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Original

IL-33 and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) in BAL Fluid in Asthma Patients Without Cigarette Smoking

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SUMMARY

Background : Inflammatory cytokines and chemokines have been reported to play important roles in the pathogenesis of bronchial asthma. However, no criteria for the classification of 'smoker' and 'atopic' in bronchial asthma have been defined. In this study, we compared the levels of several cytokines found in the bronchoalveolar lavage (BAL) fluid of patients classified as having bronchial asthma.

Methods : Cell subpopulations in BAL fluid were counted. BAL fluid levels of interleukin (IL)-4, -5, -13, -17, and -33 and RANTES (regulated on activation, normal T cell expressed and secreted were measured using a bead suspension array in 36 asthma patients (13 males, 23 females ; mean age, 39.5±92.8 years) who were non-smokers, 18 asthma patients (11 males, 7 females ; mean age, 30.7±2.7 years) who were ex or current smokers (Brinkman index (BI) : 1－399), and 10 asthma patients (9 males, 1 female ; mean age, 50.2±5.5 years) who were current heavy smokers (BI : ≥400). Relationships were assessed by Spearman's rank correlation analysis.

Results : The number of lymphocytes in BAL cell subpopulations of non-smokers ($25\pm7\times10^3$/ml) were significantly ($p<0.05$) higher than those of heavy smokers ($12\pm3\times10^3$/ml). The number of neutrophils was significantly ($p<0.05$) higher in heavy smokers ($18\pm9\times10^3$/ml) than in non-smokers ($4\pm2\times10^3$/ml). Levels of IL-33 and RANTES were significantly ($P<0.05$) higher in non-smokers (26.1±7.3 pg/ml and 42.8±10.3 pg/ml, respectively) than in heavy smokers (13.7±4.5 pg/ml and 27.4±5.4 pg/ml, respectively). In addition, the levels of IL-33 and RANTES in non-smokers were significantly ($P<0.05$) higher in atopic asthma patients (33.0±9.8 pg/ml and 47.8±14.0 pg/ml, respectively) than in non-atopic asthma patients (9.1±3.8 pg/ml and 29.5±7.8 pg/ml, respectively). A good correlation was noted between RANTES and lymphocytes ($R=0.365$, $P<0.05$) or IL-33 ($R=0.561$, $P<0.05$) in atopic asthma patients who were non-smokers.

Conclusions : Differences in the cell types of BAL fluid, as well as in the levels of IL-33 and RANTES in asthma patients with or without smoking, might reflect pathogenesis.

Key Words : BAL fluid, IL-33, RANTES, asthma, smoker, atopy

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Abbreviations used

BAL : bronchoalveolar lavage ; RANTES : regulated on activation, normal T cell expressed and secreted ; BI : Brinkman index

INTRODUCTION

Airway inflammation plays a central role in the pathogenesis of asthma. The large and medium airways of patients with asthma show evidence of chronic inflammation, including leukocyte infiltrates in bronchial tissue, excessive mucus production, epithelial damage, basement membrane thickening, and smooth muscle hypertrophy[1,2]. The inflammatory infiltrates characteristically contain substantial populations of T cells, eosinophils, monocytes, and neutrophils. Asthma is associated with atopy and recruitment of eosinophils to the airways, which has led to the hypothesis that the pathogenesis of asthma is driven by a T helper (Th) 2 response to inhaled antigens[3,4]. Th2-type immunoregulatory cytokines play an important role in orchestrating immune and inflammatory processes[5~8]. In addition, RANTES (regulated on activation, normal T cell expressed and secreted, classified as a chemotactic cytokine or chemokine, is chemotactic for T cells, eosinophils, and basophils and plays an active role in recruiting leukocytes into inflammatory sites[9,10]. IL-33 is a recently described member of the IL-1 family which induces signaling via its receptor, ST2[11,12]. ST2 is expressed abundantly on the surface of mast cells and Th2 cells[12].

Despite the strength of the Th2-eosinophil paradigm, some features of human asthma cannot be explained by this mechanism alone. For instance, neutrophilic inflammation is commonly observed in bronchial biopsy of asthma patients, and the degree of neutrophilia correlates significantly with asthma severity[13]. Previous studies have demonstrated that 50% of asthma cases are non-eosinophilic—the predominant mechanism is neutrophil inflammation in the airway[14], which can be induced by IL-1, tumor necrosis factor (TNF), and IL-17[15~17]. Furthermore, smoking also induces neutrophilic airway inflammation, and relationships have been established between smoking history, airway inflammation, and lung function in asthma patients who smoke[18]. And IL-8, interferon (IFN)-γ, and TNFα involves in pathogenesis of airway inflammation in smoking asthma[19,20].

