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ARTICLE

Inhibiting the Notch signaling pathway suppresses Th17-associated airway hyperresponsiveness in obese asthmatic mice

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Abstract

Notch signaling is crucial for the regulation of asthma and obesity. The interleukin (IL)-17-expressing $CD4^+$ T cell (Th17 cell) response and airway hyperresponsiveness (AHR) are critical features of both asthma and obesity. We previously demonstrated that inhibiting the Notch signaling pathway alleviates the Th17 response in a mouse model of asthma. However, obese asthmatic individuals show increased Th17 responses and AHR, with the underlying mechanism not currently understood. We aimed to assess the function of Notch signaling in obese mice with asthma and to determine the impact of a γ-secretase inhibitor (GSI), which inhibits the Notch signaling pathway, on the regulation of the Th17 response and AHR. C57BL/6 mice were administered ovalbumin (OVA) to induce asthma, while a high-fat diet (HFD) was used to induce mouse diet-induced obesity (DIO). GSI was then administered intranasally for 7 days in DIO-OVA-induced mice. The results showed increased Notch1 and hes family bHLH transcription factor 1 (Hes1) mRNA levels and Notch receptor intracellular domain (NICD) protein levels in obese asthmatic mice. Furthermore, these mice showed an increased proportion of Th17 cells, serum IL-17A, IL-6, and IL-1β levels, mucin 5AC (MUC5AC) mRNA level, retinoic acid-related orphan receptor-γt (RORγt) mRNA and protein levels, and increased AHR severity. Interestingly, GSI treatment resulted in reduced Notch1 and Hes1 mRNA and NICD protein levels in DIO-OVA-induced mice, with a decreased Th17 cell proportion and IL-17A quantity and alleviated AHR. These data strongly indicate that the Notch pathway is critical in obese asthmatic mice. In addition, inhibiting the Notch pathway ameliorates AHR and the Th17 response in obese mice with asthma.

Introduction

Asthma, a chronic inflammatory ailment of the airways, is commonly found in children and adults. Both airway hyperresponsiveness (AHR) and interleukin (IL)-17-expressing $CD4^+$ T (Th17) cells are key factors in asthma [1–6]. Likewise, obesity is associated with AHR and Th17 cells [7–11]. A high-fat diet (HFD) increases the number of circulating Th17 cells, increases the expression of IL-17A mRNA, and induces AHR [12, 13]. Currently, obesity is a major global health challenge [14], with more than 50% of patients with severe asthma also being obesity [15]. Obesity is considered to be critical in asthma development; indeed, it reduces the asthma treatment response and increases asthma risk [16–18]. As the relationship between asthma and obesity is growing closer, asthmatic individuals with obesity have been classified as a special phenotype termed "obese-asthma" by the International Consensus on (ICON) pediatric asthma [19]. Steroid resistance has been reported in obese asthmatic individuals [20], which indicates that they are insensitive to conventional asthma therapies, and makes the treatment of these individuals difficult. Hence, obese asthmatic individuals need increased attention. According to existing findings, AHR and Th17 responses are common features of both asthma and obesity. However, the severity of Th17 responses and AHR is increased in obese asthmatic individuals [21]. The relationship between Th17 and ovalbumin (OVA)-induced asthma is receiving increasing attention; OVA-specific Th2 responses are widely recognized. We have previously shown that there is

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no significant difference in OVA-specific IgE or IgG1 between asthmatic mice and obese asthmatic mice. However, Th17 and IL-17A are significantly increased in obese asthmatic mice compared with asthmatic mice, which indicates that the T cells linking obesity to asthma may be the Th17 subset. We have also demonstrated that Th17 and IL-17A levels are positively associated with AHR in obese asthmatic mice [22]. Nevertheless, the molecular mechanisms underlying Th17-related AHR in obese asthma have not been elucidated and require further investigation.

The Notch signaling pathway is widely prevalent in organisms ranging from invertebrates to mammals and others. Notch signaling plays vital roles in cell recognition, proliferation, differentiation, and apoptosis [23]. There are four Notch receptors (Notch1–4) and five Notch ligands (Jagged1, Jagged2, Delta-like ligand (Dll)1, Dll3, and Dll4) in mammals [24]. The Notch pathway is activated upon Notch receptor binding to a ligand. Then, multiple enzymatic reactions lead to the cleavage of the Notch receptor intracellular domain (NICD), which binds to CSL/RBP-Jk after translocating to the nucleus to recruit Mastermind-like 1 protein. The newly formed complex then induces the transcription of downstream effectors. Researchers have found that the level of Notch1 is significantly increased in asthma mice and that it plays a vital role in the pathogenesis of asthma [25]. We previously showed that the Notch signaling pathway regulates the proliferation and differentiation of $CD4^+$ T lymphocytes and further demonstrated that Notch1 plays a more important role in asthma than Notch2; furthermore, a γ-secretase inhibitor (GSI) blocks Notch signaling and alleviates airway inflammation in asthmatic mice by decreasing Th17 cell differentiation [6, 26–28]. However, the function of the Notch pathway in obesity has attracted increasing attention. Indeed, Notch has been shown to regulate energy metabolism [29, 30]. Inhibiting Notch signaling by blocking γ-secretase-mediated cleavage of NICD improves glucose tolerance and insulin sensitivity in diet-induced obese mice [31]. In addition, Notch pathway inhibition induces white adipose tissue browning and alleviates the obesity-induced activation of Notch signaling leading to HFD-induced obesity; conversely, the inhibition of Notch suppresses obesity [32]. Furthermore, Notch1-knockout mice are resistant to HFD-induced obesity. However, the exact protective mechanism of Notch signaling in obese asthma remains unknown. Accordingly, since Notch is involved in obesity and controls Th17 levels in asthma, Notch also has great potential to modulate Th17 levels in obese asthmatic individuals. Thus, Notch represents a new crucial therapeutic target for obese asthma.

Therefore, this study aimed to assess the role of Notch signaling in a mouse model of obese asthma and to determine the therapeutic effects of GSI.

