

Innate lymphoid cell population distributions and related gene expression characteristics in blood from allergic and nonallergic patients with eosinophilic asthma

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Abstract

Background: Non-allergic eosinophilic asthma (NAEA) is a distinct subtype of asthma. However, the immune mechanisms associated with NAEA are not yet clearly understood.

Objective: To gain further insight into the pathogenesis of NAEA.

Methods: The proportion of innate lymphoid cells (ILCs) in the blood of patients with allergic eosinophilic asthma (AEA) and NAEA was evaluated. Eosinophilic asthma was defined when fractional exhaled nitric oxide measured at diagnosis (before initiating anti-asthma medications) was greater than 50 ppb. We evaluated the genome-wide gene expression profiles in peripheral blood mononuclear cells obtained at enrollment (in a stable state).

Results: A total of 57 participants were enrolled (10 healthy controls, 23 patients with NAEA, and 24 patients with AEA). We found that the type 1 ILC (ILC1) proportion significantly decreased, but the type 2 ILC (ILC2) and type 3 ILC (ILC3) proportions significantly increased in the blood of both patients with NAEA and those with AEA compared with healthy controls. However, there were no significant differences in the ILC1~3 proportions between NAEA and AEA patients. We also identified distinct biological pathways in patients with NAEA (anti-viral pathway) or AEA (IL-4 and IL-13 signaling and neutrophil degranulation pathways) based on co-expressed gene modules showing significant correlations with the ILC proportions.

Conclusions: ILC proportions in the blood did not differ between NAEA and AEA patients. However, different biological pathways were related to the ILC proportions in these patients. Our results provide further insight into eosinophilic airway inflammation in allergic and non-allergic patients.

Key words: Asthma, Blood buffy coat, Eosinophils, Gene expression profiling, Innate lymphoid cell

Citation:

Kim, B. K., Lee, H. S., Lee, S. Y., Park, H. W. (2026). Innate lymphoid cell population distributions and related gene expression characteristics in blood from allergic and nonallergic patients with eosinophilic asthma. *Asian Pac J Allergy Immunol*, 44(1), 171-178. <https://doi.org/10.12932/ap-140124-1765>

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Abbreviations/Acronyms:

AEA	Allergic eosinophilic asthma
FeNO	Fractional exhaled nitric oxide
GSEA	Gene set enrichment analysis
ILC	Innate lymphoid cell
NAEA	Non-allergic eosinophilic asthma
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
TSLP	Thymic stromal lymphopoietin
WGCNA	Weighted gene co-expression network analysis

Introduction

Eosinophils are the dominant inflammatory cell type in allergic asthma but are also found in non-allergic disorders, such as non-allergic conjunctivitis and rhinitis.^{1,2} Non-allergic eosinophilic asthma (NAEA) is a distinct subtype of asthma.³ Although previous reports have recognized the possible roles of IL-23, vitamin D, or thymic stromal lymphopoietin (TSLP) in NAEA pathogenesis,⁴⁻⁶ the immune mechanisms associated with NAEA are not clearly understood.

Innate lymphoid cells (ILCs) have a lymphoid morphology but lack T and B cell receptors and thus they are not directly activated by specific antigens.⁷ A substantial amount of evidence has been accumulated on the role of ILCs in the pathogenesis of asthma with different inflammatory phenotypes.⁸⁻¹⁰ Type 2 ILC (ILC2) induces eosinophilic inflammation in allergic and non-allergic asthmatic patients via alarmins such as IL-33 and TSLP.⁹ Type 1 (ILC1) and type 3 ILC (ILC3) counts increased in sputum from both patients with eosinophilic asthma and those with non-eosinophilic asthma.¹⁰ However, knowledge on the roles of ILC1 and ILC3 in the pathogenesis of NAEA is lagging behind.

