RESEARCH ARTICLE

Increased serum IL-17A and Th2 cytokine levels in patients with severe uncontrolled asthma

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ABSTRACT. Asthma is a syndrome of chronic bronchial inflammation and airway remodelling. Initially, asthma has been categorized into atopic and nonatopic types, based on antigen-specific IgE levels. Moreover, recently, asthma has been classified into different endotypes based on its pathophysiology, leading to the selection of the most optimal and effective therapies. Although T helper cell type 2 (Th2) cytokines were proven to play critical roles in atopic asthma, IL-17A has been reported to be involved in severe refractory asthma. ***Patients and methods:*** **In this study, we measured the levels of 24 cytokines/chemokines in the sera of healthy controls (HCs) (n = 34) and patients with asthma (n = 77), that were compared among patient groups with different disease activities and characteristics.** ***Results:*** **The serum levels of nine cytokines were significantly higher in patients with asthma than in HCs, and the levels of IL-17A and SCF were significantly different between uncontrolled and well-controlled patient groups (p = 0.003). The IL-17A levels were significantly correlated with those of IL-4, IL-25, IL-10, and IFN-γ in patients with uncontrolled asthma, and the patients with the highest levels of all the above cytokines were refractory to high-dose of inhaled corticosteroid therapy and have a history of acute exacerbation within 1 year, requiring systemic steroid therapy.** ***Discussion:*** **This study examines the profiles of upregulation and downregulation of various cytokines and chemokines in relation to asthmatic control status. IL-17A was significantly upregulated in patients with the uncontrolled and refractory status. Therefore, IL-17A may play important roles in asthmatic exacerbation, and its high level, in combination with upregulated Th2 and other cytokines, may indicate the refractory endotype of asthma.**

Key words: asthma, endotype, uncontrolled asthma, serum cytokines, IL-17, Th2

Asthma is a syndrome of chronic bronchial inflammation and airway remodelling, with reversible smooth muscle constriction and hyper-responsiveness, leading to wheezing, coughing, and shortness of breath, and it is also thought to be caused by a combination of complex and incompletely understood environmental and genetic interactions. Although asthma had been considered as a single disease for a long time, recent studies have emphasized its pathophysiological heterogeneity [1].

Indeed, results from cluster analyses [2, 3] revealed several heterogenous asthmatic subgroups with different pathophysiology and distinct responses to treatment. The subgroups have been classified into "endotypes", based on various features, including serum allergen-specific IgE levels, numbers of sputum eosinophils, and fractional exhaled nitric oxide (FeNO) in a breath test [4-6].

Cytokines and chemokines play an important role in the pathogenesis of the various types of bronchial inflammation [7-9]. The Th2 type cytokines, IL-4 and IL-5, induce switch recombination leading to the production of IgE by B cells and promote the differentiation and activation of eosinophils, respectively [10-12]. IL-13, another Th2 cytokine, has pleiotropic activities different from IL-4, and enhances airway inflammation and hyper-responsiveness by goblet cell differentiation, fibroblast activation, and B cell class switch from IgM to IgE [10-12].

More recently, IL-17A, a cytokine with strong pro-inflammatory properties, has been described in multiple aspects of asthma pathogenesis. IL-17A is a pro-inflammatory cytokine produced by a subset of CCR6-expressing $CD4^+$ T (Th17) cells that induces the secretion of IL-8 and IL-6, as well as several chemokines, including CXCL1, 3, 5, and 6, resulting in the recruitment and activation of neutrophils and macrophages [13]. Increased levels of circulating IL-17A levels have been observed in patients with asthma [13-20], and IL-17A has been described to exacerbate Th2 cell-mediated eosinophilic airway inflammation and hyper-responsiveness in experimental animal models [21, 22].

Most of those reported results are based on *ex vivo* experiments [15, 23], and there are few studies analyzing

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cytokine profiles in association with pathophysiological features in patients with asthma.
In the present study, we have investigated the relation between the serum levels of cytokines and chemokines and the severity of asthma, in particular in patients with a severe and steroid-resistant phenotype.

METHODS

Subjects

Patients' and matched control subjects' characteristics included age, sex, disease duration, presence or absence, of atopic asthma [4], % forced expiratory volume (FEV), and serum IgE levels. The assessment of patient's control status was performed based on the standard medication protocols of Japanese Asthma Prevention and Management guidelines 2009.
The study was approved by the research and ethics committees of Sagamihara National Hospital and Sysmex Corporation. All the study procedures were performed in accordance with the Declaration of Helsinki. Patients with asthma and healthy controls (HCs) were recruited from Sagamihara National Hospital and Sysmex Corporation, respectively.
A total of 77 patients were included as eligible for this study. Exclusion criteria were as follows: non-observance of treatment according to the medication protocol guidelines, the presence of other clinical entities, including atopic dermatitis, hyperthyroidism, paranasal sinusitis, aspirin intolerant asthma (AIA), non-steroidal anti-inflammatory drug (NSAID) hypersensitiveness, or eosinophilic bronchitis or having a history of smoking.
The average age of the patients was 56.3 (range 23-85) years, with the group comprising 30 men and 47 women. The average respiratory function (%FEV1) was 91% (SD 19.3%, range 50-146%, n = 76), with 13 patients having a %FEV1 lower than 70%. The median serum IgE level was 231 (range 49-459, n = 72) IU/mL. The number of patients with atopic asthma was 40 (56%).
Fifty-four patients (70%) were categorized as having "uncontrolled asthma" (UA), unlike patients with controlled asthma (CA) and, therefore, received step III therapy. Patients receiving low, middle, and high doses of inhaled corticosteroid (ICS) were 9, 29, and 38, respectively. Patients receiving the daily medication step I, II, and III were 10, 25, and 42, respectively. The doses of ICS and daily medication steps were not significantly different between UA and CA patient groups (data not shown). The average age of the HC was 30 (range 20-50) years, with the group comprising 6 men and 28 women.