Materials and methods

Animals and treatments

Three- to four-week-old male C57BL/6 mice were fed an HFD (MD12032, MediScience Ltd, China) containing 45% fat energy for 16 weeks to establish a model of diet-induced obesity (DIO). Lean mice were provided normal chow (MD12031, MediScience Ltd) comprising 10% fat energy as a control group [33, 34]. The OVA-induced asthma model in DIO and lean mice was established as previously described [22, 26, 28]. Mice were obtained from the Shanghai SLAC Laboratory Animal Center (Shanghai, China) and maintained in specific pathogen-free rooms in the vivarium of the Wenzhou Medical University under a 12–12 h light dark cycle at 22 ± 1 °C and 50 ± 1% relative humidity, with access to rodent food and water ad libitum. Animal experiments (including the euthanasia procedure) received approval from the Wenzhou Medical University Institutional Animal Care and were conducted according to the Association for Assessment and Accreditation of Laboratory Animal Care. GSI L685, 458 (Calbiochem, CA) (0.3 mg/kg) was administered intranasally 30 min before each OVA challenge as previously described [6, 35]. Control animals received intranasal DMSO (vehicle). The mice were randomized into five groups, including the sham, OVA + DMSO, DIO + DMSO, DIO + OVA + DMSO, and DIO + OVA + GSI groups.

Lung function analysis

After the last OVA or NS challenge, anesthesia was carried out by intraperitoneal administration of 1% pentobarbital sodium (50 mg/kg) before tracheotomy. Airway resistance (Rn) was detected by the forced oscillation method (FlexiVent, SCIREQ Scientific Respiratory Equipment Inc., Montreal, Canada) while spontaneous breathing was completely stopped in mice. After baseline Rn measurement, mice were administered increasing amounts of methacholine (MCh) (3.125, 6.25, 12.5, 25, and 50 mg/mL), and further Rn measurements were taken. FlexiVent parameters were set at appropriate levels: ventilation, 150 breaths/min; tidal volume, 0.2 mL; and positive end-expiratory pressure, 2 cm H_2O.

Immunohistochemistry

Lung tissues were sectioned, counterstained with hematoxylin (Thermo Shandon, PA, USA), and immunohistochemically analyzed with retinoic acid-related orphan receptor-γt (RORγt), IL-17A, and NICD antibodies (Santa Cruz Biotechnology, USA). RORγt, IL-17A, and NICD

Table 1 Primer sequence for reverse transcription quantitative real-time PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Notch1	TGGATGGAGACTGCTGGAATG	TAAGGACCTCAAGGCACGGAG
RORγt	GACTTCCATTGCTCCTGCTTT	TTCAGTATGTGGTGGAGTTTGC
IL-17A	TCATGTGGTGGTCCAGCTTTC	CTCAGACTACCTCAACCGTTCC
GAPDH	AAGAAGGTGGTGAAGCAGG	GAAGGTGGAAGAGTGGGAGT
Hes1	CGGCATTCCAAGCTAGAGAAGGC	CGGCATTCCAAGCTAGAGAAGGC
MUC5AC	AATGACTCAATCTGCGTGCCTTCC	CAGGTTAGCGTGGCTTCCTTACAG

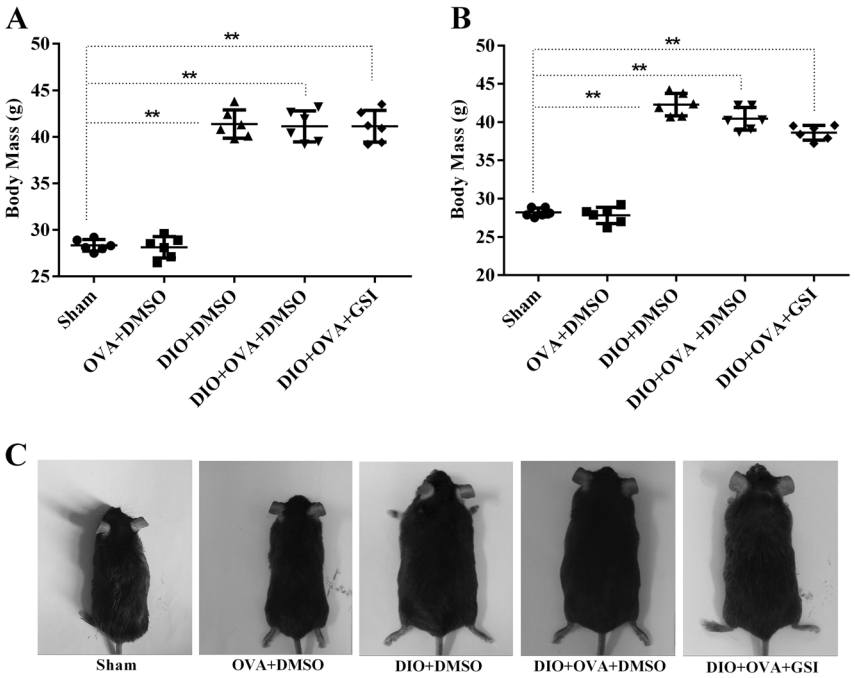

Fig. 1 Effects of GSI on body weight. C57BL/6 mice were fed a high-fat diet or normal chow for 16 weeks and sensitized/challenged with OVA. **a** Body weights were obtained pretreatment with GSI. **b** Body weights after 7 consecutive days of GSI intervention. **c** Changes in appearance after GSI intervention. Data are presented as the mean ± SD ($n = 6$). $^{**}P < 0.01$ versus sham group, $^{\#\#}P < 0.01$ versus DIO + OVA + DMSO group, $^{\Delta\Delta}P < 0.01$ versus DIO + OVA + GSI group

protein levels were obtained by assessing optical densities with Image-Pro Plus 6.0 software.

Quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was prepared from the lung tissue with TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. First-strand cDNA synthesis was performed with a PrimeScript RT reagent kit (Takara Bio Inc., USA). Then, qRT-PCR was carried out on a Lightcycler 480 System (Roche, Switzerland) with SYBR Green (Roche). Glyceraldehyde-3-phosphate dehydrogenase was employed for normalization, and relative mRNA amounts were determined using the Delta–Delta Ct method. Primers are listed in Table 1.