In this study, we measured the BAL fluid levels of IL-4, -5, -13, -17, and -33 and RANTES in asthma patients who were non-smokers and in those who were ex or current smokers to determine whether a distinct profile of cell populations and the noted cytokines exist in the phenotype and to evaluate the correlations between BAL cell populations and cytokines.

METHODS

Patients

Patients enrolled in the study had undergone a medical examination between 1999 and 2011 and endobronchial fiber biopsy within one month before enrollment. Diagnosis of mild or moderate bronchial asthma was clinically established on the basis of a consensus report[21] and pathologically confirmed by endobronchial biopsy. Subjects included 36 asthma patients who were non-smokers (13 males and 23 females ; mean age, 39.5±2.8 years), 18 who were ex or current moderate smokers (Brinkman index (BI) : 1−399 ; 11 males and 7 females ; mean age, 30.7±2.7 years), and 10 who were current heavy smokers (BI : ≥400 ; 9 males and 1 female ; mean age, 50.2±5.5 years). Serum total IgE was examined, and the pulmonary function test was performed in all enrolled patients (Table 1).

None of the subjects had received oral steroid therapy or experienced acute bronchitis in the 4 weeks prior to clinical sample collection. Patients with cancer in any organ and those suspected of malignancy were excluded from the study. Each atopic patient (26 non-smokers, 13 moderate smokers, and 9 heavy smokers) tested positive (≥class 2) for *Dermatophagoides farinae*, house dust mite, cat, or candida in sera detected by CAP-RAST (Phadia, Uppsala, Sweden). Patients with chronic obstructive pulmonary disease (COPD) were excluded on the basis of diffusing capacity of CO and chest computed tomography findings.

The study protocol was approved by the Human Ethics Review Committees of Dokkyo Medical University, and a signed consent form was obtained from all subjects.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) fluid samples were obtained from all subjects. BAL was performed as described previously[22] using a flexible fiberoptic bronchoscope (Olympus 1T-200, Olympus, Tokyo, Japan) after local anesthesia of the upper airway with 4% li-

Table 1

	Age (years)	Sex (male/female)	B. I	Height (cm)	Atopy (+/−)	IgE (IU/ml)	%VC (%)	FEV1.0 (%)	%DLCO (%)
Non Smoker	39.5±2.8	13/23	0	160.1±1.5	26/8 subjects	374±99	107.5±3.0	77.4±1.6	101.6±3.1
BI=1-399	30.7±2.70	11/7	125.56±21.16	164.9±1.6	13/5	506±175.5	90.9±8.1	79.7±3.4	103.2±3.9
BI≧400	50.2±5.5	9/1	836±114.15	165.3±2.3	5/5	544±300.	99.9±14.1	72.6±5.9	97.3±8.0

Clinical characteristics of asthma patients who were non-smokers, ex or current moderate smokers (Brinkman index (BI) : 1−399), and current heavy smokers (BI : >400). Data are expressed as mean±SE values.

docaine. Briefly, the bronchoscope was wedged for lavage into one of the subsegmental bronchi of the right middle lobe. BAL was performed three times using a 50 ml aliquot of sterile physiologic saline solution. BAL fluid was passed through two sheets of gauze and then centrifuged at 500× *g* for 10 min at 4 ℃ before being centrifuged at 500× *g* for 5 min at room temperature, and the supernatant was stored at −80 ℃ for further quantification of non-cellular components. After washing twice with phosphate-buffered saline solution, cells were suspended with 10% heat-inactivated fetal calf serum and counted using a hemocytometer. Differential cell counts were determined from cell suspensions displayed on slides using a cytocentrifuge (Cytospin 2 ; Shandon Instruments ; Sewickley, PA). Cells were dried, fixed onto slides, and stained by the May-Grunwald-Giemsa method. A total of 200 cells were identified under a photomicroscope.

Measurement of IL-4, -5, -13, -17, and -33 and RANTES in BAL fluid

BAL fluid samples were concentrated using a Centriprep-3 (Millipore Corporation ; Billerica, MA), which is used to concentrate low-molecular-weight components. The cut-off value for molecular weight was 3000 Da. In this procedure, magnification of concentration was calculated using the ratio of protein consistency in nonconcentrated BAL fluid to concentrated BAL fluid, which was measured by assay (DC protein Assay, Bio-Rad Laboratories, Hercules, CA), and the original levels of cytokines were corrected with this ratio[23]. Since BAL producer has a dilutional effect on cytokine recovery, measurement results are occasionally standardized to albumin. A good correlation with IL-5 ($R=0.79$, $P<0.05$) and IL-33 ($R=0.78$, $P<0.05$) was observed between the nonstandardized and standardized albumin concentrations in BAL fluid of 7 patients with bronchial asthma (data not shown). Consequently, the cytokine levels reported in the text are those of measured concentrations rather than those relative to albumin concentration. BAL fluid levels of IL-4, -5, -13, -17, and -33 and RANTES were measured using a bead suspension array. Detection limits were 0.2 pg/ml, respectively.

