



The effect of blocking Notch signaling by γ -secretase inhibitor on allergic rhinitis



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ABSTRACT

Objective: This study aimed to investigate the effect of blocking Notch signaling by γ -secretase inhibitor (GSI) on allergic rhinitis.

Method: GSI, N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butylester (DAPT) was administered to ovalbumin-induced AR mice models intranasally. We observed symptoms of sneezing and nose rubbing. To detect the inflammatory state, the serum OVA-specific-IgE, IFN- γ , IL-4, and IL-5 were analyzed by ELISA, and Th cell cytokines in nasal mucosa were analyzed by RT-PCR, including T-bet, IFN- γ , GATA-3, IL-4, and IL-5. In addition, hematoxylin-eosin (HE) and periodic acid-Schiff (PAS) were applied for histopathological examination. As for the evaluation of Notch signaling, we analyzed the Notch-1, Notch signaling target Hes-1, and Hes-5 in mucosa by RT-PCR, besides, used western blotting and immunohistochemistry to assess NICD (Notch intracellular domain).

Results: The results showed that the DAPT ameliorated the development of AR and suppressed Th2 cytokine levels significantly, alleviating eosinophils infiltration and goblet cells metaplasia, suggesting that the GSI can regulate Th2 response and weaken airway inflammation in AR.

Conclusion: Our findings provide evidence that blocking Notch signaling by GSI offers high value in treating AR.

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1. Introduction

Allergic rhinitis (AR) is a chronic hyper responsive upper respiratory disease, the morbidity of which in children and adult has all been rising in recent years. The prevalence of AR in different regions varies, such as ranging from 2.9% in 10- to 18-year-old children in Turkey to 54.1% in 13- to 14-year-old children in Nigeria [1]. However, the mystery of how AR occurs is not yet discovered. Its pathophysiology is characterized by epithelia turbulence, including eosinophils infiltration, mucosa edema, mucin hypersecretion, immune imbalance, and inflammatory mediator infiltration. And AR is hardly cured. Numerous studies showed that an imbalance between T helper (Th) 1 and Th2 cells immune response, is a key step in AR [2–4], increased Th2 cytokine level (interleukin-4 and interleukin-5, IL-4 and IL-5), which may contribute to the

enhancement in the secretion of IgE and mucosal eosinophils infiltration, and decreased Th1 cytokine level (interferon- γ , IFN- γ), together with other inflammatory cells, such as mast cells, goblet cells, eosinophils, and B cells. Therefore, it is necessary to find a therapeutic method to reverse the response of Th1 and Th2.

Notch signaling is a highly conservative single transmembrane protein that plays a crucial role in cellular activities such as proliferation, differentiation, and cell function, and takes part in organs' formation and development. At present, four receptors (Notch1, Notch2, Notch3, and Notch4) and five ligands (Delta1, Delta3, Delta4, Jagged1, and Jagged2) have been identified in mammals. When activated, the intracellular domain of Notch (NICD) is released by γ -secretase and is translocated to the nucleus to interact with CSL to regulate the target genes, such as Hes-1 and Hes-5, expression of which can reflect the activation state of Notch signaling. Also, Notch signaling can interact with other signaling such as NF- κ B and TGF- β to broaden the target gene spectrum [5,6].

So far, researches on Notch signaling have mainly been concerned with immunological diseases, inflammation, tumors, and so on. Accumulating evidence has shown that Notch signaling affects the behavior of Th cells, especially Th1 and Th2 [6]. Studies

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demonstrated that blockage of Notch signaling is beneficial for asthma models, inhibiting inflammation by restoring the balance of Th1/Th2 and downregulating airway remodeling [7–9]. In addition, repressing Notch signaling can inhibit the function of eosinophils and reduce the mucin production [10–12]. Even so, whether the Notch signaling is involved in the pathogenesis of AR is still unknown. In the present study, we explore the effect of [N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butylester (DAPT), a GSI, on the development of AR in mice to detect if the inference of Notch signaling could be a new therapeutic strategy for AR.

2. Materials and methods

2.1. Animals and groups

6-week-old female BALB/c mice were purchased from SLAC Laboratory Animal Co. Ltd (Shanghai, China) and kept in the Department of Laboratory Animal Science of Shanghai Medical college of Fudan University. Mice protocol was approved by the Animal Care and Use Committee of Eye and ENT Hospital of Fudan University.

