

Eosinophilic Bronchitis, Eosinophilia Associated Genetic Variants, and Notch Signaling in Asthma

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While much has indeed been learned about the biology and role of eosinophils, the paradigm of eosinophils has the pros and cons in development of asthma. To answer the questions in the black box, this review firstly discusses the biological and morphological differences between asthma and eosinophilic bronchitis (EB). EB is an interesting clinical manifestation of eosinophilic airway disease that does not involve airway hyperresponsiveness (AHR), demonstrating that airway eosinophilia alone is insufficient to merit a diagnosis of asthma. Secondly, I will describe and discuss the effect(s) of single-nucleotide polymorphisms (SNPs) in the genes *CCR3*, *IL-5 RECEPTOR ALPHA (IL5RA)*, and *IL1RL1*, and finally the in vitro and in vivo effects of Notch inhibition on both eosinophil differentiation and experimental asthma. Eosinophilic airway inflammation is not as important in the pathogenesis and maintenance of asthma as had previously been thought. However, the role of eosinophils in other asthma subphenotypes, including refractory or severely remodeled asthma, needs to be evaluated further. High-throughput methodologies such as genomics will facilitate the discovery of new markers of inflammation; these, in turn, will aid in the evaluation of the role of eosinophils in asthma and its various subphenotypes.

Key Words: Eosinophils; bronchitis; polymorphism, single nucleotide; notch; asthma

INTRODUCTION

Asthma is an airway disorder characterized by inflammation, variable airflow obstruction, and airway hyperresponsiveness (AHR). The infiltration of eosinophils, mast cells, and CD4 lymphocytes, in addition to airway remodeling (including subepithelial fibrosis and hyperplasia of goblet cells and smooth muscle fibers), are all characteristic of asthma.¹ Excessive production of interleukin (IL)-4, -5, and -13 by T helper type 2 (Th2) cells has been implicated in the pathogenesis of allergic asthma. IL-5 mobilizes eosinophils from the bone marrow pool while chemokines such as eotaxin-1 induce the recruitment of eosinophils to the airway.² Eosinophils are multifunctional leukocytes that release a plethora of immunomodulatory compounds, including potent inducers of the inflammatory and immune responses observed in asthma, eczema, rhinitis, and other autoimmune diseases.³ Airway eosinophils, which release mediators that damage the airway epithelium, have been thought of as a major contributor to AHR in asthmatic patients⁴; however, this paradigm has been challenged by data obtained from studies in humans. In one such study, the administration of an anti-IL-5 antibody decreased peripheral blood and sputum eosinophilia in asthmatic patients but did not affect airway

hyperreactivity.⁵ Subsequent analysis of bronchial biopsies confirmed these findings and highlighted the role of eosinophils in airway tissue remodeling.⁶ Thus, while much has indeed been learned about the role of eosinophils in asthma, many unanswered questions remain. In this manuscript, I shall first describe the results of studies performed in my laboratory and discuss the biological and morphological differences between asthma and eosinophilic bronchitis (EB). EB is an interesting clinical manifestation of eosinophilic airway disease that does not involve AHR, demonstrating that airway eosinophilia alone is insufficient to merit a diagnosis of asthma. Second, I will describe and discuss the effect(s) of single-nucleotide polymorphisms (SNPs) in the genes *CCR3*, *IL-5 RECEPTOR ALPHA (IL5RA)*, and *IL1RL1*, and the in vitro and in vivo effects of Notch inhibition on both eosinophil differentiation and experimental asthma.

