

Letter to the Editor

Levels of circulating IL-33 and eosinophil cationic protein in patients with hypereosinophilia or pulmonary eosinophilia

To the Editor:

IL-33, a member of the IL-1 family, induces eosinophilia in mice and is involved in eosinophilic inflammation, as well as production of IL-5, a major cytokine that can lead to eosinophilia.¹⁻³ ST2, the IL-33 receptor, is expressed on eosinophils and T_H2 cells and is necessary for induction of T_H2 responses by IL-33.¹⁻³ Previous studies reported increased levels of a soluble form IL-33 receptor (soluble form ST2, sST2) during asthma exacerbation and in a case of eosinophilic pneumonia.^{3,4} Currently, no clinical evidence exists that shows altered levels of circulating IL-33 in patients with hypereosinophilia.

We aimed to investigate whether circulating IL-33 is elevated in patients with hypereosinophilia or pulmonary eosinophilia (PE) and to evaluate the clinical value of circulating IL-33 levels as a diagnostic or severity indicator for eosinophilic pulmonary disease.

This study was a prospective cross-sectional, selected case-controlled study that examined at a single time point the circulating IL-33 levels in 182 patients with hypereosinophilia (blood eosinophil numbers >1500/ μ L; $n = 82$) or PE (sputum eosinophils >15%; $n = 100$) and 194 controls. Two longitudinal studies included 20 patients with hypereosinophilia and 6 patients with idiopathic acute eosinophilic pneumonia (IAEP) from the cross-sectional study. The recruitment and selection of patients and controls, statistical analyses, and blood and sputum analysis protocols are described in this article's Methods and Table E1 in the Online Repository at www.jacionline.org.

For the cross-sectional study, blood eosinophil numbers were counted, and the plasma levels of IL-33, sST2, IL-5, and eosinophil cationic protein (ECP) were measured by ELISAs for 376 subjects.

For the longitudinal study, the levels of 5 blood markers from 20 patients with hypereosinophilia whose eosinophil numbers normalized within 6 months of the cross-sectional enrollment day were compared during the 2 states of hypereosinophilia and normal eosinophil numbers. In addition, 6 patients with IAEP were monitored over time for the 5 markers, C-reactive protein (CRP) levels, and symptomatic severity (scores of fever, dyspnea, and cough).

The median levels of IL-33 were marginally elevated in patients with hypereosinophilia (114 pg/mL; $P = .02$) and PE (76 pg/mL; $P = .03$) compared with the controls (37 pg/mL; Fig 1). The median difference for IL-33 levels between patients with hypereosinophilia and controls was not as significant or consistent as the other markers examined. Although the median levels of ECP were greatly elevated in patients with hypereosinophilia and PE, the median difference between patients with PE and controls was the most significant.

In our correlation analysis, blood eosinophil numbers and the proportion of sputum eosinophils demonstrated significant positive correlations with ECP levels (see this article's Table E2 in the Online Repository at www.jacionline.org). This correlation did not consistently hold true for IL-33. Taken together, plasma IL-33 levels are not a good reflection of tissue/local eosinophilia. In receiver operating characteristic (ROC) curve analyses for identifying PE, IL-33 had a poor overall diagnostic accuracy with an area under the ROC curve of 0.58, which is unsuitable for practical use (see this article's Fig E1 in the Online Repository