BALB/c mice were randomly divided into five groups ($n = 20$ per group) as follows: A: control group, sensitized and challenged with normal saline; B: AR group; C: AR + vehicle group (since dimethylsulfoxide (DMSO) is poisoned, we dissolved DAPT in a vehicle containing 5% DMSO and 95% corn oil); D: OVA + GSI (1 mg/kg) group; and E: OVA + GSI (5 mg/kg) group. Because the research we did demonstrated that the vehicle (DMSO plus corn oil) had no effect on airway function (see Supplement), we only show group C to represent AR group. To induce allergic rhinitis model, mice were sensitized on Day 1, Day 8, and Day 15 by intraperitoneal injection of 0.2 ml suspension, containing 500 μ g/ml ovalbumin (OVA, Sigma, USA) and 20 mg/ml aluminum hydroxide (Sigma, USA). Then, mice were challenged daily by intranasal instillation with 20 μ l OVA (40 mg ova dissolved in 1 ml normal saline) from Day 22 to Day 28. In GSI treatment group, GSI-DAPT (Sigma, USA) was administered intranasally 1 h before each OVA challenge. At the same time, in AR + vehicle group, GSI was instead by vehicle (Fig. 1). After the final OVA stimulation, we observed nasal symptoms including frequency of sneezing and nose rubbing behavior for 15 min. Sneezing was characterized by an explosive expiration apnea. Nose rubbing was characterized by external perinasal scratching with either one or both of the rat's paw, every time mice scratching their nose count 1 score. Mice were sacrificed 4 h after the last OVA provocation.

2.2. ELISA assay

After anesthetization (25 mg xylazine dissolved in 2 ml ketamine, diluted ten-fold, 0.1 ml/g), mouse blood was harvested from post-glomus venous plexus and then centrifuged for 15 min at

3000 rpm. Serum OVA-specific IgE, IFN- γ , IL-4, and IL-5 were assayed using specific ELISA kit (Weiao, China). Each sample was analyzed in duplicate.

2.3. Histopathology of nasal mucosa

At the time of sacrifice, the mouse heads were removed and fixed in 4% paraformaldehyde (PFA) for 24 h, then decalcified in 10% EDTA (pH 7.2–7.4) for 20 days. The head tissue was embedded in paraffin, and 4 μ m coronal sections were cut. The sections were stained with hematoxylin-eosin (HE) for histopathological examination and periodic acid-Schiff (PAS) staining mucin production to identify goblet cells. PAS staining was observed in 4 fields of nasal septum mucosa, mean values were calculated, and HE staining was observed in all fields by two pathologists. Besides, samples were stained with the immunohistochemistry method for assessing NICD expression. Following deparaffinization and rehydration, the sections were rinsed for 5 min using PBS three times, boiled in citrate buffer for 30 min at 95 °C. After incubating the primary antibody (rabbit anti-mouse NICD, 1:50, Abcam) at 4 °C for 36 h, the sections were rinsed and the second antibody was incubated for 20 min. Then, 3,3-diaminobenzidine (DAB) was used for visualization.

Immunostaining was analyzed using semi quantitative histologic score (H-score). Specifically, the staining intensity of NICD, ranging from low (score 1) to moderate and high (score 2 and 3), was multiplied by the percentage of positive epithelial cells (thus obtaining value from 0 to 300) [13,14]. Normal mesothelial cells served as positive control for NICD-positive cells. Immunohistochemical observation was evaluated by two pathologists, and ambiguous cases were reassessed until a consensus was reached.