Flow cytometry

Single-cell suspensions were obtained from spleen samples; the positive selection method was used to isolate $CD4^+$ T cells with a mouse $CD4^+$ T cell isolation kit (MACS, Miltenyi Biotec, Germany) by magnetic cell sorting. The quantities of Th17 cells were detected on an FC500 flow cytometer (Beckman Coulter, USA) using FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse IL-17A (BD Bioscience, USA).

In vitro mouse $CD4^+$ T cell differentiation

For Th17 cell differentiation, naive $CD4^+$ T cells were isolated from mouse spleen using a mouse naive $CD4^+$ T cell isolation kit II (Miltenyi Biotec). Purified naive $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (5 μg/mL, eBioscience), anti-CD28 (2 μg/mL, eBioscience), TGF-β (2 ng/mL), IL-6 (30 ng/mL), IL-1β (10 ng/mL), and IL-23 (20 ng/mL) in the presence of anti-IFN-γ (10 μg/mL) and anti-IL-4 (10 μg/mL) for 3 days. The cells were cultured in 24-well plates with a total volume of 1 mL/well of culture medium with 1×10^6 $CD4^+$ T cells in the presence or absence of different concentrations of GSI (1, 5, and 10 μM). Finally, RORγt

Table 2 The body mass changes of GSI-treated group during the GSI treatment period

Mice	Body mass (g)							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
G1	42.6	42.3	41.9	41.6	41.4	40.6	40.0	39.5
G2	41.2	41.0	40.6	40.3	39.8	39.6	39.6	39.2
G3	43.5	43.1	42.5	42.4	41.1	40.8	40.1	39.6
G4	39.2	39.0	38.5	38.2	37.9	37.8	37.4	37.2
G5	40.9	40.2	40.0	39.7	39.6	39.0	38.7	38.4
G6	39.4	39.1	38.9	38.5	38.4	38.1	37.9	37.9

Body weights during 7 consecutive days of intervention of GSI. G1–6 represent each mice of DIO + OVA + GSI group

Table 3 GSI reduce the baseline airway resistence (Rn) of obese asthmatic mice

Group	*n*	Rn (cm H_2O/mL/s)
Sham	6	0.5531 ± 0.0272
OVA + DMSO	6	0.7501 ± 0.0240*
DIO + DMSO	6	0.7070 ± 0.0404*
DIO + OVA + DMSO	6	0.8268 ± 0.0347*
DIO + OVA + GSI	6	0.5887 ± 0.0408**

Baseline Rn was assessed at the stage of aerosolized saline nebulizing with a mouse ventilator by the forced oscillation technique. Data are mean ± SD ($n = 6$)

*$P < 0.01$ versus sham group

**$P < 0.01$ versus DIO + OVA + DMSO group

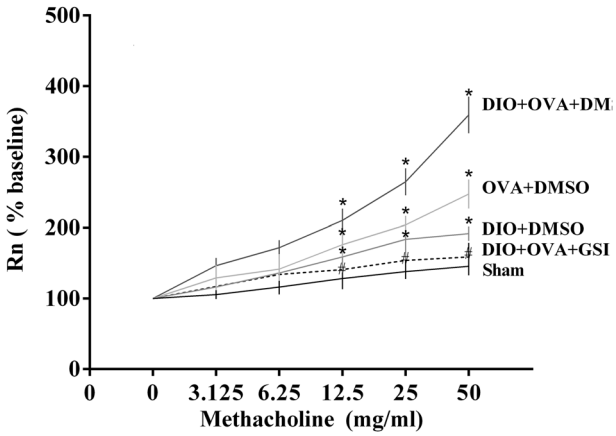

Fig. 2 GSI alleviates AHR in obese asthmatic mice. After baseline Rn measurement, mice were administered increasing amounts of methacholine (MCh) (3.125, 6.25, 12.5, 25, and 50 mg/mL), and further Rn measurements were taken; percentages relative to baseline values were determined. Data are presented as the mean ± SD ($n = 6$). *$P < 0.05$ versus sham group; #$P < 0.05$ versus DIO + OVA + DMSO group

and hes family bHLH transcription factor 1 (Hes1) mRNA levels in $CD4^+$ T cells were assessed by using qRT-PCR.

Enzyme-linked immunosorbent assay (ELISA)

IL-6, IL-1β, and IL-17A levels in mouse serum were determined with an ELISA kit (Boyun, Shanghai, China) as directed by the manufacturer.

Histopathological examination

The middle segment of the left lung was fixed in formalin, paraffin embedded, sectioned (4 μm), and submitted to hematoxylin–eosin or periodic acid-Schiff staining. Airway inflammation scoring was carried out as previously reported by our team [6].

Statistical analysis

All data are presented as the mean ± standard deviation (SD) and were assessed by one-way ANOVA. $P < 0.05$ indicated statistical significance. Experiments were performed in triplicate.

Results

Effects of GSI on body mass

To assess the effects of GSI on body weight in the DIO-OVA-induced model, the animals were weighed pre and post treatment. Before drug administration, increased body masses were found in animals fed a HFD (DIO + DMSO, DIO + OVA + DMSO, and DIO + OVA + GSI groups) compared with those of sham animals (OVA + DMSO groups). However, body weights of GSI-treated mice were not significantly reduced (from 41.13 ± 1.706 g to 38.63 ± 0.965 g)

