

γ -Secretase Inhibitor Reduces Allergic Pulmonary Inflammation by Modulating Th1 and Th2 Responses

Jin Hyun Kang¹, Byung Soo Kim¹, Tae Gi Uhm¹, Shin-Hwa Lee², Gap Ryol Lee³, Choon-Sik Park², and Il Yup Chung¹

¹Division of Molecular and Life Sciences, College of Science and Technology, Hanyang University, Seoul; ²Genome Research Center for Allergy and Respiratory Diseases, Division of Allergy and Respiratory Medicine, Soonchunhyang University Hospital, Bucheon; and ³Department of Life Science, Sogang University, Seoul, South Korea.

Rationale: γ -Secretase inhibitor (GSI) has been used to effectively block Notch signaling, which is implicated in the differentiation and functional regulation of T helper (Th) effector cells. In asthma, a subset of CD4⁺ T cells is believed to initiate and perpetuate the disease.

Objectives: The aim of this study was to evaluate the therapeutic potential of GSI against allergic asthma.

Methods: GSI was administered to an ovalbumin-sensitized mouse via an intranasal route at the time of ovalbumin challenge.

Measurements and Main Results: The administration of GSI inhibits asthma phenotypes, including eosinophilic airway inflammation, goblet cell metaplasia, methacholine-induced airway hyperresponsiveness, and serum IgE production. GSI treatment of bronchoalveolar lavage cells stimulated via TCR or non-TCR pathways led to a decrease in Th2 cytokine production with a concomitant increase in Th1 cytokine secretion. Expression of Hes-1, a target of Notch signaling, was down-regulated in conjunction with a reduction of Notch intracellular domain and GATA-3 levels after GSI treatment of bronchoalveolar lavage cells. GSI treatment resulted in an inhibition of NF- κ B activation, and combined treatment with GSI and an NF- κ B inhibitor augmented IFN- γ production in a synergistic manner.

Conclusions: These data suggest that GSI directly regulates Th1 and Th2 responses in allergic pulmonary inflammation through a Notch signaling-dependent pathway and that GSI is of high therapeutic value for treating asthma by inhibiting airway inflammatory responses.

Keywords: asthma; GATA-3; NF- κ B; notch; γ -secretase inhibitor

It is widely accepted that a shift toward T helper (Th) 2 responses orchestrates cardinal features of asthma, including eosinophilia, airway inflammation, mucin hypersecretion, and airway hyperresponsiveness (AHR) (1). Numerous studies performed in subjects with asthma and animal models of asthma have shown increased Th2 cytokine levels and decreased Th1 cytokine levels within the affected airways. Specifically, Th2 cells, together with resident cells and other inflammatory cells, such as eosinophils, mast cells, and B cells, play major roles in the initiation and pathological development of asthma (2, 3). Therefore, targeted therapies have been directed toward sup-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

γ -Secretase inhibitor was initially developed to treat neurological diseases by blocking aberrant cleavage of amyloid precursor protein. Additionally, it has been used to block Notch signaling, which plays a crucial role in T-helper cell differentiation.

What This Study Adds to the Field

In an experimental model of asthma, γ -secretase inhibitor directly up-regulated Th1 and down-regulated Th2 cytokines via a Notch signaling-dependent mechanism, suggesting the therapeutic potential of γ -secretase inhibitors in asthma.

pressing the production and action of Th2 cytokines and/or enhancing Th1 responses (4–6).

Notch is an evolutionarily conserved transmembrane protein that regulates a broad spectrum of cell fate decisions and differentiation (7). Four mammalian Notch receptors (Notch1–4) and five ligands (Jagged1 and -2 and Delta-like1, -3, and -4) have been identified (8, 9). Notch signaling is initiated when Notch receptors contact their ligands. The intracellular domain of Notch (NICD) is then liberated from the final cleavage of the transmembrane domain by a γ -secretase. NICD is translocated to the nucleus, where it interacts with CSL/RBP-J κ to recruit Mastermind-like 1 protein. This interaction results in the trans-activation of target genes, such as Hes-1 (10, 11), that negatively regulate the expression of target genes such as tissue-specific transcriptional activators (12). It has been argued that Notch signaling regulates lineage commitment of naive Th cells to effector Th cells; however, there is no consensus regarding the role of Notch in Th1 or Th2 differentiation. Exposure of naive T cells to soluble Delta or surface Delta on APC induces IFN- γ production (13, 14) and suppresses Th2 cell development via a block in the downstream effects triggered by IL-4 (15). Notch 1 can directly regulate T-bet, which is a key transcription factor for Th1 differentiation (16, 17). Treatment with a γ -secretase inhibitor (GSI) results in the inhibition of Th1 differentiation and Th1-mediated disease progression (18). Furthermore, NICD up-regulates T-bet and IFN- γ expression (13). Taken together, these studies support a role for Notch signaling in Th1 differentiation. In marked contrast, conditional inactivation of CSL/RBP-J κ or the expression of dominant negative Mastermind-like protein leads to impaired Th2 differentiation (14, 19) or enhanced Th1 responses (20). Jagged1 on APC constitutes an instructive signal for Th2 differentiation (14), and the selective silencing of Jagged 1 in human dendritic cells (DCs) led to a significant reduction in Th2 polarization (21). These studies suggest that the blockage of Notch signaling provides a potential strategy for treating Th2-type

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Correspondence and requests for reprints should be addressed to Il Yup Chung, Ph.D., Division of Molecular and Life Sciences, College of Science and Technology, Hanyang University, 1271 Sa-1-dong, Ansan, Gyeonggi-do 426-791, South Korea. E-mail: iychu@hanyang.ac.kr

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diseases, such as asthma. It has not been determined whether interference of the Notch signaling pathway is a feasible treatment option for airway inflammatory disease. In addition, increasing evidence has demonstrated that Notch plays a role in the functional regulation of mature immune cells, including T-cell activation (22, 23) and NF- κ B activation (22, 24). In particular, NF- κ B activation is required for the expression of GATA-3 (25), a key transcription factor essential for the differentiation of Th2 cells (26).

Previously, we demonstrated that the blockage of Notch signaling by GSI promotes cord blood-derived eosinophil differentiation (27). Subsequently, we showed that the functionality of eosinophilic cells is severely impaired, as judged by reduced chemotaxis. In addition, we observed reduced Th2 cytokine production and blunted ECP degranulation (28). Given that Notch signaling is essentially involved in effector Th cell differentiation and that eosinophils contribute to the pathophysiologic development of asthma, we investigated whether GSI has therapeutic effects on the development of diseases associated with eosinophilic inflammation. Some of the results of these studies have been previously reported in the form of an abstract (29).