2.4. Real-time PCR

Nasal mucosa was harvested under microscope. Total RNA was extracted by TRIzol reagent (Invitrogen). The purity and integrity were assessed by measuring absorbance ratios at 260/280 nm (1.9–2.0 was considered eligible). cDNA was synthesized applying PrimeScript™ RT Master Mix (Takara), and SYBR Premix Ex Taq™ (Takara) was used for real-time PCR with ABI PRISM 7500 Sequence Detection System (Applied Biosystems) to detect Hes-1, Hes-5, T-bet, IFN- γ , GATA-3, IL-4, IL-5, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each sample was analyzed in triplicate. The real-time PCR primer sequences used were as follows: Notch-1 F: TCGTGCTCTGTTCTTTGTG R: CTCTCCGCTTCTTCTGCTG; Hes-1 F: AAGGTTTTGGCGGCTTC R: CCCACTGTGCTGGTGTAGA; Hes-5 F: ATGCTCAGTCCCAAGGAGAA R: GGCTTTGCTGTGTTTCAGGT; T-bet F: AACACACACGCTTTTACTTTCCA R: CGTATCAACAGATGCGTACATGG; IFN- γ F: ATGAACGCTACACACTGCATC R: CCATCCTTTTGC-CAGTTCCTC; GATA-3F: AAGCTCAGTATCCGCTGACG R: GATACCTCTGCACCGTAGCC IL-4 F: ATCATCGGCATTTTGAACGAGG R: TGCAGCTCCATGAGAACAATA; IL-5 F: CAAGCAATGAGACGATGAGG R: CCCACGGACAGTTTGAT; GAPDH: GGTGTCTCTGCGACTTCA R: TGGTCCAGGTTTCTTACTCC. The relative mRNA level was calculated by $2^{-\Delta\Delta Ct}$, of which $\Delta\Delta Ct = \Delta Ct(\text{Sample}) - \Delta Ct(\text{Calibrator})$, each $\Delta Ct = \text{mean } Ct(\text{Target}) - \text{mean } Ct(\text{GAPDH})$ and sample that dealt with normal saline was designed as a calibrator.

2.5. Western blotting

Nasal mucosa tissues (two animals as one sample) were homogenized and lysed in RIPA buffer (Sigma, USA), 40 μ g of protein was loaded into each lane of gel, and proteins were separated by 10% SDS polyacrylamide. The proteins were electro blotted onto a polyvinylidene fluoride membrane (PVDF, Immobilon-P, Millipore,

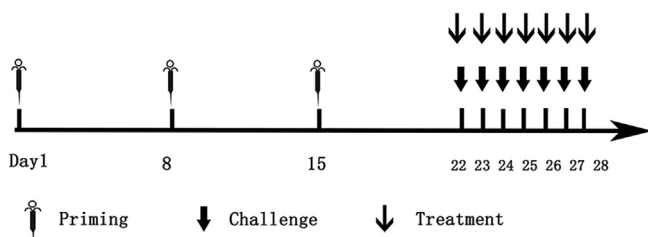


Fig. 1. Schematic diagram of AR mice model and GSI treatment.

Billerica, MA). The membrane was incubated with primary antibodies—rabbit anti-NICD (1:500, Sigma, USA) and rabbit anti- β -actin (1:2000, Weiao, China)—overnight at 4 °C. The membrane was then immunoblotted with a secondary anti-rabbit IgG-HRP (1:2000, Jackson, USA) at room temperature for 2 h. Signals in the linear range of X-ray film were captured digitally and densitometry was performed using Kodak Molecular Imaging Software (Kodak, Shinkawa, Japan). We used β -actin level as a loading control, and the target protein levels were normalized against β -actin levels.

2.6. Statistical analysis

All data are presented as mean $\pm$ SD. GraphPad Prism 6.0 and SPSS version 20.0 software were applied to statistical analyzes. One-way ANOVA test was used for intergroup comparison. The Mann-Whitney U- test was used to analyze the H-score of immunohistochemistry staining. $P < 0.05$ was deemed statistically significant.

3. Results

3.1. Notch signaling expression is enhanced in AR mice, and GSI affects the expression of Notch signaling components

To investigate the expression of Notch signaling in AR mice and the effect of GSI on Notch signaling in vivo, we generated AR models and treated them with GSI, DAPT. We assessed the mRNA expression of Notch-1; the target genes of Notch, Hes-1, and Hes-5; and the NICD protein level in mice nasal mucosa. As shown in Fig. 2, we observed that there is enhanced Notch1, Hes-1, Hes-5 mRNA expression, and NICD protein level in AR mice compared with the control group. On the other hand, the GSI, DAPT is an effective blockage of Notch singling and the influence is dose-dependent. Furthermore, immunohistochemical data confirmed the observation of RT-PCR and western blotting; there is downregulation of NICD in mucosa in a dose-dependent way (see Fig. 3).

