2, and JNK signaling pathways in 16-HBE cells. As shown in **Fig. 1F**, only the phosphorylation levels of STAT3 and ERK1/2 were increased in response to IL-24 stimulation, peaking at 30 and 20 minutes, respectively. To further determine whether IL-24 upregulates IL-17A by activating the STAT3 or ERK1/2 pathways, 16-HBE cells were pretreated with a JAK inhibitor (tofacitinib) or an ERK1/2 inhibitor (PD98059) prior to 1 hour of IL-24 stimulation. Tofacitinib and PD98059 pretreatment reduced the mRNA (**Fig. 1G and H**) and protein (**Fig. 1I-L**) levels of IL-17A induced by IL-24, respectively. Overall, these results indicated that IL-24 elicited a dose-dependent increase in epithelial-derived IL-17A expression, which was dependent on the activation of the STAT3 and ERK1/2 signaling pathways.
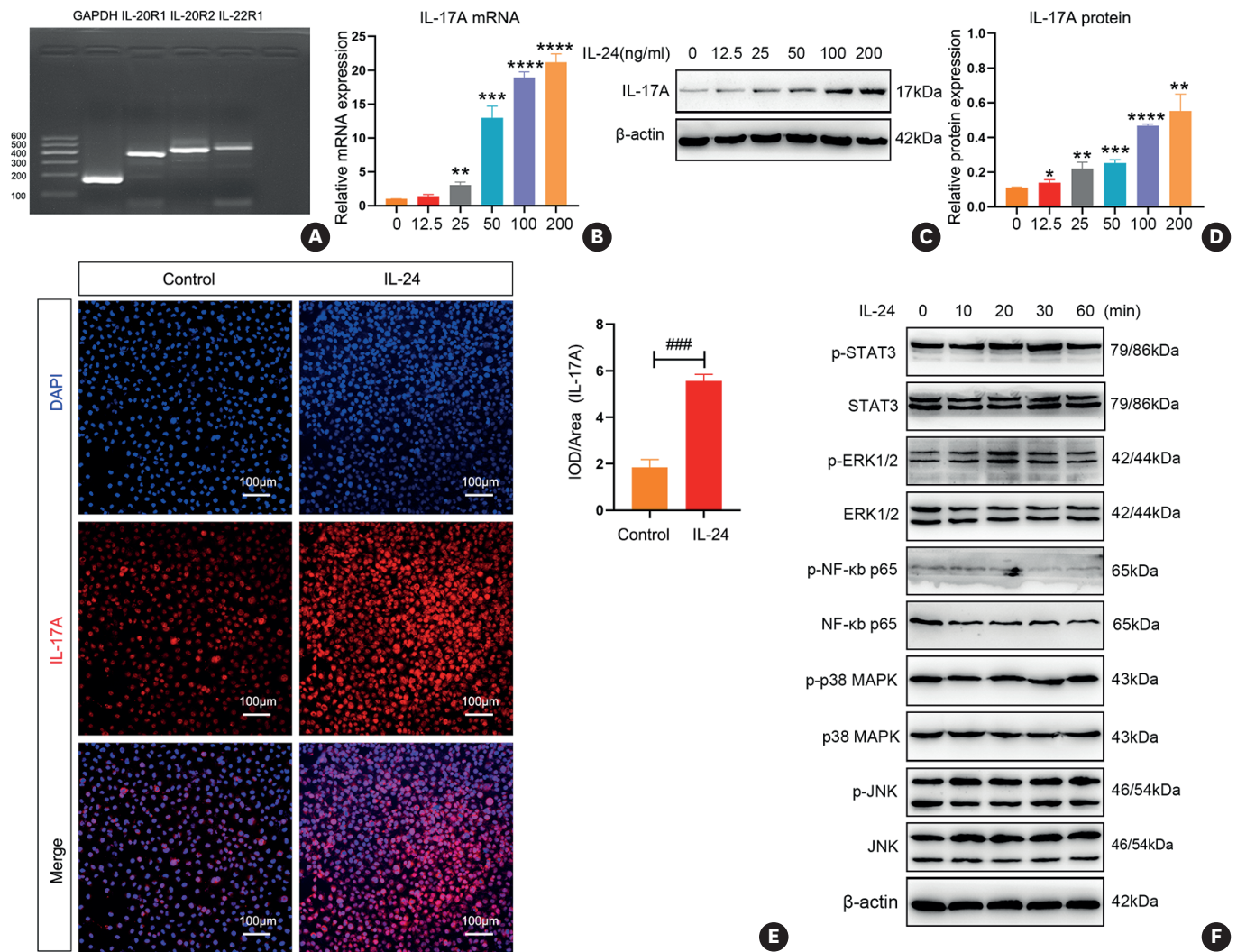
Fig. 1. IL-24 promotes IL-17A production in 16-HBE cells by promoting the phosphorylation of p-STAT3 and p-ERK1/2 *in vitro*. (A) IL-24 receptors (IL-20R1, IL-20R2, and IL-22R1) were expressed in 16-HBE cells, as determined by agarose gel electrophoresis. (B) The mRNA level of IL-17A was determined in 16-HBE cells, followed by rhIL-24 (0–200 ng/mL) stimulation for 24 hours by RT-qPCR. The protein level of IL-17A was determined in 16-HBE cells, followed by rhIL-24 (0–200 ng/mL) stimulation for 48 hours by western blotting (C, D) and immunofluorescence (E). The blue fluorescence of DAPI indicated nuclear localization (200×, scale bar = 100 μm). (F) 16-HBE cells were treated with 100 ng/mL IL-24 for gradient times (0–60 minutes), and the activation of various signaling pathways was determined by western blotting. The mRNA level of IL-17A was quantified in 16-HBE cells after treatment with IL-24 (100 ng/mL) for 24 hours with or without tofacitinib (G) or PD98059 (H) pretreatment for 1 hour by RT-PCR. The activation of p-STAT3/STAT3 or p-ERK1/2/ERK1/2 and IL-17A was quantified in 16-HBE cells after treatment with IL-24 for 48 hours with or without tofacitinib (I, J) or PD98059 (K, L) pretreatment for 1 hour by western blotting. Data are presented as the mean ± standard deviation and represent 3 independent experiments.
IL, interleukin; HBE, human bronchial epithelial; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; rhIL, human recombinant interleukin; DAPI, 4′,6-diamidino-2-phenylindole; ns, no significance; tofacitinib, a specific JAK inhibitor; PD98059, a specific ERK1/2 inhibitor; DMSO, dimethyl sulfoxide.
vs. Control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; vs. IL-24 group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$.

(continued to the next page)

