

Blockade of Notch Signalling by γ -Secretase Inhibitor in Lung T Cells of Asthmatic Mice Affects T Cell Differentiation and Pulmonary Inflammation

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Abstract—Notch is a single-pass transmembrane receptor protein expressed by T cells, which contributes to the pathogenesis of asthma through regulation of the development and differentiation of T cells. γ -Secretase inhibitor (GSI) acts as an effective blocker of Notch signalling. The present study aimed to investigate the role of GSI MW167 in T cell differentiation and antigen-induced airway inflammation. An OVA-induced airway inflammation mouse model was established. Blockade of Notch signalling was achieved using MW167. The expression of IL-4, IL-5, IFN- γ , Notch1 signalling and pro-inflammatory transcription factors in activated lung T cells was evaluated. Finally, the therapeutic effect of MW167 was investigated by haematoxylin and eosin staining, real-time PCR and ELISA. The expression of IL-4 and IL-5 decreased and that of IFN- γ increased significantly, and the protein expression levels of pro-inflammatory transcription factors reduced in active lung T cells after administration of MW167, compared to the control group. MW167 treatment prevented OVA-induced airway inflammation and histological changes. The serum and bronchoalveolar lavage fluid (BALF) levels of IL-4 and IL-5 in MW167-treated mice decreased significantly, whereas those of IFN- γ increased, relative to the levels in OVA-challenged animals treated with PBS. Our findings indicate that Notch signalling plays an important role in the pathogenesis of asthma and that MW167 may be a potential therapeutic target for allergen-induced airway inflammation.

KEY WORDS: asthma; Notch signal; γ -secretase inhibitor; T-lymphocyte; inflammation.

INTRODUCTION

T lymphocytes are among the main effector cells in asthma, which is a chronic inflammatory disorder of the airways. Activation of CD4⁺ T cells is the key step in the pathogenesis of asthma [1]. Numerous studies have demonstrated that Th2 polarization and Th1/Th2 imbalance play important roles in inflammation during asthma and airway remodelling, which are characterized by

increased Th2 cytokine levels (IL-4, IL-5, IL-6 and IL-10) and decreased Th1 cytokine levels (IFN- γ and IL-2) [2, 3]. Suppression of Th2 and/or enhancement of Th1 responses may thus be helpful in controlling asthma [4].

Notch is a single-pass transmembrane receptor protein that regulates a broad spectrum of cell fate decision and differentiation, proliferation and apoptosis. Four Notch receptors (Notch1–4) and at least five ligands have been identified [5]. The mechanism of activation and transduction of Notch signalling is complex; however, in essence, cytoplasmic Notch is cleaved by γ -secretase, leading to release of Notch intracellular domain (NICD), which possesses a nuclear localization signal (NLS), and this NICD then translocates to the nucleus to regulate the expression of related genes. Notch signalling has been shown to function in the development, differentiation and activation of T cells [6–8]. Because differentiation of Th2 cells can be inhibited by blockade of Notch signalling [9], we presume

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that restriction of Th2 polarization by γ -secretase inhibitor (GSI) may be a potential therapeutic strategy for allergen-induced airway inflammation. Therefore, in this study, we investigated whether allergen-induced airway inflammation is related to Notch signalling and evaluated the therapeutic effects of GSI injection in a mouse model for asthma.

MATERIALS AND METHODS

Animal Model

Ninety BALB/c mice (6–8 weeks old; equal number of males and females; mean weight, 20 ± 2 g) purchased from Shanghai Xipuer-Bikai Experimental Animal Limited Company were maintained under specific pathogen-free conditions at the animal centre of Xinhua Hospital. Among these mice, 48 mice were used for *in vitro* experiments and 42 mice were used for *in vivo* experiments (T cells isolated from two mice as one sample). All animal research was approved by the Animal Care and Use Committee of Shanghai Jiaotong University School of Medicine.