METHODS

Treatment with GSI in Ovalbumin-Induced Asthma

To induce asthma, 6-week-old male BALB/c mice (Charles River Technology Inc., Seoul, Korea) were sensitized on Days 0 and 14 by intraperitoneal injection of 100 μ g of ovalbumin (OVA) (Sigma, St. Louis, MO) emulsified with 20 mg of aluminum hydroxide in a total volume of 200 μ L. Mice were then challenged with 150 μ g of OVA by intranasal instillation once a day for three consecutive days from Day 21. Three different concentrations of GSI L685,458 (Calbiochem, La Jolla, CA) were used for this experiment: 0.003, 0.03, and 0.3 mg/kg, which were approximately equivalent to 0.1, 1, and 10 nmol per mouse, respectively. GSI was administered intranasally 30 minutes before each OVA challenge. Sham mice were sensitized and challenged with phosphate-buffered saline (PBS) and 20, 2, and 0.2% dimethylsulfoxide (DMSO) (vol/vol) in 50 μ L as a control for GSI. The GSI used in this study is highly selective to intramembranous γ -secretase processing of Notch (30) and displays a higher selectivity (i.e., 50-fold or greater) as compared with other aspartyl proteases, such as HIV protease and cathepsin D (31). The Institutional Animal Care and Use Committee of Soonchunhyang University approved this study.

Measurement of Airway Hyperresponsiveness to Methacholine

Airway Hyperresponsiveness (AHR) was evaluated as the change in airway resistance (cm H₂O/ml/s) after challenge with aerosolized methacholine. Anesthetized (pentobarbital sodium, 70–90 mg/kg, intraperitoneal), tracheostomized (stainless steel cannula, 18 gauge) mice were mechanically ventilated, and lung function was assessed using methods described previously (32). Lung resistance and compliance were measured using the FlexiVent system (Scireq, Montreal, Canada). We also measured AHR in unrestrained mice by noninvasive whole-body plethysmography (All Medicus, Inc., Seoul, South Korea) according to the manufacturer's protocol.

Preparation of Bronchoalveolar Lavage Cells, Lung Tissues, and Histologic Staining

To collect bronchoalveolar lavage fluid (BALF), PBS was slowly infused into the lungs and withdrawn via a cannula inserted into the trachea. The total number of cells in the BALF was counted on slides prepared by cyto-centrifugation and Diff-Quick staining (Scientific Products, Gibbstown, NJ). The BALF was centrifuged, and the supernatant was kept at -70°C for cytokine measurement. Trachea and lung tissues were removed from the mice and fixed with 4% paraformaldehyde. The specimens were dehydrated and embedded in

paraffin. For histologic examination, 4- μ m sections of embedded tissues were deparaffinized and stained with hematoxylin and eosin or periodic acid-Schiff. Histopathology analysis and goblet cell counts were performed in a blind fashion.

The Effects of GSI on BAL Cell Cytokine Production

The collected BAL cells were stimulated with phorbol myristate acetate (PMA) (100 ng/ml) and ionomycin (1 μ M) in the presence or absence of different concentrations of GSI. BAL cells were also stimulated with anti-CD3 and anti-CD28 antibodies (BD Pharmingen, San Diego, CA) to activate the T cells in the BAL cells. The culture supernatants were measured for the presence of IL-4, IL-5, IL-13, IFN- γ , and eotaxin.

Partial Purification of CD4⁺ Cells from BAL Cells and Immunocytochemistry Analysis

The CD4⁺ cells were partially purified with a MACS negative selection CD4 isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity of the CD4⁺ cells was approximately 70%, as evidenced by microscopic observation after Diff-Quick staining. Cytospins were prepared with 4×10^4 cells. The cells were permeabilized and reacted with anti-mouse IFN- γ (Abcam, Cambridge, UK) and treated with biotinylated goat anti-mouse IgG. Color reaction was developed by staining with DAB (Zymed Laboratories, San Francisco, CA).

ELISA Assay

Cytokine concentrations and serum IgE were determined by using an ELISA kit (see online supplement).

Electrophoretic Mobility Shift Assay of NF- κ B

An electrophoretic mobility shift assay was performed according to the gel shift assay system protocol (Promega, Madison, WI). Additional detail is provided in an online supplement.

Real-Time Polymerase Chain Reaction

Real-time quantitative polymerase chain reaction (RT-PCR) was performed with SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Additional detail is provided in the online supplement.

Western Blot Analysis

Cells were lysed in RIPA buffer. Cell lysate samples (20 μ g) were resolved by SDS-PAGE and blotted to PVDF membranes. The blots were probed with anti-NICD (Abcam), anti-GATA-3 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-T-bet antibodies (Santa Cruz Biotechnology), followed by anti-rabbit HRP-conjugated antibody (Cell Signaling, Beverly, MA). Immunostained proteins were detected by ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

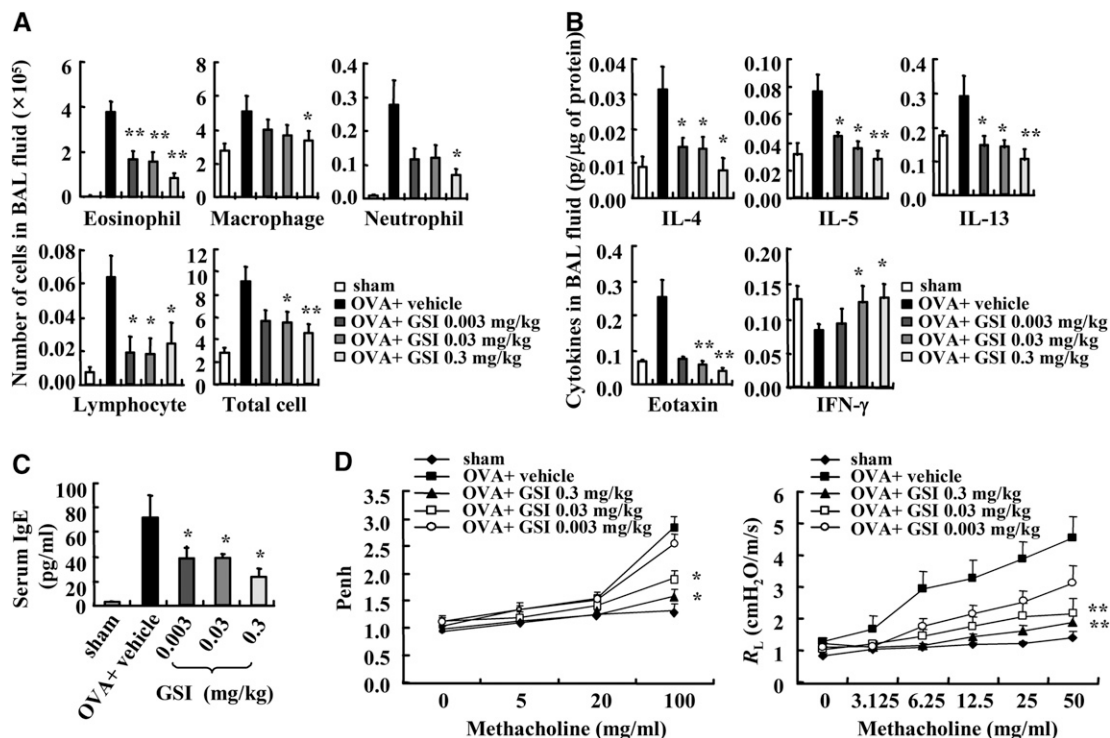
Statistical Analysis

All statistical analyses were performed using the Microsoft Excel data analysis program, or the SPSS statistical analysis program for the Wilcoxon signed rank test. Differences with a *P* value < 0.05 were considered statistically significant. The results are expressed as the mean $\pm$ SEM.