Epithelial-derived IL-17A is required for IL-24-induced neutrophil migration

Considering that IL-17A is a representative cytokine contributing to neutrophilic airway inflammation, we postulated that epithelial-derived IL-17A induced by IL-24 might contribute to neutrophil migration. To address this issue, we applied a coculture system of 16-HBE cells and neutrophils using a Transwell assay with a neutralizing antibody against IL-17A or an isotype control antibody. Initially, primary human neutrophils were isolated from the peripheral blood of healthy volunteers. Diff-Quik staining confirmed the morphology of neutrophils (**Fig. 2A**), and flow cytometry was used to assess neutrophil viability (up to 95.3%)
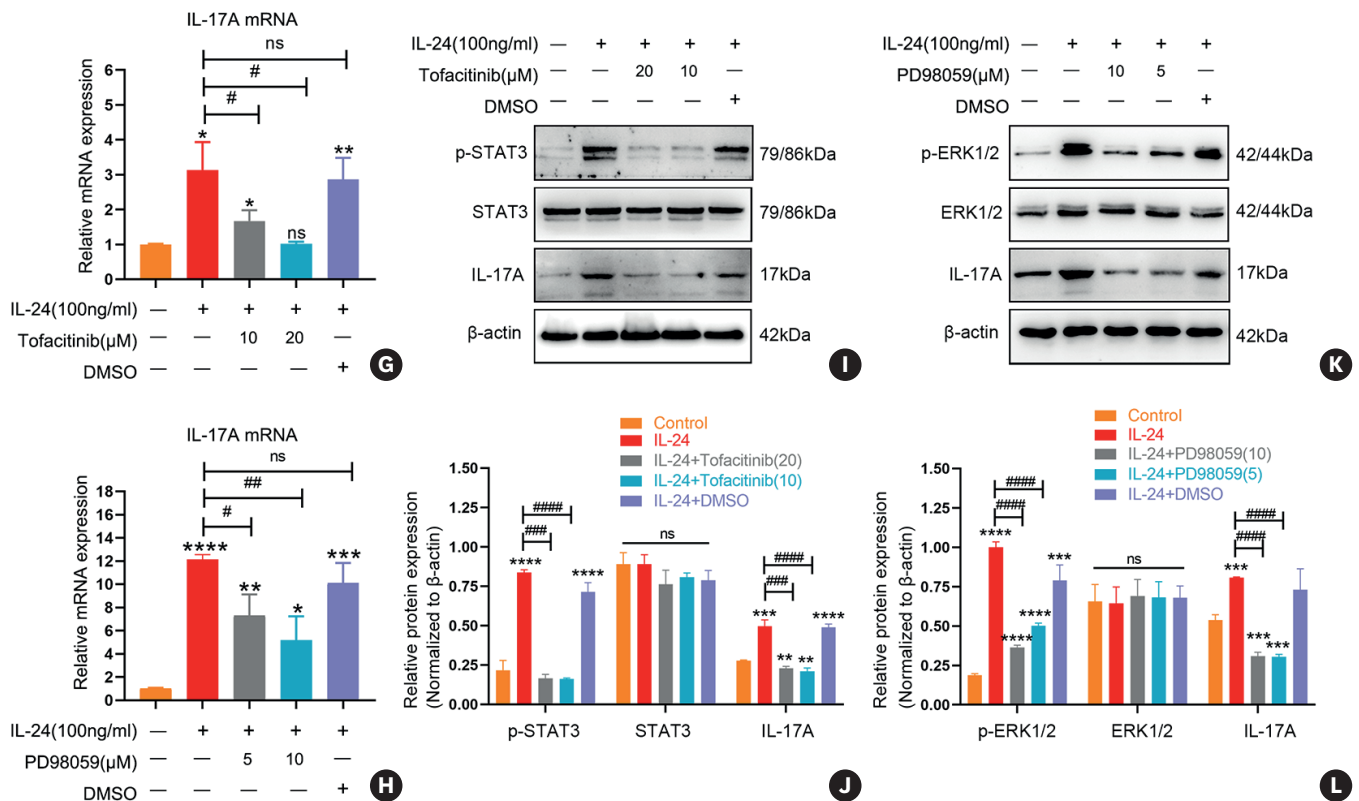
Fig. 1. (Continued) IL-24 promotes IL-17A production in 16-HBE cells by promoting the phosphorylation of p-STAT3 and p-ERK1/2 *in vitro*. (A) IL-24 receptors (IL-20R1, IL-20R2, and IL-22R1) were expressed in 16-HBE cells, as determined by agarose gel electrophoresis. (B) The mRNA level of IL-17A was determined in 16-HBE cells, followed by rhIL-24 (0–200 ng/mL) stimulation for 24 hours by RT-qPCR. The protein level of IL-17A was determined in 16-HBE cells, followed by rhIL-24 (0–200 ng/mL) stimulation for 48 hours by western blotting (C, D) and immunofluorescence (E). The blue fluorescence of DAPI indicated nuclear localization (200×, scale bar = 100 μm). (F) 16-HBE cells were treated with 100 ng/mL IL-24 for gradient times (0–60 minutes), and the activation of various signaling pathways was determined by western blotting. The mRNA level of IL-17A was quantified in 16-HBE cells after treatment with IL-24 (100 ng/mL) for 24 hours with or without tofacitinib (G) or PD98059 (H) pretreatment for 1 hour by RT-PCR. The activation of p-STAT3/STAT3 or p-ERK1/2/ERK1/2 and IL-17A was quantified in 16-HBE cells after treatment with IL-24 for 48 hours with or without tofacitinib (I, J) or PD98059 (K, L) pretreatment for 1 hour by western blotting. Data are presented as the mean ± standard deviation and represent 3 independent experiments.

IL, interleukin; HBE, human bronchial epithelial; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; rhIL, human recombinant interleukin; DAPI, 4′,6-diamidino-2-phenylindole; ns, no significance; tofacitinib, a specific JAK inhibitor; PD98059, a specific ERK1/2 inhibitor; DMSO, dimethyl sulfoxide.

vs. Control group, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; vs. IL-24 group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$.

and purity (up to 96.1%, human neutrophils were identified as CD15^{+}CD66b^{+}) (**Fig. 2B and C**). As shown in **Fig. 2D and E**, IL-24-treated 16-HBE cells dramatically enhanced the number of migrated neutrophils *in vitro*, which could be partly suppressed by pretreatment with an anti-IL-17A antibody rather than an isotype control antibody. Therefore, these findings indicated that IL-24 may recruit neutrophils by releasing epithelial-derived IL-17A.

IL-37 prevents the upregulation of IL-17A induced by IL-24 by blocking the STAT3 and ERK1/2 signaling pathways in 16-HBE cells

IL-37 is a negative immunosuppressant and involved in the pathogenesis of diverse inflammatory disorders. Next, we demonstrated that pretreatment with IL-37 diminished the IL-24-induced IL-17A mRNA and protein levels (**Fig. 3A-E**). To delineate which signaling pathways were involved in IL-37 downregulation of IL-24 downstream signaling, we analyzed several pathways in response to IL-24 in the presence or absence of IL-37 in 16-HBE cells. We discovered that exogenous IL-37 selectively inhibited IL-24-mediated activation of the STAT3 and ERK1/2 signaling pathways (**Fig. 3F**). Taken together, these data provide evidence that
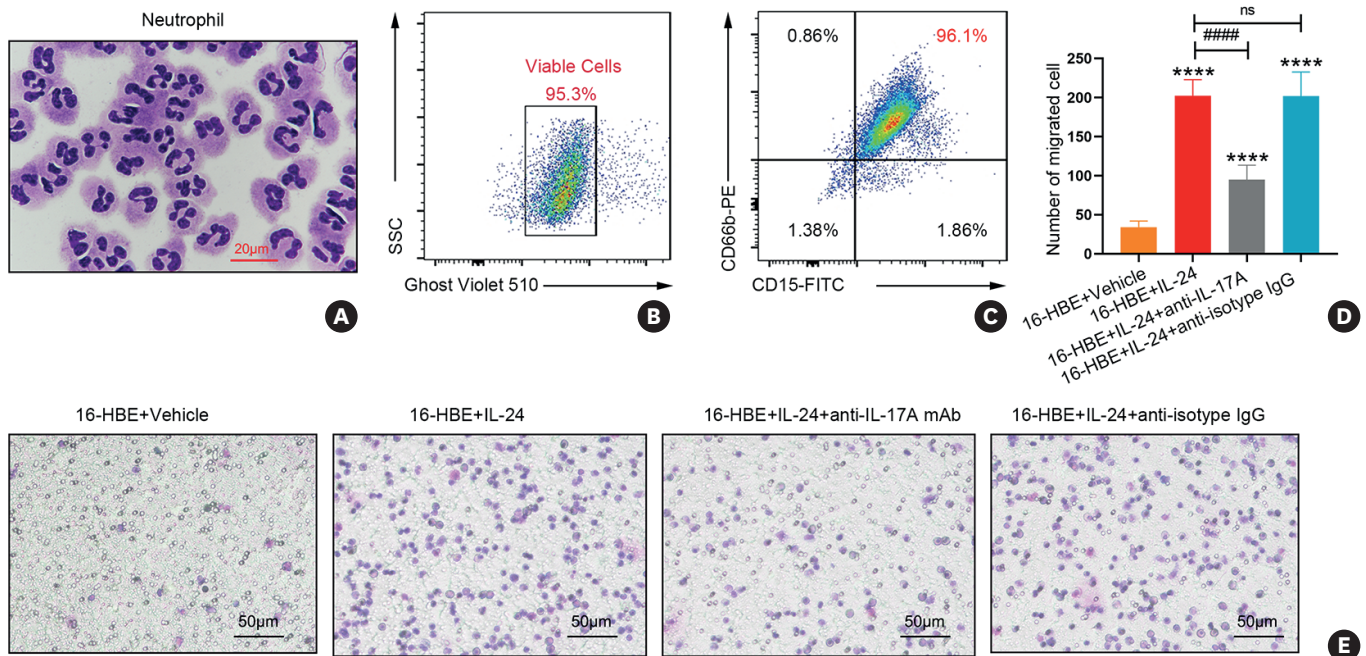
Fig. 2. Epithelial-derived IL-17A is essential for IL-24-induced neutrophil migration *in vitro*. (A) The morphology of human peripheral neutrophils was determined by Diff-Quik staining (1,000×, scale bar = 20 μm). The frequency of living cells (B) and purity (C) of isolated human neutrophils were analyzed by flow cytometry. (D, E) The effect of anti-IL-17A mAbs or isotype control IgG antibody on the neutrophil migration ability of IL-24-stimulated 16-HBE cells in a Transwell coculture chamber (200×, scale bar = 50 μm). Data are presented as the mean ± standard deviation and represent 3 independent experiments.
IL, interleukin; HBE, human bronchial epithelial; IgG, immunoglobulin G; ns, no significance; mAb, monoclonal antibody.
vs. 16-HBE+Vehicle group, ****P < 0.0001; vs. 16-HBE+IL-24 group, ####P < 0.0001.

IL-24-induced IL-17A production depends on STAT3 and EKR1/2 phosphorylation levels in 16-HBE cells and is suppressed by IL-37.

