Methods

Study participants

Patients were enrolled from Soonchunhyang University Bucheon Hospital (11). Asthma was diagnosed by physicians based on the Global Initiative for Asthma guidelines (12). The diagnosis was supported by one or more of the following criteria: an increase in forced expiratory volume in one second (FEV_1) of >15%, or FEV_1 of 200 mL and 12% after 400 μg albuterol inhalation; a positive methacholine bronchial provocation test (MBPT) result, which was as defined as a concentration of methacholine that caused a 20% decrease in FEV_1 (PC_{20}) ≤10 mg per ml; or more than 20% increase in FEV_1 after 2-week treatment with inhaled or systemic corticosteroids. Asthmatic patients who were current smokers or ex-smokers with more than 10 pack-years were excluded. All included patients with COPD had pre-bronchodilator forced expiratory volume in 1 s/forced vital capacity (FEV_1/FVC) values of <0.7 and a smoking history of >10 pack-years. Control participants were selected using the criterion of normal spirometric values.

All enrolled patients were in stable condition when serum and sputum samples were collected. Patients with COPD or asthma exacerbations were excluded. Demographic characteristics including smoking status, results of induced-sputum analyses, pre-bronchodilator lung function tests, and eosinophil/neutrophil counts in peripheral blood were collected for the study populations. Serum levels of total IgE and results of induced sputum examination were collected for control participants. For patients with asthma, the following information was collected: onset age of asthma, asthma duration, post-bronchodilator spirometry, results of the methacholine provocation test PC_{20}, and number of subsequent exacerbations. Asthma exacerbation was defined as a short-term treatment with a systemic corticosteroid for increased typical asthmatic symptoms with FEV_1 < 80% of the personal best (12,13).

Informed written consent was obtained from all participants. All samples were obtained and processed according to the protocol of the Soonchunhyang University Bucheon Hospital biobank. Induced Sputum and specimen processing were performed as previously described protocols (14). The study was approved by the ethics committee of Kangwon National University Hospital (KNUH-2015-02-004-001) and Soonchunhyang University Bucheon Hospital (SCHGM 2014-16).

Quantitative measurement of cytokines

A sandwich ELISA kit (YbdY Biotechnology, Seoul, Korea) was used to measure IL-32γ (15) and IL-33 in duplicate according to the manufacturer's instructions with the lower limit of detection 0.082 ng/mL. The values less than the lower limit of detection were regarded as 0 ng/mL in statistical analyses. Cytokines were measured in duplicate and presented as mean values.

Immunocytochemistry

Peripheral blood mononuclear cells (PBMCs) were extracted from the buffy coat using Ficoll-Paque™ PREMIUM (17-5442-02, GE Healthcare, Chicago, IL, USA). To examine the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) or tumor necrosis factor alpha (TNF-α) on the induction of IL-32 or IL-33 expression, PBMCs were cultured in 24-well culture plates with or without TNF-α (ab9642, Abcam, Cambridge, MA, USA) and GM-CSF (sc-4585, Santa Cruz Biotechnology Inc., Dallas, TX, USA) for 24 hours. PBMCs were seeded in RPMI-1640 medium containing 10% fetal bovine serum, streptomycin, and penicillin with 1×10^6 cells/mL density. For immunocytochemical analyses, cells were fixed in 4% formaldehyde. The cell were washed with phosphate-buffered saline before permeabilization with 0.1% Triton X-100 (Sigma-Aldrich Inc., St. Louis, MO, USA), and then probed using a mouse monoclonal antibody to IL-32 (sc-517408, Santa Cruz Biotechnology Inc.) or IL-33 (ab207737, Abcam) for 60 min at room temperature. After repeated washes with phosphate-buffered saline, the cells were then probed with a secondary polyclonal antibody to mouse IgG1 conjugated to Alexa-488 (A32723, Thermo Fisher Scientific, Waltham, MA, USA) and rabbit IgG1 conjugated to rhodamine (R-6394, Thermo Fisher Scientific) for 40 min. The cells were washed again with phosphate-buffered saline and mounted on microscope slides with a mounting medium containing DAPI (DUO82040, Sigma-Aldrich Inc.) for nuclear staining. An Olympus FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) was used to obtain fluorescence images.

