

Original article

Elevated interleukin-18 levels in bronchoalveolar lavage fluid of patients with eosinophilic pneumonia

Background: Interleukin (IL)-18 can induce Th2 cytokine production particularly in collaboration with IL-2. Accumulation of Th2 cells and increased levels of Th2 cytokines are found in bronchoalveolar lavage fluid (BALF) from patients with eosinophilic pneumonia (EP). To evaluate the role of IL-18 in the pathogenesis of EP, we measured the concentration of IL-2, IL-12, IL-18, and Th2 cytokines in BALF from patients with EP.

Methods: The concentrations of interferon (IFN)- γ , IL-2, IL-5, IL-10, IL-12, IL-13, and IL-18 in BALF were measured in patients with idiopathic acute eosinophilic pneumonia (AEP), with idiopathic chronic eosinophilic pneumonia (CEP), with sarcoidosis and healthy volunteers (HV).

Results: The BALF concentrations of Th2 cytokines, IL-5, IL-10, and IL-13, were higher in patients with EP than in sarcoidosis and control. The IL-2 level in BALF was higher in EP than in sarcoidosis and control. The IL-18 and IL-12 (p40 + p70) levels were higher in patients with EP than sarcoidosis, while the level of IL-12 (p70) was below the detection limit in patients with EP. There was a significant correlation between IL-2 level and both IL-5 and IL-13 in BALF of patients with EP.

Conclusions: Our findings suggest that IL-18 may contribute to Th2 cytokine-dominant responses in patients with EP in collaboration with IL-2.

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Eosinophilic pneumonia (EP) is an inflammatory lung disease characterized by infiltration of eosinophils into the alveolar space and interstitium of the lung (1). Increased influx of eosinophils into the lung can be caused by fungal and helminthic infection as well as various drug reactions (2). Idiopathic eosinophilic pneumonia, which has no known cause, includes two clinical types, acute eosinophilic pneumonia (AEP) and chronic eosinophilic pneumonia (CEP) (3, 4). We have reported that local production of interleukin (IL)-5 and eotaxin might be important in patients with EP including AEP and CEP (5–7). Previous studies have also suggested that activated T lymphocytes are responsible for pulmonary eosinophilia (8, 9). Recent studies including those from our laboratory have demonstrated the accumulation and activation of T helper 2 (Th2) cells at the sites of inflammation in EP (10, 11).

The IL-18 was identified as an interferon (IFN)- γ -inducing factor that plays an important role in Th1 cell development especially in collaboration with IL-12. It induces the production of IFN- γ from T, B and natural killer (NK) cells, particularly in the presence of IL-12 (12–14). However, IL-18 also acts as a potent inducer of atopic

responses. Native T cells and NK cells can produce IL-4 and IL-13 in response to IL-18, particularly in collaboration with IL-2 (15, 16). Recent studies have indicated that IL-18 can also induce the production of Th2 cytokines, e.g. IL-4, IL-5, IL-10, IL-13, and IgE (17–19). Furthermore, a complex pleiotropic role for IL-18 has also been reported in human clinical studies. High concentrations of IL-18 have been detected in patients with pulmonary sarcoidosis as well as in patients with acute asthma (20, 21). The balance of IL-18, IL-2, and IL-12 seems to be critical for the development of Th1 and Th2 cells.

The present study was designed to determine the role of Th2 and proinflammatory cytokines in the pathogenesis of EP. For this purpose, we measured the concentrations of IFN- γ , IL-2, IL-5, IL-10, IL-12, IL-13, and IL-18 in the bronchoalveolar lavage fluid (BALF) of patients with EP, and results were compared with those of healthy volunteers (HV) and patients with sarcoidosis, which is considered a Th1-type disease.

Material and methods

Patient population

Included in this study were 29 untreated patients with EP consisting of 12 patients with AEP (four women and eight men;

Abbreviations: AEP, acute eosinophilic pneumonia; BALF, bronchoalveolar lavage fluid; CEP, chronic eosinophilic pneumonia; ELISA, enzyme-linked immunosorbent assay; IL, interleukin.

age 24.2 ± 4.4 years, mean $\pm$ SEM) and 17 patients with CEP (12 women and five men; age 53.4 ± 3.2 years), 15 with sarcoidosis (11 women and four men; age 55.3 ± 3.3 years), and 12 HV (four women and eight men; age 44.5 ± 4.4 years). None of the patients in this study was treated with corticosteroids at the time of the investigation. Eleven patients with AEP, four with sarcoidosis, and two of the volunteers were smokers.