The mice in the asthmatic group were immunized by intraperitoneal administration of 50 μ g of OVA (grade V; Sigma-Aldrich, St. Louis, MO) with 100 μ g of aluminium hydroxide (Shanghai Experiment Reagent Co., China) on days 0, 7 and 14, followed by aerosol challenge with nebulized OVA (2 %; 800 mg of OVA in 40 ml of PBS) for 30 min per day from day 14 to day 21. For the control group, normal saline was administered instead of OVA. Mice were bled and killed 24 h after the last aerosol challenge on day 21. Subsequently, the peripheral serum and bronchoalveolar lavage fluid (BALF) were collected and the lungs were removed.

Isolation and Culture of Lung T Lymphocytes and Treatment with MW167

T lymphocytes were isolated from lung tissues, and its purity was determined by flow cytometry. The isolated cells were cultured according to our previous protocol and were separated into three groups: the MW167 group, DMSO group and blank control group [10]. T cells were incubated for 24 h before treatment with MW167 (molecular weight 705.8; Merck; 1 mg dissolved in 141.7 μ l of DMSO). The medium was replaced with fresh CRPMI-1640 (Sigma-Aldrich), and the cells were transferred to another 24-well plate, of which, each well was coated with 1.0 μ l each of purified anti-mouse CD3 (1.0 μ g/ μ l) and

purified anti-mouse CD28 (1.0 μ g/ μ l), and incubated for 24 h. Then, 2.0 μ l of MW167 solution was added to the MW167 group. After incubation for another 48 h, cells and supernatants were collected.

Real-Time PCR

Total RNA was extracted from 1×10^6 to 5×10^6 cultured T cells by using the TRIzol reagent (Invitrogen, USA) and reverse transcribed to generate first-strand complementary DNA (cDNA) (SuperScript II reverse transcriptase; Invitrogen, USA). SYBR Green Master Mix kit (Takara) was used for real-time PCR with the Bio-Rad iCycler iQ system (Bio-Rad). The real-time PCR was carried out under the following conditions: 95 °C for 1 min, followed by 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. As a control, each cDNA sample was simultaneously subjected to β -actin amplification. The real-time PCR primer sequences used were as follows: IL-4, 5'-GCTAGTTGTCATCCTGCTC-3' (sense) and 5'-GTGATGTGGACTTGGACTC-3' (antisense); IL-5, 5'-GCACAGTGGTGAAAGAGACC-3' (sense) and 5'-TAATCCAGGAAGTGCCTCGT-3' (antisense); IFN- γ , 5'-ATGAAATCCTGCAGAGCC-3' (sense) and 5'-GGACAATCTCTCCCCACCC-3' (antisense); and β -actin, 5'-AAGTGTGACGTTGACATCCGTAAAG-3' (sense) and 5'-CAGCTCAGTAACAGTCCGCCTAGA-3' (antisense). The threshold cycle (Ct) for each sample was determined according to the manufacturer's instructions, and the relative level of each transcript ($2^{\Delta Ct}$) was calculated from ΔCt (test Ct-GAPDH Ct).

Measurement of Cytokine Levels

The concentrations of IL-4, IL-5 and IFN- γ in the collected supernatants were determined by ELISA according to the manufacturer's protocols (RapidBio Lab, Calabasas, California, USA).

Western Blotting

Lung T cells were lysed and denatured. The lysates were subjected to SDS-PAGE, and the separated proteins were transferred to PVDF membranes. The membranes were blocked in Tris-buffered saline, 0.1 % Tween20 and 5 % milk, and then incubated with primary antibodies overnight at 4 °C. After incubation, the membranes were incubated with specific HRP-conjugated secondary antibodies for 2 h at room temperature. Finally, the membranes were incubated in ECL reagents (Pierce) and exposed to film. For semi-quantification of the expression levels, the

blots were scanned, and the density of each band was determined using image analysis software and normalized against the density of the GAPDH band. All experiments performed in triplicate.

MW167 Treatment *In Vivo*

Mice were separated into three groups equally: MW167-treated group (I), asthmatic group (II) and control group (III). Asthma was induced in groups I and II, and MW167 (1 mg/kg) was injected into the vena caudalis 1 h before challenge with nebulized OVA in group I. In group III, OVA was replaced by normal saline. Peripheral serum, BALF supernatant, a quarter of the right lung, half of the liver and one kidney from each mouse were harvested at 48 h after the final OVA challenge. Pathological sections of the lung, liver and kidney tissue were stained by haematoxylin and eosin (HE). The messenger RNA (mRNA) expression of IL-4, IL-5 and IFN- γ in lung T cells was evaluated by real-time PCR, and the concentrations of IL-4, IL-5 and IFN- γ in the peripheral serum and BALF supernatants were detected by ELISA.