Effect of BUD on pulmonary inflammation and goblet cell metaplasia in HDM- or HDM/LPS-induced mouse models of asthma

To evaluate the responses of HDM and HDM/LPS-induced asthma mouse models to BUD treatment, we compared airway inflammation, goblet cell metaplasia, and other parameters of mice. In terms of periodic acid-Schiff (PAS) staining, mice sensitized with HDM alone or HDM plus LPS exhibited mucus accumulation, and the PAS-positive goblet cell count was considerably lower in HDM-sensitized mice than in those sensitized with HDM and LPS after BUD therapy (**Supplementary Fig. S3A**). Hematoxylin and eosin (H&E) staining results indicated that HDM plus LPS exposure increased the degree of infiltrating inflammatory cells around the bronchi and perivasculature compared to PBS or HDM exposure alone (**Supplementary Fig. S3B**). HDM-induced lung inflammation was dominated by eosinophils, and there was a substantial decrease in eosinophil counts in the HDM+BUD group compared to the HDM group. However, mice exposed to HDM plus LPS had an increased number of neutrophils in the lungs, whereas BUD treatment had no obvious effect on the increased number of neutrophils in HDM/LPS-induced asthmatic mice (**Supplementary Fig. S3C**). Immune cells in the spleen of mice sensitized by HDM plus LPS increased slightly compared to those sensitized by HDM or PBS alone, and intranasal application of BUD had no effect on inflammatory cell counts in the spleen of mice (**Supplementary Fig. S3D**). Moreover, mice sensitized with HDM alone or HDM plus LPS developed a Th2- or Th17-dominant immune response, respectively. BUD treatment elicited a decrease in Th1, Th2, and Th17 cells in the lungs of HDM/LPS-sensitized mice, but not HDM-sensitized mice; no similar
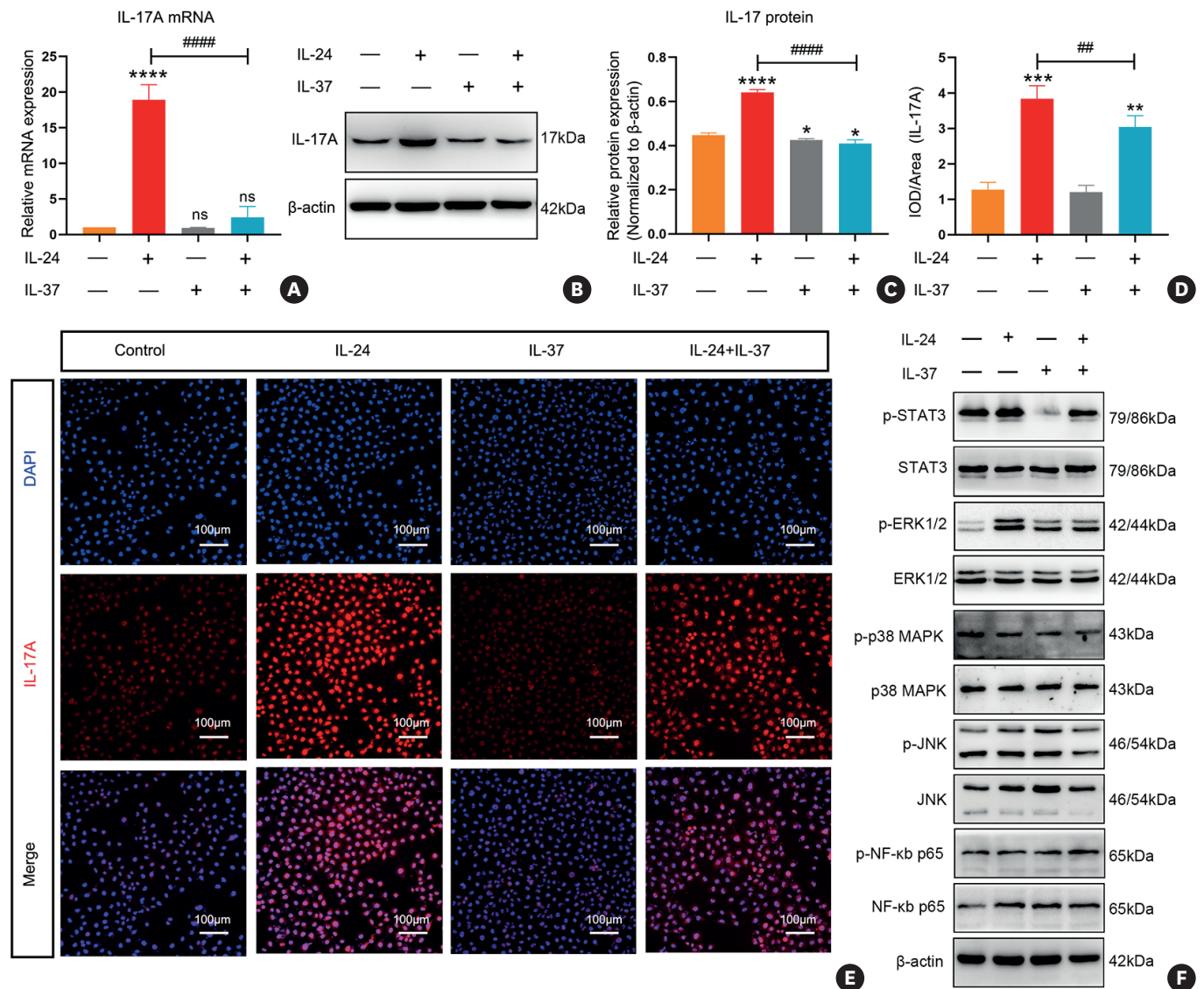
Fig. 3. IL-37 restrains IL-24-induced IL-17A production by modulating the STAT3 and ERK1/2 signaling pathways in 16-HBE cells *in vitro*. (A) The mRNA level of IL-17A was quantified in 16-HBE cells after treatment with IL-24 (100 ng/mL) for 24 hours with or without IL-37 (100 ng/mL) pretreatment for 8 hours by RT-PCR. The protein level of IL-17A was quantified in 16-HBE cells treated with IL-24 (100 ng/mL) for 48 hours with or without IL-37 (100 ng/mL) pretreatment for 8 hours by western blotting (B, C) and immunofluorescence (D, E). The blue fluorescence of DAPI indicated nuclear localization (200×, scale bar = 100 µm). (F) 16-HBE cells were treated with IL-24 (100 ng/mL) for 48 hours with or without IL-37 (100 ng/mL) pretreatment for 8 hours, and the activation of several signaling pathways was determined by western blotting. Data are presented as the mean ± standard deviation and represent 3 independent experiments.
IL, interleukin; HBE, human bronchial epithelial; RT-PCR, reverse transcription-polymerase chain reaction; DAPI, 4′,6-diamidino-2-phenylindole.
vs. Control group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; vs. IL-24 group, $^{##}P < 0.01$, $^{####}P < 0.0001$.

trend was observed in the spleens (**Supplementary Fig. S4A and B**). These results indicated that HDM exposure induced eosinophilic airway inflammation, but that HDM/LPS exposure exacerbated neutrophilic airway inflammation, which was insensitive to BUD administration.

IL-37 treatment or silencing IL-24 diminishes IL-17A expression in bronchial epithelium in an HDM/LPS-induced asthma murine model

To explore the effect of IL-24 and IL-37 *in vivo*, we established eosinophil-predominant and neutrophil-predominant asthma mouse models with HDM alone and HDM plus LPS by intranasal delivery of siRNA targeting IL-24 or IL-37 protein, respectively. We first confirmed

the knockdown efficiency of IL-24 in mice using ELISA and immunofluorescence. As shown in **Fig. 4A, B, E, and F**, HDM/LPS-induced model mice showed overproduction of IL-24 and IL-17A in the BALF and lungs, and intranasal administration of si-IL-24 and IL-37 minimized IL-24 expression in BALF and bronchial epithelium, accompanied by a lower level of IL-17A. However, these trends were not observed in serum (**Fig. 4C and D**). Interestingly, increased IL-24 was observed not only in the epithelium but also in the other cells of the submucosal layer (**Fig. 4E**). Furthermore, we noticed that si-IL-24 and IL-37 treatment diminished p-STAT3 and p-ERK1/2 activation in HDM/LPS-sensitized mouse lung tissues (**Supplementary Fig. S5A and B**). Epithelial cell-derived inflammatory mediators, such as IL-25, IL-33, and TSLP, are involved in asthma pathogenesis, so we evaluated the expression of these cytokines in the airway epithelium. As depicted in **Supplementary Fig. S6A-D**, mice sensitized by HDM or HDM plus LPS both increased the levels of IL-25, IL-33, and TSLP, whereas IL-37 administration reduced IL-33 and TSLP expression, but not IL-25. Collectively, HDM/LPS-exposed mice exhibited augmented IL-24, IL-17A, IL-33, and TSLP levels in the airway epithelium, and IL-37 treatment resulted in the downregulation of these proteins in the airway epithelium, indicating that IL-37 has the potential to regulate inflammatory mediators in neutrophilic asthma.