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LESSONS FROM A CLINICAL PHENOTYPE OF EOSINOPHILIC BRONCHITIS

There exists a poorly defined group of patients with similar levels of eosinophilic airway inflammation but normal airflow and no evidence of BHR.⁷ EB is a common cause of chronic cough syndrome.^{8,9} Morphologic and cellular changes, including alterations in basement membrane thickness and in the numbers of eosinophils, mast cells, and T-lymphocytes in the submucosa, are comparable in EB and asthma. In addition, no differences in the expression of eosinophil-active cytokines, chemokines, T-cell activation markers, or exhaled nitric oxide have been found among individuals with these conditions.^{10,11} However, in our study using thin-section CT scanning, those patients with EB showed no thickening of the large airway walls, whereas the amount of trapped air and centrilobular prominence were similar in patients with EB and asthma (Fig. 1).¹² These results suggest that eosinophilic inflammation is not always accompanied by large airway wall thickening, which may explain the normal responsiveness to methacholine in the airways of EB patients. The absence of BHR in EB is also attributed to the normal number of mast cells in airway smooth muscle as compared to asthma.¹³ The infiltration of airway smooth muscle by IL-4- and IL-13-expressing mast cells is characteristic of asthma but not EB.¹⁴ This was confirmed by assays of the IL-13 level in sputum samples. IL-13 was present at higher concentrations in the sputum of asthmatics as compared to those with EB; IL-5 levels were similar between the two groups (Fig. 2). In addition, sputum IL-13 levels were inversely correlated with the PC20 concentration in subjects with asthma.¹⁵ These findings raise the possibility that interactions between mast cells and airway smooth muscle in addition to the overproduction of cytokines contribute to bronchial wall

thickening and the development of AHR, but that eosinophils do not. However, in a long-term prospective follow-up study,¹⁶ EB recurred in one-fifth of patients who stopped using inhaled steroids. A progressive reduction in FEV1 was observed in individuals with recurrent EB, but not in those whose EB was non-recurrent (Fig. 3). This suggests that recurrent EB is accompanied by deterioration of the airflow rate. Based on these findings, it is possible that some subjects with recurrent EB also have a component of asthma or chronic asthmatic bronchitis; thus, further research is needed.

ASSOCIATION OF GENETIC VARIANTS WITH BLOOD EOSINOPHILIA

Prior to the publication of a genome-wide association study, research on candidate genes provided information on the contribution of eosinophilia-associated genetic alterations to asthma. Eosinophilic infiltration and peripheral blood eosinophilia are dependent on the cooperative action of eosinophil-specific cytokines and chemokines (e.g., IL-5 and members of the eotaxin family). CCR3, an eotaxin receptor, plays an important role in the infiltration of eosinophils into target tissues in cooperation with IL-5.² The human *CCR3* gene (MIM number 601268), located on chromosome 3p21.3, has been linked to atopic dermatitis and asthma.¹⁷ IL-5 is important for the terminal differentiation, activation, and survival of committed eosinophil precursors, via an effect mediated by IL-5 receptor (IL-5R).¹⁸ The human IL-5R complex is a heterodimer composed of alpha and beta subunits.¹⁹ The *IL5RA* gene (MIM number 147850), located on chromosome 3p26-p25, has also been linked to the development of asthma.²⁰ In a genome-wide scan using polymorphic markers, the linkage of chromosome 5q with familial eosinophilia was shown.²¹ This region contains a