RESULTS

Inhibitory Effects of GSI on Airway Inflammation, Goblet Cell Metaplasia, and AHR

The primary aim of this study was to evaluate the therapeutic potential of GSI against allergic asthma. Mice were sensitized using intraperitoneal injections of OVA (or PBS for sham group) at Days 0 and 14, and were subsequently challenged with OVA (or PBS for sham group) via an intranasal route three times 7 days later. As expected, OVA-sensitized/challenged mice showed significantly increased numbers of total cells, macrophages, eosinophils, neutrophils, and lymphocytes 48 hours after the last OVA challenge, as compared with sham mice. Three GSI concentrations (0.3, 0.03, and 0.003 mg/kg)



(n = 16–23 per group) and airway resistance in mice (n = 8–10 per group) treated with GSI (0.3, 0.03, and 0.003 mg/kg) after challenge with increasing concentrations of aerosolized methacholine; **P* < 0.05 and ***P* < 0.01, compared with the OVA-challenge group. 2% DMSO was used in OVA plus vehicle.

were prepared in 20, 2, and 0.2% (vol/vol) DMSO, respectively, in a total of 50 μ l PBS. GSI was administered three times via an intranasal route 30 minutes before OVA challenge. GSI treatment inhibited the accumulation of total inflammatory cells in the BAL compartment in a dose-dependent manner. In particular, decreases in eosinophils were prominent and substantially accounted for the changes in total cell number. Lymphocytes were also significantly reduced by GSI treatment, although they constituted the smallest fraction of total cells in the inflamed airways of this model. Macrophage and neutrophil numbers were significantly decreased only at the highest concentration of GSI used (Figure 1A). Furthermore, when GSI was intranasally administered once a day on Days 24, 25, and 26, after the last OVA challenge on Day 23, airway inflammation was significantly inhibited, as assessed at Day 28 (see Figure E1 in the online supplement). Thus, GSI impeded the development of airway inflammation when administered after the Th2 response had already been induced. The production of Th2 and Th1 cytokines was demonstrated in BALF from OVA-administered mice. GSI inhalation resulted in significant decreases in IL-4, IL-5, IL-13, and eotaxin levels in BALF, whereas IFN- γ production rose to the level of the sham group (Figure 1B). In agreement with these results, an increase in BAL IFN- γ production is observed after treatment with agents that inhibit airway inflammation in a murine asthma model (33, 34). RT-PCR analysis showed that mRNA levels for cytokines in lung tissues had a similar tendency with secreted cytokine levels in BALF (Figure E2 and Table E1). IgE titers in serum were increased in the OVA group and declined with GSI treatment (Figure 1C). Airway function was evaluated using Penh and airway resistance at 24 hours after the last antigen exposure. Allergen-challenged mice developed enhanced responses to methacholine, as compared with the sham group. In the groups treated with 0.03 and 0.3 mg/kg GSI, which were equivalent to approximately 1 and 10 nmole per mouse, respectively, lung

function significantly improved (Figure 1D). DMSO (vehicle) had no effect on airway function (data not shown). Histologic staining of lung tissues showed that GSI prevented intense perivascular and peribroncheolar eosinophilic inflammation in the lung (Figure 2A). Mucin production, as determined by periodic acid-Schiff-positive cells in the bronchial epithelium, was blocked by GSI inhalation in a dose-dependent manner (Figures 2B and 2C).

GSI Inhibits Th2 Cytokine Production but Increases Th1 Cytokine in BAL Cells

To understand the mechanism by which GSI inhibits airway responses to an antigen, we examined whether GSI altered the cytokine production profiles in BAL cells of the diseased lung. BAL cells were collected from the inflamed airways of mice 48 hours after the last OVA challenge. Unstimulated BAL cells produced minimal amounts of cytokines. Stimulation with PMA and ionomycin for 12 to 48 hours led to the induction of cytokines IL-4, IL-5, and IL-13, with slightly different kinetic patterns. The addition of GSI (10 μ M) resulted in almost complete inhibition of Th2 cytokine production; however, it strikingly enhanced IFN- γ production (Figure 3A). Likewise, GSI inhibited Th2 cytokine production in a dose-dependent manner while augmenting Th1 cytokine production (Figure 3B), indicating a GSI-related shift from Th2 to Th1 responses. Eotaxin levels remained low and unchanged, suggesting that BAL cells are not the main cellular source for the high levels of eotaxin seen in BALF from the inflamed airways of mice. Because these inflammatory cytokines are mainly T-cell products, we examined the contribution of T cells to the *ex vivo* cytokine production of BAL cells. Plate-bound anti-CD3 (2 μ g/ml) and anti-CD28 (1 μ g/ml) induced IL-5 and IFN- γ at levels comparable to those seen with PMA and ionomycin stimulation. GSI inhibited IL-5 production but augmented IFN- γ production (Figure 3C). Similarly, analysis of the intracellular

Figure 1. Therapeutic effects of γ -secretase inhibitor (GSI) administration on airway inflammation in ovalbumin (OVA)-induced mice. (A) Inhibition of inflammatory cell infiltration by GSI. Bronchoalveolar lavage fluid (BALF) was collected 48 hours after the last OVA challenge. Total and differential cell counts were performed by enumerating a minimum of 300 cells. Data represent the mean $\pm$ SEM; n = 18 per group. (B) Suppression of Th2 cytokine production by GSI. The concentrations of Th2 and Th1 cytokines in BALF were estimated by ELISA (n = 16–23 per group). (C) Decreased serum IgE with GSI treatment (n = 21 per group). (D) Airway hyperresponsiveness (AHR) measurement. AHR was assessed as Penh