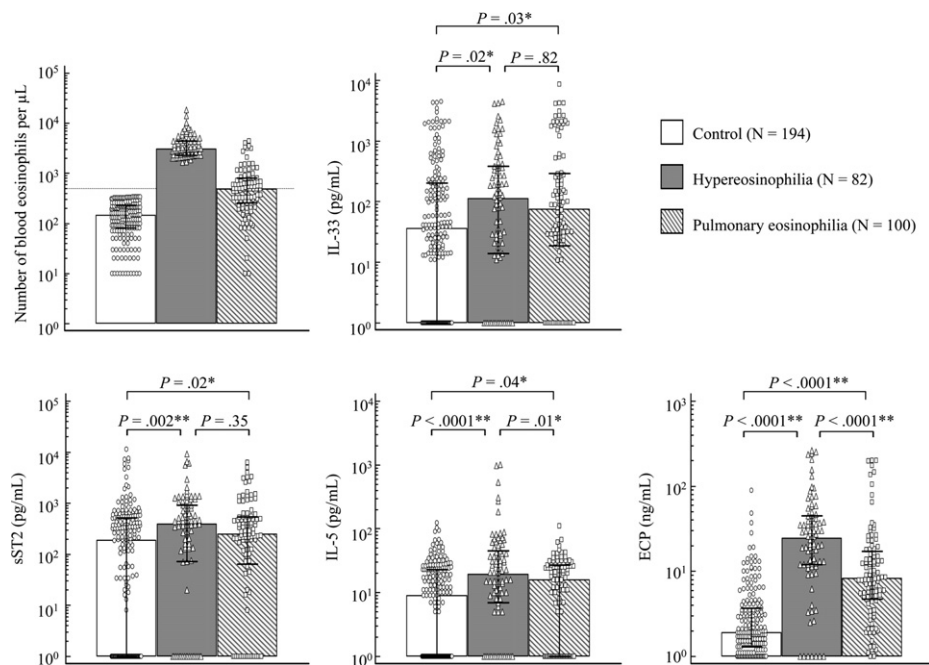


FIG 1. Levels of circulating IL-33, sST2, and eosinophilia-related markers in patients with hypereosinophilia or pulmonary eosinophilia and healthy controls. Each box indicates the median. Horizontal lines indicate interquartile ranges. The blue line indicates 500 eosinophils/ μ L blood. * $P < .05$; ** $P < .01$.

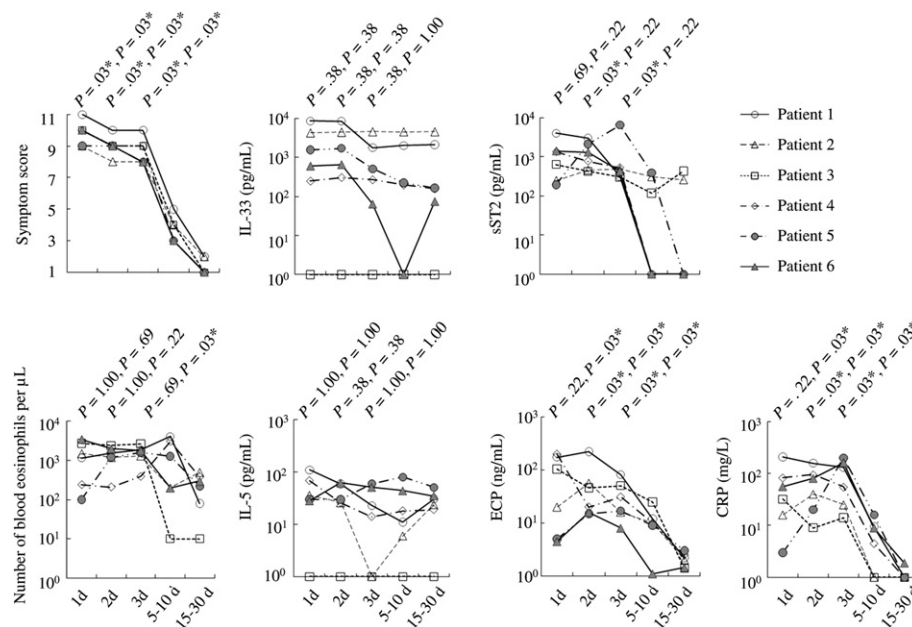


FIG 2. Changes in levels of IL-33, sST2, CRP, and eosinophilia-related markers after symptom improvement in patients with idiopathic acute eosinophilic pneumonia. First *P* values, comparison of the first 3 days with 5 to 10 days after the admission date; second *P* values, comparison of the first 3 days with 15 to 30 days after the admission date. **P* < .05.

at www.jacionline.org). When comparing the diagnostic accuracies, the ECP level (0.77) was superior to the other markers ($P \leq .001$) in all pairwise comparisons for identifying PE when blood eosinophils were normal.