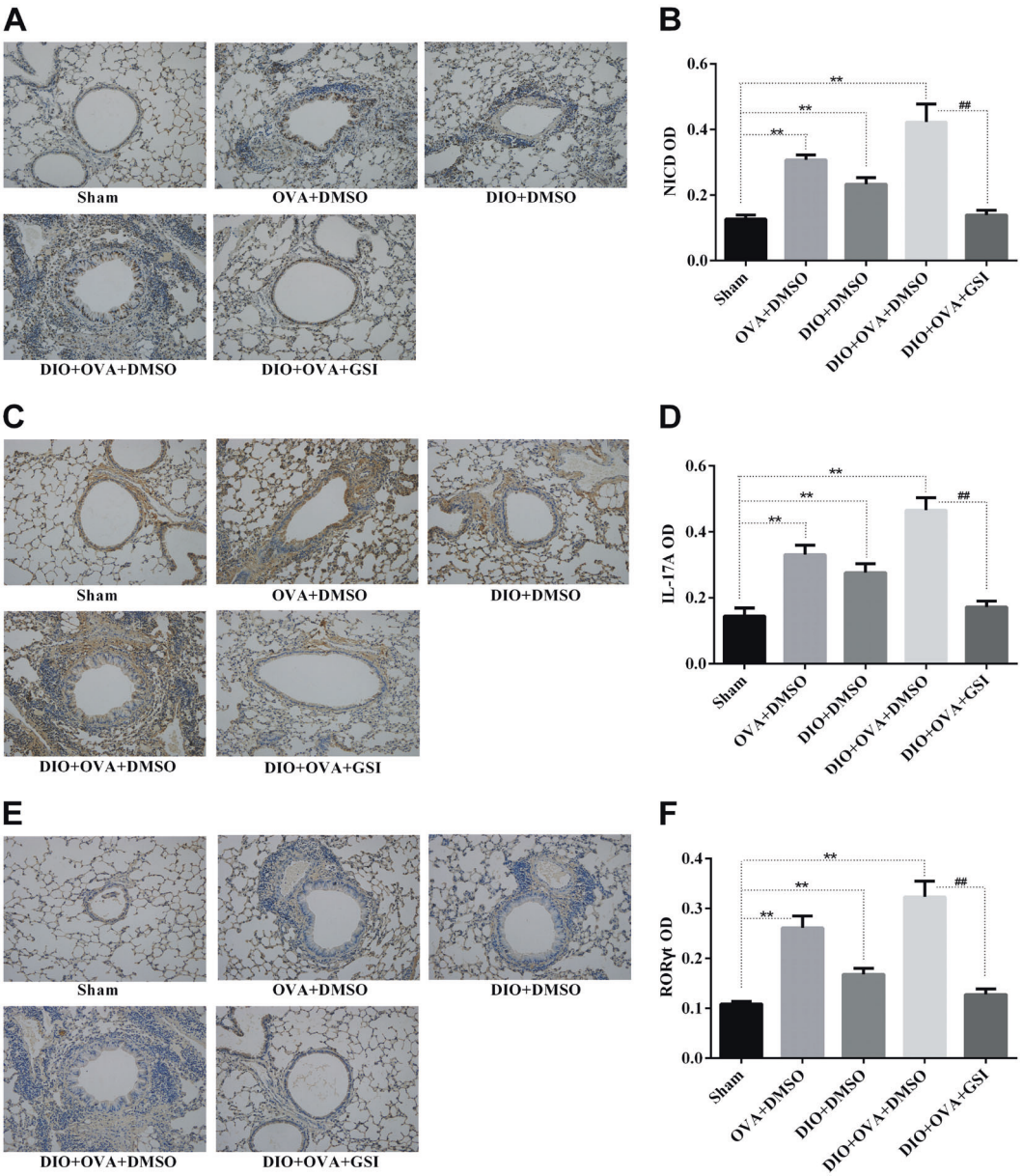

Fig. 3 GSI treatment decreases the expression levels of NICD, RORγt, and IL-17A protein in the lung tissue. **a, c, e** NICD, IL-17A, and RORγt protein levels were obtained by immunohistochemistry. **b, d, f** NICD, IL-17A, and RORγt protein levels were semiquantified with Image-Pro Plus software. Data are presented as the mean ± SD ($n = 6$). $^{**}P < 0.01$ versus sham group; $^{\#\#}P < 0.01$ compared with DIO + OVA + DMSO group

compared with those of obese mice (41.37 ± 1.508 g) and obese asthmatic mice (41.13 ± 1.671 g) ($P > 0.05$). (Fig. 1 and Table 2).

Effects of GSI on airway hyperresponsiveness in obese asthmatic mice

Higher baseline Rn and MCh AHR values were detected in the DIO + OVA + DMSO, OVA + DMSO, and DIO + DMSO groups compared with those of the sham group ($P < 0.05$). However, these parameters were markedly reduced after GSI treatment ($P < 0.05$). (Table 3 and Fig. 2).

GSI reduces NICD, RORγt, and IL-17A protein levels in the lungs of obese asthmatic mice

NICD, RORγt, and IL-17A protein levels were evaluated immunohistochemically. The protein levels in lung tissue samples from the DIO + OVA + DMSO (NICD, 0.4228 ± 0.0501; RORγt, 0.3236 ± 0.0285; IL-17A, 0.4659 ± 0.0371), OVA + DMSO (NICD, 0.3075 ± 0.0136; RORγt,

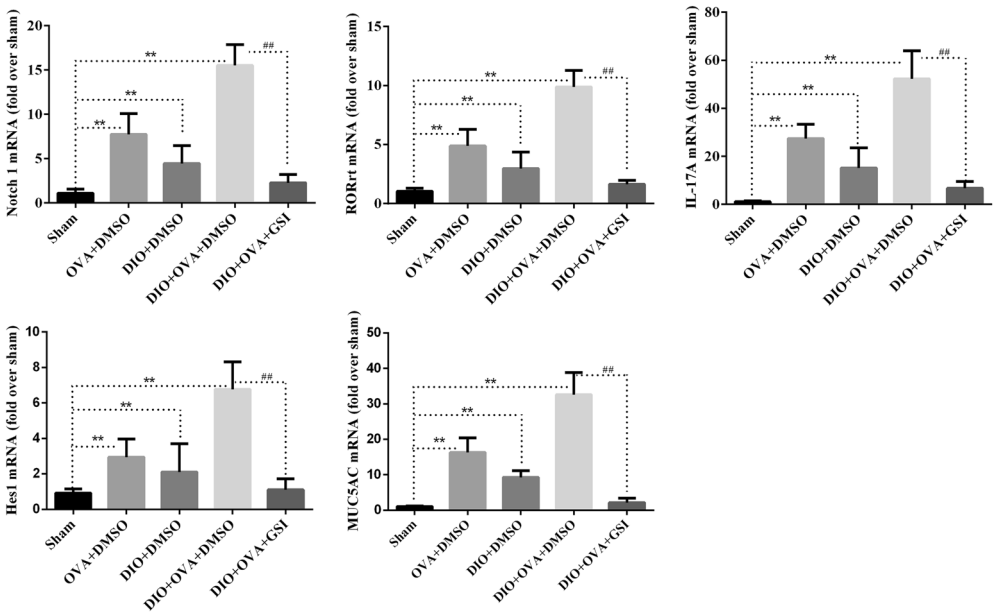

Fig. 4 GSI reduces Notch1, RORγt, IL-17A, MUC5AC, and Hes1 mRNA in the lung. Lung Notch1, RORγt, IL-17A, MUC5AC, and Hes1 mRNA levels were assessed by quantitative real-time reverse transcription PCR (qRT-PCR). Data are presented as the mean ± SD ($n = 6$). $^{**}P < 0.01$ versus sham group; $^{\#\#}P < 0.01$ versus DIO + OVA + DMSO group