3.2. GSI alleviates upper airway inflammation

As illustrated in Fig. 4, obvious elevation in frequency of sneezing and nose rubbing was observed in the AR group compared with the control group; whereas the symptoms were improved after GSI treatment. And in order to test whether GSI alters the inflammatory condition of AR mice, we examined the concentration of OVA-IgE and Th1 cytokine; IFN- γ , accompanying with Th2 cytokine; IL-4; and IL-5 in serum. In Fig. 5A, we see that OVA-IgE increased sharply and Th2 immune response was enhanced in the AR group, as expected. GSI leads to a decline in OVA-IgE and Th2 immune response, and the level of IL4 and IL-5 diminished after treatment. However, it seems that GSI has no impact on Th1 response. To detect the local state of nasal mucosal, we took a nasal mucosa sample and assessed the mRNA expression level of T-bet and GATA-3 (which are the key transcription factors for Th1 and Th2 respectively) in it. At the same time, the mRNA expression level of IFN- γ and IL-4, IL-5 was also assessed (Fig. 5B). There is a similar tendency with serum Elisa results. The mRNA level of Th2 cytokines and transcription factor ascended after OVA-challenged, which were reduced by repressing the Notch signaling pathway via GSI. Moreover, the RT-PCR result of T-bet and serum IFN- γ level showed that AR mice may sustain injury in the Th1 immune response, but the GSI can't restore it. Notably, mRNA expression level IFN- γ shows no difference between groups. In summary, GSI has the ability to alleviate upper airway inflammation.

3.3. GSI ameliorates the eosinophils infiltration and suppresses goblet cell metaplasia

Besides the measurement of cytokines, we evaluated the changes in pathology of the nose by HE staining to test eosinophils and PAS staining to test goblet metaplasia via mucin production. In Fig. 6, it is indicated that there is an overt eosinophil infiltration in the AR group and GSI can reduce it only at the highest concentration. In addition, goblet metaplasia and mucin production are responsible for AR's development, and just low concentration of GSI

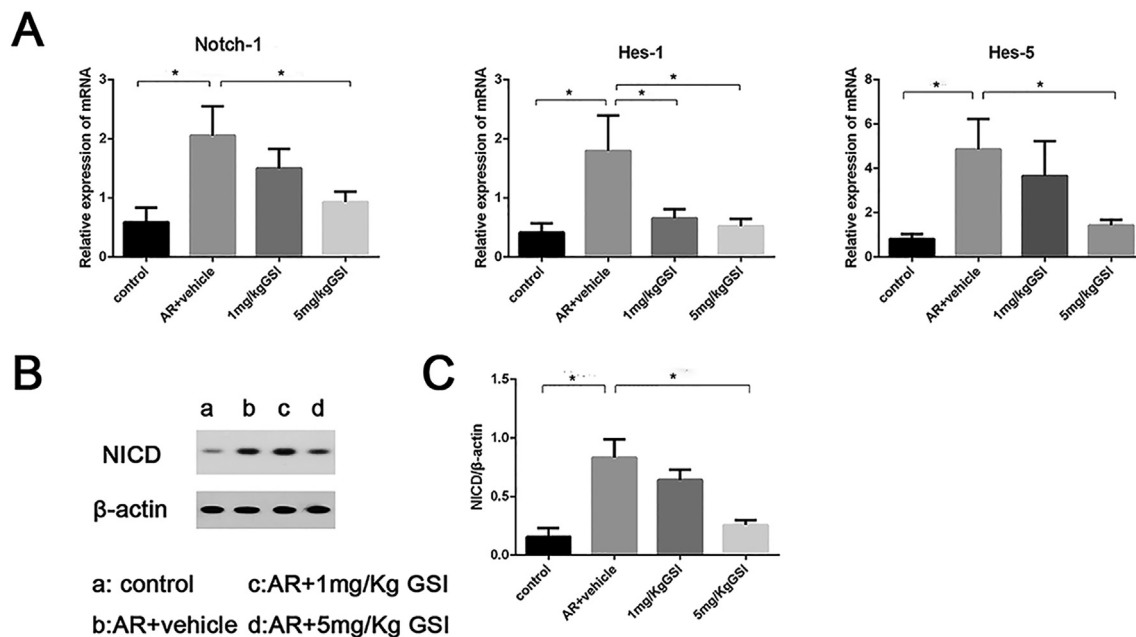


Fig. 2. Analysis of Notch signaling in AR mice and the effect of GSI. (A) Expression of Notch-1, Hes-1, Hes-5 in the nasal mucosa were assayed by Real-time PCR. (n = 5) (B). Photographs of representative samples of each group (C) Measurement of protein expression of NICD in nasal mucosal by Western blotting (n = 5) Data represent the mean $\pm$ SD. * $P < 0.05$ compared with AR + vehicle group.