Sample collection and cytokine and chemokine measurement

Serum samples were collected, frozen at −80 °C, and thawed immediately before analysis. Freeze–thaw cycles did not exceed three times.
The serum concentrations of 24 cytokines and chemokines were measured with a sandwich ELISA system using the antibodies as follows: anti-IL-4 (8D4-8 and MP4-25D2) and anti-IFN-γ (NIB42 and 4S.B3) antibodies were purchased from BioLegend (CA, USA), and anti-IL-17A (eBio64CAP17 and eBio64DEC17) and anti-IL-10 (JES3-9D7 and JES3-12G8) antibodies were purchased from eBioscience (CA, USA). HGF, angiopoietin-2 (Ang-2), SCF, MIF, IL-1RA, IFN-γ, IL-5, IL-6, IL-8 (CXCL8), IL-10, TGF-γ, IL-13, CCL11 (Eotaxin), CCL17 (TARC), TSLP, and periostin were measured with ELISA development system DuoSet (R&D Systems). IL-25 and IL-9 were measured with Human IL-17E Standard ELISA Development Kit (Peprotech, NJ, USA).
Recombinant cytokines, including IL-4 (BD Biosciences, NJ, USA), IL-10, IL-17A, IL-23 (eBioscience), and IFN-γ (BioLegend), were used as standards.
All the detection protocols were modified by using streptavidin-alkaline phosphatase (R&D Systems) and CDP-Star Substrate with Sapphire-II Enhancer (Life Technologies). The chemi-luminescence intensity was measured on an FLUOstar OPTIMA microplate reader (BMG LABTECH, Ortenberg, Germany) (Berthold, Wildbad, Germany). The within-run coefficients of variation were less than 15%.

Statistical analysis

Statistical analysis was performed using the StatFlex software (Artech Co. Ltd., Osaka, Japan).
All values were log-transformed, and the p-values were calculated using Welch's t-test (*table 1*, *figures 1 and 3*). Clinical parameters were analyzed using Fisher's exact test in R (http://www.r-project.org/) (*table 2* and *figure 4B*) . Correlation between cytokines was analyzed using Spearman's rank-order correlation analysis (*table 3* and *figure 2*). Unsupervised hierarchical cluster analysis was performed using Cluster 3.0 (University of Tokyo Human Genome Center) (*figure 4A*).
P-value <0.05 was considered to be statistically significant.

RESULTS

Various cytokines and chemokines are upregulated in patients with asthma

We measured serum levels of a very large panel of inflammatory mediators, including 24 cytokines/chemokines and periostin, in patients with asthma and HC. Serum levels of IL-6, IL-8, IL-13, IL-16, HGF, TSLP, IL-1RA, MIF, and MIP-1α were significantly higher, while those of IL-4, EGF, SCF, and Ang-2 were significantly lower, in the asthmatic patient group, as compared to the HC group (*table 1*).
To investigate the correlation between the levels of the above factors and the severity of asthmatic disease, we then compared their serum levels among HC, CA, and UA patient groups.
Serum levels of IL-16, IL-1RA, Ang-2, and MIP-1α were significantly different between UA and HC, as well as between CA and HC groups (*table 1*). Moreover, the levels of IL-17A and SCF were significantly different between UA and CA groups (*table 1*).

Higher IL-17A levels in patients with uncontrolled asthma

Because of the statistically significant difference between serum IL-17A levels of the UA and CA patient groups, we extensively analyzed the various clinical characteristics of these patients with asthma in relation to their serum

Table 1
Comparison of cytokine and chemokine levels in serum of healthy controls, controlled, and uncontrolled patients with asthma.

Cytokine Chemokine	Mean (Minimum-Maximum)				Mean (Minimum-Maximum)				**HC VS Asthma p value**	**HC VS CA p value**	**HC VS UA p value**	**CA VS UA p value**
	HC		**Asthma**		**Controlled (CA)**		**Uncontrolled (UA)**					
IL-4	14.75	(0.00- 54.53)	10.83	(0.00- 140.80)	8.89	(0.00- 64.80)	11.65	(0.00- 140.80)	0.019*	0.052	0.022*	0.906
IL-5	17.42	(1.06- 47.50)	12.54	(1.10- 59.30)	13.44	(1.30- 59.30)	12.15	(1.10- 29.30)	0.180	0.207	0.244	0.640
IL-6	0.74	(0.00- 9.62)	6.74	(0.00- 87.20)	4.73	(0.00- 23.40)	7.61	(0.00- 87.20)	0.000*	0.000*	6.074	0.709
IL-9	38.01	(0.00- 786.18)	185.35	(0.00- 4488.80)	66.42	(0.00- 913.20)	236.32	(0.00- 4488.80)	0.311	0.539	0.315	0.809
IL-10	3.82	(0.89- 11.22)	17.69	(0.00- 261.70)	16.04	(1.30- 172.60)	18.4	(0.00- 261.70)	0.118	0.114	0.186	0.617
IL-13	184.16	(0.00- 2444.28)	183.45	(0.00- 2041.20)	150.2	(0.00- 477.00)	197.7	(0.00- 2041.20)	0.018*	0.005*	0.055	0.184
IL-16	420.52	(176.01- 800.76)	658.97	(144.70- 2821.50)	760.44	(322.30- 2821.50)	615.48	(144.70- 1748.10)	0.001*	0.001*	0.007*	0.195
IL-17A	7.58	(0.17- 23.36)	74.09	(0.00- 2347.40)	4.14	(0.00- 30.70)	103.89	(0.00- 2347.40)	0.586	0.253	0.234	0.003*
IL-25	49.26	(0.00- 164.44)	149.98	(0.00- 2452.10)	54.79	(0.00- 199.20)	190.52	(0.00- 2452.10)	0.329	0.417	0.402	0.930
IL-33	43.44	(0.00- 349.18)	70.95	(0.00- 2179.00)	147.45	(0.00- 2179.00)	38.17	(0.00- 316.30)	0.702	0.993	0.595	0.654
IL-1RA	60.69	(0.00- 226.59)	267.84	(0.00- 1426.20)	184.59	(0.00- 707.30)	303.29	(0.00- 1426.20)	0.005*	0.015*	0.003*	0.344
IFN-y	143.8	(0.00- 886.66)	140.23	(0.00- 1308.00)	181.43	(0.00- 1308.00)	122.57	(0.00- 1308.00)	0.111	0.108	0.227	0.379
SCF	382.16	(271.84- 507.74)	345.53	(26.20- 1050.30)	429.46	(225.20- 1050.30)	309.78	(26.20- 594.70)	0.014*	0.767	0.002*	0.004*
EGF	720.94	(517.05- 918.92)	456.26	(64.80- 1255.30)	466.5	(69.20- 790.30)	451.89	(64.80- 1255.30)	0.000*	0.001*	2.299	0.651
HGF	645.49	(413.25- 892.35)	964.18	(461.00- 4114.60)	1035.54	(537.00- 4114.60)	933.6	(461.00- 1624.70)	0.000*	0.003*	4.434	0.843
Ang-2	600.34	(466.12- 769.21)	556.94	(167.60- 4162.80)	493.26	(325.10- 906.00)	584.07	(167.60- 4162.80)	0.018*	0.009*	0.046*	0.765
TSLP	0.92	(0.00- 6.31)	30.45	(0.00 -1271.50)	14.76	(0.00- 206.80)	37.14	(0.00- 1271.50)	0.020*	0.189	0.016*	0.448
TGF-ß	38112.66	(23771.96- 52657.96)	32331.56	(8914.70- 61925.20)	33554.3	(17985.20- 54988.60)	31810.77	(8914.70- 61925.20)	0.068	0.205	0.049*	0.395
MIP1a	38.52	(13.88- 115.61)	158.82	(13.90- 873.30)	172.44	(15.70- 833.10)	152.98	(13.90- 873.30)	0.000*	0.002*	0.001*	0.430
Eotaxin	101.11	(78.25- 130.89)	109.65	(48.70- 345.30)	116.49	(60.60- 177.60)	106.74	(48.70- 345.30)	0.566	0.097	0.998	0.079
TARC	362.44	(161.64- 1032.80)	279.58	(36.10- 900.70)	314.81	(116.60- 645.20)	264.57	(36.10- 900.70)	0.174	0.594	0.101	0.116
GROa	119.85	(85.14- 238.72)	114.48	(23.30- 618.90)	109.33	(23.80- 209.10)	116.67	(23.30- 618.90)	0.091	0.263	0.097	0.751
IL-8	9.95	(4.26- 16.73)	175.61	(5.60- 1419.90)	210.67	(7.90- 1178.70)	160.59	(5.60- 1419.90)	0.000*	3.896	1.656	0.157
MIF	1034.4	(195.76- 1711.16)	2466.51	(467.30- 10973.30)	2542.85	(1107.00- 5019.60)	2433.79	(467.30- 10973.30)	0.000*	2.131	1.471	0.416
Periostin	46747.9	(15913.42- 96010.54)	49707.99	(9014.40- 180508.80)	51812.27	(26127.90- 88610.00)	48811.73	(9014.40- 180508.80)	0.883	0.187	0.738	0.058