Blood is an easily obtainable and less strenuous alternative to sputum induction, and is thus increasingly used to study asthma. For example, it has been reported that the ILC2 proportion in peripheral blood is a reliable marker of eosinophilic airway inflammation in asthma patients, with an area under the curve of 0.88.¹¹

In this study, we measured the ILC population proportions in the blood of patients with allergic eosinophilic asthma (AEA) and NAEA. To gain further insight into the pathogenesis, we evaluated genome-wide gene expression profiles on peripheral blood mononuclear cells (PBMCs) using weighted gene co-expression network analysis (WGCNA) and searched relevant biological pathways.

Methods

This study was approved by the institutional review boards of the corresponding institution (H-1901-043-1001 and 2019AN0240), and informed consent was obtained from all study participants.

Study design

We retrospectively reviewed the medical records of two institutions (Seoul National University Hospital and Korea University Anam Hospital, Seoul, Republic of Korea) and selected adult patients with asthma who are eligible for the study. Asthma was diagnosed according to the Global Initiative for Asthma guideline based on current (past 12 months) episodic respiratory symptoms and demonstrated evidence of airway hyperresponsiveness to methacholine or positive bronchodilator response.¹² After diagnosis, all of patients were treated with medium dose inhaled corticosteroids according to their symptom severity.¹² Smokers were defined as current or former smokers whose smoking pack years ≥ 10 . We explained our study to eligible patients with asthma identified from their medical records and enrolled them if they agreed to participate.

We defined non-allergic asthma when enrolled patients showed negative results in skin tests with common inhalation allergens or had no specific IgE to common inhalation allergens,⁵ and eosinophilic asthma when the fractional exhaled nitric oxide (FeNO) of enrolled patients at diagnosis (before initiating anti-asthma medications) was greater than 50 ppb.¹³ FeNO concentration was measured with a Niox Mino unit (Circassia AB, Solna, Sweden) based on the standardization procedure.¹³ Healthy controls were defined as those without airway symptoms, showing normal lung function and negative skin test results. All participants were recruited voluntarily from each institute. Blood was drawn to measure ILC distribution and gene expression at a stable state, that is, no changes in anti-asthma medications and no acute exacerbations (short-term oral prednisone burst, unexpected clinic visits, emergency room visits, or hospitalization due to asthma symptom aggravation) within four weeks prior to blood sampling.

ILC population proportion measurements

Heparinized peripheral blood was layered over an equal volume of Histopaque 1077 (Sigma-Aldrich) and centrifuged according to the manufacturer's instructions. Mononuclear cells were collected from the interface between Histopaque and plasma. After washing, PBMCs (5×10^6 cells/mL) were pre-incubated with Human TruStain FcX™ TM (Fc Receptor Blocking Solution) (Biolegend 422301, 1:50; Biolegend, CA, US) and stained with antibodies to lineage markers¹⁴ (CD3, CD14, CD16, CD19, CD20 and CD56; Lineage marker kit, Biolegend 422301; 1:5, FITC-conjugated anti-human CD11c; Biolegend 301604; 1:20, FITC-conjugated anti-human CD123; Biolegend 306014; 1:20) and Percp/cy5.5-conjugated anti-human CD45 (Biolegend 368503; 1:50, Isotype control Biolegend 400149; 1:250), PE/cy7-conjugated anti-human CD127 (Biolegend 351319; 1:50, Isotype control Biolegend 400125; 1:200), Pacific Blue-conjugated anti-human CD161 (Biolegend 339925; 1:50, Isotype control Biolegend 339925; 1:200), PE-conjugated anti-human CRTH2 (Biolegend 350105; 1:20, Isotype control Biolegend 400507; 1:250) and APC-conjugated anti-human CD117 (Biolegend 313205; 1:20, Isotype control Biolegend 400119; 1:200). ILC1s were gated as CD45⁺lin⁻CD127⁺CD161⁺CD117⁻CRTH2⁻,¹⁵ ILC2s as CD45⁺lin⁻CD127⁺CD161⁺CD117^{+/-}CRTH2⁺,^{16,17} ILC3s as CD45⁺lin⁻CD127⁺CD161⁺CD117⁺CRTH2⁻.^{17,18} At least 500,000 cells were read using BD LSRFortessa™ (BD Biosciences, CA, US), and the results were analyzed using the FlowJo software (BD Biosciences).