Statistical Analysis

Statistical analysis was performed using SPSS 17.0 program (SPSS Inc, Chicago, IL). One-way analysis of variance (ANOVA) was used to determine significant differences between groups. Significance was defined as $P < 0.05$.

RESULTS

Classification of BALF Cells and Quantity of IgE in the Peripheral Serum

Mouse BALF smears were evaluated, and the quantity of IgE in the peripheral serum was detected by ELISA. Compared with the control group, the asthmatic group demonstrates significant increases in total BALF cells ($3.03 \pm 0.62 \times 10^5$ vs. $1.36 \pm 0.51 \times 10^5$ cells/ml; $P < 0.01$), lymphocytes (percentage, 0.22 ± 0.06 vs. 0.06 ± 0.01 ; $P < 0.01$) and eosinophils (percentage, 0.03 ± 0.01 vs. 0.02 ± 0.01 ; $P < 0.01$) (Fig. 1a, b). The concentration of IgE was 6.83 ± 1.08 ng/ml, which represented a significant increase relative to that in the control group (4.82 ± 1.66 ng/ml) ($P < 0.01$) (Fig. 1c).

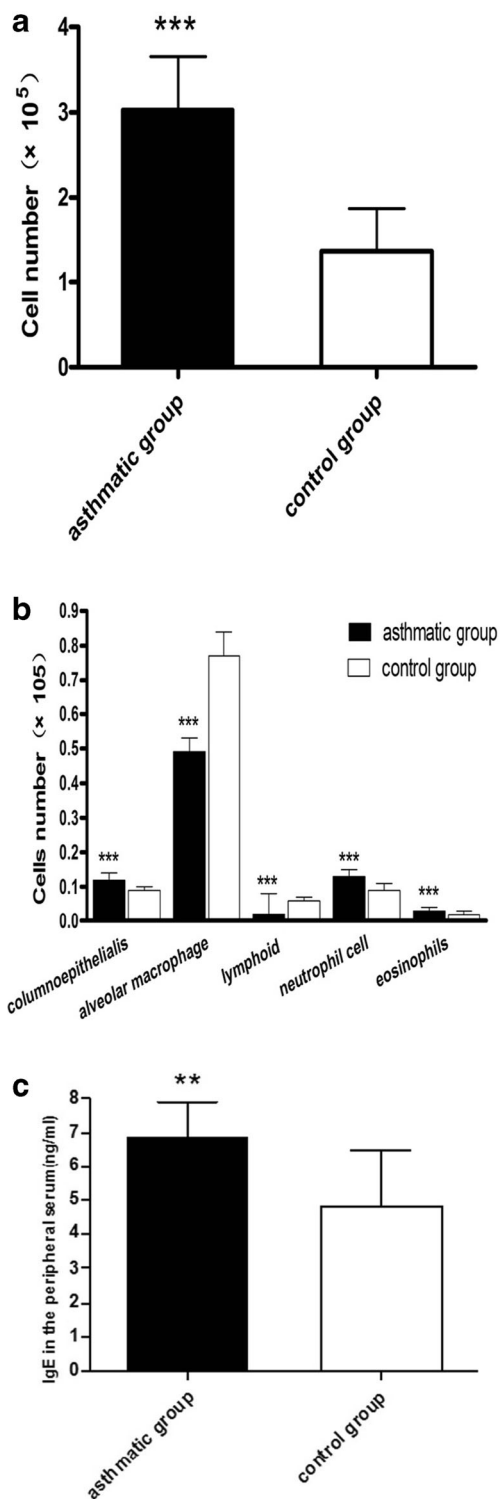


Fig. 1. a, b Cell counts and categorization of mouse BALF in asthmatic group and control group ($***P < 0.001$ vs. control group, $n = 10$) c Quantity of IgE in peripheral serum in asthmatic group and control group ($**P < 0.001$ vs. control group, $n = 10$).