IL-37 treatment or silencing IL-24 ameliorates lung function, goblet cell hyperplasia, and pulmonary neutrophilia in an HDM/LPS-induced asthma model

We next investigated the effect of silencing IL-24 or IL-37 treatment on AHR using an invasive measurement of murine lung function. The results showed that both the IL-24-silenced and IL-37-treated groups resulted in a down-regulation of airway reactivity at a dose of Mch (50 mg/mL) when compared to the HDM/LPS-treated group (**Fig. 5A**). Since mucus hyperplasia is a significant pathological hallmark of asthma, we wanted to further confirm whether IL-24 and IL-37 were involved in mucus hypersecretion using PAS staining. As shown in **Fig. 5B and C**, allergen-challenged mice developed abundant mucus hyperplasia in the bronchial epithelium, but the IL-24 silencing group exhibited fewer PAS-positive mucous cells in the airways than the si-NC group. Likewise, IL-37 treatment had an obvious inhibitory effect on
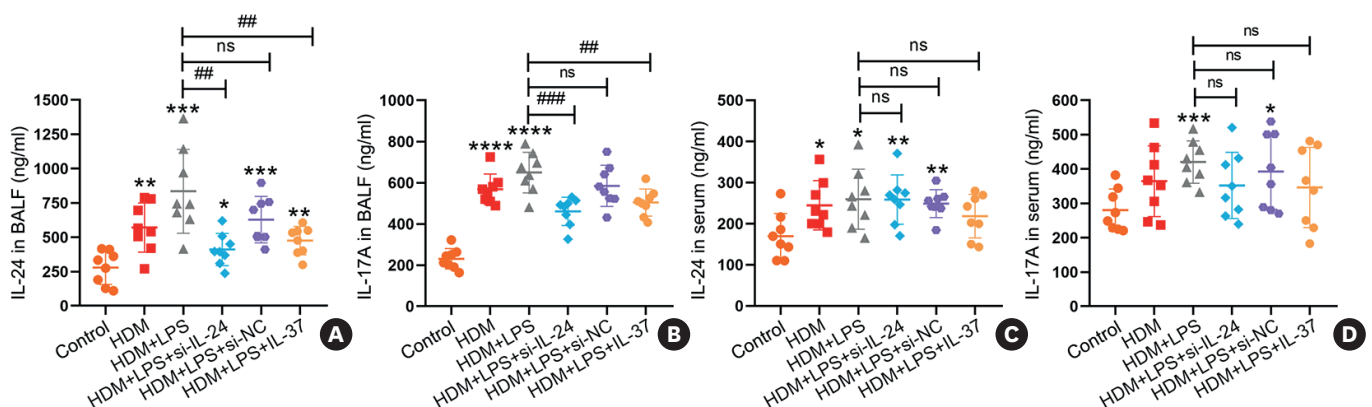

Fig. 4. The effect of si-IL-24 or IL-37 on IL-17A expression in an HDM/LPS-induced murine asthma model. (A, B) Quantification of IL-24 and IL-17A expression in the BALF of mice. (C, D) Quantification of IL-24 and IL-17A expression in the serum of mice. (E, F) The expression and quantification of IL-24, EpCAM and IL-17A in the lungs of mice were evaluated by immunofluorescence. The blue fluorescence of DAPI indicated nuclear localization (630×, scale bar = 100 μm). Data are presented as the mean ± standard deviation and represent 3 independent experiments, n = 8/group.
si-IL, small interfering interleukin; si-NC, si-negative control; IL, interleukin; HDM, house dust mite; LPS, lipopolysaccharide; DAPI, 4′,6-diamidino-2-phenylindole; BALF, bronchoalveolar lavage fluid; ns, no significance; EpCAM, epithelial cell adhesion molecule, an epithelial marker; si-IL, small interfering interleukin.
vs. Control group, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; vs. HDM+LPS group; ##P < 0.01, ###P < 0.001.
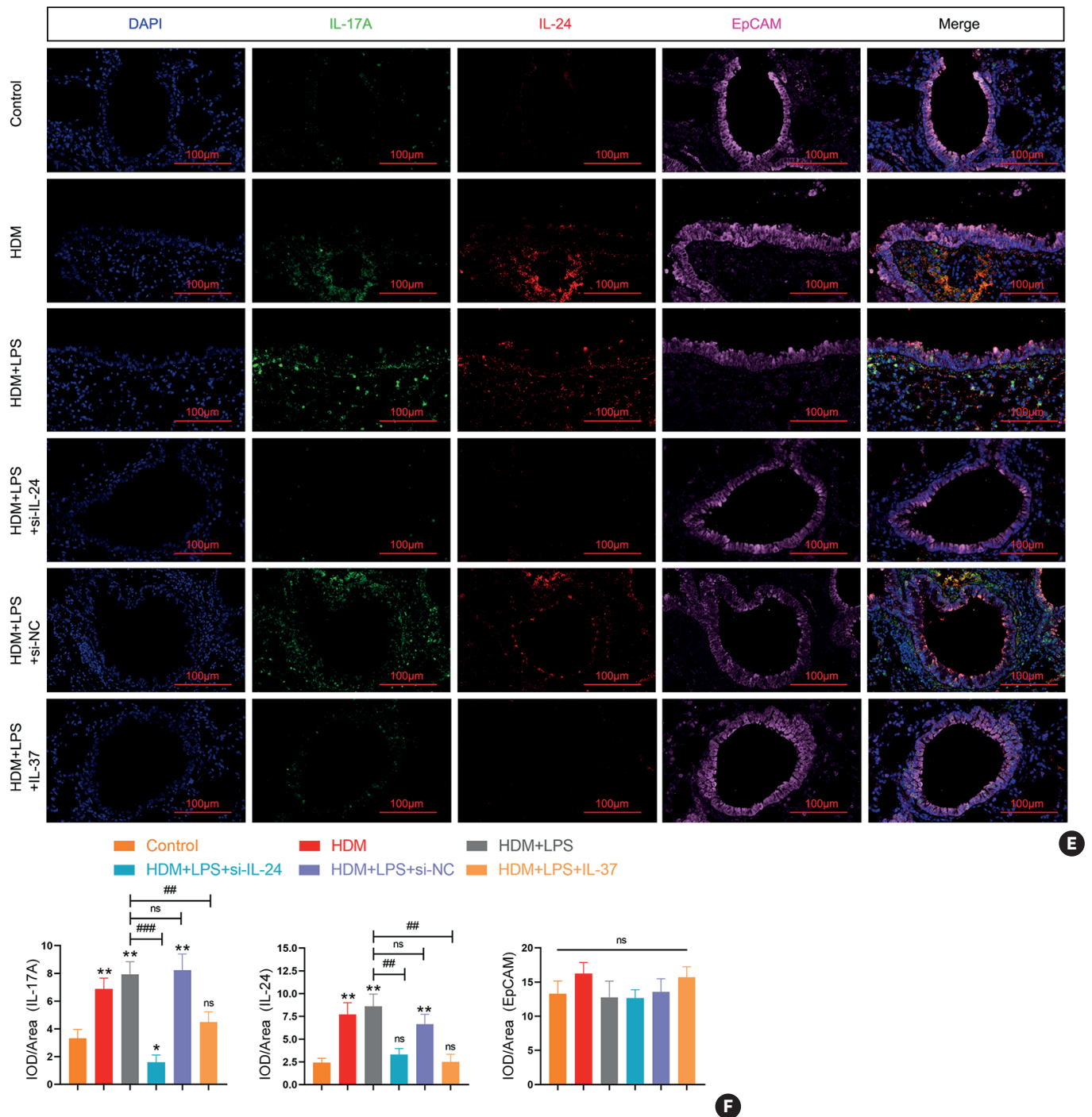
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Fig. 4. (Continued) The effect of si-IL-24 or IL-37 on IL-17A expression in an HDM/LPS-induced murine asthma model. (A, B) Quantification of IL-24 and IL-17A expression in the BALF of mice. (C, D) Quantification of IL-24 and IL-17A expression in the serum of mice. (E, F) The expression and quantification of IL-24, EpCAM and IL-17A in the lungs of mice were evaluated by immunofluorescence. The blue fluorescence of DAPI indicated nuclear localization (630×, scale bar = 100 μm). Data are presented as the mean ± standard deviation and represent 3 independent experiments, n = 8/group.

si-IL, small interfering interleukin; si-NC, si-negative control; IL, interleukin; HDM, house dust mite; LPS, lipopolysaccharide; DAPI, 4′,6-diamidino-2-phenylindole; BALF, bronchoalveolar lavage fluid; ns, no significance; EpCAM, epithelial cell adhesion molecule, an epithelial marker; si-IL, small interfering interleukin.