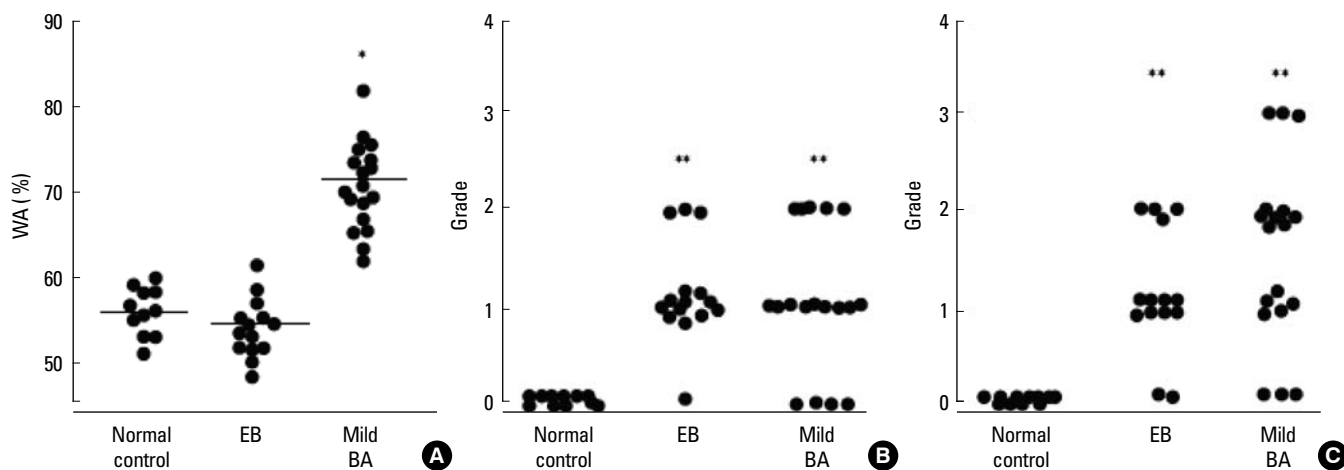


Fig. 1. Comparison of the (A) percentage of wall area (WA%), (B) prominence of centrilobular structures, and (C) air trapping on HRCT scans between patients with eosinophilic bronchitis (EB), mild asthma, and normal controls. The WA% was comparable in patients with EB than in normal controls (A). The prominence of centrilobular structures (B) and airway trapping (C) were comparable in patients with EB and mild asthma.¹²