MW167 Treatment Affected the Expression of IL-4, IL-5 and IFN- γ in Activated T Cells Isolated from Lungs of Asthmatic Mice

The mRNA levels of IL-4, IL-5 and IFN- γ were detected by real-time PCR in the MW167 group, DMSO group and blank control group at 48 h after costimulation of asthmatic lung T cells with anti-mouse CD3 and anti-mouse CD28. Relative to the other two groups, the MW167 group showed significant decrease in the mRNA expression of IL-4 and IL-5 ($P < 0.05$). There was no significant difference in the mRNA expression of IL-4 and IL-5 between the DMSO group and the blank control group ($P > 0.05$). In contrast, the MW167 group showed a significant increase in the mRNA expression of IFN- γ relative to the other two groups ($P < 0.01$). The mRNA expression of IFN- γ was not significantly different between the DMSO group and the blank control group ($P > 0.05$) (Fig. 2a).

The presence of IL-4, IL-5 and IFN- γ in the supernatants obtained from cell culture was also investigated by ELISA in the three groups. Relative to the other two groups, the MW167 group showed a significant decrease of IL-4 and IL-5 (39.769 ± 7.160 and 14.323 ± 3.375 pg/ml, respectively; $P < 0.05$). There was no significant difference in the level of IL-4 and IL-5 between the DMSO group (58.069 ± 8.216 and 19.650 ± 4.559 pg/ml, respectively) and the blank control group (71.991 ± 18.992 and 21.310 ± 5.109 pg/ml, respectively; $P > 0.05$). Relative to the other two groups, the MW167 group showed significant increase of IFN- γ (26.810 ± 5.154 pg/ml; $P < 0.01$). There was no significant difference in the level of IFN- γ between the DMSO group (19.547 ± 3.681 pg/ml) and the blank control group (18.367 ± 0.834 pg/ml; $P > 0.05$). The MW167 group also demonstrated a significant decrease in the IL-4/IFN- γ ratio relative to the other two groups (1.552 ± 0.490 ; $P < 0.01$). There was no significant difference

in the IL-4/IFN- γ ratio between the DMSO group (3.058 ± 0.713) and the blank control group (3.915 ± 1.008 ; $P > 0.05$) (Fig. 2b).

MW167 Effects on Expression of Notch Signalling, GATA-3 and T-bet

To explore the effects of MW167 on Notch signalling, Western blotting was used to detect the protein expression of NICD and Hes-1. Results showed that MW167 treatment led to a decrease in NICD and Hes-1 protein, suggesting that MW167 inhibits Notch signalling effectively in asthmatic lung T cells. We also examined the protein expression of GATA-3 and T-bet, which are transcription factors for the development of Th2 and Th1, respectively. The T-bet protein expression increased significantly, while GATA-3 protein expression decreased significantly (Fig. 3).

HE Staining of Lung, Liver and Kidney Tissues After MW167 Injection *In Vivo*

Necrosis and swelling were not seen at the injection site after MW167 treatment. Relative to the control group, the asthmatic group showed bronchial inflammation that manifested as epithelial damage, wall thickening of the tunica mucosa bronchiorum, inflammatory cell infiltration into the lower layer of the mucous membrane and the surrounding bronchus, accretion and spasm of airway smooth muscle after HE staining of asthmatic lung tissues. Bronchial inflammation and bronchospasm were reduced in the MW167-treated group relative to that in the asthmatic group. No obvious histological changes were observed in the pathological sections of the liver and kidney among the three groups (Fig. 4).