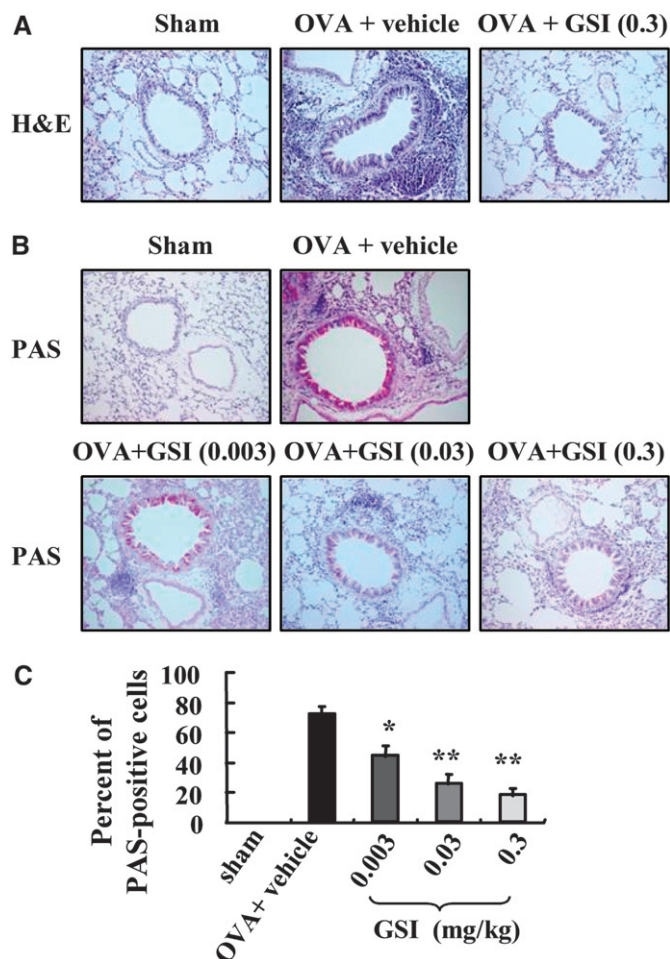


Figure 2. γ -Secretase inhibitor (GSI) reduced lung tissue eosinophilia and goblet cell metaplasia in ovalbumin (OVA)-induced mice. (A) Histologic staining of inflammatory cells in lung tissues. Tissue sections were stained with hematoxylin and eosin to analyze the severity of inflammation. The value in parentheses indicates the GSI dose in mg/kg. The results shown are representative of more than 10 independent experiments. (B) Staining of goblet cells. Tissue sections were stained with periodic acid-Schiff (PAS) for mucus production. The values in parentheses indicate the GSI doses in mg/kg. All results shown are representative of more than 10 independent experiments. (C) Results are expressed as the percentage of PAS-positive cells in epithelial areas examined ($n = 12$ mice per group). * $P < 0.05$ and ** $P < 0.01$, compared with the OVA-challenge group. 2% DMSO was used in OVA plus vehicle.

production of IL-4 and IFN- γ in BAL cells revealed that GSI inhibited IL-4 production while augmenting IFN- γ synthesis (Figure E3). Given that anti-CD3 and anti-CD28 specifically stimulated T cells among BAL cells, that the cytokine production pattern and the levels with the stimuli were similar to those with PMA and ionomycin, and that unstimulated BAL cells produced negligible amounts of cytokines (Figures 3B and 3C), these data suggest that the CD4 $^{+}$ T cells in BAL cells were largely responsible for the cytokine production in response to the stimuli. To further prove that T cells were responsible for the cytokine production in BAL cells, CD4 $^{+}$ cells were partially purified from BAL cells and stimulated via the TCR pathway. GSI treatment resulted in markedly elevated IFN- γ expression, as evidenced by immunocytochemistry analysis (Figure 3D). With a similar pattern to that seen in BAL cells, GSI modulated IL-5 and IFN- γ production in the purified CD4 $^{+}$ cells. By

contrast, the cytokine production levels were low in CD4 $^{-}$ cells, and the GSI effects were not observed (Figure E4). These data support the finding that the T cells in the BALF provide most, if not all, of the Th1 and Th2 cytokines produced in BAL cells. Furthermore, GSI effectively regulates Th1 and Th2 cytokine production by T cells.

GSI Effects on Expression of GATA-3, T-bet, and Notch Signaling Component

To investigate the involvement of Notch signaling in these GSI effects, we assessed the mRNA expression of Hes-1, a direct transcriptional target of Notch signaling (10, 11). Because we failed to obtain sufficient purified CD4 $^{+}$ cells to analyze mRNA expression, we used BAL cells collected from the inflamed airways of mice. The stimulated BAL cells displayed enhanced Hes-1 mRNA expression, and GSI treatment led to a decrease in Hes-1 mRNA (Figure 4A). In agreement with these observations, NICD generation was reduced after GSI treatment of BAL cells (Figure 4B), suggesting that GSI effectively blocks Notch signaling in BAL cells. We also examined the effects of GSI on the expression of GATA-3 and T-bet, which are key transcription factors essential for the development of Th2 cells and Th1 cells, respectively (16, 26). GATA-3 mRNA and protein levels were decreased in GSI-treated BAL cells, whereas the T-bet mRNA level was increased by GSI treatment, although the incremental change at the protein level was marginal (Figures 4A and 4B). These findings raise the possibility that GATA-3 expression is down-regulated by GSI through the blockage of the Notch signaling pathway.

GSI Inhibits the Activation of NF- κ B

Notch signaling has been shown to augment NF- κ B activity (24). Therefore, we tested whether GSI inhibited NF- κ B activation in BAL cells. When BAL cells ($5\text{--}10 \times 10^6$ cells) were treated with PMA and ionomycin for 12, 24, and 36 hours, NF- κ B activation reached a peak at 24 hours. Activation of NF- κ B was inhibited by GSI. The addition of GSI to the 24-hour cultures decreased NF- κ B activation in a dose-dependent manner (Figure 5A). To examine the effects of NF- κ B activity on cytokine production, BAL cells were treated in the absence or presence of 5 μ M GSI with N-p-tosyl-L-phenylalanyl chloromethyl ketone (TPCK). TPCK prevents the activation of NF- κ B by blocking phosphorylation and, therefore, subsequent degradation of I κ B. The effects of TPCK on IL-5 and IFN- γ production were mediocre to minimal; however, combined treatment with GSI and TPCK led to the up-regulation of IFN- γ in a synergistic manner, together with an additive down-regulation of IL-5 (Figure 5B). These results suggest that GSI-mediated regulation of cytokine production is partially dependent on its inhibitory effects on NF- κ B activity (IFN- γ) or occurs via a pathway independent of NF- κ B inhibition (IL-5).

DISCUSSION

Increasing evidence suggests that Notch signaling is required for the developmental program of Th2 immunity (14, 19, 20). Eosinophils are important effector cells for airway damage in allergic inflammatory airway disease. Our previous results demonstrate that GSI induces the differentiation of eosinophils lacking effector functions (28). It is therefore possible that the restriction of Th2 and eosinophil inflammatory responses by GSI, a pharmacological blocker of Notch signaling, can provide a means to blunt asthma. Our results suggest that GSI intranasal challenge attenuates the pathological features of asthma, including eosinophilic inflammation of the airways, goblet cell metaplasia, methacholine-induced AHR, and serum IgE pro-