Cytokine and chemokine values are shown as pg/ml. For statistical analysis, all variables were log-transformed, and p-values were calculated by Welch's t-test. *, $P < 0.05$. The numbers of samples are as follows: HC (n = 23) for IFN-γ, IL-5, IL-6, IL-8, IL-9, IL-13, IL-16, IL-25, IL-33, MIF, MIP1α, HGF, periostin; HC (n = 11) for IL-4, IL-10, IL-17A, GROα, Eotaxin, TARC, TSLP, TGF-β, SCF, Ang-2, EGF, IL-1RA; all patients with asthma (n = 70-77), controlled asthma (n = 21-23), and uncontrolled asthma (n = 49-54) for all the above cytokines and chemokines.

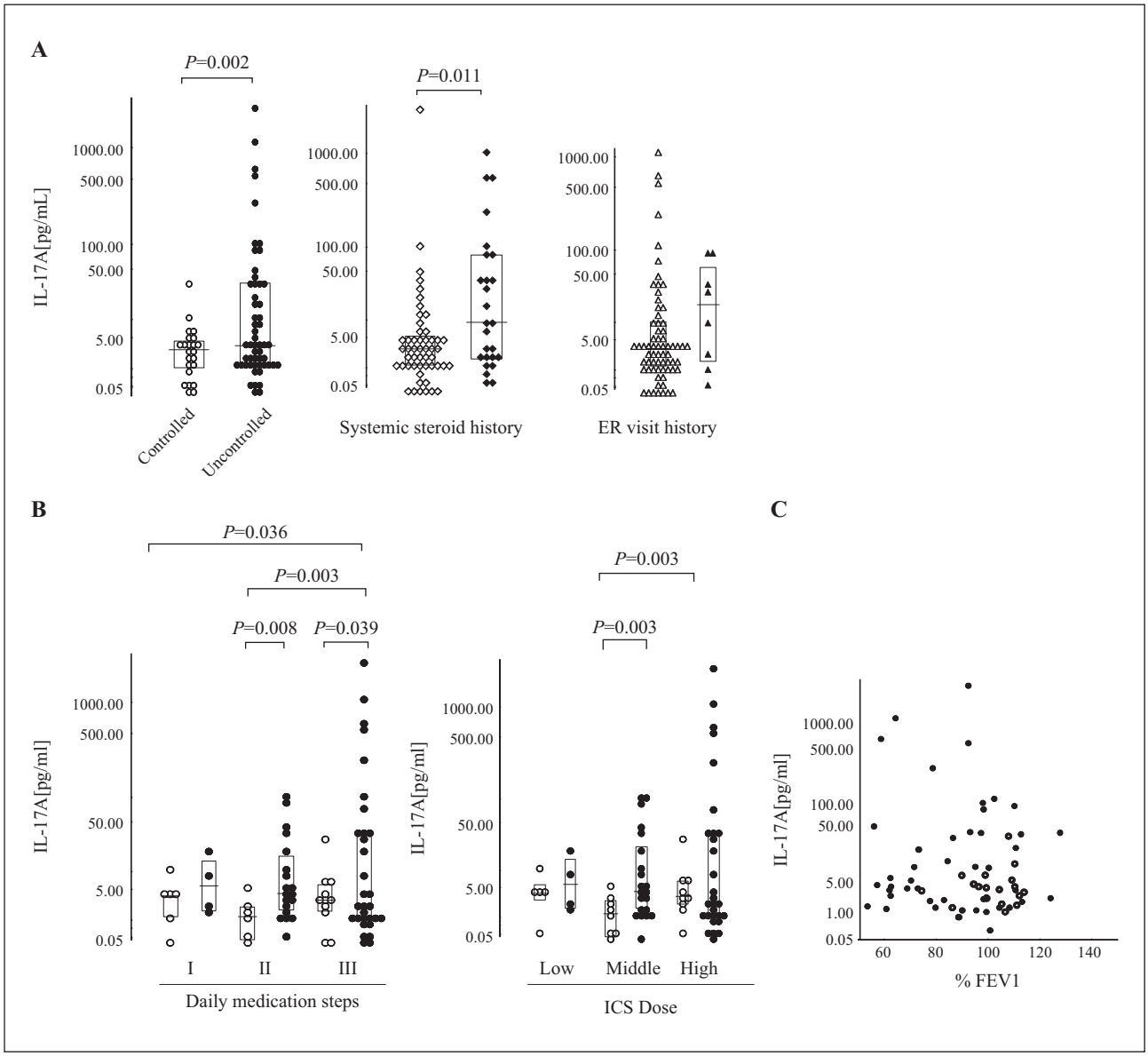

Figure 1

Serum IL-17A levels determined by ELISA. **A.** Serum IL-17A levels (pg/mL) were compared in relation with the control status, history of systemic steroid therapy, and emergency room (ER) admission within 12 months. **B.** Serum IL-17A levels were compared between patient groups with controlled and uncontrolled asthma, in relation with daily medication steps and ICS doses. **C.** The correlation between %FEV1 and serum IL-17A levels. Results are shown as individual data points with median (bars) and interquartile range (box). Open circles indicate patients with controlled asthma and closed circles indicate patients with uncontrolled asthma. Open diamond indicates the patients without steroid history, and closed diamond indicates the patients with steroid history. Open triangle indicates the patients without ER history, and closed triangle indicates the patients with ER history. P-values were calculated using Welch's t-test (**A, B**).