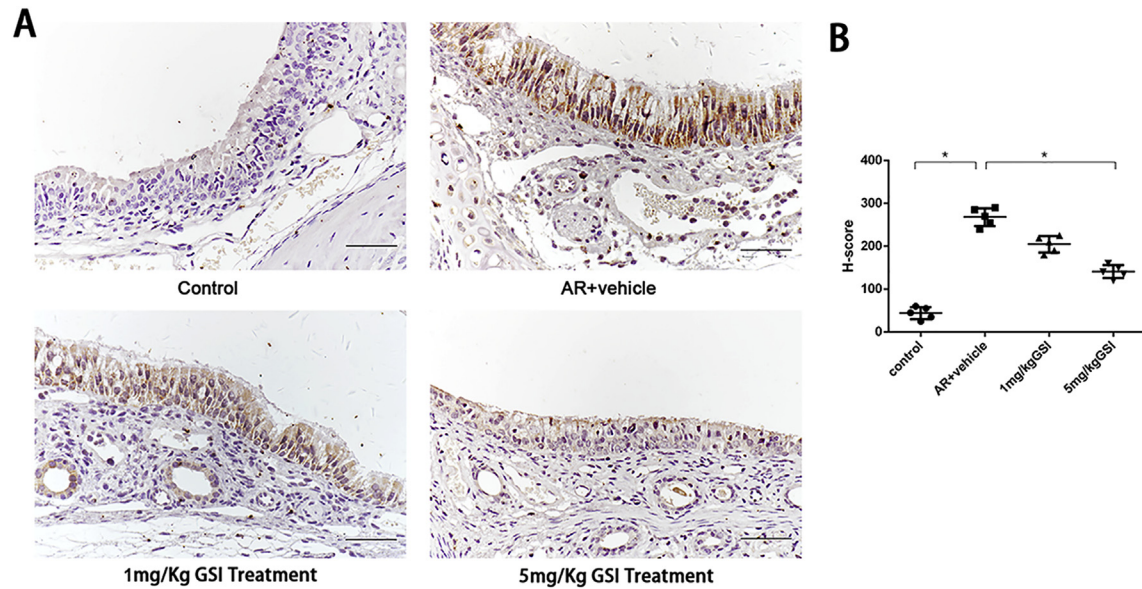


Fig. 3. (A) Examples of immunohistochemistry (IHC) for NICD. Control group: weakly stained NICD sample (H-score of 50); AR + vehicle: strongly stained NICD sample (H score of 291); 1 mg/kg GSI treatment: moderately stained NICD sample (H score of 180); 5 mg/kg GSI treatment: relatively weakly stained NICD sample compared with AR + vehicle group (H score of 90). Scale bar = 50 μ m. (B) The analysis of H-score of each group. n = 5 *P < 0.05 compared with AR + vehicle group.

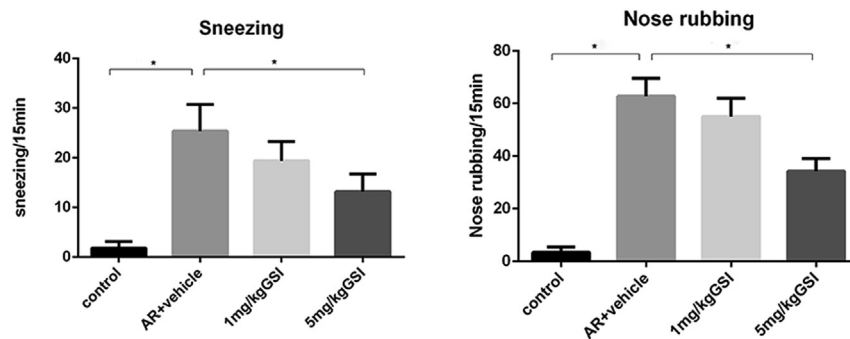


Fig. 4. Symptom scores. Symptoms of AR mice model can be ameliorated by GSI treatment Data represent the mean $\pm$ SD. *P < 0.05 compared with AR + vehicle group.

can restrain cells' activity (Fig. 7).

4. Discussion

It is well known that AR is involved in enhanced Th2 response, eosinophil infiltration, mucosa edema, and mucin hypersecretion. In the present study, the result of severe symptoms, increased OVA-IgE, strengthened Th2 immune response, and hypermetabolism of eosinophils and goblet cells compared with the normal group is consistent with previous studies.