0.2611 ± 0.0216; IL-17A, 0.3306 ± 0.0289), and DIO + DMSO (NICD, 0.2331 ± 0.0185; RORγt, 0.1677 ± 0.0114; IL-17A, 0.2756 ± 0.0270) groups were significantly higher than those in the tissue samples from the sham group (NICD, 0.1268 ± 0.0113; RORγt, 0.1085 ± 0.0047; IL-17A, 0.1448 ± 0.0247, $P < 0.01$). In addition, the protein levels in lung tissue specimens were markedly reduced in the DIO + OVA + GSI group (NICD, 0.1393 ± 0.0129; RORγt, 0.1274 ± 0.0104; IL-17A, 0.1711 ± 0.0187, $P < 0.01$) (Fig. 3).

Inhibitory effects of GSI on Notch1, RORγt, IL-17A, MUC5AC, and Hes1 mRNA in the lungs of obese asthmatic mice

Notch1, RORγt, IL-17A, mucin 5AC (MUC5AC), and Hes1 mRNA levels were examined after various treatments. Interestingly, Notch1, RORγt, IL-17A, MUC5AC, and Hes1 gene expression levels were markedly elevated in obese asthmatic mice (Notch1, 15.52 ± 2.36; RORγt, 9.88 ± 1.40; IL-17A, 52.26 ± 11.74; MUC5AC, 32.61 ± 6.24; Hes1, 6.77 ± 1.54), as well as the OVA + DMSO (Notch1, 7.73 ± 2.34; RORγt, 4.88 ± 1.40; IL-17A, 27.45 ± 5.90; MUC5AC, 16.28 ± 4.13; Hes1, 2.95 ± 1.02) and DIO + DMSO (Notch1, 4.46 ± 2.01; RORγt, 2.97 ± 1.40; IL-17A, 15.13 ± 8.46; MUC5AC, 9.27 ± 1.90; Hes1, 2.11 ± 1.59) groups, compared with those of the sham group (Notch1, 1.09 ± 0.46; RORγt, 1.03 ± 0.26; IL-17A, 1.05 ± 0.39; MUC5AC, 1.00 ± 0.17; Hes1, 0.92 ± 0.23, all $P < 0.01$); however, these values were decreased in the DIO + OVA + GSI (Notch1, 2.27 ± 0.93; RORγt, 1.63 ± 0.33; IL-17A, 6.67 ± 2.91; MUC5AC, 2.15 ± 1.27; Hes1, 1.10 ± 0.62, $P < 0.01$) group (Fig. 4).

Effects of GSI on Th17 cell quantities in obese asthmatic mice

To examine the effect of GSI on Th17 cell expansion, the proportion of Th17 cells relative to all $CD4^+$ T cells was assessed by flow cytometry. Th17 cell proportions were markedly increased in the OVA + DMSO (3.59 ± 0.37%), DIO + DMSO (2.43 ± 0.42%), and DIO + OVA + DMSO (5.85 ± 0.75%) groups compared with those of the sham group (1.18 ± 0.19%) ($P < 0.01$). However, the Th17 cell proportions were significantly lower in the DIO + OVA + GSI group (1.60 ± 0.24%) than the DIO + OVA + DMSO group ($P < 0.01$) (Fig. 5).

Effects of GSI on Hes1 and RORγt mRNA in $CD4^+$ T cells

To assess the effect of GSI on Th17 polarization in naive $CD4^+$ T cells, naive $CD4^+$ T cells isolated from mouse spleen were stimulated under Th17 polarizing conditions for 3 days. Cells were cultured in 24-well plates with a total volume of 1 mL/well of culture medium with 1×10^6 $CD4^+$ T cells in the presence or absence of different concentrations of GSI (1, 5, and 10 μM). Subsequently, the RORγt and Hes1 mRNA levels in $CD4^+$ T cells were assessed by using qRT-PCR. The results indicated that RORγt and Hes1 gene expression levels were markedly elevated in the CD3