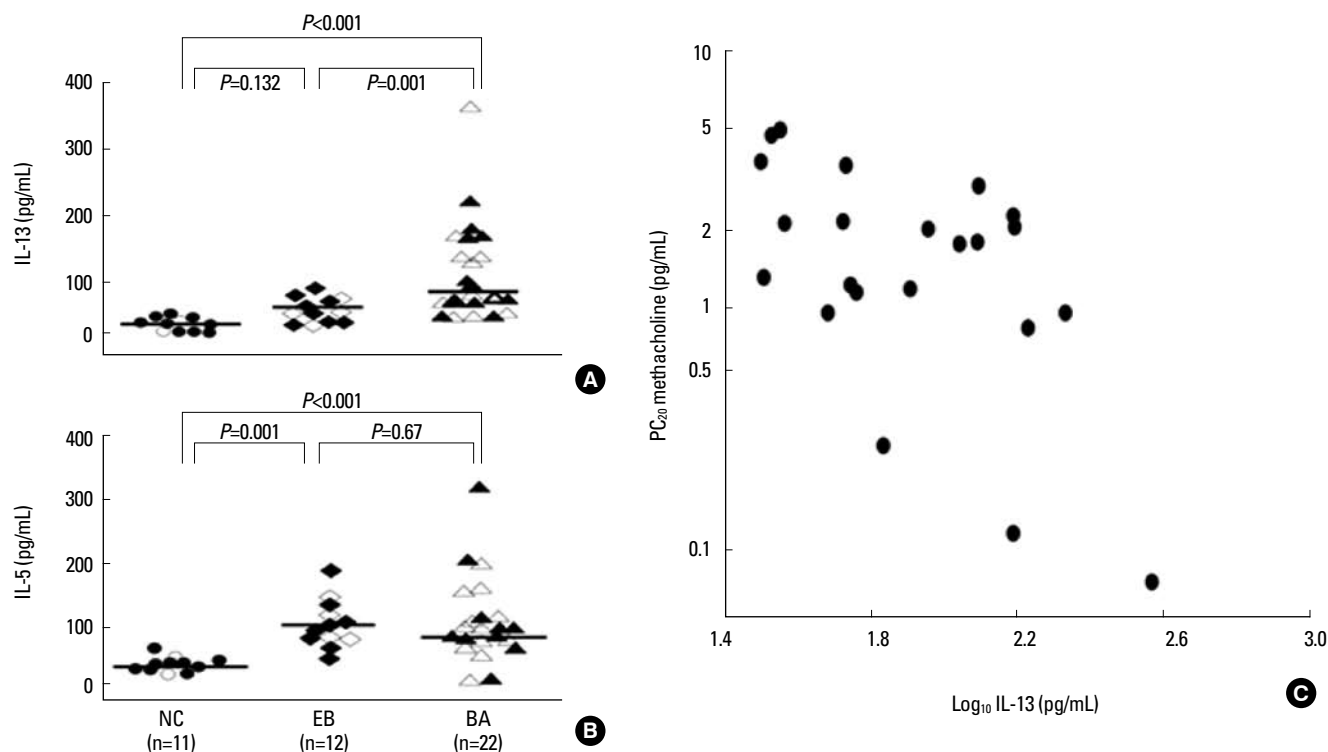


Fig. 2. IL-13 (A) and -5 (B) levels in the induced-sputum of asthmatic patients (BA), those with eosinophilic bronchitis (EB), and healthy control subjects (NC). Open and closed symbols represent atopic and non-atopic subjects, respectively. Horizontal bars represent the median values for each of the three groups. (C) Correlation between sputum IL-13 levels and the PC₂₀ for methacholine in the asthmatic patients. There was a significant, inverse correlation between the IL-13 level and PC₂₀ for methacholine ($r=0.484$; $P=0.02$).¹⁵

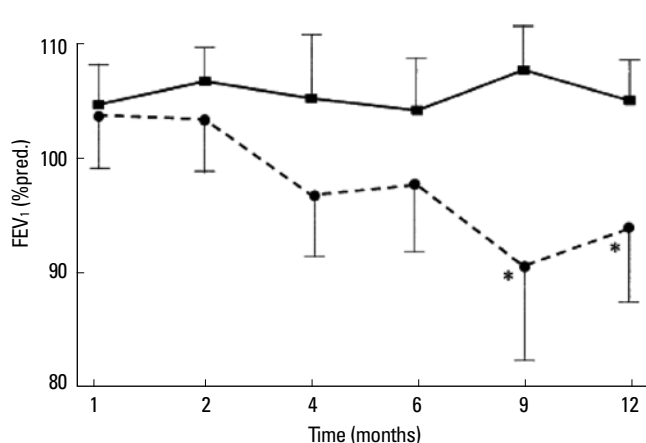


Fig. 3. Time-dependent changes in the FEV₁ of patients with non-recurrent (solid line) and recurrent (dashed line) eosinophilic bronchitis (EB). FEV₁ was significantly lower in the latter group compared with the former during months 9 and 12 of the study period. The data are presented as mean values + SEM. * $P=0.01$ compared to the group with nonrecurrent EB.¹⁶

cytokine gene cluster that includes three genes, the products of which play important roles in the development and proliferation of eosinophils: *IL3*, *IL5*, and *CSF2*. However, no other studies have demonstrated sporadic eosinophilia-associated genet-

ic variants in this region. We previously reported the association of variations in candidate genes with eosinophilia in asthma. Three completely linked SNPs (222557G>A, 2520T>G, and 2174C>T) in *CCR3* as well as the c.25091G>A polymorphism and c.2480_482ins/del in *IL5RA* were associated with eosinophil abundance in the peripheral blood of asthmatics.²² There is an intergenetic association between *CCR3* ht2 and c.25091G>A of *IL5RA*, such that the genetic effect of this combination lowers P values more than the effect of either condition alone (Table 1).

Data from a genome-wide SNP study of 9,392 Icelanders with known blood eosinophil counts suggested that an SNP (rs-1420101) in *IL1RL1* was associated with asthma; a similar phenomenon was observed in a collection of ten different populations (7,996 cases, including 1,958 Koreans and 44,890 controls; Table 2).²³ SNPs within the genes encoding *WDR36*, *IL-33*, and *MYB* that showed a suggestive association with eosinophil counts were also associated with atopic asthma in these populations. Rs1420101 on 2q12 is located in *IL1RL1*, while rs3939286 on 9p24 is located 32 kb proximal to the start codon of *IL-33*. *IL-33* encodes a cytokine belonging to the IL1 superfamily, and is the natural ligand for the IL1RL1 receptor. *IL-33* also functions as a chromatin-associated nuclear factor with transcriptional regulatory properties.²⁴ Signaling through the IL1RL1-IL-33

complex has an important role in eosinophil maturation, survival, and activation, both by direct effects on eosinophils and indirectly through the recruitment and regulation of Th2 cell effector functions. These findings are consistent with the proposed function of IL1RL1-IL-33 signaling in eosinophil-mediated

inflammation.²⁵ After functional analysis reveals how these genetic variances affect the differentiation of eosinophils and development of asthma, they may be used as targets for novel diagnostic assays and therapies.