Increasing evidence suggests that Notch signaling plays an important role in the process of complex immune response, especially the development of Th1 and Th2 [15]. It is controversial whether Notch signaling is promoting or suppressing the Th1. Maciej Jurynczyk et al. found that Notch3, but not Notch1, inhibition amended the symptom of experimental autoimmune encephalomyelitis (EAE) via suppressing Th1 response [16]. Zhijun Jiao et al. discovered the same impact in collagen-induced arthritis mice [17]; whereas some researchers reported that GSI is helpful for Th1 response in asthma model, bringing about IFN- γ level ascending [7,8]. Further exploration is needed to recognize the effect of GSI on Th1. Our data indicated that Notch signaling increased strikingly in AR mice, and GSI had no influence on Th1 function. The

different observations may be due to the different pathology condition. On the other hand, Notch signaling is of great importance for the Th2 development. IL-4 and Th2's key transcription factor, Gata-3, is the target gene of Notch signaling. Derk Amsen et al. [18], demonstrated that Th2 responses depend on expression of Notch1 and Notch2. In vitro, Notch1/2 deficient CD4 + T cells failed to make IL-4 under strong Th2-inducing conditions. Besides, they found that Notch signaling can regulate the upstream Gata-3 promoter via transcription factor Rbp-j and NICD elicited marked IL-4 production, which is not observed when cells lack Gata-3 expression, proving Gata-3 is required for induction of Th2 differentiation by Notch. One study found that neutralization of DLL4 weakens the Th2 production in vivo [19]. Two reports suggested that γ -secretase inhibitor blocking Notch signaling reduces airway inflammation by impairing Th2 response [7,8]. Based on our data, the Th2 cytokines production (IL-4, IL-5) decreased sharply compared with AR group, which is in agreement with the reports on asthma.

Also, the effect of GSI on eosinophil and goblet in AR mice is similar to conditions in asthmatic mice. Our observation revealed that GSI ameliorated eosinophil infiltration and mucin production. This tendency was also found in cord blood-derived eosinophils, and it is reported that GSI blunted eosinophils' ability to respond to eotaxin and reduce the eosinophil cationic protein (ECP)