For the first longitudinal study, we classified the 20 patients with hypereosinophilia on the basis of type of immune response leading to eosinophilia and treatment to assess changes in IL-33 and other biomarkers in different conditions: group A, eosinophil-directed treatment by complete eradication of immune activation (withdrawal of possible offending agent); group B, eosinophil-directed treatment by transient immune suppression with persistence of the underlying immune disease (corticosteroid treatment); group C, underlying disease-specific management without eosinophil-directed treatment. In these patients with hypereosinophilia (see this article's Fig E2 in the Online Repository at www.jacionline.org), ECP levels decreased ($P < .0001$) after blood eosinophil numbers returned to normal levels. In contrast, the levels of other markers did not significantly decrease on normalization of blood eosinophil numbers. On the basis of the pathogenic mechanism of hypereosinophilia,⁵⁻⁷ decreased levels of IL-33 may not be found in patients who have temporarily decreased eosinophil numbers without eradication of the underlying disease. In contrast, reduced levels of IL-33 (upstream inhibition) are found in patients who were treated by eradicating the underlying cause of their eosinophilia.¹⁻³ However, our measurements of circulating IL-33 levels did not support this mechanism.

In the serial tests of 6 patients with IAEP, their symptom scores were remarkably improved within 5 to 10 days of admission (Fig 2). The levels of ECP and CRP measured within 5 to 10 days and 15 to 30 days after admission were significantly lower than those on the second and third days after admission. However, IL-33 levels during the first 3 days after admission were not significantly different from those observed within 5 to 10 days or 15 to 30 days after admission. Notably, IL-33 levels varied

between individuals and did not correlate with symptom scores. Two of the 6 patients with IAEP (patients 1 and 2; Fig 2) presented high levels (>1000 pg/mL) of IL-33 regardless of the reduction of their symptom scores, and 1 of the 6 patients with IAEP (patient 3) presented continuously low levels. These results suggest that circulating IL-33 is not a useful severity marker for IAEP. A plausible explanation for this is that an individual heritable trait could affect the concentration of circulating IL-33 more so than the disease state. For example, *in vivo* IgE concentrations are more strongly related to individual genetic variation than to specific IgE response states.⁸

Our study for the first time identified elevated circulating IL-33 levels in patients with eosinophilia. However, on comparing IL-33 levels with previous eosinophilia-related markers and CRP levels, we found that IL-33 levels were less reliable for detecting PE or for the resolution of an eosinophil-mediated immune response and indicators of disease activity of IAEP. Further studies are required to identify the individual levels of each IL-33 form (full-length pro-form and cleaved forms) or inactive IL-33 by sST2 binding. Plasma ECP levels presented higher PE detection rates than other markers, and their progression correlated with clinical improvement of patients with IAEP. The ECP trends in this study were similar to findings of previous studies of eosinophilic disease.⁵⁻⁷ The data presented here strongly support the use of ECP, rather than IL-33, as a marker of PE and IAEP activity.

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METHODS

Study population

For the cross-sectional study, a total of 1562 adult (≥ 18 years) Korean patients were prospectively recruited who visited or were admitted to the Departments of Emergency, Internal Medicine, and Dermatology at Wonkwang University Hospital in Iksan, Korea. The patients were recruited because of blood eosinophilia ($>500/\mu\text{L}$) or a suspicion of an eosinophilia-related disease.^{E1,E2} Sputum eosinophil tests were performed on 200 patients with pulmonary symptoms. None of the patients with hypereosinophilia or PE received any corticosteroids or immune-modulating agents during the 2 weeks before the study, with the exception of 4 patients with hypereosinophilia. Two patients used topical steroids, 1 patient used systemic steroids 10 days before the study, and 1 patient took herbal medications 1 day before the study. These 4 patients were not included in the longitudinal study.