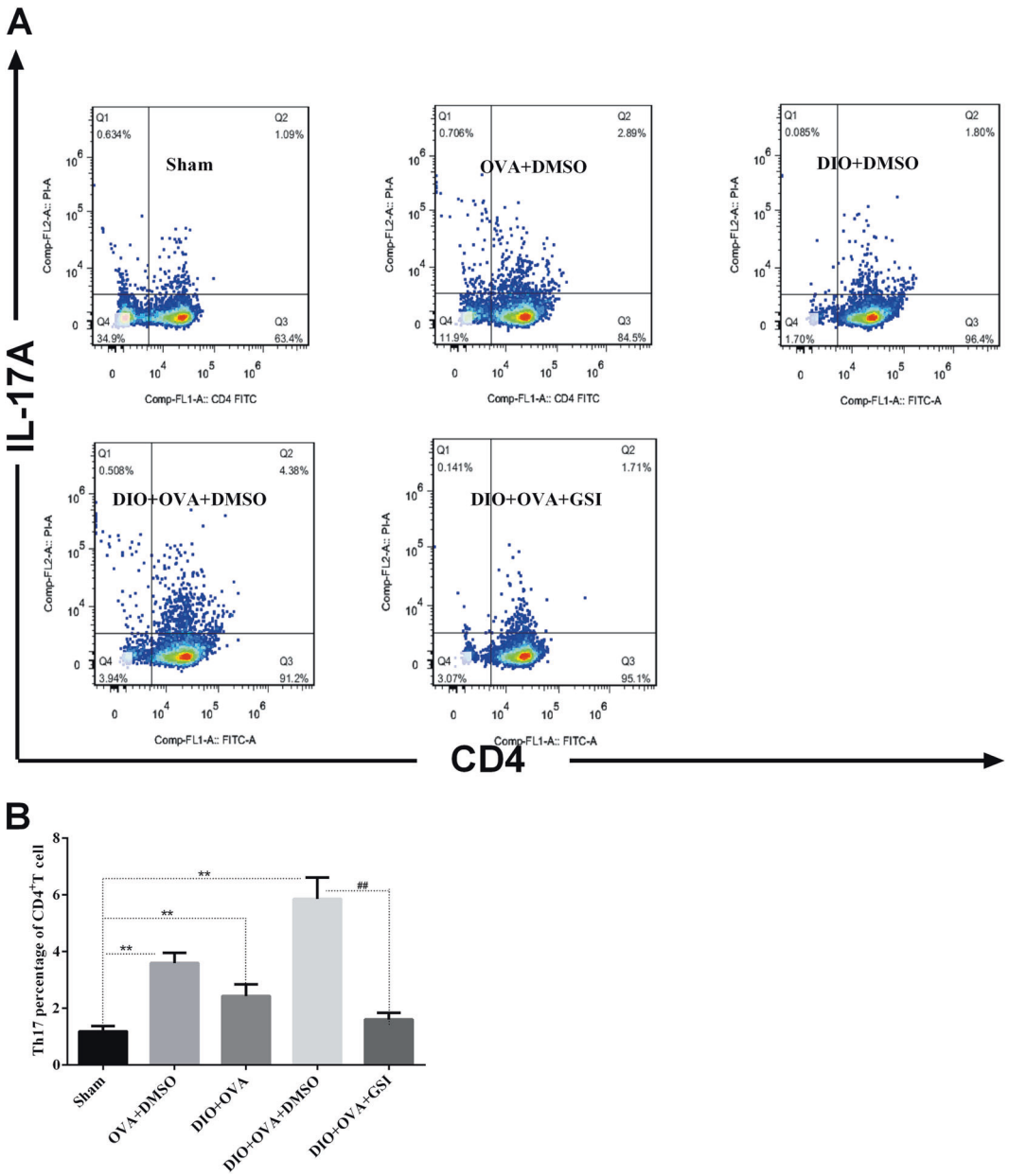

Fig. 5 GSI reduces Th17 cell proportions. C57BL/6 mice were fed a high-fat diet or normal chow diet for 16 weeks and sensitized/challenged with OVA in the presence or absence of GSI. The proportion of Th17 cells relative to all $CD4^+$ T cells was assessed by flow cytometry. **a** Dot plots show the percentage of cells positive for CD4 and IL-17A staining. **b** Th17 percentage of total $CD4^+$ T cells, data are the mean ± SD ($n = 6$). $^{**}P < 0.01$ versus sham group; $^{\#\#}P < 0.01$ versus DIO + OVA + DMSO group

+ CD28 + Th17 mixed group (RORγt, 2.75 ± 1.05; Hes1, 3.86 ± 1.49) compared with those of the CD3 + CD28 group (RORγt, 1.09 ± 0.31; Hes1, 0.99 ± 0.17, $P < 0.01$); however, these gene expression levels were decreased in the GSI groups ($P < 0.01$) (Fig. 6).

Effects of GSI on serum IL-17A, IL-6, and IL-1β levels in obese asthmatic mice

As shown in Fig. 7, serum IL-17A, IL-6, and IL-1β levels were markedly increased in the OVA + DMSO (IL-17A, 128.61 ± 9.11 pg/mL; IL-6, 170.28 ± 20.12 pg/mL; IL-1β, 256.94 ± 36.88 pg/mL), DIO + DMSO (IL-17A, 96.55 ± 7.46 pg/mL; IL-6, 159.05 ± 21.96 pg/mL; IL-1β, 203.55 ± 24.09 pg/mL), and DIO + OVA + DMSO groups (IL-17A, 147.72 ± 7.61 pg/mL; IL-6, 240.55 ± 28.62 pg/mL; IL-1β, 462.22 ± 52.90 pg/mL $P < 0.01$) in comparison with those of the sham group (IL-17A, 43.85 ± 4.32 pg/mL; IL-6, 69.43 ± 9.80 pg/mL; IL-1β, 142.35 ± 6.44 pg/mL). In contrast, the DIO + OVA + GSI (IL-17A, 75.14 ± 11.34 pg/mL; IL-6, 65.31 ± 9.84 pg/mL; IL-1β, 155.31 ± 21.96 pg/mL) group ($P < 0.01$) showed

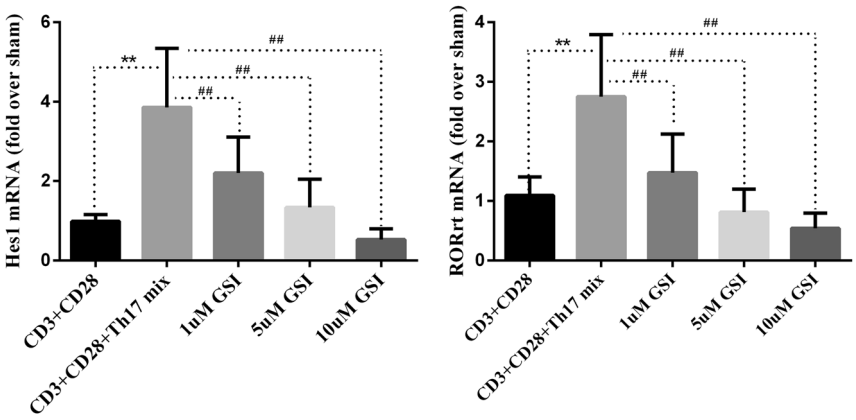

Fig. 6 GSI reduces RORγt and Hes1 mRNA in $CD4^+$ T cells. Naive $CD4^+$ T cells isolated from mouse spleen were stimulated under Th17 polarizing conditions for 3 days. Cells were cultured in 24-well plates with a total volume of 1 mL/well of culture medium with 1×10^6 $CD4^+$ T cells in the presence or absence of different concentrations of GSI (1, 5, and 10 μM). Finally, RORγt and Hes1 mRNA levels in $CD4^+$ T cells were assessed using qRT-PCR. Data are presented as the mean ± SD ($n = 6$). $^{**}P < 0.01$ versus CD3 + CD28 group; $^{\#\#}P < 0.01$ versus CD3 + CD28 + Th17 mix group