levels of these pro-inflammatory cytokines. This comparison revealed that the levels of IL-17A were significantly higher in UA (n = 44) than in CA (n = 22) group (p = 0.002, *figure 1A*). Moreover, the IL-17A levels were significantly higher in patient group with a history of systemic steroid therapy than that in the group without history (p = 0.011, *figure 1A*), whereas no differences between the emergency room visits of both groups were observed.

Similarly, when compared among distinct clinical groups, including the group II and III of the daily medication steps and the group of middle ICS dose, the IL-17A levels of UA group were significantly higher than those of CA group (*figure 1B*). However, no significant correlation was observed between IL-17A levels and respiratory functions (%FEV1) (Spearman's rank correlation test) (*figure 1C*).

Then, the patients were divided into two groups, including the group of patients with serum IL-17A levels higher (n = 20) and lower (n = 47) than the third quartile, respectively (*table 2*). The comparison of eight clinical characteristics between the two groups revealed the significant differences in control status, as well as history of systemic steroid therapy and emergency room (ER) admission within 12 months (*table 2*).

Various atopic cytokines are upregulated along with IL-17A

Several sets of cytokines and chemokines are co-induced in various control status of asthma, reflecting various immune conditions. The correlation analysis of the above 25 factors revealed the significant correlation between the levels of IL-17A and those of IL-4, IL-9, IL-10, IL-25, and IFN-γ, respectively (*figure 2A* and *table 3*).

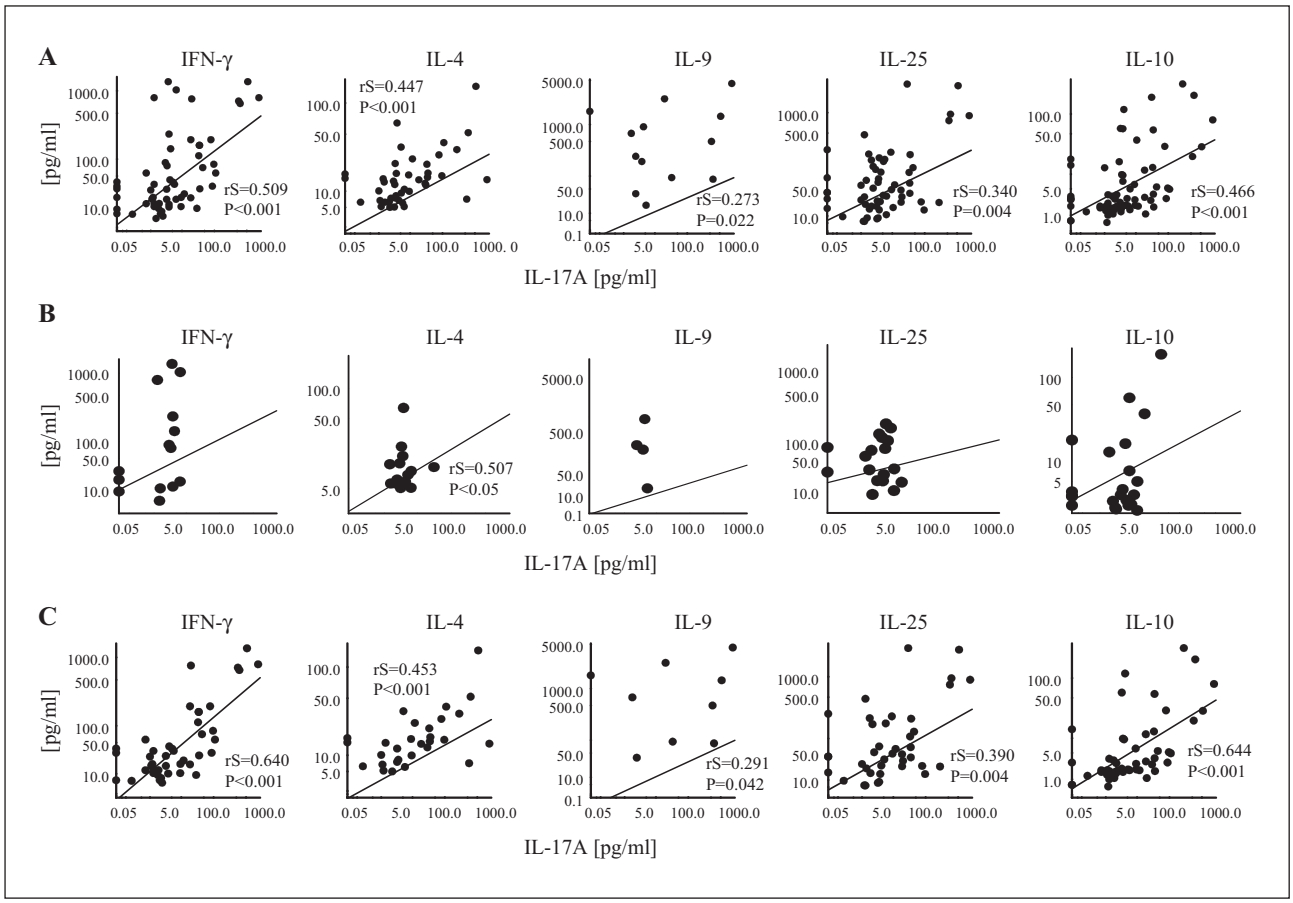

Figure 2

Correlation between IL-17A and other cytokines. The correlation diagram among IL-17A and IFN-γ, IL-4, IL-9, IL-10, and IL-25 was shown in all asthmatic patient group **(A)** and patient groups with controlled **(B)** and uncontrolled asthma **(C)**. The correlation was calculated using Spearman's rank correlation.