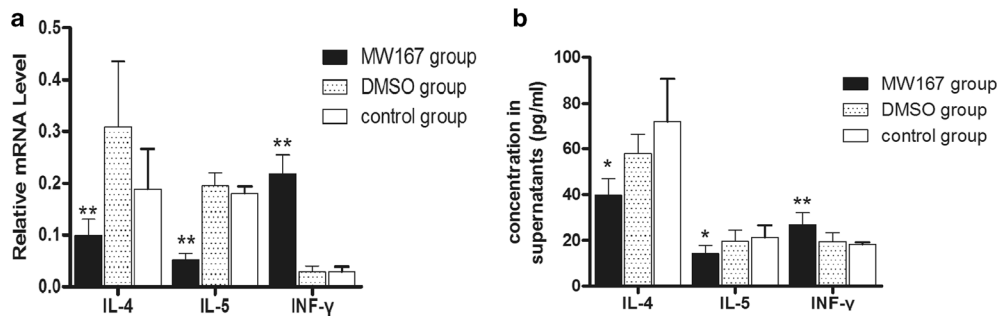


Fig. 2. **a** Real-time PCR analysis of IL-4, IL-5 and IFN- γ in activated lung T cells of asthmatic mice after blockade of Notch signalling by the γ -secretase inhibitor MW167. ** $P < 0.01$ relative to the DMSO group and blank control group, $n = 10$. **b** Quantity of IL-4, IL-5 and IFN- γ in the supernatants of cultured asthmatic lung T cells after blockade of Notch signalling by MW167. * $P < 0.05$, ** $P < 0.01$ relative to the DMSO group and blank control group, $n = 10$.

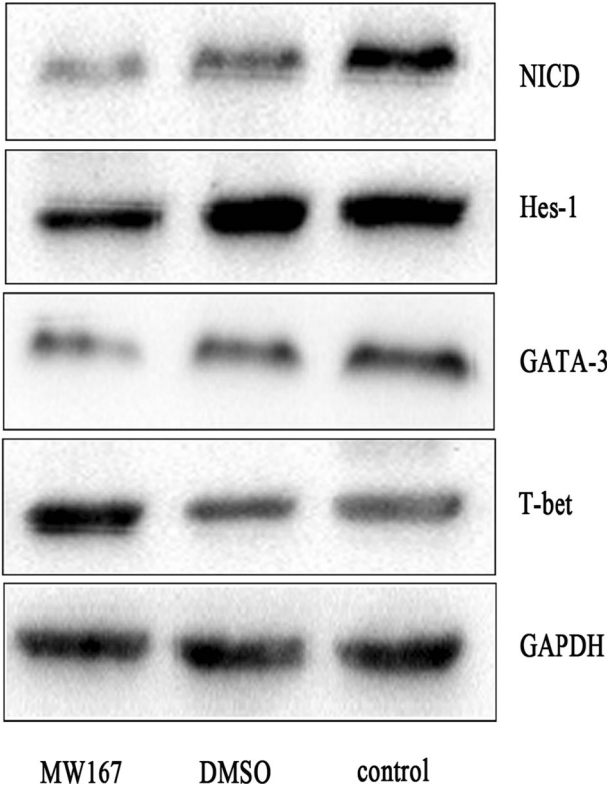


Fig. 3. The protein levels of NICD, Hes-1, T-bet and GATA-3 in asthmatic lung CD4⁺ T cells after treatment with MW167 (* $P < 0.05$, $n = 6$).

Expression of IL-4, IL-5 and IFN- γ in Lung T Cells After MW167 Injection *In Vivo*

IL-4, IL-5 and IFN- γ were expressed in all groups (MW167-treated group, asthmatic group and negative control group). However, relative to the control group, the asthmatic group showed obvious increase in the mRNA expression of IL-4 and IL-5 in the lung T cells. The mRNA expression of IL-4 and IL-5 decreased significantly in the lung T cells of MW167-treated mice relative to the asthmatic group, but the expression level was not significantly different from that in the control group. Relative to the control group, the asthmatic group showed significant decrease in the mRNA expression of IFN- γ in the lung T cells. The mRNA expression of IFN- γ in the lung T cells of MW167-treated mice increased significantly relative to that in the asthmatic group. No significant difference was observed between the MW167-treated group and the control group (Fig. 5).