Gene expression arrays

PBMCs were isolated and genome-wide gene expression levels were measured using the Affymetrix GeneChip Human Gene 2.0 ST (Affymetrix, CA, USA). Probes with poor chromosomal annotations and probes on the X or Y chromosome were removed. We then perform a variance-stabilizing transformation and quantile-normalization to reduce technical noise and distribute the expression levels for each array closer to a normal distribution.

Statistical analysis

The results are expressed as mean \pm standard deviation, and statistical differences among groups were assessed using the unpaired two-tailed t-test or nonparametric Mann-Whitney test. Using the top 4,000 genes with the highest standard deviation in gene expression profiles from whole patients, we performed WGCNA with the R package “WGCNA” (R Foundation, Austria)¹⁹ in patients with AEA and in those with NAEA, separately. Modules were defined as groups of highly interconnected genes.¹⁹ We computed the eigengene values of the identified modules and performed multivariate linear regression analysis adjusted for age, sex, and smoking status to identify a module whose eigengene value was significantly associated with the ILC proportions in the AEA or NAEA groups. To assign biological meaning to interpretability of the gene module identified, we then performed pathway overrepresentation analysis (gene set enrichment analysis; GSEA) using “g:Profiler” (<https://biit.cs.ut.ee/gprofiler/>, database version: e106_eg53_p16_65fcd97) which provides an adjusted P-value calculated in a manner that accounts for the hierarchical relationships among the tested gene sets.²⁰ Among biological pathways implemented in the g:Profiler, we selected the Reactome database, because it provides more and diverse signaling pathways including immunological, developmental and kinase, signaling pathways, the drug or target-based, stress activated, or lipid-mediated signaling pathways compared to the other databases.²¹ All analyses were performed with R version 4.2.3 (www.r-project.org; R Foundation).

Results

Table 1 summarizes the characteristics of the 57 participants (10 healthy controls, 23 patients with NAEA, and 24 patients with AEA). Compared with patients with NAEA, those with AEA were significantly younger and showed higher FeNO levels at diagnosis and less obstructive pulmonary function at enrollment. However, other features, including sex, proportion of smokers, and blood eosinophil count at diagnosis, were not different between the two groups. The distribution of the ILC population in the blood is presented in **Figure 1**. Compared to healthy controls, the ILC1 proportions in both patients with NAEA and those with AEA were significantly lower ($P = 0.017$ and $P = 0.013$, respectively), whereas the ILC2 and ILC3 proportions were significantly higher (ILC2: $P = 0.045$ and $P = 0.028$, respectively, ILC3: $P = 0.037$ and $P = 0.022$, respectively). However, no significant differences in the proportions of ILC1, ILC2 and ILC3 were observed between NAEA and AEA patients. The ILC1 proportion was significantly negatively correlated with the FeNO levels in patients with NAEA ($P = 0.0072$), and the ILC2 proportion was significantly positively correlated with the FeNO levels in patients with AEA ($P = 0.047$), although the FeNO levels were measured at diagnosis (ILC proportions were measured at enrollment and thus there was a time interval between the two measurements). WGCNA identified 12 gene modules in patients with NAEA and 16 in those with AEA (**Figure 2**). In patients with NAEA, the eigengene value of the magenta module showed a significantly positive correlation with the ILC1 proportion ($P = 0.00021$) (**Figure 3A**).

Table 1. Characteristics of participants in this study.