vs. Control group, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; vs. HDM+LPS group; $^{##}P < 0.01$, $^{###}P < 0.001$.

mucus hyperplasia. Together, these results demonstrated that silencing IL-24 can ameliorate the degree of goblet cell hyperplasia and AHR in mice in response to allergen sensitization.
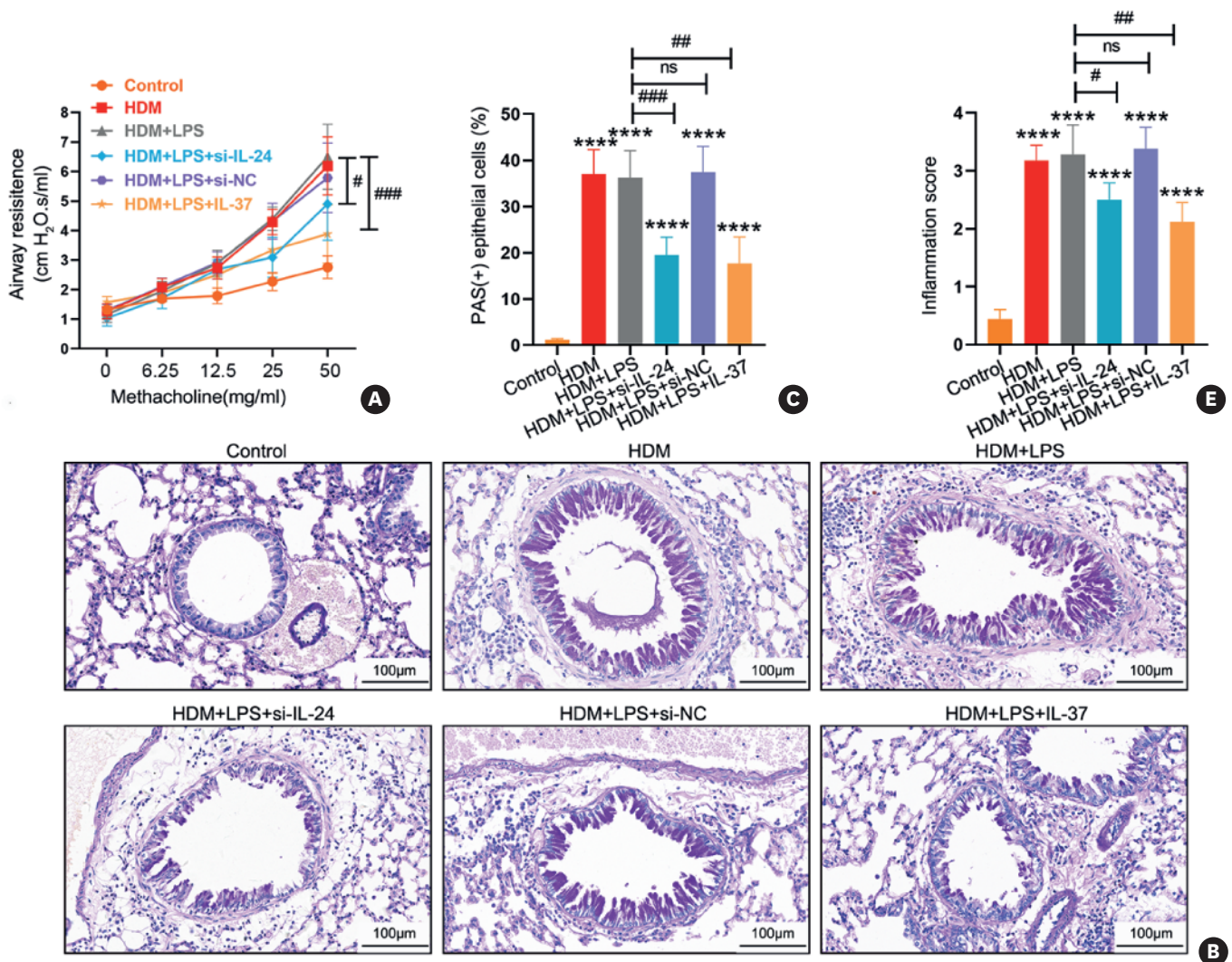
Fig. 5. The effect of si-IL-24 or IL-37 on airway hyperresponsiveness, mucus production and airway inflammation in an HDM/LPS-induced asthma murine model. (A) Airway resistance was measured with increasing doses of nebulized methacholine by an invasive mouse lung function instrument. (B, C) Histologic analysis of goblet cell hyperplasia was determined by PAS staining (400×, scale bar = 100 μm). (D, E) Histologic analysis of pulmonary inflammation was determined by H&E staining (scale bar = 100 μm for 200×; scale bar = 100 μm for 730×). Data are presented as the mean ± standard deviation and represent 3 independent experiments, n = 8/group.

si-IL, small interfering interleukin; si-NC, si-negative control; IL, interleukin; HDM, house dust mite; LPS, lipopolysaccharide; PAS, periodic acid-Schiff; H&E, hematoxylin and eosin; ns, no significance.

vs. Control group, $^{****}P < 0.0001$; vs. HDM +LPS group, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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H&E staining of lung samples indicated that both HDM-treated mice and HDM/LPS-treated mice developed robust inflammatory cell infiltration into the peribronchiolar and perivascular regions compared to PBS-challenged mice (**Fig. 5D and E**). However, in contrast to HDM/LPS-treated mice, this shift was significantly attenuated by IL-37 or si-IL-24 treatment (**Fig. 5D and E**). To clarify whether IL-24 production after allergen challenge is linked to neutrophilia, we analyzed the populations of neutrophils and eosinophils in murine BALF, lungs and spleen by flow cytometry (**Fig. 6A**). We observed that HDM and HDM combined with LPS sensitized mice exhibited robust airway inflammation dominated by eosinophils and neutrophils, respectively. However, administration of either si-IL-24 or IL-37 significantly decreased the number of neutrophils, but not eosinophils, in the BALF and lungs in HDM/LPS-sensitized mice (**Fig. 6B and C**). There were no obvious differences in the
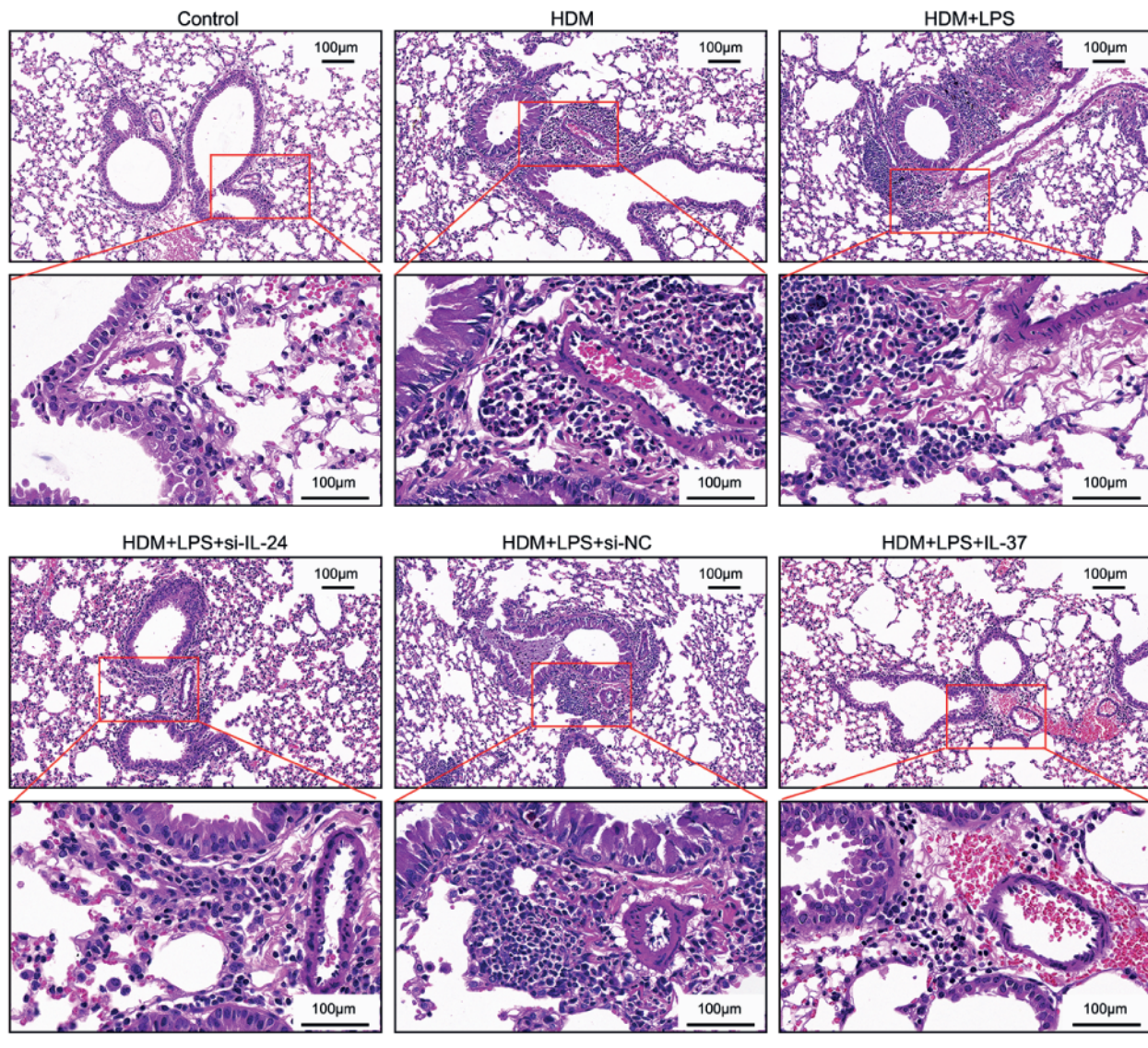
Fig. 5. (Continued) The effect of si-IL-24 or IL-37 on airway hyperresponsiveness, mucus production and airway inflammation in an HDM/LPS-induced asthma murine model. (A) Airway resistance was measured with increasing doses of nebulized methacholine by an invasive mouse lung function instrument. (B, C) Histologic analysis of goblet cell hyperplasia was determined by PAS staining (400×, scale bar = 100 μm). (D, E) Histologic analysis of pulmonary inflammation was determined by H&E staining (scale bar = 100 μm for 200×; scale bar = 100 μm for 730×). Data are presented as the mean ± standard deviation and represent 3 independent experiments, n = 8/group.
si-IL, small interfering interleukin; si-NC, si-negative control; IL, interleukin; HDM, house dust mite; LPS, lipopolysaccharide; PAS, periodic acid-Schiff; H&E, hematoxylin and eosin; ns, no significance.
vs. Control group, $^{****}P < 0.0001$; vs. HDM +LPS group, $^{#}P < 0.05$, $^{##}P < 0.01$, $^{###}P < 0.001$.