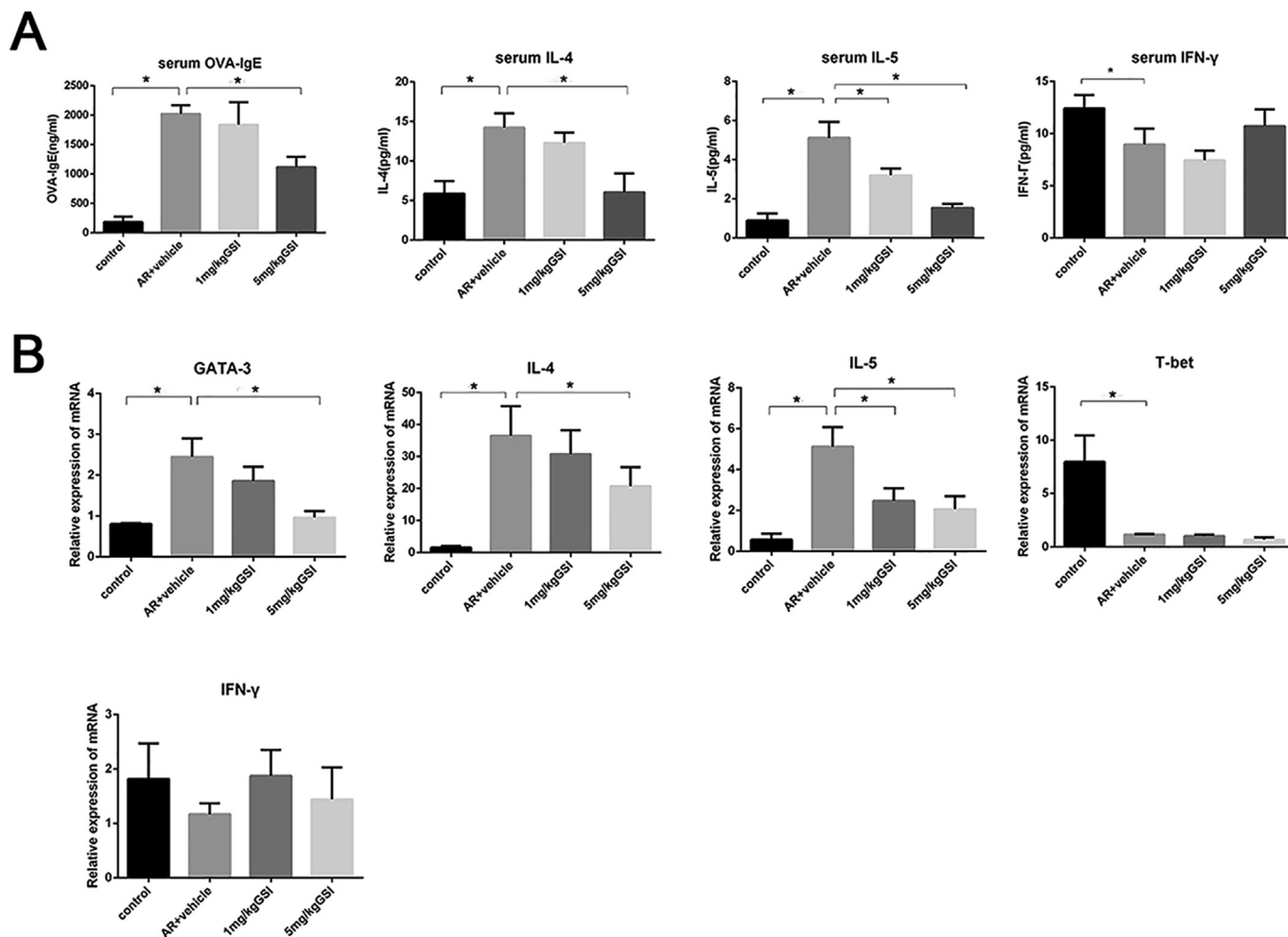


Fig. 5. The evaluation of inflammation state. GSI has inhibition effect on Th2 response but not Th1 response in AR mice (A) Concentration of OVA-IgE, IL-4, IL-5 and IFN-γ in serum were estimated by Elisa (n = 15 per group) (B) mRNA expression level of T-bet, IFN-γ, Gata-3, IL-4, and IL-5 in nasal mucosa were assessed by Real-time PCR. Data represent the mean ± SD * P < 0.05 compared with AR + vehicle group.

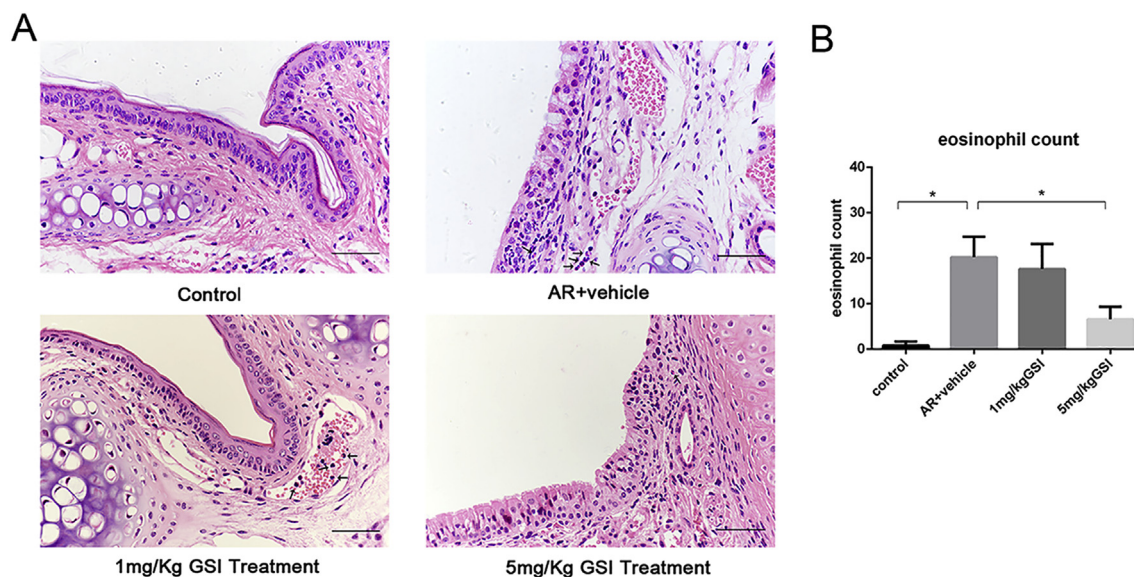


Fig. 6. GSI alleviated nasal mucosal eosinophil infiltration in AR mice. (A) Tissue sections were stained with HE to analyze the severity of inflammation. Photographs of representative nasal mucosal in each group. (B) Eosinophil count of each group (n = 5 per group). Data represent the mean ± SD Scale bar = 50 μm *P < 0.05 compared with AR + vehicle group.