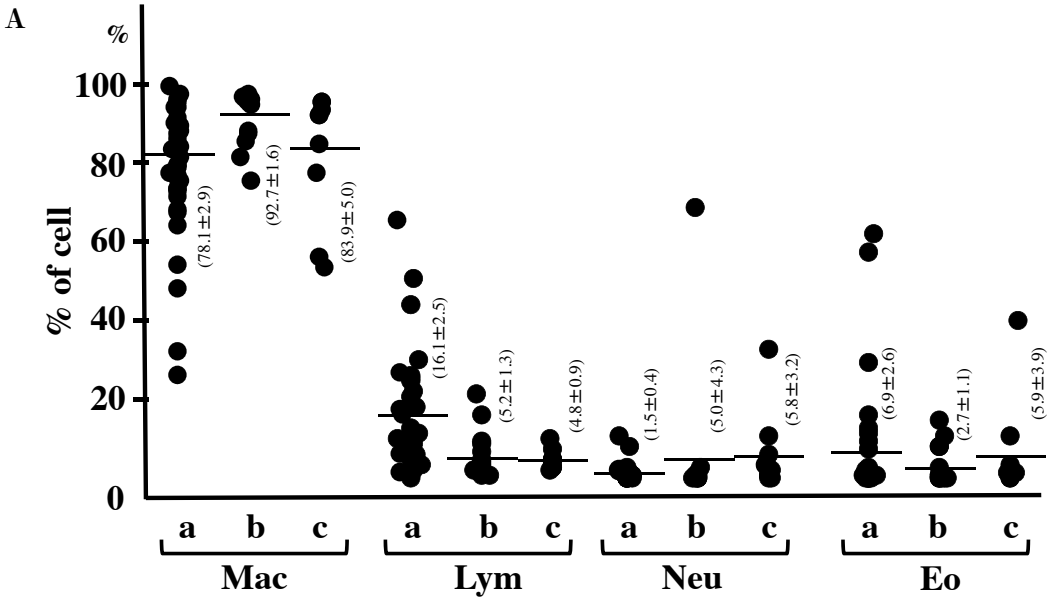
Statistical analysis

All data are expressed as mean±standard error (SE) values. Differences between groups were compared by one-way analysis of variance. Fisher's PLSD test was used as the post hoc test. We also used Spearman's rank correlation analysis to examine relationships. Statistical analysis was performed using JMP software (Cary, NC). Statistical significance was defined by $P<0.05$.