Statistical analyses

Continuous variables were presented as means and standard

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deviations (SD) and relative frequencies are presented for categorical variables in baseline characteristics. The normalities in data distributions were evaluated via the Shapiro–Wilk test, and the body mass index (BMI), FVC, and FEV_1 followed normal distributions. Plasma levels of cytokines, cell counts of induced sputum, and other variables which did not follow a normal distribution were analyzed with Spearman's rank correlation coefficient analysis. Kruskal–Wallis test and analysis of variance were used for comparisons in nonparametric and parametric variables, and then Mann–Whitney U tests or t-test were performed for post hoc analyses. Differences in plasma IL-32γ and IL-33 levels were compared among patients with asthma, patients with COPD, and healthy controls after adjustment for age and FEV_1 (%) using a general linear model. Statistical significance was accepted for P-values <0.05. All statistical analyses were performed using IBM SPSS Statistics, ver. 20.0 (IBM Corp., Armonk, NY, USA).

References

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Table S1 Characteristics of the study population

Characteristics	Asthma (n=103)	COPD (n=40)	Controls (n=51)	P value
Sex (M/F)	29/74	39/1	39/12	<0.0001
Age (range) in years	50.03 (17–80)	65.51 (47–84)	64.60 (40–86)	<0.0001
Onset age in years	42.80±16.19	–	–	
Duration of asthma in years	7.24±9.30	–	–	
Smoking History (NS/ES/SM)	85/13/5	0/22/18	17/10/24	<0.0001
Smoking amount (pack-year)	3.81±10.71	36.54±17.85	19.90±23.39	<0.0001
FVC, %	71.01±16.88	62.73±15.11	99.24±12.91	<0.0001
FEV_1, %	64.68±22.03	44.90±16.49	111.59±13.75	<0.0001
FEV_1/FVC	67.80±12.54	47.58±10.67	81.20±5.62	<0.0001
BMI	23.35±3.24	21.61±2.53	23.47±4.34	0.014
Bronchodilator FEV_1, %	12.94±11.51	–	–	
Bronchodilator FEV_1, L	0.19±0.18	–	–	
PC_{20}	6.33±9.16	–	–	
Total IgE	442.83±859.72	–	150.70±209.28	0.004
Exacerbation	65 (63.11%)	–	–	
Exacerbation (Y/N/ND)	65/18/11	–	–	
Sputum neutrophils %	62.28±32.89	84.77±17.20	53.99±37.10	0.003
Sputum eosinophils %	27.36±32.53	3.14±12.07	0.22±0.58	0.006
Detection rates of plasma IL-32γ	98 (95.1%)	40 (100%)	48 (94.1%)	0.323
Detection rates of plasma IL-33	82 (79.6%)	26 (65.0%)	25 (49.0%)	0.001

Mean±SD. FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second; NS, non-smoker; ES, ex-smoker; SM, smoker; Y, yes; N, no; ND, not determined