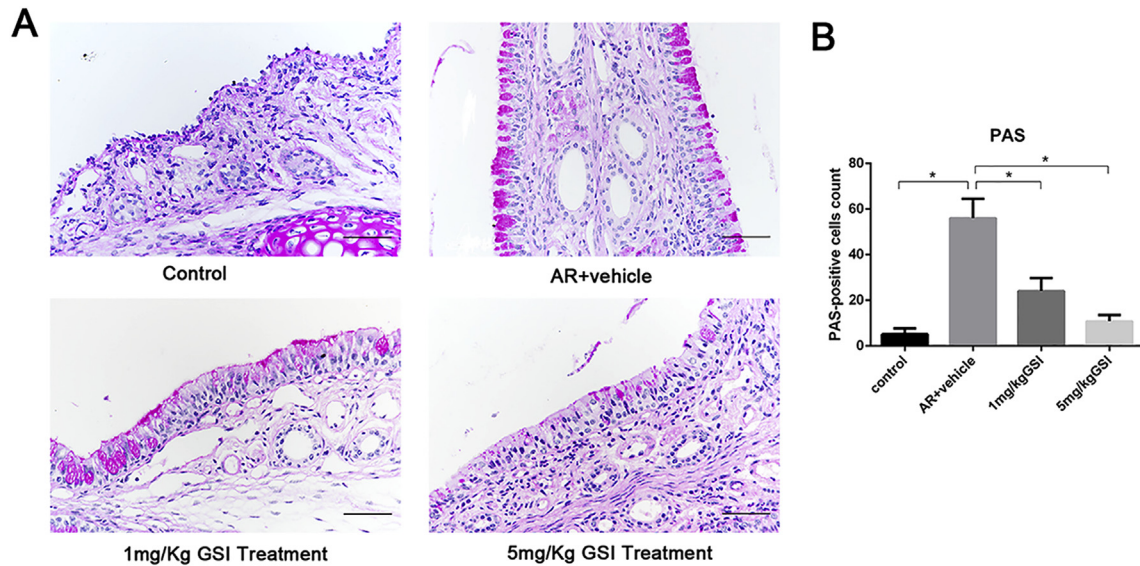


Fig. 7. GSI reduced goblet cell metaplasia in AR mice. (A) tissue sections were stained with PAS for mucus production. Photographs of representative nasal mucosal in each group. (B) PAS-positive cells count of septum mucosa of each group. (n = 5 per group) Scale bar = 50 μ m Data represent the mean $\pm$ SD *P < 0.05 compared with AR + vehicle group.

degranulation through ERK pathway [10]. The crucial mucin gene, MUC5AC, is the target gene of Notch signaling and its expression is induced by NICD by activating EGFR pathway [12], which may explain the mucin production's decrease after GSI treatment in the AR model.

Other than the effect on Th2 response, eosinophils, and goblet cells, the beneficial influence to AR mice, such as symptom weakening and decreased OVA-IgE is possibly associated with the disturbance of Notch signaling, which results in the function changes of dendritic cells (DCs) and mucosal mast cells, inducing tolerogenic DCs activity and suppressing mast cell hyperplasia [20,21]. Some limitations of our study should be acknowledged. Even though we testify that Notch signaling pathway participated in AR disease, we did not find the exact mechanism underlying GSI effect on AR. Second, we used a relatively small sample size; there is a need to investigate using a larger sample size and representative populations.

The children with AR is easy to get asthma [22]. So it will be of great help to find ways cure AR. Collectively, in this study, we used GSI, DAPT, to hamper Notch signaling in OVA-induced AR mice, and to our best of knowledge provided evidence for the first time that GSI can restore the Th2 response skewing, and restrain inflammation cells, such as eosinophils and goblet cells, in AR mice. It was demonstrated that GSI has the potential to be applied in allergic rhinitis clinical treatment.

Conflicts of interest

All authors declare no conflicts of interest in this research.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ijporl.2017.04.036>.

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