Characteristics	Control	NAEA	AEA	P values		
	N = 10	N = 23	N = 24	Control vs. NAEA	Control vs. AEA	NAEA vs. AEA
Male, N (%)	3 (30.0)	7 (30.4)	11 (45.8)	NS	NS	NS
Age, year	49.2 \pm 14.1	55.3 \pm 13.9	43.3 \pm 13.9	NS	NS	5.0 \times 10 ⁻³
Smoker, N (%)	0 (0)	2 (8.7)	3 (12.5)	NA	NA	NS
BEC, / μ L	141.0 \pm 86.6	434.4 \pm 256.7	548.9 \pm 271.8	1.4 \times 10 ⁻³	6.1 \times 10 ⁻⁵	NS
FeNO, ppb	NA	61.4 \pm 11.5	83.2 \pm 32.0	NA	NA	3.5 \times 10 ⁻³
FEV1, L	2.5 \pm 0.6	2.2 \pm 0.7	2.8 \pm 0.6	NS	NS	1.7 \times 10 ⁻³
FEV1p, %	100.3 \pm 12.0	80.4 \pm 18.8	88.9 \pm 15.9	4.4 \times 10 ⁻³	0.04	NS
FVC, L	2.7 \pm 0.6	3.0 \pm 0.9	3.7 \pm 0.6	NS	6.2 \times 10 ⁻³	7.9 \times 10 ⁻³
FVCp, %	104.4 \pm 9.9	90.7 \pm 12.4	96.2 \pm 11.5	4.2 \times 10 ⁻³	NS	NS
FEV1/FVC ratio, %	83.7 \pm 6.1	72.7 \pm 8.3	76.0 \pm 8.6	7.5 \times 10 ⁻⁴	0.031	0.037

Data are presented as mean \pm standard deviation. BEC and FeNO level were measured at diagnosis and pulmonary function was measured at enrollment. NAEA, nonallergic eosinophilic asthma; AEA, allergic eosinophilic asthma; N, number; BEC, blood eosinophil count; FeNO, fractional exhaled nitric oxide; FVC, forced vital capacity; FVCp, FVC predicted value; FEV1, forced expiratory volume in a second; FEV1p, FEV1 predicted value; NA, not applicable; NS, not significant

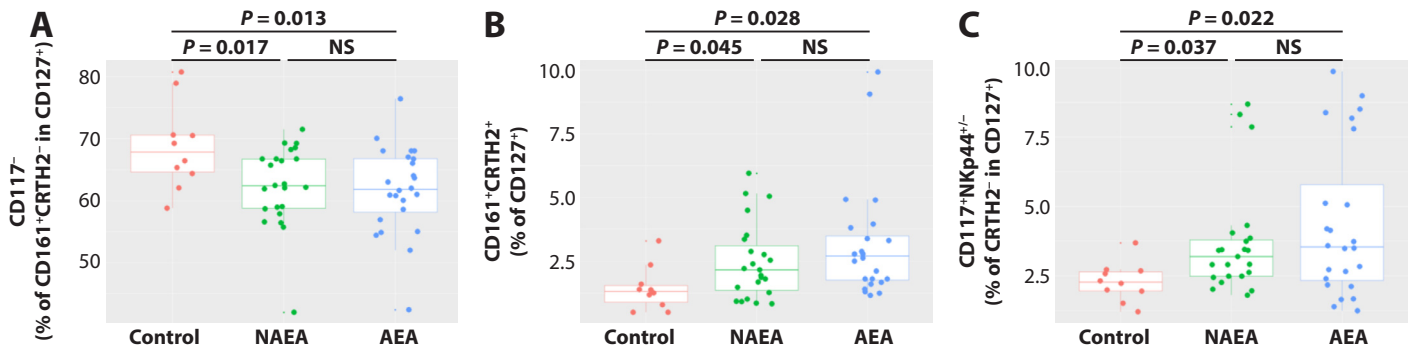


Figure 1. Distributions of ILC population in blood.