Table 1. Association of the *IL5RA* c.25091G>A genotype and *CCR3* ht2 with the peripheral blood eosinophil count in asthmatic patients²²

		<i>IL5RA</i> c.25091 G>A				Pvalue [†]
		GG		GA or AA		
		N	GM* (95% CI)	N	GM* (95% CI)	
<i>CCR3</i> ht2	+/+	6	213.7 (82.1-556.6)	10	181.7 (113.5-290.7)	0.643
	-/+	58	270 (223.1-327.6)	30	314.6 (240.8-411.1)	0.426
	-/-	150	318.4 (273.3-371.0)	92	444.9 (378.8-522.6)	0.003
Pvalue [‡]	Cod		0.444		0.001	
	Dom		0.344		0.002	
	Rec		0.283		0.002	

*Values are presented as geometric means.

[†]The P values for the association of *IL5RA* c.25091G>A were obtained using a general linear model type III SS, controlling for age and sex as covariables.

[‡]The P values for the association of *CCR3* ht2 were obtained using a general linear model type III SS, controlling for age and sex as covariables.

Cod, codominant model; Dom, dominant model; Rec, recessive model.

Table 2. Results of a genome-wide search for SNPs associated with peripheral blood eosinophil counts and replication²³

SNP	Chr.	Position	Freq.	Gene	Icelandic discovery (n=9,392)		Icelandic follow-up (n=4,458)		Other European (n=7,660)		Combined European (n=21,510)		East Asian (n=5,212)		
					Effect	P	Effect	P	Effect	P	Effect (95% CI)	P	Freq	Effect	P
SNPs satisfying the criteria for genome-wide significance															
rs1420101[A]	2	102,324,148	0.41	<i>IL1RL1</i>	6.4	2.2×10 ⁻⁶	10.6	8.5×10 ⁻¹¹	4.4	0.0064	6.4	5.3×10 ⁻¹⁴	0.37	4.8	0.046
rs12619285[G]	2	213,532,290	0.74	<i>IKZF2</i>	6.8	5.1×10 ⁻⁶	4.1	0.024	7.0	7.6×10 ⁻⁵	6.3	5.4×10 ⁻¹⁰	0.36	5.9	0.017
rs4857855[T]	3	129,743,240	0.82	<i>GATA2</i>	8.3	1.5×10 ⁻⁶	9.6	3.6×10 ⁻⁶	11.0	1.3×10 ⁻⁷	9.4	8.6×10 ⁻¹⁷	0.70	8.4	0.00057
rs4143832[C]	5	131,890,876	0.16	<i>IL5</i>	10	4.6×10 ⁻⁸	12.3	1.0×10 ⁻⁸	2.0	0.34	7.1	1.2×10 ⁻¹⁰	0.17	10.2	0.0039
rs3184504[T]	12	110,368,991	0.38	<i>SH2B3</i>	7.7	1.3×10 ⁻⁸	10.6	8.5×10 ⁻¹¹	6.6	3.2×10 ⁻⁵	7.6	6.5×10 ⁻¹⁹	0.00		
SNPs that did not satisfy the criteria for genome-wide significance															
rs2416257[G]	5	110,463,389	0.85	<i>WDR36</i>	7.9	2.5×10 ⁻⁵	6.1	0.0070	4.4	0.062	6.1	1.0×10 ⁻⁶	0.95	-4.4	0.27
rs2269426[T]	6	32,184,477	0.76	<i>MHC</i>	6.0	1.9×10 ⁻⁵	5.1	0.0023	1.5	0.48	4.6	2.9×10 ⁻⁶	0.30	-0.4	0.89
rs9494145[T]	6	135,474,245	0.33	<i>MYB</i>	7.4	1.5×10 ⁻⁶	6.6	0.00037	-0.9	0.66	4.3	1.2×10 ⁻⁵	0.70	2.5	0.38
rs748065[A]	8	21,734,049	0.69	<i>GFRA2</i>	6.0	2.9×10 ⁻⁵	2.7	0.11	1.6	0.36	3.8	0.00012	0.43	-0.5	0.84
rs3939286[A]	9	6,200,099	0.25	<i>IL33</i>	6.8	8.2×10 ⁻⁶	2.8	0.13	2.7	0.14	4.4	1.8×10 ⁻⁵	0.03	-1.9	0.72