percentages of neutrophils and eosinophils in the spleen of each group of mice (**Fig. 6D**). As mentioned above, silencing IL-24 in the epithelium or IL-37 administration resulted in less neutrophil influx into the airways.

IL-37 treatment or silencing IL-24 diminishes the Th17 immune response in an HDM/LPS-induced NA model

To further address whether IL-24 contributes to the modulation of T lymphocyte differentiation, we detected the frequencies of different lineages of CD4+ T lymphocytes (Th1, Th2, and Th17) in mouse BALF, lungs and spleen (**Fig. 6E**). Treatment with si-IL-24

The gate of neutrophil and eosinonphil.
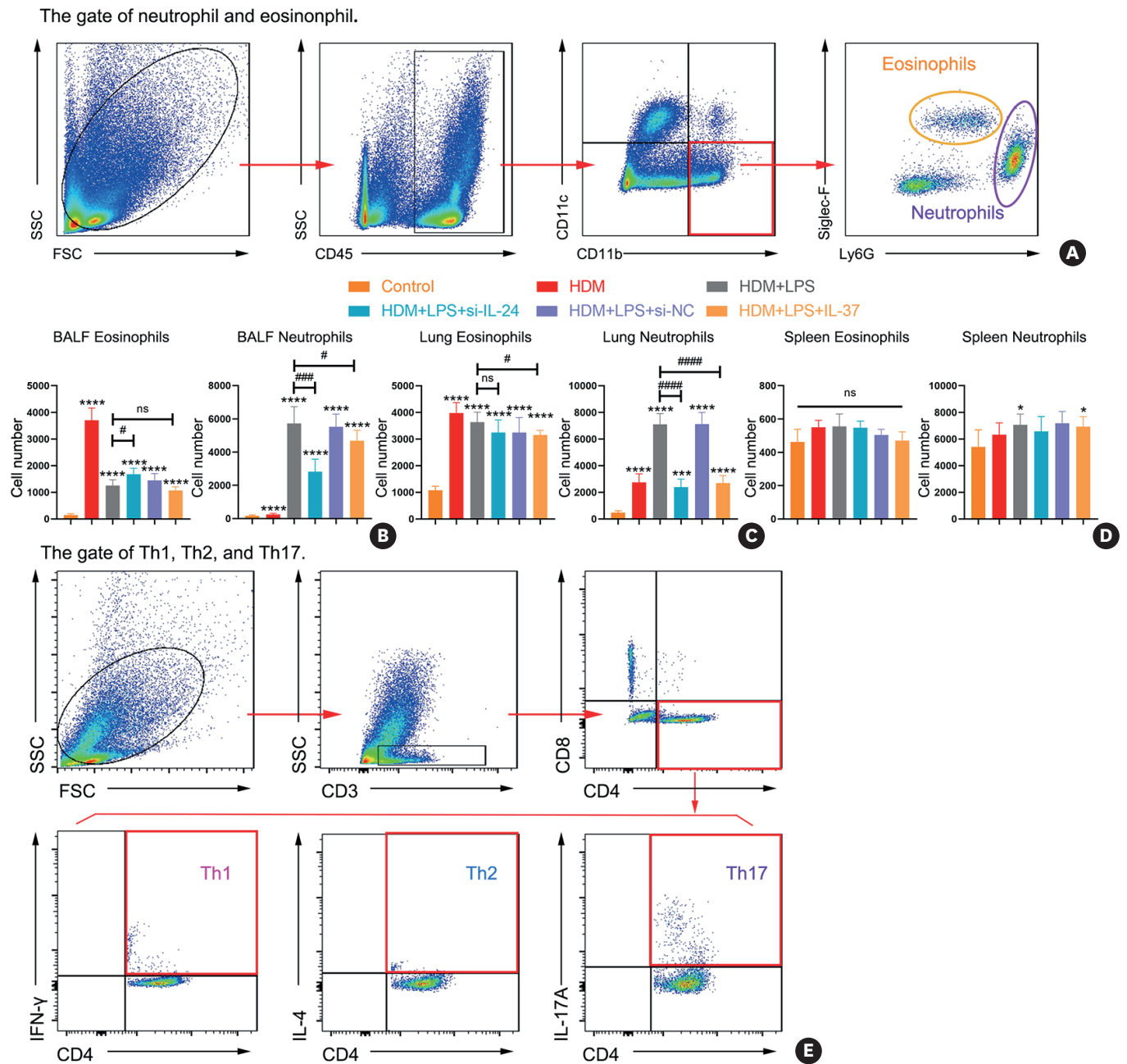

The gate of Th1, Th2, and Th17.

Fig. 6. The effect of si-IL-24 or IL-37 on the population of neutrophils, eosinophils and lymphocytes in an HDM/LPS-induced murine asthma model. (A) Gating strategies of neutrophils and eosinophils by flow cytometry. The quantification of eosinophils and neutrophils in the BALF (B), lungs (C) and spleens (D) of mice was determined by flow cytometry. (E) Gating strategies of Th1 (CD4$^+$ IFN-γ^+), Th2 (CD4$^+$ IL-4$^+$), and Th17 (CD4$^+$ IL-17A$^+$) cells by flow cytometry. The quantification of Th1, Th2 and Th17 in the BALF (F), lungs (G) and spleens (H) of mice was determined by flow cytometry. (I) Expression of the mRNA levels of T-bet, GATA-3, and RORγt in lung tissues of mice were detected by RT-qPCR. Data are presented as the mean ± standard deviation and represent 3 independent experiments, n = 8/group.
si-IL, small interfering interleukin; si-NC, si-negative control; IL, interleukin; HDM, house dust mite; LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid; ns, no significance; Th, T-helper; IFN, interferon; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
vs. Control group, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; vs. HDM+LPS group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$.