The correlation between IL-17A and all the above factors was also significant in the UA patient group (*figure 2C*), whereas, in the CA patient group, only IL-4 levels significantly correlated with IL-17A levels (*figure 2B*).

Atopic asthma is a subtype with increased serum Th2 cytokines and sputum eosinophils. As described above, the levels of several Th2 cytokines, including IL-4, IL-9, IL-10, and IL-25, as well as IFN-γ, correlated significantly with those of IL-17A. Therefore, we focused on atopic status of asthma, and revealed that the levels of IL-4, IL-9, IL-25, and IFN-γ, but not IL-10 and IL-17A, were significantly higher in atopic than in nonatopic asthmatic patient group (*figure 3A*). Moreover, we showed that the levels of IL-4 and IL-25 of the atopic group were significantly higher than those of the nonatopic group, respectively, in the patients with UA (*figure 3B*). Contrarily, the serum IL-17A levels of UA group were significantly higher than those of CA group, in both atopic and nonatopic patient groups (*figure 3B*).

Steroid-resistant patients with high IL-17A cytokine profiles

As the serum levels of IL-9, IL-4, IL-25, IL-10, and IFN-γ correlated significantly with those of IL-17A in all asthmatic patients as well as in the group of patients with UA (*figure 2*), we examined their correlation by cluster analysis (*figure 4A*). A subgroup of patients (G4), who showed significantly higher levels of all the six cytokines, was identified (*figure 4A, C*). All the four patients in this subgroup showed uncontrolled status in spite of receiving high dose inhaled corticosteroid (ICS) therapy (*figure 4B*). Moreover, three (75%) of them suffered acute exacerbation that required systemic steroid therapy within 12 months (*figure 4B*).

DISCUSSION

Asthma is a heterogeneous and genetically complex disease whose pathogenesis is classically thought to be driven by a Th2 cell-skewed immune response, referred to as $Th2^{high}$ asthma. Indeed, results from various studies have underscored the critical role of cytokines and chemokines in asthma pathogenesis [7-9]. Airway epithelial cells activated by allergens, viruses, or oxidants, produce TSLP, IL-25, and IL-33, leading to the increased production of Th2-type cytokines, including IL-4, IL-5, IL-9, and IL-13, which, in turn, trigger disease-associated downstream events, such as IgE-triggered hypersensitivity to allergens, activation of airway epithelium, recruitment of eosinophils, neutrophils, and mast cells, as well as airway remodeling [11, 12].

Measurement of cytokine levels provides useful information for understanding the pathologic process and monitoring of disease progression and inflammation [24-26]. Various cytokines are produced locally and have a very short half-life [27].

Therefore, the detection of cytokines in the affected tissues is theoretically optimal, but it requires a biopsy and

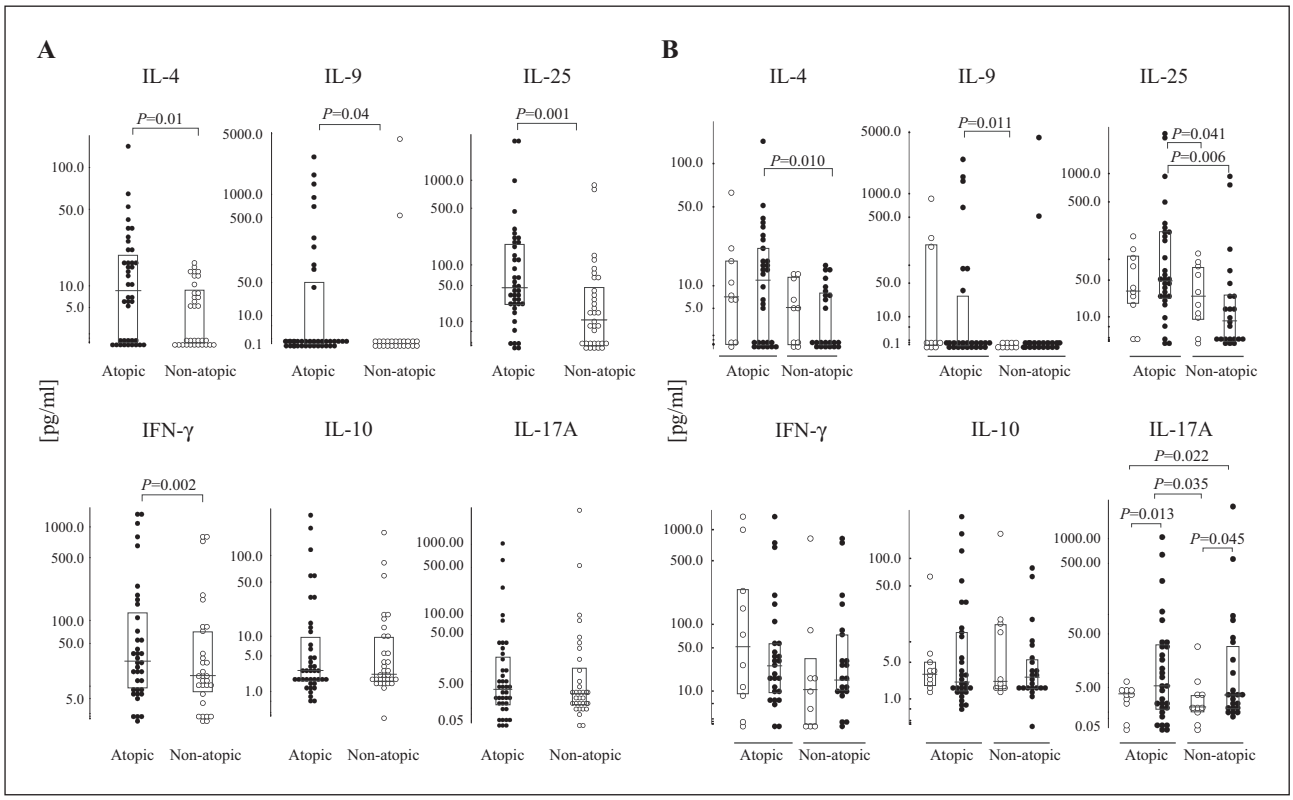

Figure 3

Serum levels of IL-17A and other cytokines. **A.** The levels of IL-17A and related cytokines were compared between patient groups of atopic asthma (open circle) and no-atopic asthma (closed circle). **B.** The levels of serum cytokines were compared between patient groups with controlled (open circle) and uncontrolled asthma (closed circle) and between patient groups with atopic and non-atopic asthma. P-values were calculated using Welch's t-test.

may be difficult to perform. Moreover, cytokines produced by peripheral blood mononuclear cells (PBMC) can be measured by ELISA of culture supernatants, intracellular cytokine detection by flow cytometry, and the measurement of cytokine messenger RNAs (mRNAs) [25]. Using the ELISA method, Abbal *et al.* examined the kinetics of circulating allergen-specific T cells in patients with allergy and revealed the rapid disappearance of the above T cells after the allergen challenge *in vivo* [28].