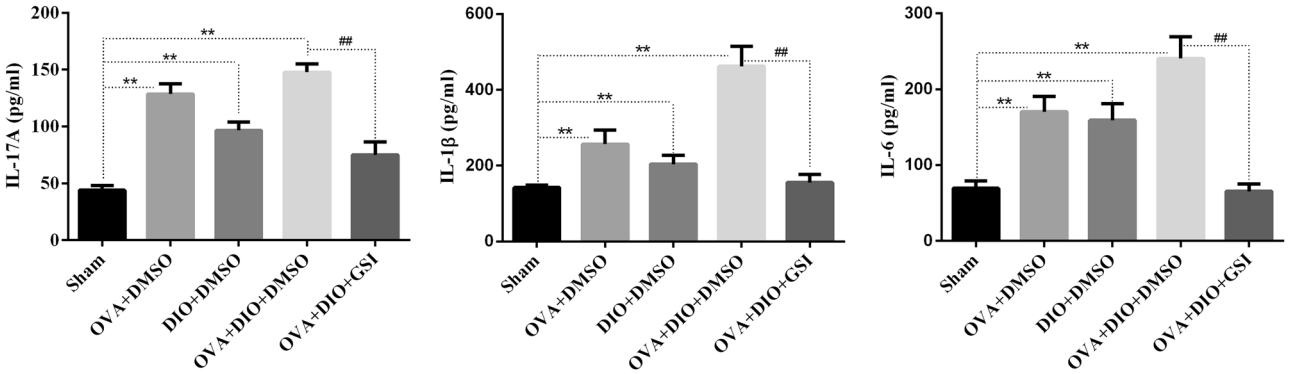

Fig. 7 GSI reduces serum IL-6, IL-1β, and IL-17A levels. Serum IL-6, IL-1β, and IL-17A levels were detected by the ELISA. Data are presented as the mean ± SD ($n = 6$). $^{**}P < 0.01$ versus sham group, $^{\#\#}P < 0.01$ versus DIO + OVA + DMSO group

significantly reduced serum IL-17A, IL-6, and IL-1β levels.

GSI ameliorates airway inflammation and goblet cell metaplasia in obese asthmatic mice

As indicated in Fig. 8a, b, compared with the sham group, the OVA + DMSO, DIO + DMSO, and DIO + OVA + DMSO groups showed more severe inflammatory cell infiltration around the trachea and small perivascular space of the lung. In contrast, the DIO + OVA + GSI animals showed markedly decreased inflammation and airway wall thickening compared with that of the obese animals with asthma. In addition, goblet cell proliferation was increased in the DIO + OVA + DMSO animals, but this proliferation increase was reversed by GSI treatment (Fig. 8c).

Discussion

In this study, we examined the role of GSI in the Notch pathway in obese asthmatic mice. The results indicate that the Notch pathway is induced in obese asthmatic mice, and GSI administration significantly reduced Notch1 and Hes1 mRNA and NICD protein levels in obese asthmatic mice. Moreover, GSI treatment reduced the protein and mRNA levels of RORγt, IL-17A, and MUC5AC, Th17 cell quantities, and IL-6 and IL-1β protein levels in the serum, ameliorating AHR by inhibiting Notch signaling.

The incidence rates of obesity and asthma in children and adults are increasing year by year. It has been shown that obesity is a potent risk factor for asthma. Asthma in patients with obesity is generally worse than that in nonobese patients. Obesity might be a determinant of reduced exacerbation-free time in asthma [36]. However, clinical