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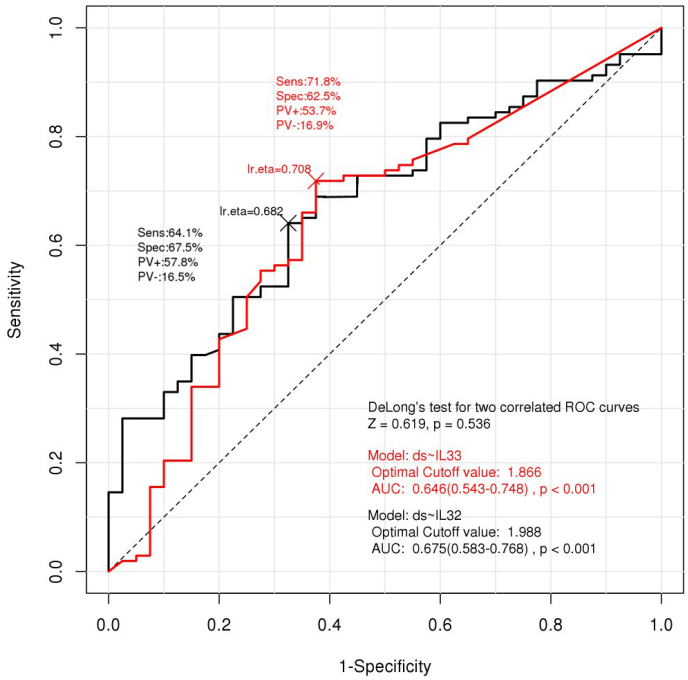
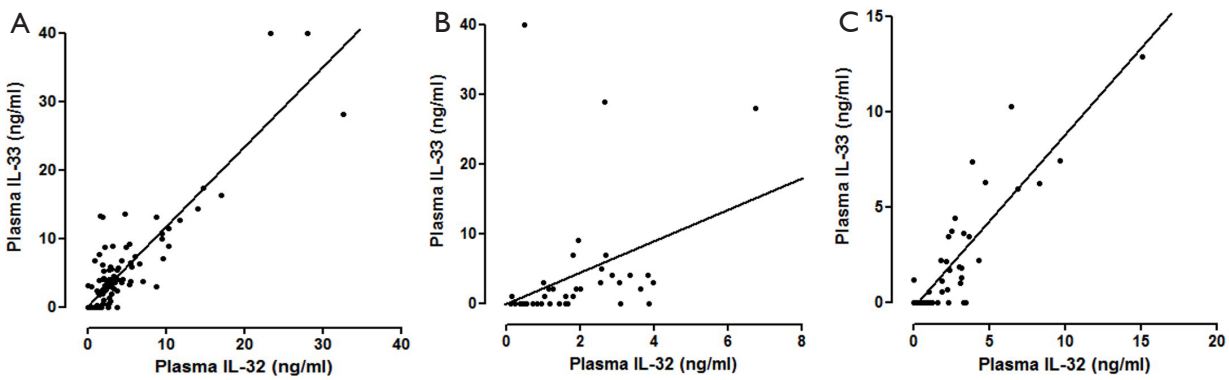


Figure S1 ROC curve to distinguish asthma from COPD.



Patients with asthma (r = 0.720, p < 0.0001) Patients with COPD (r = 0.512, p = 0.001) Healthy controls (r = 0.784, p < 0.0001)

Figure S2 Correlation between the levels of plasma IL-32γ and IL-33. (A) Asthma patients, (B) COPD patients, and (C) Healthy controls.

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Table S2 Characteristics of the study population

			Age	BMI	FVC%	FEV1%	FEV1/FVC	Sputum Neutrophili (%)	Sputum Eosinophil (%)	Total_IgE	PC20
Asthma	hIL-32 (ng/mL)	Correlation Coefficient	–0.395**	–0.230*	-0.061	-0.086	0.095	-0.133	0.122	0.019	-0.086
		P value	0.000	0.020	0.537	0.388	0.340	0.347	0.387	0.850	0.450
		Number	103	103	103	103	103	52	52	99	80
	hIL-33 (ng/mL)	Correlation Coefficient	–0.336**	–0.257**	–0.157	–0.215*	–0.017	–0.079	–0.022	0.039	-0.183
		P value	0.001	0.009	0.112	0.029	0.862	0.578	0.880	0.701	0.105
		Number	103	103	103	103	103	52	52	99	80
COPD	hIL-32 (ng/mL)	Correlation Coefficient	–0.038	–0.147	0.011	0.022	0.080	–0.269	0.358*		
		P value	0.816	0.365	0.945	0.894	0.623	0.112	0.032		
		Number	40	40	40	40	40	36	36		
	hIL-33 (ng/mL)	Correlation Coefficient	0.264	-0.150	0.149	0.220	.385*	–0.219	0.122		
		P value	0.100	0.354	0.358	0.172	0.014	0.199	0.478		
		Number	40	40	40	40	40	36	36		
Control	hIL-32 (ng/mL)	Correlation Coefficient	–0.042	–0.108	–0.221	–0.180	0.211			0.109	
		P value	0.770	0.452	0.120	0.205	0.137			0.467	
		Number	51	51	51	51	51			47	
	hIL-33 (ng/mL)	Correlation Coefficient	–0.270	–0.179	–0.204	–0.220	0.210			0.058	
		P value	0.056	0.208	0.150	0.121	0.140			0.696	
		Number	51	51	51	51	51			47	
All	hIL-32 (ng/mL)	Correlation Coefficient	-0.354**	-0.130	-0.128	-0.102	0.075	–0.251*	.250*	0.076	
		P value	0.000	0.071	0.076	0.157	0.301	0.019	0.019	0.362	
		Number	194	194	194	194	194	88	88	146	
	hIL-33 (ng/mL)	Correlation Coefficient	-0.356**	-0.190**	–0.218**	–0.212**	-0.014	-0.182	0.100	0.104	
		P value	0.000	0.008	0.002	0.003	0.851	0.089	0.353	0.213	
		Number	194	194	194	194	194	88	88	146	