Contrarily, cytokine levels can be measured more easily in the serum or plasma [8]. As they are influenced by various processes, including local production by various cell types, secretion into tissues and circulation, tissue absorption, and degradation (half-lives) [24-26], many cytokines, except for TNF-α, IL-1β and IL-6, are not easily detectable in the circulation.

In spite of those limitations, the detection and profiling of serum cytokines have led to a better understanding of the inflammatory responses in several diseases [25, 29].

In the present study, we investigated the serum levels of a large panel of cytokines and chemokines in relation to asthmatic endotypes. The serum levels of nine cytokines were found to be significantly higher in patients with asthma than in HCs (*table 1*), and, when compared between uncontrolled and controlled asthmatic patient groups, serum levels of IL-17A and SCF were significantly different. Moreover, the results show that serum levels of IL-4, IL-9, IL-25, and IFN-γ were significantly higher in atopic (n = 40) than in nonatopic (n = 33) patients with asthma (*figure 3*), compatible with the previous reports [11, 12, 30].

Furthermore, cluster analyses of large cohorts identified several asthmatic phenotypes with distinct pathophysiology and responses to treatment, called "endotypes", based on allergen-specific IgE levels, sputum eosinophils, and respiratory functions [1-6, 31, 32].

More specifically, Wenzel identified five endotypes [1], including three Th2-dominant, referred to as early-onset allergic, late-onset eosinophilic, and exercise-induced asthma, and two non-Th2-dominant subtypes, neutrophilic and obesity-related asthma, respectively. Among these endotypes, $Th2^{high}$ asthma is a more atopic and allergic condition with childhood-onset, higher amounts of tissue IL-13 and IL-5 mRNAs, as well as greater number of eosinophils and mast cells that respond well to classical steroid therapy. In contrast, $Th2^{low}$ asthma is a less atopic condition with adult-onset, responding poorly to steroid therapy [33]. Of the $Th2^{high}$ endotypes, early-onset allergic asthma is corticosteroid-responsive, whereas late-onset eosinophilic asthma is often severe and refractory to corticosteroid therapy, albeit responsive to specific antibody therapy directed against IgE, IL-5, IL-13, etc. Of the latter subtypes, neutrophilic asthma shows lung neutrophilia, in addition to the above-mentioned characteristics of $Th2^{low}$ asthma [1].

Taking into account these various endotypes, the results from our study show that, out of the 25 factors, only IL-17A was significantly higher in patients with steroid-resistant asthma (UA), as compared to CA patients (*table 1* and *figure 1*). The upregulation of IL-17A levels was prominent in patients with severe asthma with history of systemic