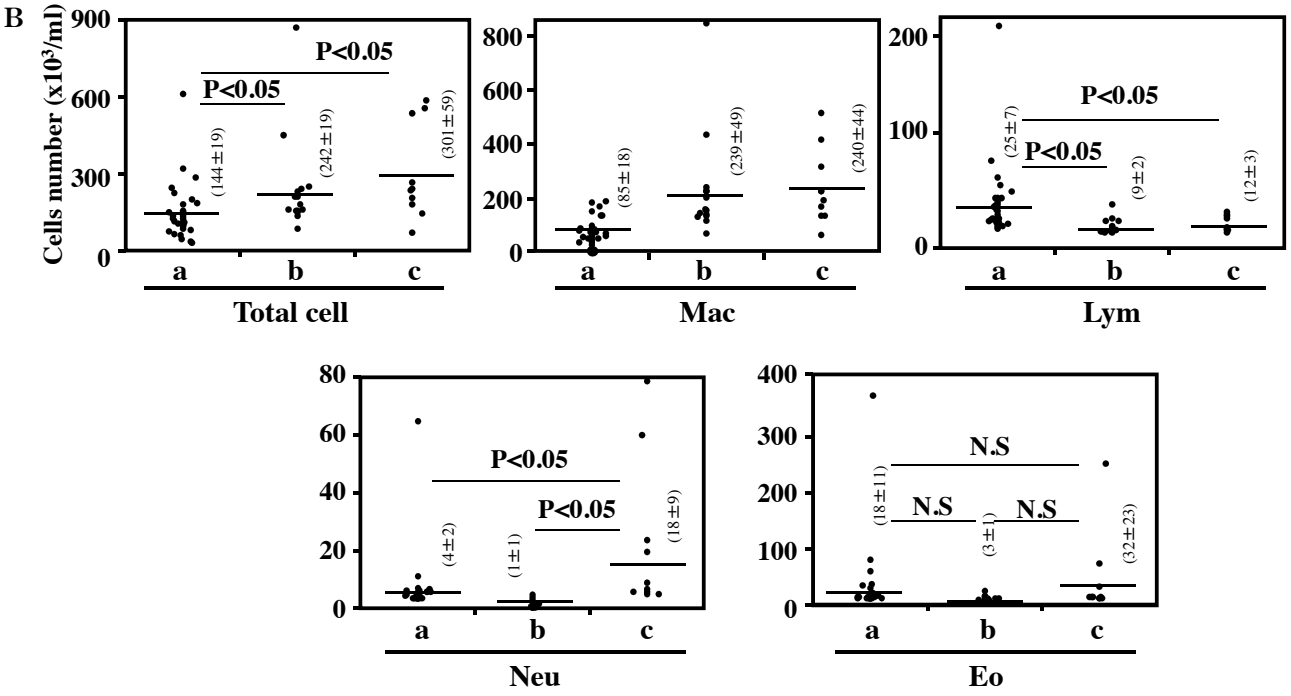
RESULTS

Cell count in BAL fluid of asthma patients with or without smoking

Assessment of cell subpopulations (Fig. 1A) and cell number (Fig. 1B) in BAL fluid revealed that the number of total cells was significantly ($p<0.05$) greater in current heavy smokers ($301\pm59\times10^3$/ml) and ex or current moderate smokers ($242\pm19\times10^3$/ml) than in non-smokers ($144\pm19\times10^3$/ml). Neutrophils were significantly ($p<0.05$) more prevalent in current heavy smokers ($18\pm9\times10^3$/ml) than in ex or current moderate smokers ($1\pm1\times10^3$/ml) and in non-smokers ($4\pm2\times10^3$/ml). On the other hand, the number of lymphocytes was significantly ($P<0.05$) greater in