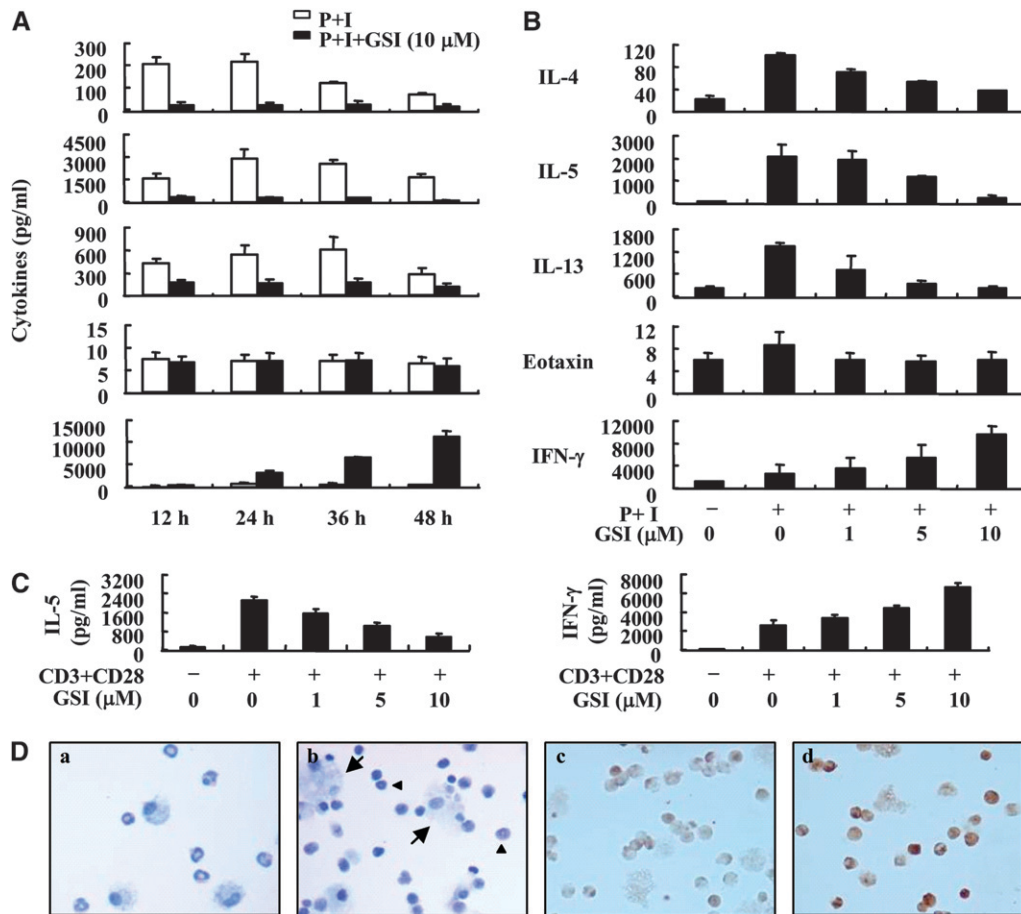


Figure 3. Effects of γ -secretase inhibitor (GSI) on cytokine production of bronchoalveolar lavage (BAL) cells. (A and B) Effects of incubation time and GSI dose on cytokine production by BAL cells. BAL cells were collected from the airways of mice sensitized and challenged with ovalbumin (OVA). The cells were stimulated with PMA (P) and ionomycin (I) in the presence of different concentrations of GSI for the indicated time intervals. The results are expressed as the mean $\pm$ SEM in triplicate from a representative of three to five independent experiments. (C) BAL cells were stimulated with plate-bound anti-CD3 and anti-CD28 in the presence or absence of GSI. The results are expressed as the mean $\pm$ SEM in triplicate from a representative of three to five independent experiments. (D) IFN- γ expression in partially purified CD4⁺ cells from BAL cells was analyzed by immunocytochemistry. (a) BAL cells and (b) partially purified CD4⁺ cells were stained with Diff-Quick. Arrows and arrowheads denote macrophages and eosinophils, respectively. The partially purified CD4⁺ cells were stimulated with anti-CD3 and anti-CD28 in (c) the absence or (d) presence of GSI for 36 hours and then stained with anti-IFN- γ .

duction (see Figures 1 and 2). The results from the *ex vivo* GSI treatment of stimulated BAL cells showed that GSI has dual effects: the inhibition of Th2 cytokine production and the augmentation of Th1 cytokine production (see Figure 3). Thus, GSI directly induces a dramatic shift in the profile of cytokine production in airway inflammatory cells that have been generated *in vivo*. In line with these observations, Liotta and colleagues recently reported that GSI indeed inhibits IL-4 production in CD4⁺ T cells cocultured with dendritic cells while promoting IFN- γ production (21). Given that the balance between Th1 and Th2 cytokines regulates the aforementioned asthmatic characteristics, it seems likely that the skewing of the cytokine production profile from Th2 to Th1 responses provides a basis for the therapeutic value of GSI as an antiasthma agent.

Although the role of Notch in the regulation of naive T-cell differentiation into effector T-cell lineages remains controversial, there is evidence to support the role of Notch signaling in the induction of Th2 differentiation. Analyses of genetically modified mice that are defective in Notch signaling components, such as CSL/RBP-J κ and Mastermind-like protein, reveal that Notch signaling promotes Th2 differentiation (14, 19). The RBP-J κ -mediated Th2 differentiation was shown to occur via the engagement of conserved noncoding sequence-2 (CNS-2) located downstream of the IL-4 locus. CD4⁺ T cells that exhibited active CNS-2 were actively infiltrated into the inflamed airways of OVA-induced mice (35). Subsequent studies have demonstrated that Notch directly induced transcription of GATA-3. Furthermore, in the absence of GATA-3,

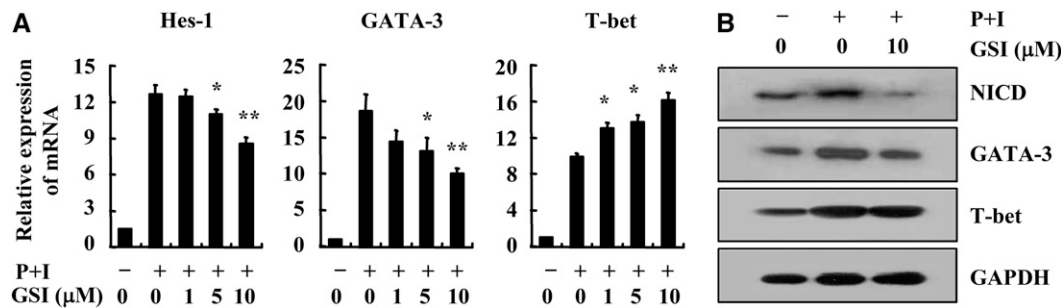


Figure 4. γ -Secretase inhibitor (GSI) treatment reduced NICD generation and GATA-3 protein expression in bronchoalveolar lavage (BAL) cells. BAL cells isolated from airways of experimental mice were treated with PMA (P) and ionomycin (I) in the presence or absence of GSI for 24 hours. (A) The expression of mRNAs for Hes-1, GATA-3, and T-bet

was analyzed by real-time polymerase chain reaction ($n = 5$). * $P < 0.05$; ** $P < 0.01$. (B) The immunoblot analysis of NICD, GATA-3, and T-bet protein. The blot was probed with the respective antibodies. GAPDH was used as a loading control. The results shown are representative of three independent experiments.

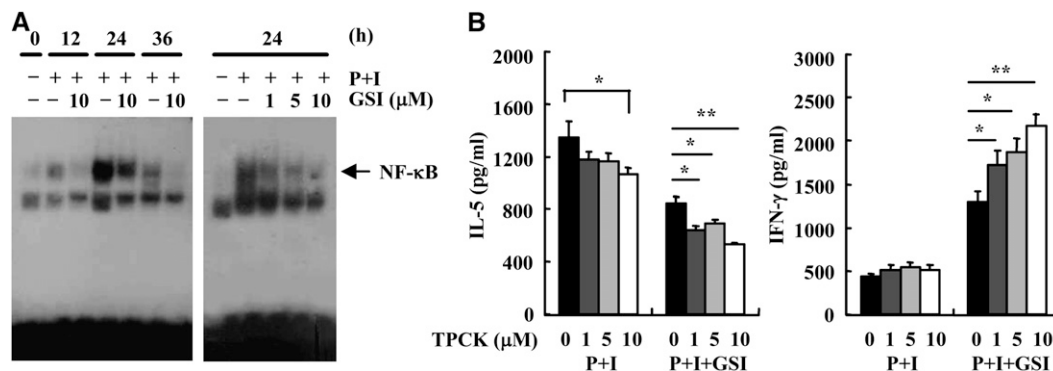


Figure 5. Inhibition of nuclear factor- κ B (NF- κ B) activation by γ -secretase inhibitor (GSI). Bronchoalveolar lavage (BAL) cells isolated from the inflamed airways of mice were treated with PMA (P) and ionomycin (I) in the presence or absence of GSI for the indicated times. (A) Nuclear extracts were isolated and subjected to electrophoretic mobility shift assay analysis. Two micrograms of the nuclear ex-