- A. ILC1
- B. ILC2
- C. ILC3

NAEA, nonallergic eosinophilic asthma; AEA, allergic eosinophilic asthma; NS, not significant

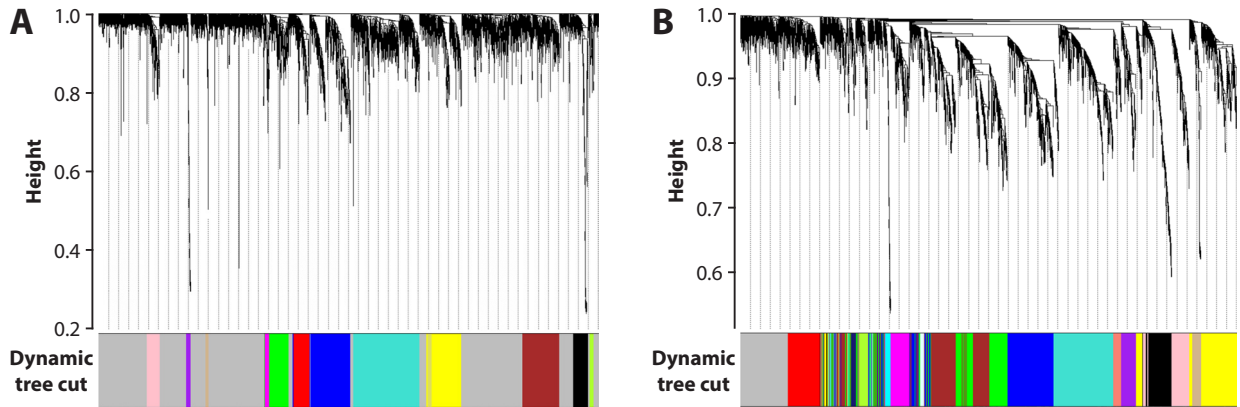


Figure 2. Co-expression modules identified in the gene expression profiles on PBMCs.

- A. Gene modules identified in patients with NAEA
- B. Gene modules identified in patients with AEA

The top panel shows a dendrogram of 4,000 genes with the highest standard deviation in the gene expression profiles from all patients. The bottom panel shows the colors corresponding to the cluster membership labels for each gene module.

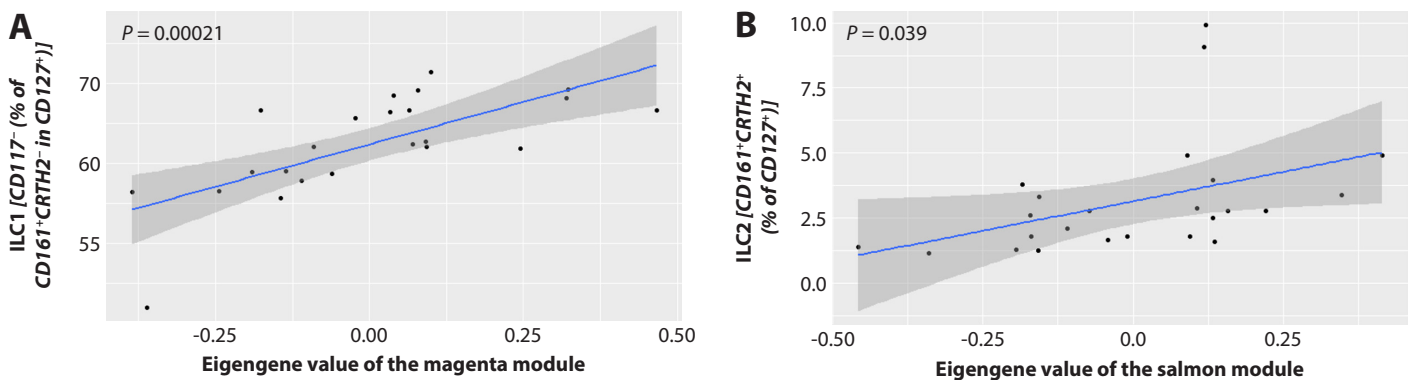


Figure 3. Gene modules showing a significant correlation with the ILC proportion.