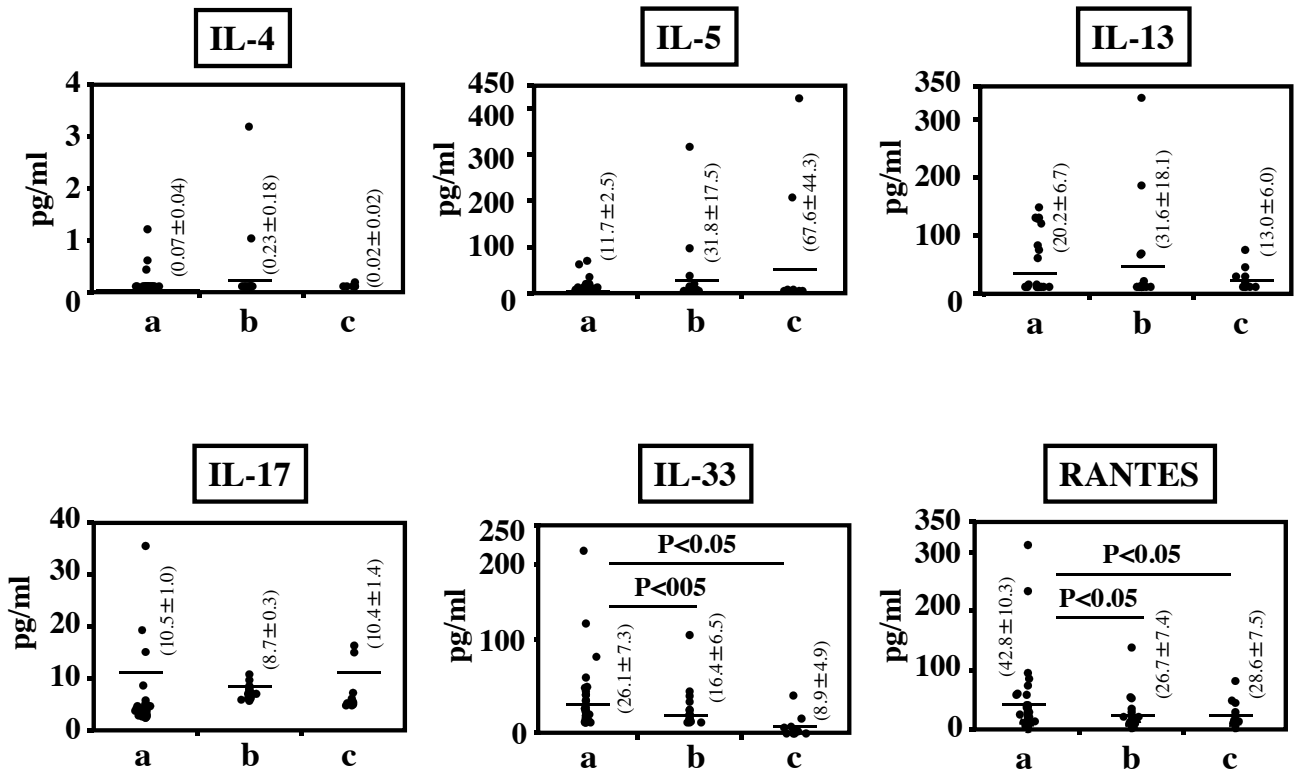


Mac; macrophages, Lym; lymphocytes, Neu; neutrophils, Eo; eosinophils
a: non-smoker , b: 0<BI<399, c: BI ≧400



Mac; macrophages, Lym; lymphocytes, Neu; neutrophils, Eo; eosinophils
N.S; not significance, a: non-smoker, b: 0<BI<399, c: BI ≧400

Figure 1 Cell subpopulations (**A**) and cell number (**B**) in BAL fluid of asthma patients who are non-smokers (a), ex or current moderate smokers (BI : 1－399) (b), and current heavy smokers (BI : ≥400) (c). Data are expressed as mean±SE values. P<0.05 compared with non-smokers or moderate smokers. Eo, eosinophils ; Ly, lymphocytes ; Neu, Neutrophils ; Mac, macrophages.



N.S; not significance, a: never smoker , b: 0<BI<399, c: BI ≧400

Figure 2 BAL fluid levels of IL-4, -5, -13, -17, and -33 and RANTES in asthma patients who are non-smokers (a), moderate smokers (BI : 1－399) (b), and heavy smokers (BI : ≥400) (c). Cytokines were measured using a bead suspension array. Detection limits were 0.2 pg/ml. Data are expressed as mean ± SE values. P <0.05 compared with non-smokers.

non-smokers ($25 \pm 7 \times 10^3$/ml) than in ex or current moderate smokers ($9 \pm 2 \times 10^3$/ml) and in current heavy smokers ($12 \pm 3 \times 10^3$/ml). There were no significant differences in the number of eosinophils between smokers and non-smokers.

BAL fluid levels of IL-4, -5, -13, -17, and -33 and RANTES

We measured the levels of IL-4, -5, -13, -17, and -33 and RANTES in BAL fluid of asthma patients who were non-smokers, ex or current moderate smokers, and current heavy smokers (Fig. 2). Levels of IL-4, -5, -13, and -17 were similar between smokers and non-smokers. On the other hand, the levels of IL-33 and RANTES were significantly ($p<0.05$) higher in non-smokers (26.1 ± 7.3 pg/ml and 42.8 ± 10.3 pg/ml, respectively) than in heavy smokers (8.9 ± 4.9 pg/ml and 28.6 ± 7.5 pg/ml, respectively). In addition, the levels of RANTES were significantly ($p<0.05$) higher in non-smokers than in ex or current moderate smokers (26.7 ± 7.4 pg/ml).

IL-33 and RANTES concentrations in BAL fluid of atopic or non-atopic asthma patients and relationship with smoking

To determine the significance of the high levels of IL-33 and RANTES in non-smokers compared with smokers, we measured IL-33 and RANTES concentrations in BAL fluid of atopic or non-atopic non-smokers (Fig. 3). The levels of IL-33 and RANTES in non-smokers were significantly ($P<0.05$) higher in atopic asthma patients (33.0 ± 9.8 pg/ml and 47.8 ± 14.0 pg/ml, respectively) than in non-atopic asthma patients (9.1 ± 3.8 pg/ml and 29.5 ± 7.8 pg/ml, respectively).