REGULATION OF EOSINOPHIL DIFFERENTIATION AND NOTCH SIGNALING

During eosinophil development, the early action of IL-3 and GM-CSF results in the proliferation and differentiation of stem cells into multipotent myeloid and eosinophil progenitor cells, while IL-5 supports the expansion, terminal differentiation, and functional activation of eosinophils. Notch is an evolutionarily conserved transmembrane protein involved in the regulation of a broad spectrum of cell fate decisions and differentiations.²⁶ Notch signaling regulates the terminal differentiation and subsequent effector phenotypes of eosinophils, partly through modulation of the ERK pathway.²⁷ Enzymatic cleavage of Notch by γ -secretase is essential for Notch-mediated signaling. Gamma-secretase inhibitor (GSI) has been used to effectively block Notch signaling; the pharmacological equivalent of

a loss of Notch function. We previously demonstrated that GSI induces the differentiation of eosinophils lacking effector functions.²⁸ Notch signaling is also required for the development of Th2-type immunity.²⁹ It is, therefore, possible that the restriction of Th2 and eosinophil inflammatory responses by GSI will ameliorate the symptoms of asthma. GSI administration has been shown to inhibit many of the pathological and histological characteristics of asthma, including eosinophilic airway inflammation, goblet cell metaplasia, methacholine-induced AHR, and elevated serum IgE levels (Fig. 4A and B).³⁰ An ex vivo study of the effect of GSI on stimulated BAL cells showed both the inhibition of Th2 cytokine production and the augmentation of Th1 cytokine production (Fig. 4C). This skewing of the cytokine production profile from Th2- to Th1-type responses provides an empirical basis for the use of GSI as an antiasthma agent. Notch has been shown to directly induce the transcrip-