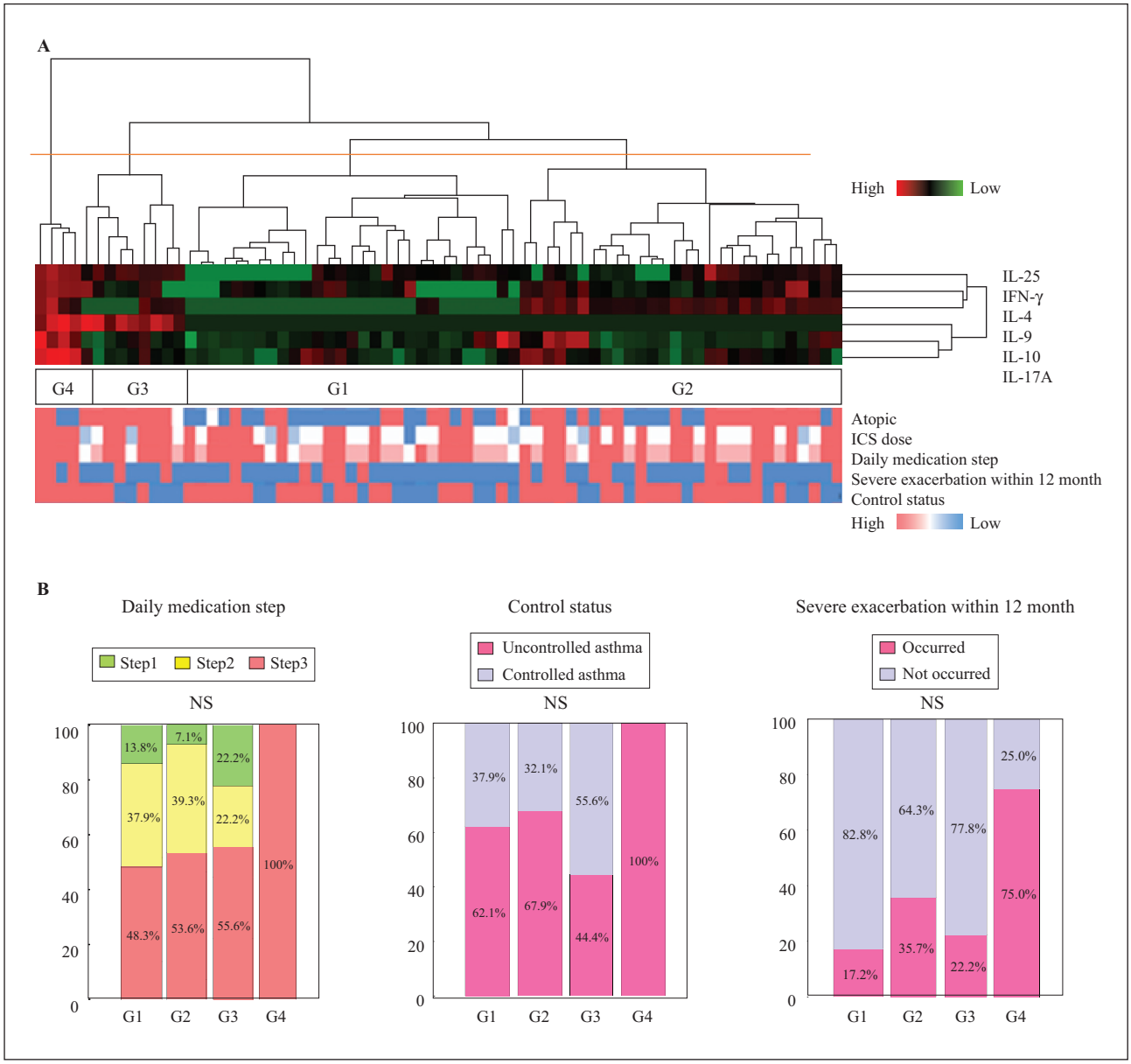

Figure 4

Clustering patients using IL-17A and other cytokines. **A.** Unsupervised hierarchical clustering analysis of IL-17A and related cytokine levels. Cluster analysis was performed by complete linkage based on Euclidean distance. **B.** The patient characteristics, including daily medication step, control status, and occurrence of severe exacerbation within 12 months, were compared among the groups identified in A. Statistical significance was calculated using Fisher's exact test. **C.** The serum levels of cytokines among the four groups identified in A. Results are shown as individual data points with median (bar) and interquartile range (box). The groups are as follows: G1 (blue dot), G2 (green dot), G3 (orange dot), and G4 (red dot).

steroid therapy and ER admission, higher daily medication group, and higher ICS doses (*figure 1*).

IL-17A is an inflammatory cytokine produced by Th17 and type 3 innate immune (ILC3) cells that induce the recruitment of neutrophils into the lungs through activation of epithelial cells to secrete various cytokines and chemokines [13]. In animal models, IL-17A exacerbates Th2 cell-mediated eosinophilic airway inflammation and hyper-responsiveness [21, 22]. IL-17A also counterbalances the effect of glucocorticoid on airway epithelium [34,35] and induces airway fibrosis by activating epithelial, fibroblastic, and smooth muscle cells [36]. Moreover, increased IL-17A levels were observed in patients with severe and steroid-resistant asthma [13-20]. Therefore, IL-17A is likely to play a critical role in the exacerbation of asthma, through neutrophilic infiltration, IL-8 production and induction of airway hyper-responsiveness [17].

Several previous large studies have suggested a positive correlation between IL-17A production and asthma severity [15, 17, 19, 37-40]. Our results confirm and extend these observation by showing higher IL-17A levels in UA, as compared to patients with CA, and in those with a more frequent history of systemic steroid administration and ER admission (*table 2*).

Our results, furthermore, indicate that increased serum IL-17A levels positively correlate with those of IL-4, IL-25, IL-10, and IFN-γ, especially in patients with UA (*figure 2*). Although Th2 cytokines, such as IL-4, IL-9, and IL-25, were significantly upregulated in atopic, and, in particular, atopic UA patients, the levels of IL-17A, IFN-γ, and

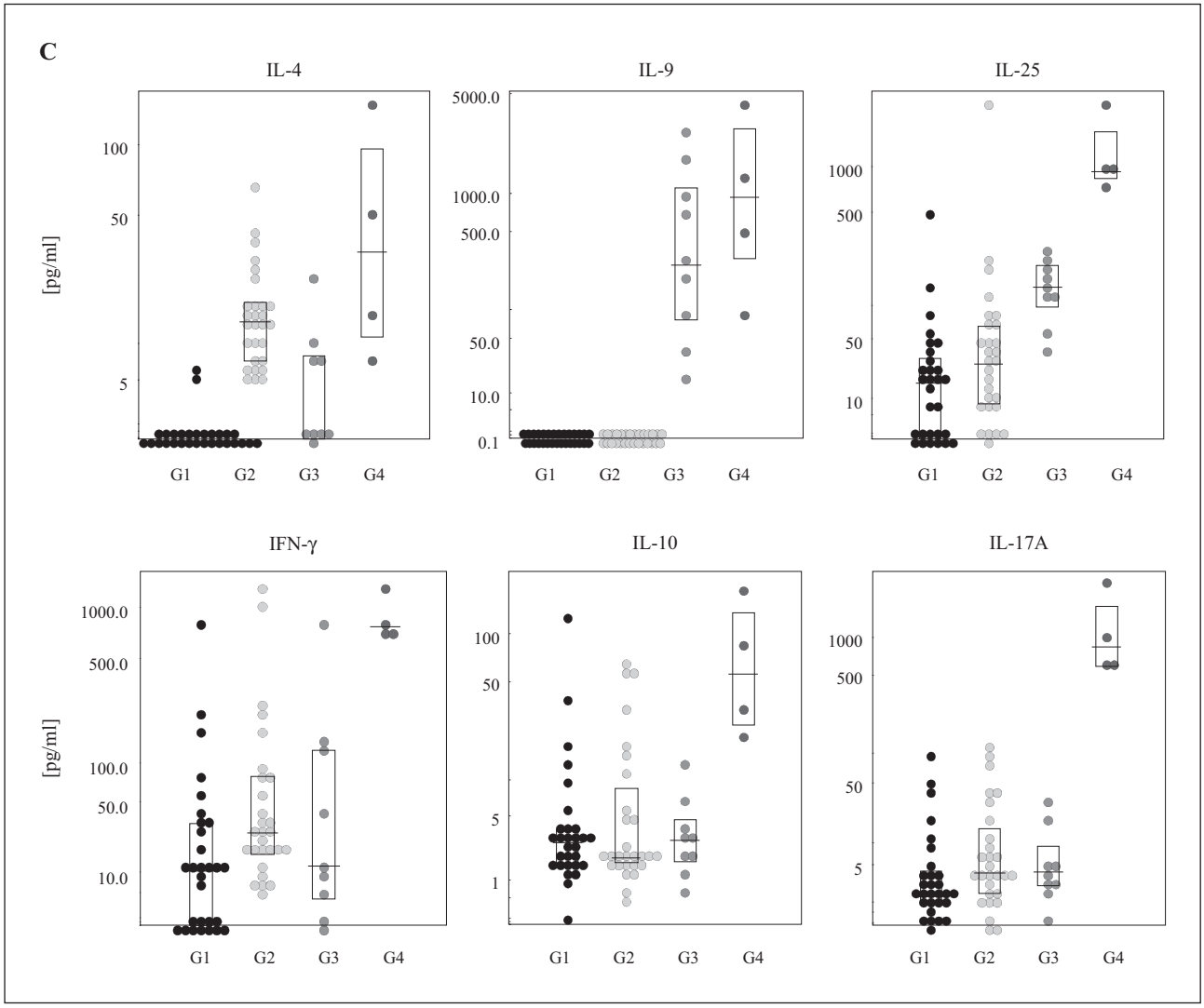

Figure 4

(*Continued.*)

IL-10 were not different between patients of atopic and nonatopic types (*figure 3*). In contrast, IL-17A was significantly upregulated in patients with UA, of both atopic and nonatopic phenotypes (*figure 3B*), suggesting that IL-17A may affect the severity of asthma, irrespective of the atopic subtypes.