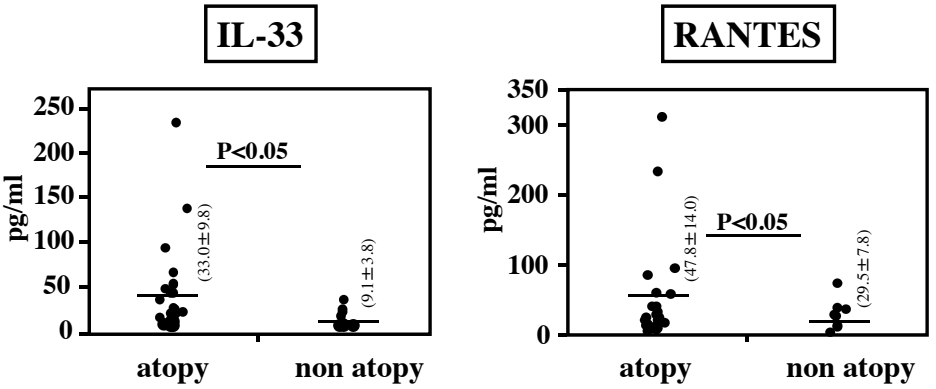


Figure 3 BAL fluid levels of IL-33 and RANTES in atopic or non-atopic asthma patients who were non-smokers. Cytokines were measured using a bead suspension array. Detection limits were 0.2 pg/ml. Data are expressed as mean ± SE values. P < 0.05 compared with non-atopic patients.

Table 2 Sperman's rank correlation

	IL-4	IL-5	IL-13	IL-17	IL-33	RANTES
Macrophages	−0.263	0.105	0.250	0.220	−0.248	0.073
Lymphocytes	−0.185	0.330	0.067	0.272	−0.056	0.365 *
Neutrophils	−0.118	0.264	0.037	0.050	−0.231	0.048
Eosinophils	0.143	0.317	−0.002	0.012	−0.202	0.038
IL-4	—	−0.263	−0.184	0.034	0.014	0.249
IL-5	−0.263	—	0.099	0.125	0.077	−0.087
IL-13	−0.184	0.099	—	0.212	−0.093	−0.071
IL-17	0.034	0.125	0.212	—	0.060	0.253
IL-33	0.013	0.077	−0.093	0.060	—	0.561 *
RANTES	0.249	−0.087	−0.071	0.253	0.561 *	—

Correlation between cytokines and differential cell counts.
* < 0.05

Correlation between each cytokine and cell type in BAL fluid

Finally, we analyzed the correlations between the levels of IL-4, -5, -13, -17, and -33, RANTES, and cell type in BAL fluid using Spearman's rank correlation test (Table 2). A good correlation was noted between RANTES and lymphocytes ($R = 0.365$, $P < 0.05$) and IL-33 and lymphocytes ($R = 0.561$, $P < 0.05$) in atopic asthma patients who were non-smokers (Fig. 4). No significant correlations were observed between the levels of other cytokines and cell type.

DISCUSSION

The pathogenesis of allergic asthma has indicated the involvement of Th2[5~8]. In this study, the finding of increased levels of IL-33 and RANTES in atopic asthma patients who were non-smokers is very important. The results suggest that the lymphocytes which infiltrate bronchial tissue play an important role in the pathogenesis of asthma and might be associated with IL-33, induction of Th2, RANTES, and the recruitment of T cells into bronchial tissue.

Airway inflammation in allergic asthma is characterized by increased numbers of eosinophils and mast cells, hypersecretion of mucus, and activation of T cells[24,25]. Classically, the recruitment of Th2 lymphocytes, accompanied by the recruitment of eosinophils to the airways, has been considered integral to the pathogenesis of asthma[26]. Eosinophils play an important role in the pathogenesis of allergic airway inflammation[3,4]. Our results showed that the number of eosinophils in BAL fluid was similar between asthma patients, irrespective of smoking status, suggesting that eosinophils play an important role in the induction