- A. Correlation between the ILC1 proportion and the eigengene value of the magenta module identified in patients with NAEA
- B. Correlation between the ILC2 proportion and the eigengene value of the salmon module identified in patients with AEA
- C. Correlation between the ILC2 proportion and the eigengene value of the brown module identified in patients with AEA

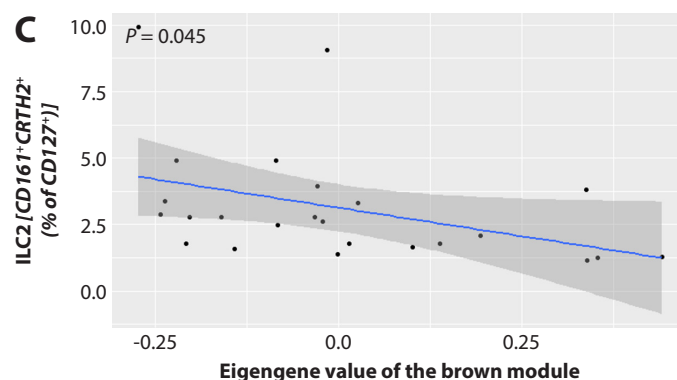


Figure 3. (Continued)

Table 2. Reactome pathways enriched in the gene modules showing a significant correlation with the ILC proportion.

Reactome pathway*	adjusted P value**
Magenta module (38 genes) identified in patients with NAEA	
Interferon alpha/beta signaling (R-HSA-909733)	7.2×10^{-22}
Interferon Signaling (R-HSA-913531)	5.8×10^{-21}
Antiviral mechanism by IFN-stimulated genes (R-HSA-1169410)	8.3×10^{-13}
Cytokine Signaling in Immune system (R-HSA-1280215)	8.9×10^{-13}
Immune System (R-HSA-168256)	5.9×10^{-8}
Salmon module (67 genes) identified in patients with AEA	
Interleukin-4 and Interleukin-13 signaling (R-HSA-6785807)	9.4×10^{-7}
Nuclear Events (R-HSA-198725)	5.8×10^{-6}
Interleukin-10 signaling (R-HSA-6783783)	2.6×10^{-5}
Signaling by NTRK1 (TRKA) (R-HSA-187037)	4.0×10^{-4}
Signaling by Interleukins (R-HSA-449147)	6.1×10^{-4}
Brown module (502 genes) identified in patients with AEA	
Neutrophil degranulation (R-HSA-6798695)	4.0×10^{-28}
Innate Immune System (R-HSA-168249)	1.0×10^{-26}
Immune System (R-HSA-168256)	3.1×10^{-23}
MyD88 deficiency (TLR2/4) (R-HSA-5602498)	2.8×10^{-11}
IRAK4 deficiency (TLR2/4) (R-HSA-5603041)	7.1×10^{-11}

*Analysis was performed using g:Profiler (version e106_eg53_p16_65fcd97; <https://biit.cs.ut.ee/gprofiler/gost>). **P value was adjusted by 'g:SCS algorithm' implemented in g:Profiler.

This also showed a significantly negative correlation with the FeNO level measured at diagnosis ($P = 0.019$). In patients with AEA, the eigengene value of the salmon module (67 genes) was significantly positively correlated with the ILC2 proportion ($P = 0.039$) (Figure 3B), whereas that of the brown module (502 genes) was significantly negatively correlated with the ILC1 proportion ($P = 0.045$) (Figure 3C). Eigengene value of each module also showed a significant correlation with the FeNO level measured at diagnosis in the same direction; a positive correlation in the salmon module ($P = 0.0098$)

and a negative correlation in the brown module ($P = 0.049$). Table 2 lists the top-5-ranked Reactome pathways enriched in genes of the magenta module identified in patients with NAEA and the salmon and brown modules identified in patients with AEA. Genes in the magenta module were significantly enriched in biological pathways related to anti-viral mechanisms. The most significantly enriched biological pathways in the salmon module and brown modules were the IL-4 and IL-13 signaling pathways and the neutrophil degranulation pathway, respectively.