Choy *et al.* have reported the dichotomous, mutually exclusive, pattern of Th2 and Th17 lymphocyte signatures by airway tissue gene expression analysis [41]. However, several other reports revealed the presence of dual-positive Th2/Th17 cells in broncho-alveolar lavage fluid (BAL) from patients with asthma [40, 42, 43]. Compared with classical Th17 or Th2 cells, dual-positive Th2/Th17 cells induced a profound effect in the recruitment of inflammatory leukocytes and exacerbation of asthma [43]. And the Th2/Th17-predominant subgroup manifested the most severe form of asthma, compared with the Th2-predominant or (Th2/Th17)-low subgroups [40]. Moreover, the levels of messenger RNAs of IL-4, IL-5, and IL-13 are significantly increased in sputum cells from asthmatic patients [44], and the 'IL-5, IL-17A, and IL-25-^{high}' airway inflammatory pattern is often observed in patients with more severe, UA [45, 46]. Serum levels of IL-17A and IL-22 tend to increase with the severity of allergic asthma [47], and IL-17A may act in synergy with Th2 cytokines, including IL-4, IL-13, IL-25, IL-33, and TGF-β, to modulate airway inflammation and remodeling [48-51]. Our results are in line with previously reported findings that IFN-γ is upregulated in the airway epithelial cells and peripheral blood lymphocytes of patients with severe chronic asthma [20, 52, 53], thus confirming the correlation of the expression of this cytokine and IL-17A in the pathogenesis of asthma. In contrast, IL-10 is an immunosuppressive cytokine, which is produced by both adaptive and innate immune cells [54]. The polymorphism of IL-10 is correlated with the decline of respiratory function in pediatric asthma [55], and IL-10 produced by Treg cells and mesenchymal stem cells (MSC) is capable of inhibiting the differentiation and function of effector Th cells [56], which suggests a protective function of IL-10 in allergic asthma. Unexpectedly, however, the results from the present study point to an upregulation of serum IL-10 levels in patients with UA, as compared to patients with CA (*figure 3B*). These results might be explained by the induction of an ongoing immunosuppressive counter-response in order to dampen sustained inflammation [57, 58].

The results from our cluster analysis identified a subgroup of patients with high levels of all six cytokines, who suffered from severe UA attacks in spite of high dose ICS medication (*figure 4*: G4 subgroup). Three of the four

Table 2
Association of serum IL-17A levels with clinical characteristics of asthmatic patients.

	IL-17A		P value
	Lower 75%†	**Higher 25%‡**	
Onset period			
Childhood	6	1	0.667
Adult	47	18	
Control status#			
Uncontrolled	35	19	0.004*
Controlled	22	1	
Exacerbation at the sample collection			
Yes	7	5	0.28
No	50	15	
Atopic status			
Yes	28	12	0.61
No	25	8	
ICS dose§			
High	26	12	0.5
Middle	22	7	
Low	8	1	
Daily medication step			
Step 1	9	1	0.443
Step 2	19	6	
Step 3	29	13	
History of systemic steroid therapy within 12 months			
Yes	14	12	0.006*
No	43	8	
History of ER visit within 12 months			
Yes	2	4	0.042*
No	52	16	

* P-values were calculated in Fisher exact test ($\alpha = 0.05$); † number of patients whose serum IL-17A levels were lower than the third quartile; ‡ number of patients whose serum IL-17A levels were higher than the third quartile; # Controlled and uncontrolled conditions were defined in accordance with physician's diagnosis based on Japanese Asthma Prevention and Management Guideline 2009; § Low Dose, 100-200 μg/day FP-HFA, Middle dose, 400 μg/day FP-HFA, High dose, 800 μg/day FP-HFA; ¶ Daily medication step according to the Japanese Asthma Prevention and Management Guideline 2009.

patients in this subgroup suffered acute severe exacerbation requiring systemic steroid therapy. Approximately 5-10% of patients with asthma are shown to be refractory and poorly controlled despite maximal inhaled steroid therapy [2].

Considering these characteristics, the above subgroup of patients in our study, with high levels of all the six cytokines, including IL-17A, IL-10, IFN-γ, and Th2-type cytokines, may fall into the endotype of severe late-onset eosinophilic asthma [1]. This endotype is reportedly refractory to corticosteroid treatment, but may respond to anti-IL-5 antibody and cysteinyl leukotriene modifiers [1, 59, 60]. Recently, Liang *et al.* hypothesized that asthmatic endotypes are related to systemic inflammation, and revealed three distinct endotypes by cluster analysis on the profiles of circulating cytokines [29].

Table 3
Correlation between IL-17A and other cytokines.

	rS	**P-value**
IL-4	0.447	<0.001*
IL-5	0.225	0.061
IL-6	0.139	0.252
IL-8	−0.014	0.911
IL-9	0.273	0.022*
IL-10	0.466	<0.001*
IL-13	−0.035	0.776
IL-16	0.179	0.138
IL-25	0.340	0.004*
IL-33	0.116	0.338
IL-1RA	0.085	0.484
IFN-γ	0.509	<0.001*
SCF	0.061	0.614
EGF	−0.060	0.623
HGF	0.203	0.093
Ang-2	−0.182	0.131
TSLP	0.232	0.053
TGF-β	−0.051	0.677
MIP1α	0.026	0.830
Eotaxin	−0.186	0.124
GROα	0.148	0.220
TARC	0.070	0.563
MIF	−0.104	0.391
Periostin	0.189	0.116

rS is Spearman's rank correlation coefficient. * P-values were calculated using Spearman's rank correlation coefficients ($P = 0.05$).

As discussed above, the precise categorization into distinct endotypes could facilitate the better-personalized therapy of severe refractory patients with asthma.

Taken together, the results presented here are compatible with and extend the previously reported subtyping of atopic and non-atopic asthma and underline the modulation of specific cytokine and chemokine levels in the circulation in association with the control of the disease. Moreover, we revealed the importance of IL-17A in the severity and exacerbation, beyond the atopic and non-atopic endotypes and identified a group of patients with severe refractory asthma with upregulation of all the cytokines, including IL-17A, IFN-γ, and Th2-type ones.

The cytokine profiling in our study may substantiate the endotying of asthma, based on clinical characteristics and facilitate the development of better-personalized and phenotype-specific therapies.

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