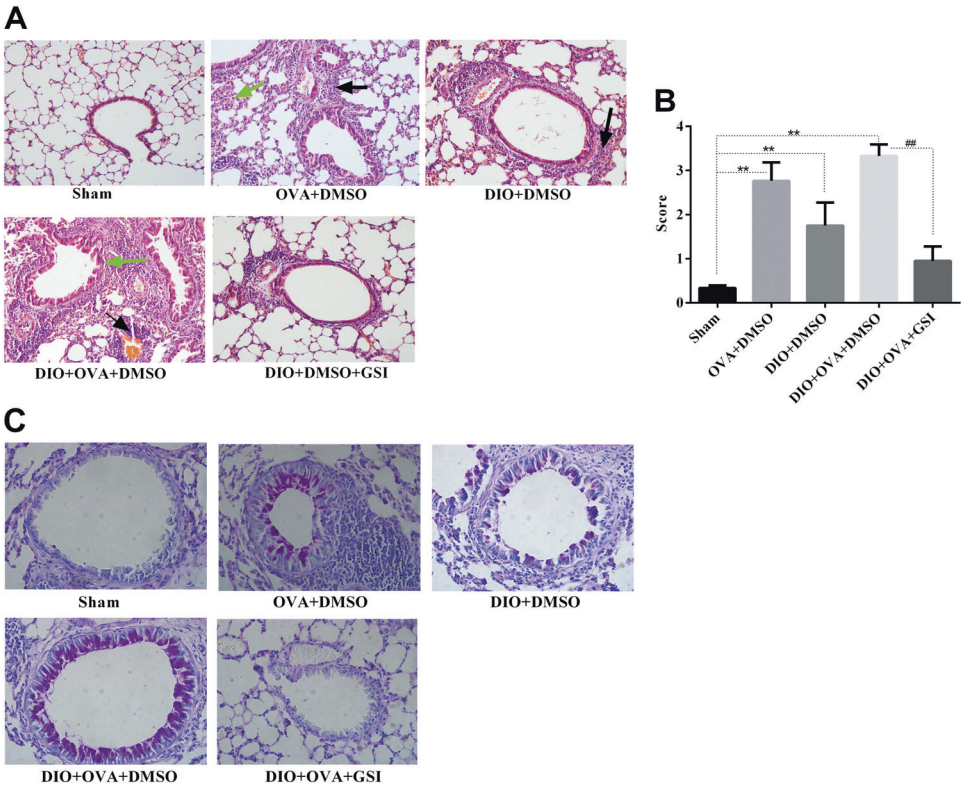

Fig. 8 GSI alleviates airway inflammation and goblet cell metaplasia in obese asthmatic mice. **a** Representative photomicrographs of lung tissue samples after H&E staining (×200 magnification). The black arrow indicates neutrophils, and the green arrow indicates eosinophils. **b** Scores of airway inflammation. **c** Staining of goblet cells. Tissue sections were stained with periodic acid-Schiff (PAS) for mucus production. Data are presented as the mean ± SD ($n = 6$). $^{**}P < 0.01$ versus sham group; $^{\#\#}P < 0.01$ versus DIO + OVA + DMSO group

data indicate that obese individuals respond poorly to conventional treatments, especially corticosteroid inhalation [20]. Therefore, obese asthma requires increased attention. We previously demonstrated that the severity of AHR and Th17 cell-mediated inflammation is increased in a mouse model of obese asthma [22]. Researchers have determined that blockading IL-17 in HFD and OVA-induced mice decreases AHR and airway inflammation [37], which suggests that IL-17 plays a key role in AHR in obese asthmatic individuals. Nonetheless, the molecular mechanism underlying the Th17 response and AHR in obese asthma should be further investigated.

Numerous studies have determined the role of Notch signaling in asthma. Notch signaling is enhanced in asthma patients [38]. Small interfering RNA-mediated knockdown of Notch1 leads to reduced IL-4 and IFN-γ levels in OVA-induced asthmatic mice [30]. Inhibiting Notch signaling alleviates formalin-inactivated respiratory syncytial virus-enhanced AHR [39]. In addition, treatment with the Notch signaling inhibitor GSI alleviates allergic inflammation in asthma [35]. Recently, increasing evidence has shown that Notch signaling is also involved in obesity. Indeed, Notch signaling is considered a new metabolic regulator [29] and might constitute an efficient treatment target in obesity [30, 32]. It has been reported that obesity activates the leptin-Notch signaling pathway in breast cancer [40]. Nevertheless, the role of the Notch pathway in obese asthma has not been clarified. As shown above, Notch1 and Hes1 mRNA and NICD protein levels were increased in OVA-induced asthmatic and diet-induced obese animals in comparison with those of the sham animals. Interestingly, Notch1 and Hes1 mRNA and NICD protein levels were highest in obese asthmatic mice. These results indicate that the Notch pathway is activated in obese asthmatic mice. Notch1-knockout mice were found to be leaner than wild-type mice after being fed a HFD for 4 weeks [32]. However, in our study, mean body mass values were reduced slightly after 7 days of treatment with GSI, but the difference between obese asthmatic mice and GSI-treated mice was not significant. Extended GSI treatment may reduce body mass to the normal level, but requires further investigation.

Notch signaling plays a crucial role in $CD4^+$ T cell differentiation and activation [26, 41, 42]. Our previous studies proved that GSI inhibits Th17 cell differentiation by decreasing Notch1 mRNA and NICD protein levels; anti-Dll4 antibodies inhibit Th17 cell differentiation in mice with asthma [6, 27]. Others have demonstrated that inactivation of the Notch signaling pathway decreases the amount of Th17 cells and the Th17/Treg ratio, accompanied by a reduction in IL-17 and RORγt mRNA expression in immune thrombocytopenia patients [43]. However, how the Notch signaling pathway affects Th17 cell differentiation in patients with obese asthma remains unclear. RORγt functions as a major transcription factor for Th17 cell differentiation in mice and humans [44–47], as a deficiency in RORγt profoundly affects the production of Th17

cytokines. In addition, obesity drives Th17 cell differentiation involving the activation of RORγt [8], and since Notch1 plays a crucial role in asthma and obesity, we detected the levels of RORγt protein, Notch1 mRNA, and NICD protein in obese asthmatic mice. We found the highest Th17 cell quantities, RORγt and IL-17A protein and mRNA levels in the lung, and serum IL-17A levels in obese asthmatic animals, which also displayed the highest Notch1 and Hes1 mRNA and NICD protein levels. Furthermore, we treated obese asthmatic mice with GSI, which reduced Notch1 and Hes1 mRNA and NICD protein levels. In addition, the proportion of Th17 cells, RORγt and IL-17A protein and mRNA levels in the lung, and serum IL-17A levels were also decreased. These findings suggest that the inhibition of Notch signaling also suppresses Th17 cell differentiation in obese mice with asthma.

Studies have shown that Th17 plays an important role in AHR. Mice fed a HFD display AHR, which is associated with Th17 cell and IL-17A quantities [9]. IL-17A, which is synthesized by Th17 cells, participates in allergen-associated AHR by directly affecting the smooth muscle of airways [48]. Th17 cell-associated airway inflammation and AHR show resistance to steroids, pointing to a possible function of Th17 cells in steroid-resistant asthma [1]; furthermore, elevated IL-17A levels cause AHR [5]. In addition, AHR likely depends on IL-17A and is not found in obese IL-17A$^{-/-}$ mice [49]. Furthermore, we previously revealed that Th17 and Th17-associated cytokines are crucial for AHR in obese asthmatic mice [22]. Consequently, inhibiting the Notch signaling pathway may also alleviate Th17-related AHR in obese asthma. As shown above, decreased Rn and MCh AHR was found in GSI-treated mice, which indicates that Th17-related AHR could be ameliorated by inhibiting the Notch pathway. Therefore, these findings suggest that inhibiting Notch signaling might be an efficient treatment tool for Th17-related AHR in obese asthma. However, this hypothesis should be confirmed in future studies.

Overall, our results show that the inhibition of Notch signaling suppresses the Th17 response and alleviates AHR in obese asthmatic mice. GSI treatment induces such effects. Therefore, these findings provide novel insights into obese asthma treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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