Discussion

This study evaluated the ILC population distributions in the blood of patients with NAEA and those with AEA and discovered several interesting findings. First, in addition to ILC2, other types of ILCs showed significantly different proportions in blood between healthy controls and patients with NAEA or AEA. However, no significant differences in the ILC1, ILC2, or ILC3 proportions were found between NAEA and AEA patients. Second, WGCNA using the gene expression profiles on PBMC identified specific gene modules that showed significant associations with ILC proportion in patients with NAEA or AEA. Although a time gap existed, these gene modules also showed significant associations with the FeNO level measured at diagnosis. Third, GSEA using genes belonging to the identified gene modules suggested that the anti-viral pathway related to the ILC1 proportion may play a role in NAEA. Meanwhile, the IL-4 and IL-13 signaling and the neutrophil degranulation pathways related to the ILC2 proportion may be important in AEA. To the best of our knowledge, this is the first study to report ILC population distributions in the blood of patients with NAEA and AEA and related gene expression features and biological pathways.

The mechanisms on how ILC2 contributes to the development of NAEA remain unclear, although it is thought that an interplay between epithelial-derived cytokines and ILC2s may contribute in part.³ In high-fat diet-fed obese mice, IL-1 β signaling had a possible role in obesity-induced ILC2 activation.²² Previous studies have consistently shown that the ILC2 proportion is elevated in the blood of patients with AEA despite variations in the gating strategies for enumerating ILC2.²³ However, there has been scarce information on the ILC2 proportion in the blood of patients with NAEA. In this study, we observed that the ILC2 proportion in the blood of patients with NAEA was significantly higher than that of healthy controls but was comparable to that of patients with AEA, suggesting that ILC2 contributes to the development of eosinophilic airway inflammation irrespective of allergic status. In WGCNA, gene modules significantly associated with the ILC2 proportion were found only in patients with AEA. Genes in the module showing a positive correlation with the ILC2 proportion were significantly enriched in the IL-4 and IL-13 signaling pathways, and those in the module with a negative correlation were enriched in the neutrophil degranulation pathway. However, we did not identify any gene module associated with the ILC2 proportion in the blood of patients with NAEA. This means that the mechanisms intervening in ILC2 and eosinophilia or eosinophilic airway inflammation in NAEA may be so heterogeneous that they cannot converge on a few biological pathways. The small number of patients with NAEA enrolled in this study must be considered for proper interpretation of our observations.

Other biological pathways related to the ILC2 proportion are also worth noticing. NTRK1 (Neurotrophic Receptor Tyrosine Kinase 1), known as high-affinity receptor for nerve growth factor, is an early transcriptional target of IL-13 and is elevated in inflamed tissue of patients with eosinophilic esophagitis and asthma.^{24,25} In addition, neuro-immune pathways exist that regulate ILC2 responses and the development of airway inflammation.²⁶ Meanwhile, MyD88 and IRAK4 (IL-1R-associated kinase 4) are involved in the IL-33 signaling pathway and it was reported that inhibition against these molecules improved the pathologic features of IL-33-mediated endometriosis.²⁷ It is well-known that IL-33 stimulates ILC2 and the resultant eosinophilic inflammation.^{3,28} Further studies are needed to evaluate the biological pathways identified in this study, as a potential therapeutic target for the treatment of eosinophilic inflammation in asthma.

Interestingly, the magenta module identified from NAEA patients harbors *SERPING1* gene. *SERPING1* gene codes C1 esterase inhibitor (a serine protease inhibitor) that is critical for inhibition of complement activation and the contact system and thus may affect viral infections such as SARS-CoV-2.²⁹ So far, there have been no reports showing the role of *SERPING1* gene in the pathogenesis of NAEA. Viral infection is thought as a risk factor for the development of non-allergic asthma³⁰ and eosinophils play a distinct role in viral infection by releasing mediators with anti-viral activity.³¹ Considering these findings, *SERPING1* gene connecting other genes belong to the magenta module may have its own role in the development of NAEA.