tract were allowed to interact with ^{32} P-labeled, double-stranded oligonucleotide probe containing an NF- κ B binding site. The arrow indicates DNA-bound NF- κ B complexes. (B) BAL cells were stimulated with TPCK alone or TPCK plus GSI for 36 hours. IFN- γ and IL-5 levels in the culture supernatant were measured by ELISA. Data are presented as the mean $\pm$ SEM of five independent experiments. * P < 0.05; ** P < 0.01.

Notch strongly elicited the conversion from Th2 to Th1 differentiation (36, 37). These results suggest a straightforward, linear path from Notch to Th2 responses via GATA-3; Notch induces the transactivation of GATA-3, which in turn induces the Th2 phenotype. These observations are fairly well matched to our results, which show that short-term (12–48 hours) GSI exposure of Th2 effector cells that have been fully developed in airways during antigen provocation induces a swift conversion from Th2 to Th1 phenotypes (see Figure 3). Moreover, the GATA-3 level is decreased concomitantly with a decrease in NICD and Hes-1 levels (see Figure 4). Stimulated BAL cells exhibit strong expression of GATA-3 and Th2 cytokines. The absence of GATA-3, which is thought to result from GSI treatment, induces the conversion to Th1 cytokine production. Hence, it is reasonable to infer that the reduction of GATA-3 levels in BAL cells by GSI treatment is responsible for the decreased production of Th2 cytokines via a Notch-dependent pathway and thereby inhibits Th2-associated airway responses.

In addition to the conflicting role of Notch signaling for Th1/Th2 cell fate decisions, there is controversy over whether Th2 and Th1 phenotypes are stable. Introduction of dominant negative GATA3 to developing Th2 cells has been shown to impair IL-4 production, whereas its introduction to mature Th2 cells does not affect IL-4 production (38). In addition, deletion of GATA3 in mature T cells has a minimal effect on the maintenance of IL-4 production (39). These results suggest that the Th2 cell phenotype is initially plastic but later becomes fixed during differentiation. In contrast, an analysis of GATA3 genetic deletion showed that GATA3 expression is required to maintain the Th2 phenotype (40) and to produce IL-5 and IL-13 (39). Furthermore, committed Th1 cells that are retrovirally transduced with GATA3 can change their phenotype (41). These results suggest that the phenotypes of committed Th2 and Th1 cells can be changed. Our data show that GSI causes the rapid shift from the Th2 to Th1 phenotype. Specifically, GSI inhibited all Th2 cytokine production and enhanced Th1 cytokine in unfractionated BAL cells when T-cell-specific stimulation was applied. Furthermore, by using an approximately 70% purified CD4 $^{+}$ T-cell subset (see Figure 3), we showed that IFN- γ expression is augmented. Additionally, GSI treatment led to a decrease in GATA3 levels, concomitant with an increase in T-bet levels (see Figure 4). Therefore, we conclude that the Th2 phenotype in inflamed airways is not fixed. Furthermore, GATA3 may be involved in the swift conversion of the Th2 to Th1 phenotype.

Our data are in contrast to previously reported results that GSI effectively blocks Th1 differentiation by inhibiting T-bet and IFN- γ expression and attenuates experimental autoimmune encephalomyelitis (EAE), a Th1-mediated disease (18). The

reason for this discrepancy is not clear; however, it is possible that the differences in the experimental conditions may have affected the outcome of the Th1/Th2 differentiation pathways by differentially modulating Notch signaling. First, it depends on whether the effector Th cells used to assess GSI effects were developed *in vivo* or *in vitro*. The study showed the inhibition of Th1 differentiation by GSI using an *in vitro* polarizing procedure. In contrast, BAL cells, which are directly isolated from the inflamed airways of mice, include Th2 cells that have already committed and developed during antigen-sensitization and challenge. A second difference is the route of GSI administration. In an EAE model, Minter and colleagues orally treated mice with GSI (18), whereas we treated the mice intranasally. The different routes of GSI administration may initially influence different types of cells, causing differential effects on Th1/Th2 differentiation. Finally, different disease models (asthma vs. EAE) in different mouse strains are used to evaluate the therapeutic activity of GSI. Balb/c mice, which were used for the present study, have been shown to predominantly exhibit Th2 responses to antigens, whereas C57BL/6 mice, which were used for inhibiting Th1 differentiation by GSI (18), exhibit Th1-dominant responses to the same antigen (42). In this context, it is worthwhile to note that Notch is able to promote a Th1 or Th2 response depending on the presence of critical factors such as GATA-3 (36). It is possible that differential levels of GATA-3 would occur in different effector T cells of mice with different diseases, resulting in the opposite effects of GSI on Th1 and Th2 responses. If this is the case, the *in vivo* effects of GSI could be exerted regardless of which effector Th response is developed.

NF- κ B activity plays a central role in allergic airway inflammation; NF- κ B activity is elevated in the asthmatic lungs of murine models and patients (43, 44), and the inactivation of NF- κ B by I κ B kinase inhibitor or NF- κ B inhibitory protein ABIN-1 results in a decrease in the inflammatory airway response (45, 46). Furthermore, mice deficient in NF- κ B genes are unable to mount appropriate airway eosinophilic inflammation (25). A few studies have revealed a direct relationship between Notch signaling and NF- κ B activity. Splenocytes from Notch antisense-transgenic mice or normal mice-derived splenocytes treated with GSI exhibit reduced NF- κ B activity upon stimulation (22, 47). Subsequently, NICD is shown to promote nuclear retention of NF- κ B by a physical association with NF- κ B, which is reduced by GSI treatment (24). These data are in agreement with our electrophoretic mobility shift assay results that GSI strongly inhibits NF- κ B activity in stimulated BAL cells (Figure 5A). However, the functional consequences of NF- κ B inhibition are not clear. Inhibition of NF- κ B activity by itself modestly or minimally reflects effects of GSI; however, combined treatment

with Notch and NF- κ B inhibitors led to the regulation of IFN- γ and IL-5 production in a synergistic and additive manner, respectively (Figure 5B). Hence, it seems likely that the therapeutic effects of GSI on airway inflammation partially overlap with the effects of NF- κ B inhibition on increased Th1 cytokine production.

Although we argue GSI-induced conversion from Th2 to Th1 responses as the principal therapeutic effect of GSI, this study does not exclude the possibility that unidentified effects of GSI in other cell types may contribute to these changes. For instance, because DCs provide instructive signals for differentiation of effector Th cells by expressing different Notch ligands upon stimulation (14, 15, 21), GSI might exert its effects by modulating Notch ligand expression on lung DCs or DC maturation and migration to peripheral lymphoid organs. In addition, GSI may restrict the functions of infiltrated eosinophils, as shown in our previous study (28), and could counteract the proinflammatory role of airway epithelial cells by directly modulating the differentiation to goblet cells and inhibiting eotaxin production (Figures 1 and 2).