*P<0.05, **P<0.01. BMI, body mass index; FVC, forced vital capacity; FEV1, forced expiratory volume in 1 second.

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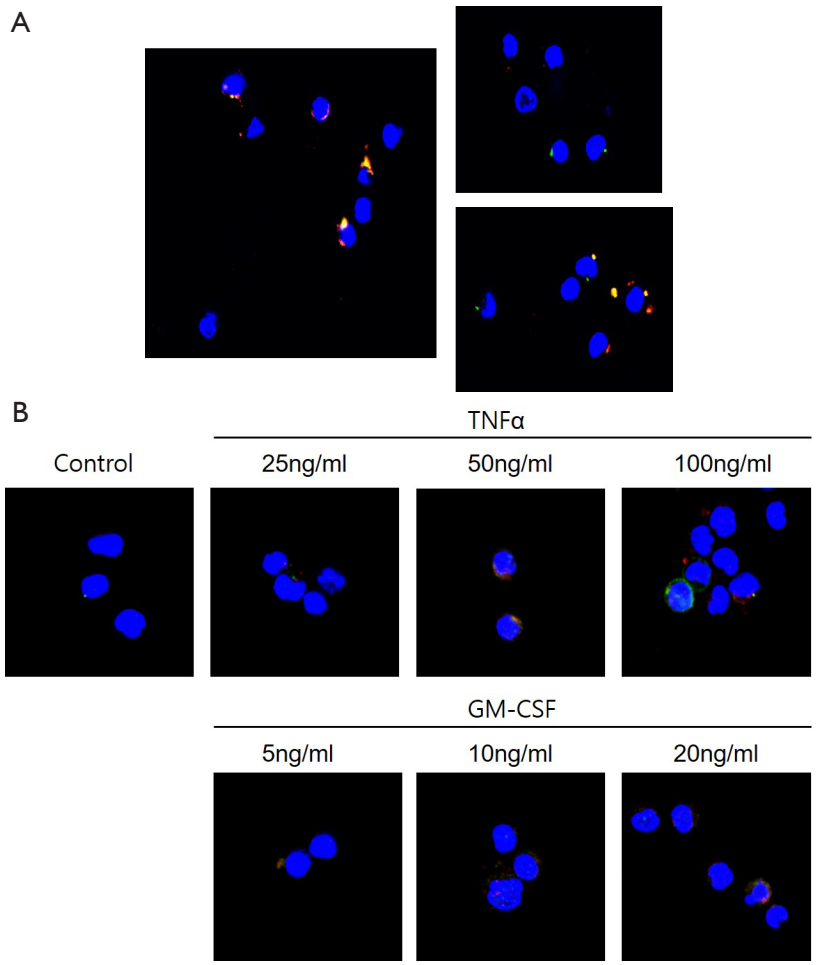

Figure S3 Immunofluorescence assay for peripheral blood mononuclear cells. (A) IL-32γ and IL-33 were expressed in monocytes. (B) Expression levels of IL-32γ and IL-33 were upregulated in the cytoplasm of monocytes among PBMCs stimulated with TNF-α and GM-CSF for 24 hours. IL-32: Alexa 488 (green), IL-33: rhodamine (red).

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