A total of 265 age-matched Korean adult volunteer controls were recruited from a population that attended a health screening in the Health Promotion Center of our hospital and were enrolled following exclusion and inclusion criteria. The exclusion criteria were (1) history or presence of eosinophil-related disease or eosinophilia,^{E1,E2} (2) presence of other general medical or other respiratory disease, (3) any medication history during the previous 2 weeks, and (4) more than 1 pack-year of smoking history or any smoking history during the previous 3 months. Inclusion criteria were (1) <350 eosinophils per μL blood and $<3\%$ eosinophils in induced sputum, (2) normal blood glucose and CRP (<3 mg/L) levels and normal liver and renal function tests (aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, and creatinine), and (3) normal chest radiography, electrocardiography, and spirometric results.

Through the evaluation process, 182 patients with hypereosinophilia (blood eosinophil numbers $>1500/\mu\text{L}$ with undetermined [74/82, 90%] or less than 15% sputum eosinophils) or PE (sputum eosinophils $>15\%$) and 194 age-matched controls were enrolled in the study. The final diagnosis of the patients was established by specialists. IAEF was diagnosed by a previously described method.^{E3}

For the longitudinal study, the blood eosinophil numbers of 25 patients with hypereosinophilia were serially monitored after 3 days, 7 days, 15 days, 1 month, 3 months, and 6 months or until the patient had normalized eosinophil numbers ($<350/\mu\text{L}$) for 2 consecutive tests after the cross-sectional enrollment day. If a patient had normalized eosinophil numbers within the full follow-up period, no more follow-up examinations were performed. If a patient had normalized eosinophil numbers at 6 months, 1 more confirmative test was performed within 1 week for the normalization of eosinophil numbers and plasma was obtained. Twenty patients had normalized eosinophil numbers during the follow-up period. Plasma samples were obtained during the 2 states of hypereosinophilia and normal eosinophil numbers.

In addition, the symptomatic severity of 6 patients with IAEF who started corticosteroid treatments within 3 days of admission was assessed on the day of admission (before the treatment), the second day, the third day, once during days 5 to 10 (just before discharge), and once during days 15 to 30 (after discharge). Serial plasma samples were also obtained for IL-33, sST2, IL-5, ECP, and CRP tests. Symptomatic severity was assessed by fever examination, verbal description of dyspnea, and cough by using a grading system. Fever was evaluated ranging from 0 to 2 (0, normal; 1, low-grade fever [37.5 – 38.2°C]; 2, high-grade fever [$>38.2^\circ\text{C}$]). Dyspnea was evaluated ranging from 0 (no dyspnea except with strenuous exercise) to 4 (dyspnea with minimal activity such as getting dressed or too dyspneic to leave the house) according to the Medical Research Council breathless scale.^{E4} Cough was evaluated ranging from 0 (no cough) to 5 (could not perform most usual daytime activities because of severe coughing).^{E5}

Study protocols were approved by the institutional review board, and all subjects gave written informed consent.

Blood and sputum analysis

Blood of the enrolled patients and controls was collected into EDTA-containing tubes. Eosinophil numbers in the blood were measured within 30 minutes after collection. Blood samples were centrifuged after cell counts. All separated plasma specimens were aliquoted and stored at -80°C and thawed immediately before use in ELISAs.