For the ILC1 and ILC3 proportions in the blood of patients with eosinophilic asthma, there has been only one report.³² In this study, authors measured ILC1 (lin⁻CRTH2⁻CD127⁺), ILC2 (lin⁻CRTH2⁺CD127⁺), and ILC3 (lin⁻CRTH2⁻CD127⁺CD117⁺CD56^{+/-}) proportions in the blood from 22 non-smoking stable asthmatics and reported that no significant differences were found in the ILC1 or ILC2 proportions when comparing asthmatics with eosinophilia and those without eosinophilia, whereas ILC3 was significantly higher in asthmatics without eosinophilia.³² These results may not be directly comparable to ours, because we defined eosinophilic asthma based on the FeNO level at diagnosis. Interestingly, similar to our study, they found no significant differences in any ILC proportions between allergic and non-allergic asthmatics irrespective of eosinophilia.³² ILC1s consist of a subgroup of natural killer (NK) cells and non-NK cells and produce a cytokine profile that mimics that of Th1 cells.³³ However, the role of ILC1s in asthma, especially eosinophilic asthma, has not yet known. ILC3s produce IL-17 and IL-22, in response to IL-1 β and IL-23, making them similar to Th17 cells and thus may play a role in driving a neutrophilic asthma phenotype.^{34,35}

One previous study found adult-onset severe asthma to be associated with a distinct gene profile consisting of eosinophils, mast cells, and ILC3s.³⁶ Another study showed higher ILC1 in the sputum of non-smoking severe asthmatics with neutrophilic inflammation, whereas ILC2s and ILC3s were higher in eosinophilic asthma.³⁷ These heterogeneous results may be due to differences in gating strategies of ILC populations, target samples (blood or sputum), and patient characteristics (e.g. medication and smoking status, and co-morbidities). Interestingly, we have identified a gene module in which interferon-related biologic pathways were enriched in patients with NAEA. This module showed a significantly positive correlation with the ILC1 proportion. ILC1 confers early host protection at the initial sites of viral infection,³⁸ which supports our findings. Presently, we are unsure whether this identified gene module has a specific role in the pathogenesis of NAEA, or is an epiphenomenon.

This study has some limitations. First, there were few participants, and our findings could not be confirmed in an independent population, both of which limit the generalizability of the results. In addition, ongoing medications may have affected the gene expression profiles, as the participants in this study did not stop their prescribed medications. Furthermore, this study was performed using peripheral blood and not airway samples, although it has been reported that the ILC2 proportion in peripheral blood can be a reliable marker of eosinophilic airway inflammation in asthma patients.¹¹ In addition, there exist a time gap between FeNO measurements and blood samplings for the gene expression analysis. This gap needs to be acknowledged before generalizing our findings. Previous reports showed that our strategies based on the expression of lineage markers could isolate functionally relevant ILC1 (predominantly IFN- γ -producing cells), ILC2 (predominantly IL-5- and IL-13-producing cells) and ILC3 (predominantly IL-17- and IL-22-producing cells).^{39,40} However, further functional measurements based on the expression of transcription factors and the cytokine receptors would be helpful to assess fine differences in ILC1~3 proportions between NAEA and AEA patients.

We found that the ILC1 proportion significantly decreased, but the ILC2 and ILC3 proportions significantly increased in the blood of both patients with NAEA and those with AEA compared to those in healthy controls. However, there were no significant differences in ILC1~3 proportions between NAEA and AEA patients. We identified distinct biological pathways in patients with NAEA or AEA based on the co-expressed gene modules that showed significant correlations with ILC proportions. Our results provide further insight into eosinophilic airway inflammation in allergic and non-allergic patients with asthma.

Conflict of interest declaration

All authors declare that they have no personal or professional conflicts of interest related to this research, and they have not received financial support from the companies that produce and/or distribute the drugs, devices, or materials described in this report.

Funding disclosure

This work was supported by the National Research Foundation of Korea grant funded by the Korea government (NRF-2017R1A2B4004772) and the Seoul National University Hospital Research Fund (03-2021-0050).

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