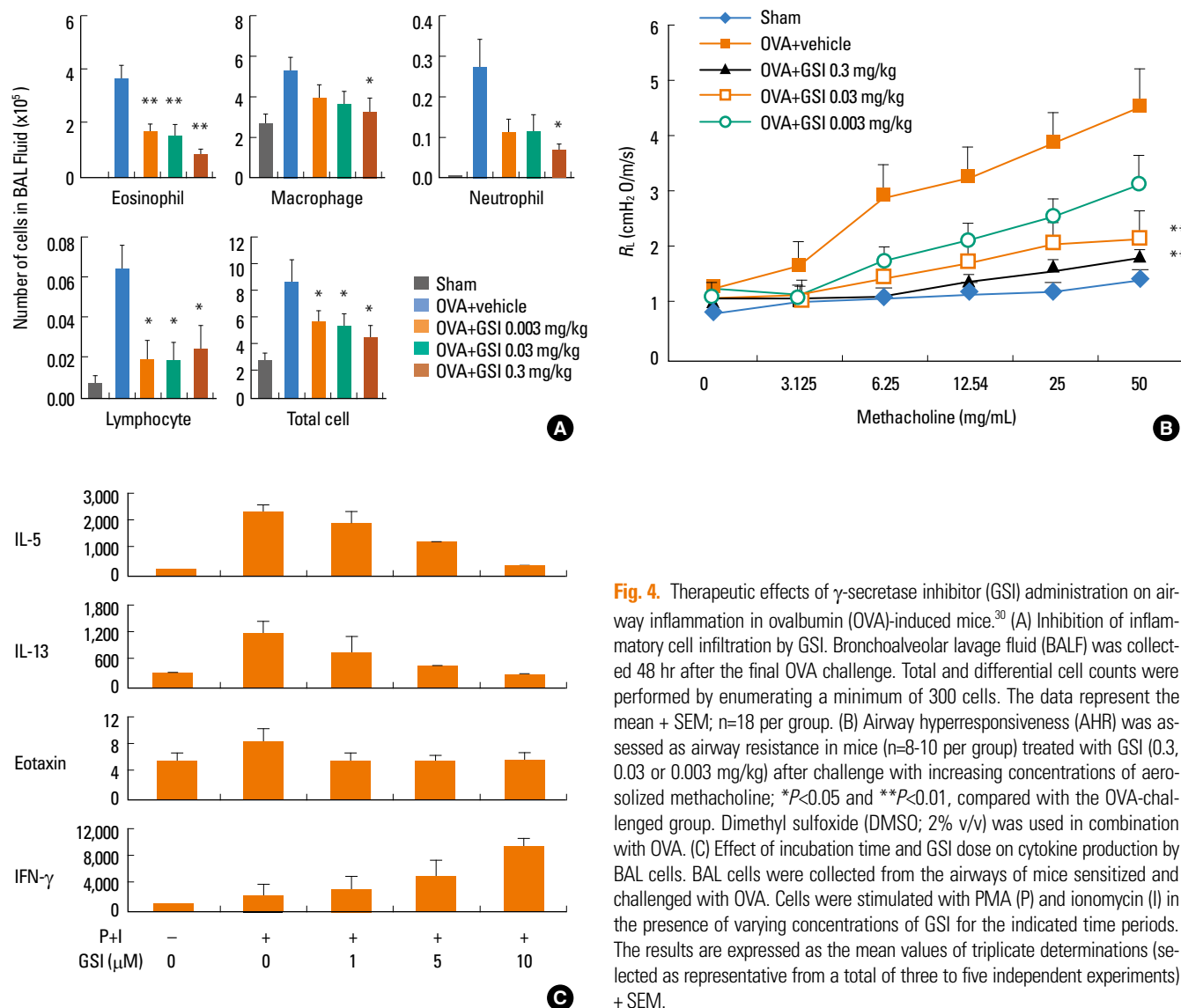


Fig. 4. Therapeutic effects of γ -secretase inhibitor (GSI) administration on airway inflammation in ovalbumin (OVA)-induced mice.³⁰ (A) Inhibition of inflammatory cell infiltration by GSI. Bronchoalveolar lavage fluid (BALF) was collected 48 hr after the final OVA challenge. Total and differential cell counts were performed by enumerating a minimum of 300 cells. The data represent the mean + SEM; n=18 per group. (B) Airway hyperresponsiveness (AHR) was assessed as airway resistance in mice (n=8-10 per group) treated with GSI (0.3, 0.03 or 0.003 mg/kg) after challenge with increasing concentrations of aerosolized methacholine; **P*<0.05 and ***P*<0.01, compared with the OVA-challenged group. Dimethyl sulfoxide (DMSO; 2% v/v) was used in combination with OVA. (C) Effect 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 OVA. Cells were stimulated with PMA (P) and ionomycin (I) in the presence of varying concentrations of GSI for the indicated time periods. The results are expressed as the mean values of triplicate determinations (selected as representative from a total of three to five independent experiments) + SEM.

tion of *GATA-3*.³¹ Reduced *GATA-3* levels in BAL cells due to GSI treatment cause decreased Th2 cytokine production via a Notch-dependent pathway, thereby inhibiting Th2-associated responses in the airway. GSI treatment also increases T-bet levels, which promote a Th1 response, as evidenced by enhanced IFN- γ expression. The marked effect of GSI on eosinophil differentiation and the balance of Th1- and Th2-type responses mediated by Notch-dependent signaling demonstrate the potential therapeutic value of this protein in the treatment of asthma.

In summary, the data presented herein suggest that eosinophilic airway inflammation is not as important in the pathogenesis and maintenance of asthma as had previously been thought. However, the role of eosinophils in other asthma subphenotypes, including refractory or severely remodeled asthma, needs to be evaluated further. High-throughput methodologies such as genomics and proteomics will facilitate the discovery of new markers of inflammation; these, in turn, will aid in the evaluation of the role of eosinophils in asthma and its various subphenotypes. Biomarkers detected as important in airway eosinophilia will help highlight processes involved in the pathogenesis of subphenotypes of asthma, including the possible participation of eosinophils.

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