The number of blood eosinophils was measured by an automated hematology analyzer (Sysmex Co, Kobe, Japan). Routine chemistry tests and CRP levels were analyzed by an automated chemical analyzer (Roche Diagnostics, Mannheim, Germany). IL-33, sST2 (R&D Systems, Minneapolis, Minn), IL-5 (PeproTech, Rocky Hill, NJ), and ECP (MBL, Nagoya, Japan) levels were measured by using commercial ELISA kits as per the manufacturers' instructions. Measurements were performed in duplicate and the results were averaged. The limit of detection for each cytokine was as follows: IL-33, 10 pg/mL; sST2, 6 pg/mL; IL-5, 5 pg/mL; and ECP, <1 ng/mL. A value of 1 was assigned to results that were below the assay's limit of detection. The linearity of each cytokine was evaluated based on the Clinical and Laboratory Standards Institute guidelines.^{E6} The maximum linear ranges were as follows: IL-33, 1500 pg/mL; sST2, 2000 pg/mL; IL-5, 2000 pg/mL; and ECP, 40 ng/mL. A sample with values over the linear range was diluted 10-fold and reanalyzed. For the ECP test, samples were initially diluted 5-fold as per the manufacturer's protocol and then diluted 10-fold if the values were over the linear range. Dilution assessment of each sample by using 5 different concentrations of each patient sample indicated a dose response for each cytokine. Spike and recovery assessment of each sample demonstrated acceptable performances for each cytokine. The IL-33 test indicated no significant cross-reactivity with or interference by 50 ng/mL of ST2, IL-1 α , IL-1 β , IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, IL-17, IL-18, IFN- γ , or TNF- α . For dilution assessment, spike and recovery, and interference evaluation, 10% of the allowable limit was used. The manufacturer of the IL-33 test declared that the IL-33 antibody recognizes full-length pro-IL-33 (amino acids 1–270) and the cytokine domain (amino acids 112–270) but not the homeodomain containing part (amino acids 1–111).^{E7,E8} We confirmed the IL-33 detection with a recombinant human IL-33 (from 2 manufacturers: PeproTech and R&D Systems) corresponding to amino acids 112 to 270 that have been used in most previous studies. Detection of the ST2 complex form of IL-33 was confirmed by mixing tests (incubation of IL-33 with ST2).

Sputum eosinophils were examined by using Diff quick (Sysmex Co) staining of smears of induced sputum samples from controls and expectorated sputum samples from patients with pulmonary symptoms. Induced sputum was obtained as previously described with slight modifications.^{E9}

Statistical analyses

The median levels and interquartile ranges of plasma IL-33, sST2, IL-5, and ECP were compared by using nonparametric tests because the concentrations were not normally distributed. The differences among 3 groups were compared by Kruskal-Wallis tests and then by individual testing by the Mann-Whitney *U* test if the differences were significant. Spearman correlations were used to determine the relationships between the number of blood eosinophils, the proportion of sputum eosinophils, and IL-33, sST2, IL-5, and ECP levels.

The comparison of diagnostic accuracy among the plasma markers for identifying pulmonary eosinophilia was performed by constructing ROC curves. Paired nonparametric Wilcoxon signed-rank tests were performed to compare serial changes of the markers. Data were analyzed with SPSS software version 15 (SPSS Inc, Chicago, Ill) and MedCalc version 7.5 (MedCalc software, Mariakerke, Belgium). The statistical significance was defined as $P < .05$.

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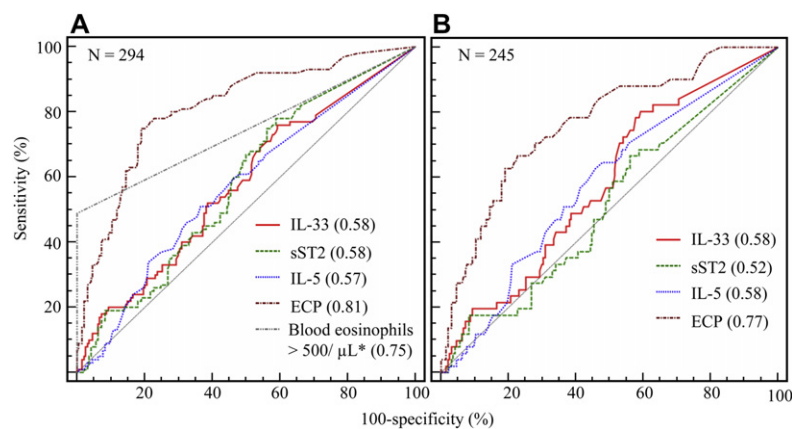


FIG E1. Comparison of diagnostic accuracies of IL-33, sST2, and eosinophilia-related markers for identifying PE using ROC curves. Numbers in parentheses indicate diagnostic accuracies (area under the ROC curves) for total PE (**A**) or PE with a normal number of blood eosinophils (**B**; less than 500 per μL). *Controls ($N = 194$) were selected from subjects with less than 350 eosinophils/ μL blood.