Quantity of IL-4, IL-5 and IFN- γ in the Peripheral Serum and BALF Supernatant After MW167 Injection *In Vivo*

The concentrations of IL-4, IL-5 and IFN- γ in the peripheral serum and BALF supernatant were determined by ELISA in the three groups mentioned above. Relative to the control group (21.644 ± 3.331 and 16.700 ± 1.394 pg/ml in peripheral serum, 77.580 ± 19.229 and $48.592 \pm$

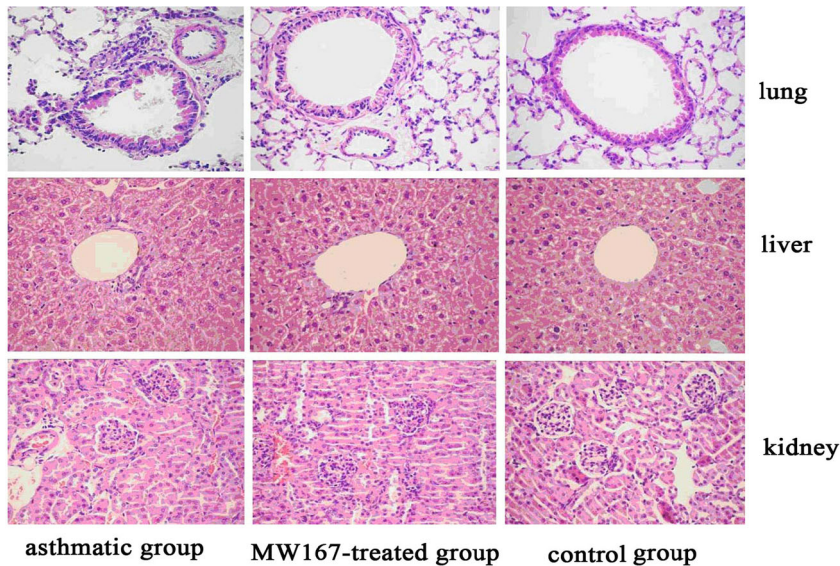


Fig. 4. HE staining of mouse lung, liver and kidney tissues in the asthmatic group, MW167-treated group and control group (original magnification $\times 400$).

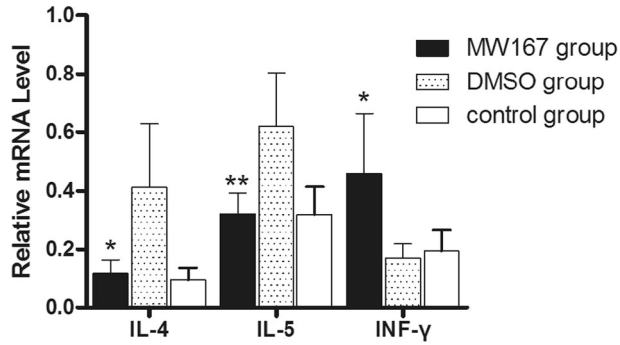


Fig. 5. Real-time PCR analysis of IL-4, IL-5 and IFN- γ in lung T cells in the three groups (MW167-treated group, asthmatic group and control group) after treatment with the γ -secretase inhibitor MW167. * P <0.05, ** P <0.01 compared with asthmatic group, n =6.

7.773 pg/ml in BALF supernatants), the asthmatic group showed a significant increase in IL-4 and IL-5 levels in the peripheral serum (26.046 ± 2.211 and 31.534 ± 9.485 pg/ml; P <0.01) and BALF supernatant (113.478 ± 8.628 and 85.431 ± 10.037 pg/ml; P <0.01). The MW167-treated group showed significant decrease in the IL-4 and IL-5 levels in peripheral serum (19.601 ± 0.870 and 15.244 ± 1.512 pg/ml; P <0.01) and BALF supernatant (75.077 ± 14.438 and 46.373 ± 5.007 pg/ml; P <0.01) relative to the asthmatic group. Relative to the control group (8.512 ± 2.019 pg/ml in peripheral serum, 27.526 ± 6.585 pg/ml in BALF supernatant), the asthmatic group showed significant decrease in the IFN- γ levels in peripheral serum (5.119 ± 2.230 pg/ml; P <0.05) and BALF supernatant (21.299 ± 2.830 pg/ml; P <0.05). The MW167-treated group showed significant increase in the IFN- γ levels in peripheral serum (10.644 ± 3.338 pg/ml; P <0.01) relative to that in the asthmatic group, as well as slight increase in the BALF supernatant, although this difference was not statistically significant (P >0.05). Accordingly, the IL-4/IFN- γ ratio in the asthmatic group was significantly higher than that in control group (P <0.01), and the IL-4/IFN- γ ratio in peripheral serum and supernatants of BALF were significantly lower in the MW167-treated group than in the asthmatic group (P <0.05 and P <0.01, respectively) (Fig. 6).