Collectively, our data, to the best of our knowledge, provide for the first time compelling evidence that GSI inhibits asthma, a Th2-type disease, through a Notch signaling-dependent pathway. Short-term exposure of BAL cells to GSI causes a dramatic shift from Th2 to Th1 responses, and this phenotypic change is commensurate with changes in the expression and generation of Notch signaling-associated molecules, including GATA-3, Hes-1, and NICD, in a collection of inflammatory cells. Our findings should provide the opportunity for a clinical study of the effectiveness of GSI for the treatment of asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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γ -Secretase inhibitor reduces allergic pulmonary inflammation by modulating Th1 and Th2 responses

Jin Hyun Kang, Byung Soo Kim, Tae Gi Uhm, Shin-Hwa Lee, Gap Ryol Lee, Choon-Sik Park, and Il Yup Chung

Online data supplement

Methods

ELISA assay. Cytokine concentrations were determined using ELISA kits from BD Biosciences to measure IL-4, IL-5, and IFN- γ . Additionally, a kit from R&D Systems was used to detect IL-13 and eotaxin concentrations. Serum IgE was determined by using an ELISA kit (BD Biosciences) according to the manufacturer's protocol.

EMSA of NF- κ B. After the BAL cells were treated with PMA and ionomycin in the presence or absence of GSI, nuclear extracts were prepared and incubated with a ^{32} P-labeled oligonucleotide probe (5'-AGTTGAGGGGACTTTCCCAGGC-3') for 30 min at room temperature. The DNA-protein complex was analyzed by electrophoresis in a 6% native polyacrylamide gel and visualized by autoradiography.

Real-time PCR. After the extraction of total RNA from the BAL cells that had been stimulated with PMA and ionomycin, the expression of mRNAs for Hes-1, GATA-3, and T-bet was analyzed using the following primers: Hes-1 forward, 5'-CTGCATGACCCAGATCAACG-3' and reverse, 5'-AAAGGCAAATTGGCCGTCAG-3'; GATA-3 forward, 5'-GAAAGAGTGCCTCAAGTATCAG -3' and reverse, 5'-CCTCGACTTACATCCGAACC-3'; T-bet forward, 5'-CGGGAGAACTTTGAGTCCATG -3' and reverse, 5'-TCCGCTTCATAACTGTGTTCC-3'. Real-time quantitative PCR was performed in a 20 μ l reaction that contained 0.5 μ l of cDNA, 0.8 μ l of 10 pM of each primer, and 10 μ l of SYBR Green Master Mix, using the Applied Biosystems Prism 7900 Sequence Detection System (Applied Biosystems). The following PCR conditions were used: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec. The PCR was performed in triplicate in 384-well plates. A standard curve was prepared to test the sensitivity of the assay and as a reference (β -actin) for the quantification.

The standard curve was derived from five points that corresponded to a two-fold cDNA dilution series. For normalization of the quantitative results, a reference gene was amplified.

Figure legends

Figure E1. Inhibition of airway inflammation by GSI treatment following asthma induction. GSI was intranasally administered once a day on days 24, 25, and 26 after the last OVA was challenged on day 23. The BAL cells were collected and counted on day 28; $n = 6$ mice per group. * $P < 0.05$ and ** $P < 0.01$, compared with the OVA-challenge group.

Figure E2. Expression of mRNAs for cytokines in lung tissues. Total RNA was extracted from lungs of each group and subjected to RT-PCR analysis using primers listed in Table E1. Data are the mean $\pm$ SEM; $n = 8-12$ mice per group. * $P < 0.05$ and ** $P < 0.01$, compared with the OVA-challenge group.

Figure E3. Intracellular staining of IL-4 and IFN- γ in BAL cells. BAL cells were stimulated with anti-CD3 and anti-CD28 for 36 h. The BAL cells were then subjected to three color-staining with FITC-anti-IFN- γ , PE-anti-IL-4 and PerCP-anti-CD4. After R2 cells and CD4⁺ cells were sequentially gated, a quadrant dot plot was displayed for IL-4 and IFN- γ expression. R2, R3, and R4 cells are predominantly lymphocytes, eosinophils, and macrophages and dendritic cells, respectively. The result shown is a representative of three independent experiments.

Figure E4. The effect of GSI on IL-5 and IFN- γ production in CD4⁺ and CD4⁻ subset. BAL cells were fractionated to CD4⁺ (A) and CD4⁻ (B) cells by MACS, stained with Diff-Quick, and stimulated with anti-CD3 and anti-CD28 in the presence of GSI for 36 h. The cytokine levels in the culture supernatant were measured. The results shown are representatives of three independent experiments. The results are expressed as the mean $\pm$ SEM in triplicate from representative of two independent experiments.

Table E1. Primers used for mouse cytokine mRNA expression analysis by RT-PCR

gene	forward primer	reverse primer
IL-4	TGTCATCCTGCTCTTCTTTCTC	GTGCAGCTTATCGATGAATCC
IL-5	TCACCGAGCTCTGTTGACAA	CCACACTTCTCTTTTTGGCG
IL-13	AACATCACACAAGACCAGACTC	AAGTGGGCTACTTCGATTTTGG
Eotaxin	AGAGGCTGAGATCCAAGCAG	CAGATCTCTTTGCCCAACCT
IFN- γ	GCTCTGAGACAATGAACGCT	AAAGAGATAATCTGGCTCTGC
β -actin	TGGAATCCTGTGGCATCCATGAAAC	AAAACGCAGCTCAGTAACAGTCCG