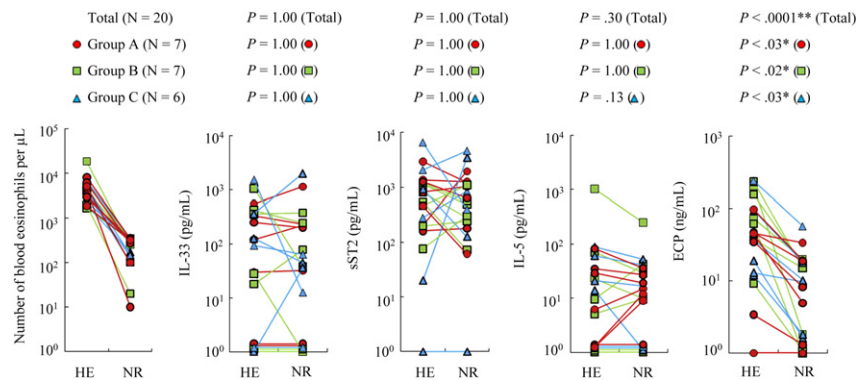


FIG E2. Changes in the levels of IL-33, sST2, and eosinophilia-related markers after normalization of blood eosinophil numbers in patients with hypereosinophilia. * $P < .05$; ** $P < .01$. HE, State of hypereosinophilia ($>1500/\mu\text{L}$); NR, state of normal eosinophil numbers ($<350/\mu\text{L}$). Group A, Patients with drug reaction; Group B, patients with immune-mediated disease (2 with skin disease, 2 with vasculitis, 2 with eosinophilic gastroenteritis, and 1 with asthma); Group C, patients with solid tumors.

TABLE E1. Subject characteristics

Group	Age (y), mean $\pm$ SD	Male sex, N (%)	Percentage of sputum eosinophils (mean $\pm$ SD)	Identifiable and/or possible causes of eosinophilia, [subtotal N], (N)
Hypereosinophilia, N = 82	53 $\pm$ 14	48 (58.5)	Undetermined or less than 15%	Allergy and skin disease [N = 25]
				Atopic dermatitis (2); other skin disease (9); asthma (3); drug reaction (10); bronchopulmonary aspergillosis (1)
				Neoplastic and myeloproliferative disease and syndromes [N = 22]
				Solid tumors (15); leukemia (3); lymphoma (2); idiopathic hypereosinophilic syndrome (2)
				Parasite infection [N = 2]
Pulmonary eosinophilia, N = 100	48 $\pm$ 18	48 (48.0)	>15 (42 $\pm$ 25)	Others [N = 19]
				Chronic dialysis (10); eosinophilic gastroenteritis (4); vasculitis (3); pneumothorax (1); Addison disease (1)
				Unknown [N = 14]
				Asthma (70)
				Eosinophilic bronchitis (20)
Control, N = 194	49 $\pm$ 18	98 (50.5)	<3 (0.2 $\pm$ 0.4)	Idiopathic acute eosinophilic pneumonia (6)
				Churg-Strauss syndrome (1)
				Unknown (3)

TABLE E2. Correlations among levels of IL-33, sST2, and eosinophilia-related markers and eosinophilia

Blood marker	Spearman correlation and P value	No. of blood eosinophils (N = 376)	Proportion of sputum eosinophils (N = 302)	IL-33 (N = 376)	sST2 (N = 376)	IL-5 (N = 376)
IL-33	<i>r</i>	−0.01	0.03			
	<i>P</i>	.92	.58			
sST2	<i>r</i>	0.11	0.08	0.28		
	<i>P</i>	.03*	.16	<.0001**		
IL-5	<i>r</i>	0.16	−0.02	0.16	0.06	
	<i>P</i>	.002**	.76	.002**	.28	
ECP	<i>r</i>	0.56	0.44	0.09	0.12	0.17
	<i>P</i>	<.0001**	<.0001**	.08	.02*	.001**

P* < .05; *P* < .01.