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significantly diminished the proportions of Th17 cells in the BALF and lungs in the HDM/LPS-induced model (**Fig. 6F and G**), but not Th1 and Th2 cells. In contrast, no significant differences were detected in the proportions of Th1, Th2, and Th17 cells in the spleens between any 2 groups (**Fig. 6H**). T-bet, GATA-3, and RORγt are critical transcription factors
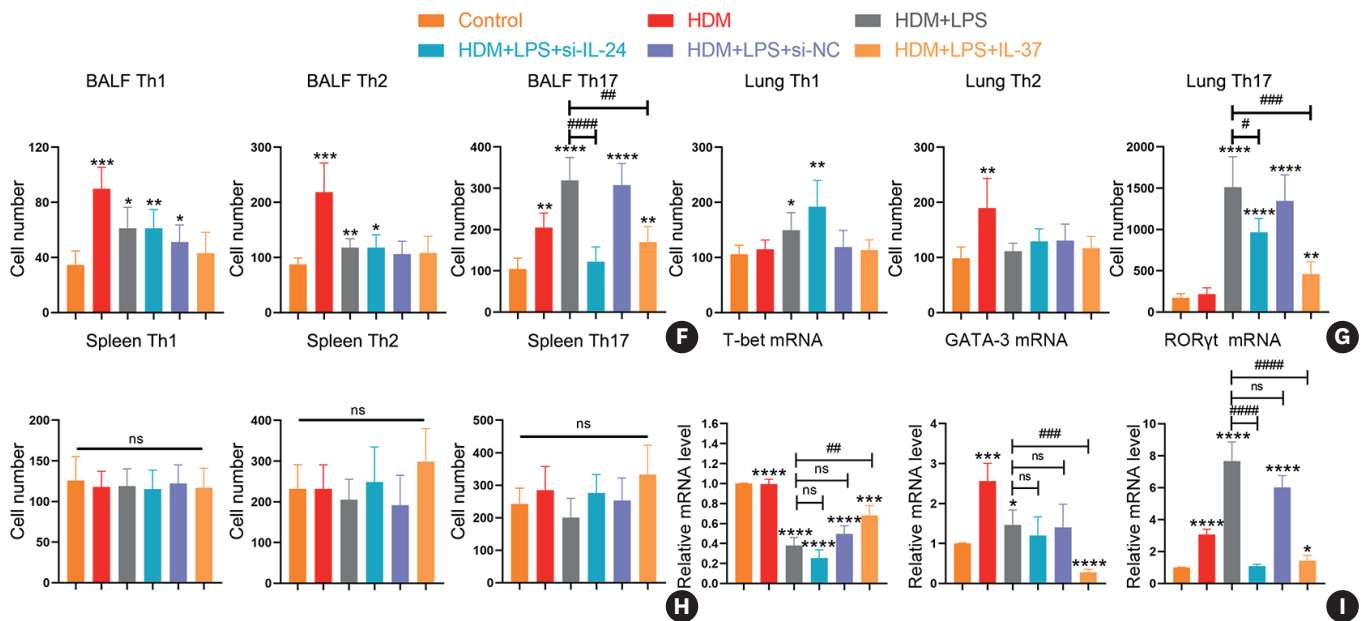
Fig. 6. (Continued) The effect of si-IL-24 or IL-37 on the population of neutrophils, eosinophils and lymphocytes in an HDM/LPS-induced murine asthma model. (A) Gating strategies of neutrophils and eosinophils by flow cytometry. The quantification of eosinophils and neutrophils in the BALF (B), lungs (C) and spleens (D) of mice was determined by flow cytometry. (E) Gating strategies of Th1 (CD4⁺ IFN-γ⁺), Th2 (CD4⁺ IL-4⁺), and Th17 (CD4⁺ IL-17A⁺) cells by flow cytometry. The quantification of Th1, Th2 and Th17 in the BALF (F), lungs (G) and spleens (H) of mice was determined by flow cytometry. (I) Expression of the mRNA levels of T-bet, GATA-3, and RORγt in lung tissues of mice were detected by RT-qPCR. Data are presented as the mean ± standard deviation and represent 3 independent experiments, n = 8/group.

si-IL, small interfering interleukin; si-NC, si-negative control; IL, interleukin; HDM, house dust mite; LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid; ns, no significance; Th, T-helper; IFN, interferon; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.
vs. Control group, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; vs. HDM+LPS group, $^\#P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$.

for Th1, Th2, and Th17 differentiation, respectively, which determine the swing of airway inflammation phenotypes. We next conducted RT-qPCR to assess their mRNA transcript levels in the lungs to address the effect of si-IL-24 and IL-37 on regulating transcription factors. The results indicated that HDM/LPS-sensitized mice displayed a significant increase in the gene level of RORγt in the lungs. Silencing IL-24 in the bronchial epithelium caused an obvious decrease in the RORγt mRNA levels compared to the asthma model group (**Fig. 6I**). Consistent with the decrease in cytokine production in IL-24-silenced mice, the levels of GATA3 and RORγt in the lungs of the IL-37 treatment group were also down-regulated (**Fig. 6I**). In general, these findings indicated that intranasal administration of HDM/LPS elicited a significant elevation of the Th17 immune response in mouse lung tissues in an IL-24 signaling-dependent manner, which could be mitigated by IL-37 treatment.

DISCUSSION

In the current study, our data showed that airway epithelial cells increased IL-24 production following HDM and LPS exposure via the NF-κB signaling pathway *in vivo*. Additionally, we observed that 16-HBE cells constitutively expressed IL-24 and IL-24 receptors, and that IL-24 exerted its effects by regulating epithelium-derived IL-17A levels in a JAK/STAT3 and ERK1/2 signaling pathway-dependent manner. Next, in the coculture system containing 16-HBE cells and human neutrophils, IL-24-stimulated epithelial cells promoted neutrophil migration, but this effect was significantly inhibited by anti-IL-17A monoclonal antibodies. Herein, we established HDM-sensitized and HDM plus LPS-sensitized asthma models and observed

robust eosinophil or neutrophil accumulation in mouse lungs. Intranasal administration of si-IL-24 reduced IL-17A production and pulmonary neutrophilia, with concomitant decreases in AHR, goblet cell metaplasia and the Th17 immune response, in the murine model of HDM/LPS-induced asthma. Importantly, we also demonstrated the therapeutic efficacy of IL-37 in neutrophilic airway inflammation, as IL-37 effectively mitigated the upregulation of IL-24 and IL-17A in the bronchial epithelium and the inflammatory infiltration of neutrophils. However, there was no obvious difference in IL-17A levels in the BALF and lungs between these 2 model groups. The extent to which LPS reverses asthma phenotypes and significantly increases IL-17A production has not been determined. Taken together, these results support the conclusion that IL-24 is a central driver of neutrophilic airway inflammation, and imply that targeting the IL-24/IL-17A signaling axis in epithelial cells may be a novel therapeutic approach for NA.

Our data indicated that 16-HBE cell exposure to HDM and LPS resulted in the activation of the NF-κB pathway. Similarly, another study showed that HDM or LPS exposure alone activated the NF-κB pathway in human bronchial Beas-2B cells.[23] Other studies have demonstrated that Derp1, Derp5, and Derp13 (one of the main components of HDMs) trigger the NF-κB pathway *in vitro* and *in vivo* in asthma.[24-26] These data indicated that NF-κB signaling may be one of the main downstream signaling pathways of HDMs. Since bronchial epithelial cells are one of the target cells of IL-24, we investigated different cytokines produced by epithelial cells in response to IL-24 signaling. Our data showed a significant increase in IL-17A expression in bronchial epithelial 16-HBE cells in response to IL-24 stimulation. We further found that silencing IL-24 in mouse models of asthma reduced epithelium-derived IL-17A expression and the influx of neutrophils and Th17 cells, suggesting that the IL-24-IL-17A axis plays an important role in the pathogenic mechanism of NA. Consistent with our results, previous studies have demonstrated that IL-24 plays an important role in regulating IL-17A expression and Th17 responses. For instance, knockdown of mouse IL-20R (an IL-24 receptor subunit) has been shown to limit IL-17A-producing dermal γδ T-cell accumulation and Th17 responses in psoriatic inflammation.[27] Higher rather than lower concentrations of IL-24 were found to increase the Th1 frequency and T-bet mRNA levels, but not the Th17 or RORγt mRNA levels, in colorectal adenocarcinoma.[28] In fact, T cells and other immune cells do not express IL-24-related receptors, indicating that immune cells are unlikely to the target cells of IL-24. However, silencing IL-24 *in vivo* regulated the frequency of Th17 cells in this study. These results of the effect of IL-24 on Th17 cell polarization *in vivo* and *in vitro* seem to be paradoxical and have not been not fully elucidated. Interestingly, Chong *et al.*[29] verified that IL-17A binds to IL-17R on Th17 cells, thereby inducing IL-24 production by activating the NF-κB pathway. IL-17A can also promote IL-24 expression in human skin fibroblasts.[30] IL-17ra^{-/-} mice exhibited lower IL-24 expression in the healing oral mucosa than WT mice.[31] These findings underscore that IL-24 and IL-17A may be regulated in a reciprocal manner. The discovery of the relationship between IL-24 and IL-17A in bronchial epithelial cells enhances a clear understanding of the complex mechanism of NA.

Many studies, including ours, have shown that IL-24 exhibits proinflammatory properties in asthma and other allergic or autoimmune diseases.[32-34] However, recent literature has confirmed the presence of an elevated IL-24 level in the affected mucosa of patients with inflammatory bowel disease, and IL-24 has a protective role against mucosal inflammation, which is paradoxical to our result.[35,36] Wang *et al.*[37] recently reported that IL-24 preferred to use IL-20R2/IL-22R1 when mediating protective effects on hepatocytes, but exerted harmful effects mainly through the IL-20R1/IL-20R2 receptor complex using WT and IL-20R1- and IL-

20R2-deficient mice. Hence, we inferred that IL-24 might exert different functions depending on the disease and receptor expression context; the controversial biological function of IL-24 deserves further studies.