Figure E1

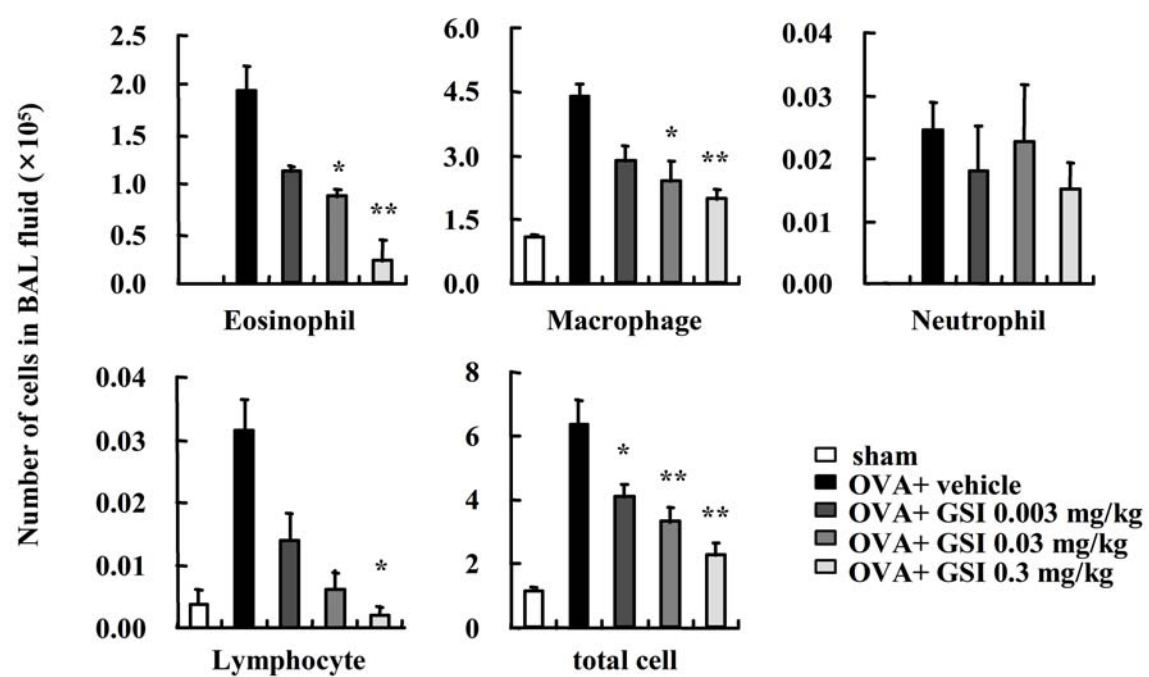


Figure E2

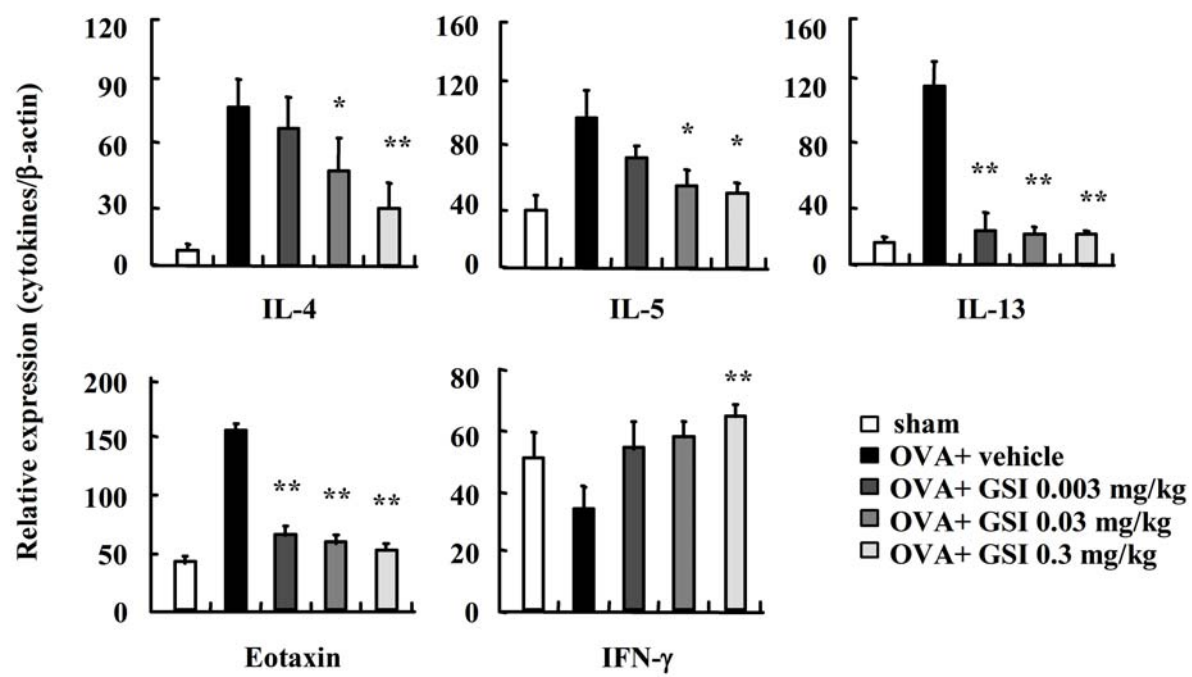


Figure E3

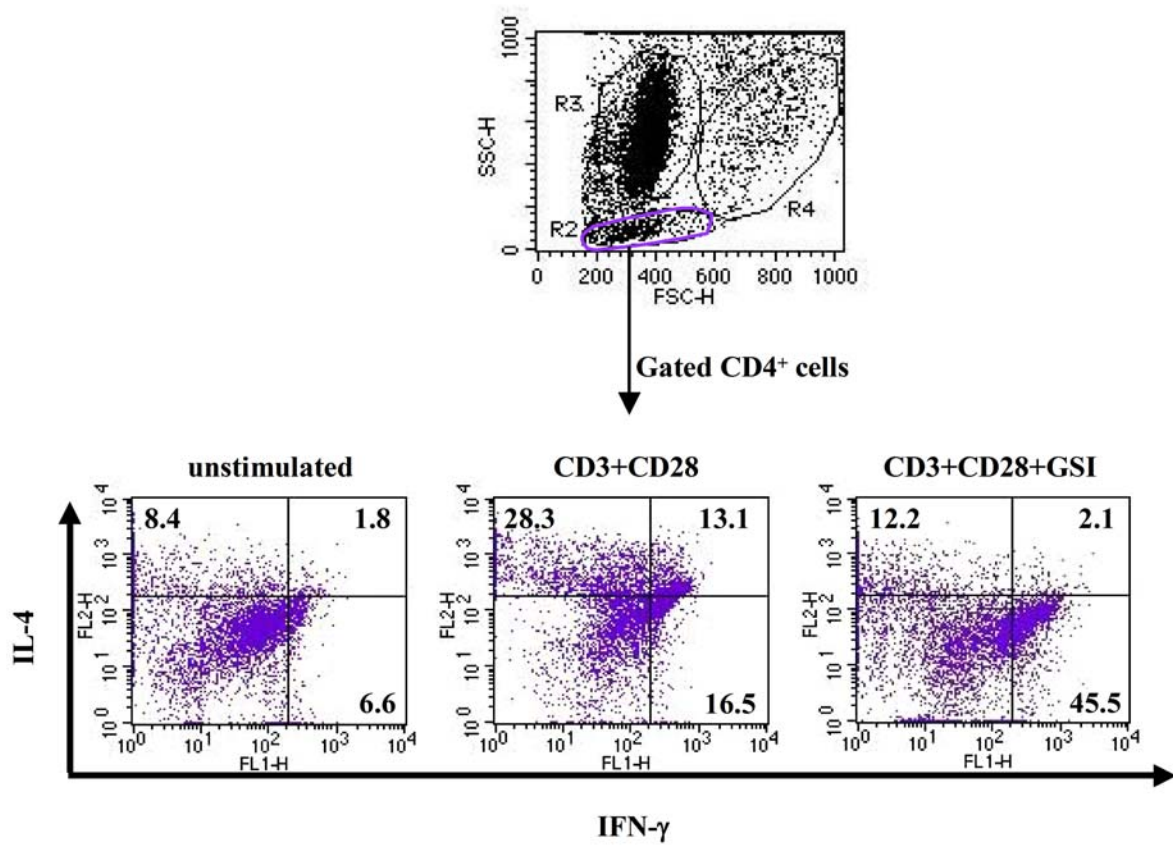


Figure E4