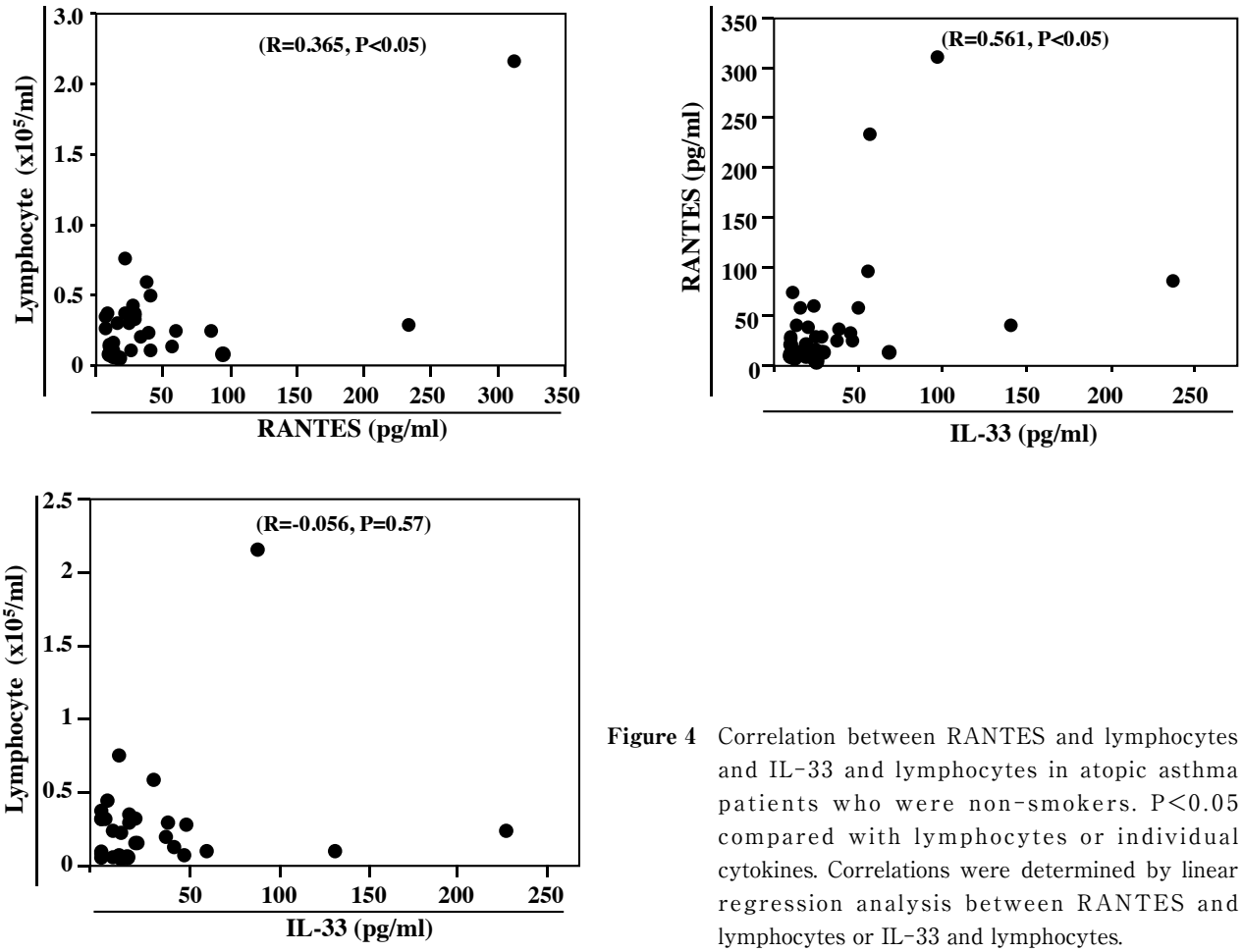


Figure 4 Correlation between RANTES and lymphocytes and IL-33 and lymphocytes in atopic asthma patients who were non-smokers. $P<0.05$ compared with lymphocytes or individual cytokines. Correlations were determined by linear regression analysis between RANTES and lymphocytes or IL-33 and lymphocytes.

of allergic airway inflammations regardless of asthmatic phenotype. On the other hand, several investigators have reported that neutrophil infiltration into bronchial and lung tissues plays an important role in the pathogenesis of COPD and asthma in smokers[27,28]. Our results revealed a similarly increased number of lymphocytes and neutrophils in asthma patients who were non-smokers and in heavy smokers, which is a similar finding to the above reports. In addition, the number of total BAL cells was increased in heavy smokers compared with those who were non-smokers. COPD is associated with a chronic inflammatory response, predominantly in the small airways and lung parenchyma, and is characterized by increased numbers of total cells, such as macrophages, neutrophils, and T lymphocytes[29,30]. The present results suggest that the mechanism of airway inflammation is similar between asthma patients with COPD and those who smoke.

Recruitment of Th2 lymphocytes, which secrete IL-4, IL-5, and IL-13, accompanied by the recruitment of eosinophils to the airways, has been considered integral to the pathogenesis of asthma[5~8]. IL-4 is a key cytokine in the development of allergic inflammation. IgE-dependent mast cell activation induced by IL-4 has a pivotal role in the development of immediate allergic reactions[31]. An additional mechanism by which IL-4 contributes to airway obstruction in asthma is through the induction of mucin gene expression and the hypersecretion of mucus[32]. IL-4 increases the expression of eotaxin and other inflammatory cytokines from fibroblasts that might contribute to inflammation and lung remodeling in chronic asthma[33]. In general however, IL-4 in BAL is difficult to detect[34]. Furthermore, although no IL-4 was detected in the present study, it has been shown to be important for allergic airway inflammation in the pathogenesis of asthma. IL-5 produced by Th2 cells and mast cells has long been associated with the cause of several allergic diseases, including allergic rhinitis and asthma, wherein a large increase in the number of circulating, airway tis-

sue, and induced sputum eosinophils has been observed[35,36]. Given the high concordance of eosinophils and, in particular, allergic asthma pathology, it is widely speculated that eosinophils have an important role in the pathology of this disease[35,36]. IL-13 produced by inflammatory cells, especially Th2 cells, induces many features of allergic lung disease, including airway hyperresponsiveness, goblet cell metaplasia, and mucus hypersecretion, which all contribute to airway obstruction[37,38]. In our study, the levels of IL-5 and IL-13 were similar between non-smokers and ex or current smokers, likely because both interleukins play important roles in the pathogenesis of asthma, regardless of smoking.