It is worth mentioning that in this study, we confirmed that IL-24-treated epithelial cells significantly enhanced neutrophil chemotaxis, and that this indirect effect could be partially suppressed by neutralizing antibodies specific for IL-17A. In this observation, we do not exclude the possibility that other epithelium-derived neutrophil chemokines also play a similar role. In addition, Buzas et al.[38] demonstrated that rhIL-24 promoted human monocyte and neutrophil migration in vitro. Hsu et al.[39] also substantiated that a low concentration of IL-24 had a direct chemotactic effect in human neutrophils and that IL-20 (another member of the IL-10 family) had a direct effect in neutrophil chemotaxis. These findings suggest that IL-24 both directly and indirectly promotes neutrophil migration. In fact, most of immune cells, including neutrophils, do not express IL-24-related receptors on their surface.[33] Considering that IL-20 and IL-24 have similar biological functions and share some receptors, we speculated that neutrophils may have some unknown receptors for IL-10 superfamily cytokines on their surface.

In our NA model, the intensity of TSLP and IL-33 in the bronchial epithelial layer was higher than that of IL-25. Although TSLP, IL-33, and IL-25 are considered key cytokines that contribute to Th2-type immune responses, IL-25 seems to play a weaker role in the pathogenesis of asthma. Evidence indicates that higher levels of IL-33 and TSLP, but not IL-25, can be detected in exhaled breath condensate and BALF in asthmatic patients.[40,41] Moreover, chitin (a component in the outer shell of HDMs)-induced asthmatic mice showed up-regulated expression of TSLP and IL-33, but not IL-25.[42] HDM and peanuts exposure mainly promoted IL-33 elevation, but not TSLP and IL-25; a similar trend can be observed in silica exacerbation of OVA-induced asthmatic mice.[43,44] In addition, evidence has shown that IL-25 is associated with eosinophilic asthma and that elevated IL-25 levels can be detected in virus-exacerbated asthmatic models and clinical patients.[45,46] These findings suggest that the levels of TSLP, IL-33, and IL-25 in different asthma models, and that the importance of their contribution to the pathogenesis of asthma may be different, possibly depending on the type of allergens. The exact mechanisms need to be clarified. We also found that IL-37 inhibited the elevated levels of TSLP and IL-33 in the mouse bronchial epithelium, but had no effect on IL-25. It may be that the expression of IL-25 in neutrophilic asthma is so low that it is difficult to observe the inhibitory effect of IL-37 on epithelial-derived IL-25. Taken together, we hypothesized that IL-25 contributes less to NA than IL-33 or TSLP, but is more important in eosinophilic asthma and virus-worsening asthma.

Earlier studies from our team and other groups have shown that IL-37 exerts anti-inflammatory functions by suppressing the production of inflammatory mediators in eosinophilic asthma and eosinophilic chronic rhinosinusitis.[18,47,48] We further observed that IL-37 prevented IL-24-induced epithelium-derived IL-17A production in 16-HBE cells by modulating the p-STAT3 and p-ERK1/2 pathways. Additionally, a growing body of studies also reported that IL-37 can inhibit the STAT3 and ERK1/2 signaling pathways in allergic inflammatory diseases.[18,49] Recently, Charrada et al.[17] demonstrated that IL-37 inhibited IL-17A production in CD4+ T cells in the sputum of asthmatic patients in vitro. In agreement with these results, i.n. administered rhIL-37 reduced the IL-17A level and Th17 immune response in the lungs of HDM/LPS-sensitized asthmatic mice. The results of this study may further expand our understanding of the role and mechanism of IL-37, a powerful inhibitor of inflammation, in relation to airway inflammation in different asthma phenotypes.

Additionally, IL-37 is expected to be a potential therapeutic agent for improving asthma airway inflammation. However, the safety and feasibility of IL-37 in clinical settings remain to be confirmed by future clinical studies.

Indeed, there are some limitations to this study that need to be considered. First, to better define the biological role of IL-24 in asthma, we plan to collect more sputum and serum specimens from asthma patients in subsequent studies and to further analyze the correlations of IL-24 expression with sputum cytology, lung function parameters, and even the inflammatory phenotypes of asthma patients. On the other hand, we will attempt to further explore the exact role of IL-24 in asthma mouse models using a mouse recombinant IL-24 protein and IL-24 knockout mice. Finally, in addition to airway epithelial cells, we noticed that immune cells and other structural cells in the submucosa can also secrete IL-17A in mouse lungs. Given that epithelial cells are not the main source of IL-17A, further research focusing on the mechanism between IL-24 and the immune cells that produce IL-17A is essential.

Collectively, these findings provide convincing evidence for the neutrophil-dependent connection between increased IL-24 expression and treatment-refractory asthma. Mechanistically, IL-24 contributes to the production of IL-17A, which attracts abundant neutrophils to inflamed sites during the process of asthma; this implies that modulating the epithelium-derived IL-24-IL-17A axis may be a pivotal strategy for alleviating pulmonary neutrophilic inflammatory responses, and that IL-37 may be a promising immunotherapeutic agent in asthma. Importantly, IL-24 can be used as a biomarker for predicting asthma exacerbation or severe refractory asthma in the future.

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIALS

Supplementary Data S1
Methods

Click here to view

Supplementary Table S1
Details of antibodies for flow cytometry analysis

Click here to view

Supplementary Table S2
The sequences of target genes

Click here to view

Supplementary Table S3
Lists of antibodies used in the western blot, IHC and IF

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Supplementary Fig. S1
Schematic diagram of the allergen-exposed murine model of asthma and drug treatment.

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Supplementary Fig. S2
HDM exposure increases the expression of IL-24 and its receptors (IL-20R1, IL-20R2, and IL-22R1) in 16-HBE cells. (A) The protein levels of IL-24 and IL-24 receptors (IL-20R1, IL-20R2, and IL-22R1) were determined in 16-HBE cells followed by HDM (1 μg/mL), LPS (10 μg/mL) or HDM plus LPS stimulation for 48 hours. (B) The protein levels of IL-33, IL-25, and TSLP were determined in 16-HBE cells followed by HDM (1 μg/mL), LPS (10 μg/mL) or HDM plus LPS stimulation for 48 hours. (C) 16-HBE cells were treated with 1 μg/mL HDM and 10 μg/mL LPS for gradient times (0–120 minutes), and the activation of various signaling pathways was determined. (D) The protein levels of p-NF-κB, NF-κB and IL-24 were quantified in 16-HBE cells after treatment with HDM (1 μg/mL) and 10 μg/mL LPS for 48 hours with or without BAY 11-7082 (5 μM) or DMSO pretreatment for 1 hour. Data are presented as the mean ± standard deviation and represent 3 independent experiments.

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Supplementary Fig. S3
The effect of BUD on airway inflammation and mucus production in a HDM or HDM/LPS-induced murine model of asthma (A) Histologic analysis of goblet cell hyperplasia was determined by PAS staining (10×, scale bar = 200 μm). (B) Histologic analysis of pulmonary inflammation was determined by H&E staining (2×, scale bar = 1 mm). The percentages and quantification of eosinophils and neutrophils in the lungs (C) and spleens (D) of mice were determined by flow cytometry. Data are presented in 3 independent experiments, n = 8/group.

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Supplementary Fig. S4
The effect of BUD on Th1, Th2, and Th17 immune responses in a HDM or HDM/LPS-induced murine model of asthma. The percentages and quantification of Th1 (CD4^{+} IFN-γ^{+}), Th2 (CD4^{+} IL-4^{+}), and Th17 (CD4^{+} IL-17A^{+}) cells in the lungs (A) and spleens (B) of mice were determined by flow cytometry. Data are presented in 3 independent experiments, n = 8/group.

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Supplementary Fig. S5
The effect of si-IL-24 or IL-37 on p-STAT3 and p-ERK1/2 activation *in vivo*. The expression and quantification of p-STAT3, p-ERK1/2 and IL-24 in the lungs of mice were evaluated by immunofluorescence. The blue fluorescence of DAPI indicated nuclear localization (630×, scale bar = 100 μm). Data were quantified randomly in 5 fields and are presented as the mean ± standard deviation, n = 8/group. IL, interleukin; HDM, house dust mite; LPS,

lipopolysaccharide; DAPI, 4′,6-diamidino-2-phenylindole; ns, no significance; si-IL, small interfering interleukin; si-NC, si-negative control; IOD, integral OD.

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Supplementary Fig. S6

The effect of si-IL-24 or IL-37 on IL-33, IL-25, and TSLP expression in a murine model of asthma. The expression and quantification of IL-33, IL-25 and TSLP in the lungs of mice were evaluated by immunohistochemistry (scale bar = 200 μm for 200×; scale bar = 100 μm for 730×). Data were quantified randomly in 5 fields and are presented as the mean ± standard deviation, n = 8/group.

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Supplementary References

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