DISCUSSION

Th1 and Th2 subsets develop from CD4⁺ T cells. The dynamic balance between Th1 and Th2 regulates the immune response. IFN- γ inhibits the differentiation and function of Th2 cells. In contrast, IL-4 and IL-10 restrain the differentiation and function of Th1 cells. Th2

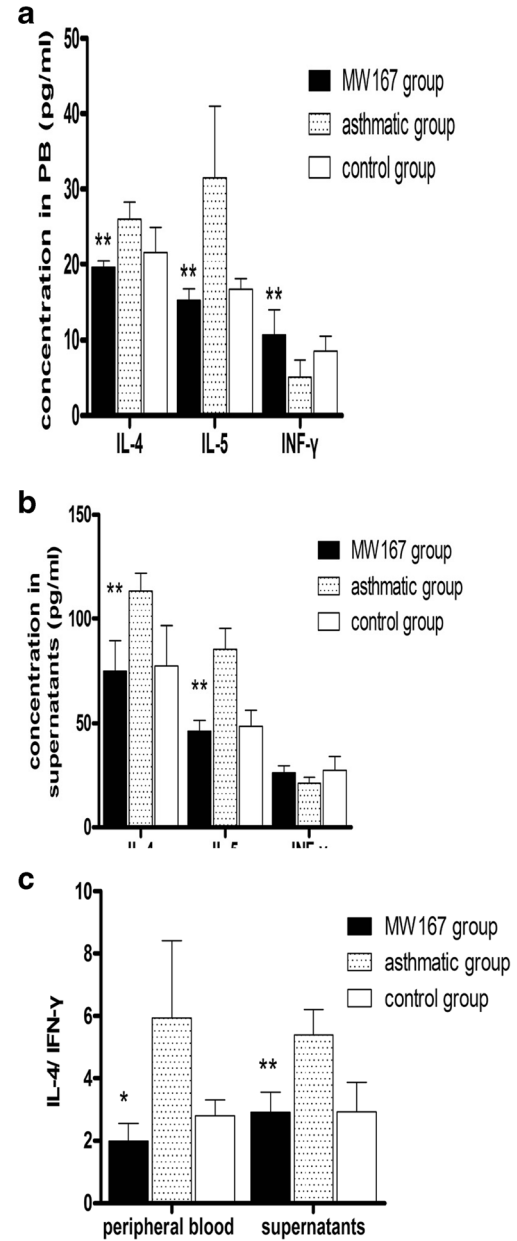


Fig. 6. **a** Quantity of IL-4, IL-5 and IFN- γ in the peripheral serum of the three groups after treatment with MW167. ** P <0.01 compared with asthmatic group, n =6. **b** Quantity of IL-4, IL-5 and IFN- γ in BALF supernatants of the three groups after treatment with MW167. ** P <0.01 for the MW167 group vs. the asthmatic group, n =6. **c** IL-4/IFN- γ ratio in peripheral serum and BALF supernatant of the three groups after treatment with MW167. * P <0.05, ** P <0.01 compared with control group, n =6.

polarization and Th1/Th2 disturbance are regarded as cardinal features of asthma pathogenesis, which includes airway inflammation, mucin hypersecretion, airway hyperresponsiveness and airway remodelling [11–13].