IL-33 is a recently described member of the IL-1 family and induces signaling via its heterodimeric receptor, ST2, and the IL-1R accessory protein[11,12]. ST2 is abundantly expressed on the surface of mast cells and Th2 cells, and like IL-33, ST2 is critical for the Th2 response[12]. IL-33 can also promote the pathogenesis of asthma by inducing the production of Th2 cells, as demonstrated in studies in which blocking IL-33 signaling suppressed the asthmatic response[39,40]. In the present study, the increase in IL-33 was especially noticeable in atopic asthma patients who were non-smokers, compared with non-atopic asthma patients who were non-smokers or those who were heavy smokers. This finding implies that the involvement of IL-33 in the pathogenesis of asthma may differ according to the phenotype of asthma patients.

RANTES, which is generated by T lymphocytes, is one of the most extensively studied C-C chemokines in allergic inflammation[41]. RANTES is likely to be important in airway inflammation because blocking antibodies to RANTES inhibited airway inflammation in a murine model of allergic airway disease[42]. A growing body of evidence suggests that many cell types present in the airways of asthma patients, such as T cells, platelets, macrophages, fibroblasts, epithelial cells, and mast cells, have the capacity to generate RANTES[43~45], which directly supports the potential role of RANTES in asthma. In our study, the increase in RANTES was especially notable in asthma patients who were non-smokers, compared with ex or current smokers. This result suggests that the involvement of RANTES in the pathogenesis in asthma may differ between asthma patients who smoke and those who were non-smokers.

IL-33 is produced by antigen- or infection-dependent apoptotic or necrotic epithelial cells[46,47]. IL-33 is activated in Th2 cells and eosinophils through the ST2 receptor and is induced by Th2 cytokines, such as IL-4, IL-5, and IL-13[12]. RANTES can be produced by several inflammatory cells including T cells and macrophages, and epithelial cells[43~45]. Moreover, RANTES is chemotactic for T cells, eosinophils, and basophils and plays an active role in recruiting leukocytes into inflammatory sites[9,10]. In our study, a good correlation was seen between IL-33 and RANTES. In addition, there was a good correlation between RANTES and lymphocytes in BAL fluid of asthma patients who were non-smokers. This finding suggests that the cellular source of this cytokine (RANTES) is bronchial epithelial cells. RANTES might also play an important role in recruiting lymphocytes for the pathogenesis of atopic asthma in non-smokers. On the other hand, no correlation was noted between individual cell populations, especially lymphocytes and IL-33, indicating that IL-33 might be induced only slightly in Th2 cells existing in lymphocytes. In general, IL-5 correlated well with eosinophils in the BAL fluid of asthma patients[48]. In our study, however, no correlation was noted between eosinophils and IL-5 in atopic asthma patients who were non-smokers and in those who currently smoked (data not shown). This discrepancy was thought to be due to the effect of eotaxin, a CC chemokine strongly recruiting eosinophils[49].

IL-17 acts as a potent mediator in delayed-type reactions by increasing chemokine production in various tissues to recruit monocytes and neutrophils to the site of inflammation[50]. IL-17 is produced by Th cells[51]. In our study, the levels of IL-17 were similar between non-smokers and current smokers, but the correlation between IL-17 and neutrophils was unclear. In general, IL-17 is chemotactic for neutrophils and plays an important role in the pathogenesis of asthma[50,51]. This discrepancy was considered to be due to the involvement of IL-8 and TNF-α inducing chemotaxis to neutrophils[19,20].

In conclusion, the effect of lymphocytes in asthma patients who were non-smokers and neutrophils in asthma patients who currently smoke might strongly

mediate the induction of airway inflammation. Furthermore, although the involvement of cytokines in smoking asthma was unclear, IL-33 and RANTES play an important role in the pathogenesis of atopic asthma patients who were non-smokers. The pathogenesis of allergic airway inflammation may differ according to smoking status, including the number of cigarettes, the duration of smoking, and phenotype of asthma.

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