The Notch pathway is an evolutionarily conserved signalling mechanism that regulates different cellular differentiation, proliferation and apoptotic events, and it has been shown to play a pathogenic role in a variety of diseases, including inflammation. GSI directly induces a shift in the profile of cytokine production in airway inflammatory cells *in vivo* and *in vitro*. Sauma studied the protein expression of Notch receptors in murine T cells and found that resting CD4⁺ and CD8⁺ T cells did not express Notch receptors, but Notch 1 and Notch 2 were upregulated shortly after activation *in vitro* and *in vivo*. Furthermore, the Notch pathway was shown to regulate IL-10 production by CD4⁺ T cells and to regulate IFN- γ and IL-17 production by CD8⁺ T cells based on experiments in which Notch signalling was blocked by GSI [14]. Liotta reported that GSI indeed inhibits IL-4 production in CD4⁺ T cells co-cultured with dendritic cells while also promoting IFN- γ production [15]. It is therefore likely that the restriction of Th2 polarization by GSI could inhibit the pathogenesis of asthma.

Although the role of Notch in T-cell differentiation remains controversial, some evidence supports the role of Notch signalling in the induction of Th2 differentiation [16, 17]. In these investigations, Notch expression was observed in the lungs and lung T cells of asthmatic BALB/C mice. Furthermore, small interfering RNA was used to block the Notch1 signalling pathway in asthmatic lung T cells *in vitro* [10], which indicated that Notch signalling plays an important role in the pathogenesis of asthma and may promote Th2 polarization [18–20].

In our study, MW167 (20 μ M) was used to block Notch signalling in lung T cells in asthmatic mice. The mRNA and protein levels of IL-4 and IL-5 in cultured T cells were decreased in the MW167 group, whereas increases were observed in the level of IFN- γ and in the IL-4/IFN- γ ratio. These data further confirm that Notch signalling plays an important role in the pathogenesis of asthma. GSI may be useful for inhibiting Th2 polarization by blocking Notch signalling. Our results are similar to those of Kang, who intranasally administered GSI to ovalbumin-sensitized mice at the time of ovalbumin challenge [21]. Furthermore, our study also preliminarily confirmed the safety of MW167 as a potential therapeutic.

MW167 was injected at a dose of 1 mg/kg through the vena caudalis into asthmatic mice. The MW167-treated group showed fewer inflammatory changes and reduced airway smooth muscle spasm. The MW167-treated group also demonstrated a significant decrease of IL-4 and IL-5 mRNA expression and a significant increase of IFN- γ mRNA expression in lung T cells relative to the asthmatic

group. The MW167-treated group also demonstrated a significant decrease of IL-4 and IL-5 expression in the peripheral serum and BALF supernatant as well as a significant increase of IFN- γ in the peripheral serum relative to the asthmatic group. Although the IFN- γ content in the BALF supernatant was not significantly different between the MW167-treated group and the asthmatic group, the IL-4/IFN- γ ratio decreased significantly. Treatment with MW167 may be helpful to inhibit Th2 polarization in asthmatic pathogenesis, thereby alleviating asthmatic inflammation.

In this study, we used GSI to block the Notch signalling pathway in lung T cells from asthmatic mice and demonstrated GSI-induced conversion from Th2 to Th1 responses. It is difficult to analyse the effect of Notch in T cell differentiation and activation because of the diversity of interactions among Notch receptors and ligands [22, 23]. Discrepancies between our results and those of previous studies may have occurred because of the different methods used and the lack of elaborate knowledge regarding regulatory mechanisms. Many signal conduction molecules are involved in the Notch signalling pathway, such as nuclear factor of activated T cells (NFAT), nuclear factor- κ B (NF- κ B), T-bet and GATA-3 [24].

A multicentre, randomized, double-blind, placebo-controlled, single-ascending dose study for GSI BMS-708163 (an oral GSI designed for selective inhibition of A β synthesis currently in development for the treatment of mild to moderate Alzheimer disease) demonstrated a favourable safety profile and pharmacokinetic effects for BMS-708163, and adverse events were predominantly mild to moderate [25–27]. In our study, the MW167-treated group did not show significant differences in liver and kidney histomorphology relative to the other two groups; however, liver and kidney damage in the earlier period cannot be excluded. Further studies are necessary to elucidate the role of the Notch pathway in the pathogenesis of asthma. It is also necessary to develop new GSIs with higher selectivity fewer side effects for clinical use.

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Conflict of Interest. No conflict of interest to disclose.

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