The diagnosis of EP was based on clinical criteria; patients with no known cause of EP (drugs, parasitic infection, and fungal disease), with acute or chronic dyspnea, interstitial infiltrates on chest radiographs, pulmonary eosinophilia, prompt resolution of clinical and radiographic abnormalities with or without corticosteroid therapy, and histopathologic evidence based on examination of transbronchial lung biopsy. The diagnosis of AEP and CEP was established based on the criteria of Allen et al. (3) and Carrington et al. (4). Briefly, the diagnosis of AEP was based on the following criteria: acute febrile illness, severe hypoxemia (partial pressure of arterial oxygen <60 mmHg), diffuse pulmonary infiltrates on chest radiographs, eosinophilia ($>25\%$) in BALF, an absence of infection and previous atopic illness, a prompt and complete response to corticosteroids and no relapse after the discontinuation of therapy with corticosteroids. All CEP patients had the clinical characteristics such as fever, cough, dyspnea, and weight loss, and the roentgenographic observations (dense infiltrates arranged in a peculiar peripheral pattern) described by Carrington et al. (4), including eosinophilia in BALF (Table 1) and histologic findings on transbronchial lung biopsy. The diagnosis of sarcoidosis was based on examination of biopsy specimens obtained from the lungs showing noncaseating epithelioid cell granulomas, with no evidence of inorganic material known to cause granulomatous diseases.

Bronchoalveolar lavage

After obtaining written informed consent from the subject, BAL was performed using a flexible fiberoptic bronchoscope under local anesthesia of the upper airway with 2% lidocaine, as described previously (22). Briefly, the bronchoscope was wedged into the subsegmental bronchus of the right middle lobe or, in patients with EP, into areas of the lung parenchyma otherwise normal on the chest roentgenogram, and 150 ml of normal saline was instilled in 50 ml aliquots. The harvested BALF was filtered through a sterile nylon mesh and centrifuged at $160 \times g$ for 10 min to obtain the cell preparation. The cells were later stained by the May-Giemsa method and a differential count was performed on 200 cells. The remaining fluid was centrifuged at $500 \times g$ for 5 min and the supernatant was stored at -80°C until analysis. The mean BALF recoveries are listed in Table 1. There were no differences in the percentage recovery of lavage fluid between the subject groups.

Measurement of IL-2, IL-5, IL-10, IL-12, IL-13, IL-18, and IFN- γ concentrations in BALF

The BALF concentrations of IL-10, IL-12 (p40 + p70), IL-12 (p70), and IFN- γ were measured using the respective enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Inc., Camarillo, CA). The IL-13 concentration in BALF was also measured by an ELISA kit (CLB, Amsterdam, the Netherlands). The IL-2 concentration was measured using an ELISA kit (R&D Systems, Inc., Minneapolis, MN). The IL-18 concentration was measured by an ELISA kit (MBL, Nagoya, Japan). The concentration of IL-5 was measured by ELISA as described previously (5). The detection limits were 0.2, 0.8, 0.5, 4.0, 0.5, 0.8, 12.5, and 2.5 pg/ml for IL-10, IL-12 (p40 + p70), IL-12 (p70), IFN- γ , IL-13, IL-2, IL-18, and IL-5, respectively. All assays were performed in duplicate. For statistical analysis, the comparison was performed using the mean of replicate measurements. Concentrations below the detection limits were assumed to be zero for the purpose of statistical analysis. The absolute concentrations of cytokines in BALF rather than those relative to proteins, are reported in this study since cytokine levels in BALF are not influenced by the concentration of albumin, as demonstrated in our previous study (22).

Statistical analysis

All data were expressed as mean $\pm$ SEM. The Kruskal-Wallis test was used to compare values of different groups. In case of a significant difference between groups, inter-group comparisons were assessed by the Mann-Whitney *U*-test. Differences with probability values of <0.05 were considered significant. Correlations between two variables were examined using Pearson's correlation coefficient.

Results

Characteristics of BALF cells

Table 1 summarizes the total number of leukocytes in BALF and their differential count in the four subject groups. The total number of cells per ml of BALF in patients with AEP and CEP were higher than that in healthy subjects. Differential cell count showed that the percentage of eosinophils in AEP and CEP, but not in sarcoidosis, was significantly higher than the control ($P < 0.01$ each, Table 1). There was no difference in eosinophil percentages between the AEP and CEP groups. Furthermore, the absolute number of eosinophils was higher in patients with AEP and CEP compared with

Table 1. Total and differential cell count in bronchoalveolar lavage fluid (BALF) of healthy volunteers, patients with acute eosinophilic pneumonia, chronic eosinophilic pneumonia, and sarcoidosis

	Total cells ($10^5/\text{ml}$)	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)	Recovery (%)
Healthy volunteers ($n = 12$)	1.5 ± 0.2	87.5 ± 1.3	9.4 ± 1.2	2.4 ± 1.1	0.7 ± 0.3 (0–3.7)	50.2 ± 3.2
Eosinophilic pneumonia						
Acute ($n = 12$)	$7.9 \pm 1.5^*$	$28.1 \pm 4.9^*$	$22.8 \pm 3.9^*$	2.6 ± 0.8	$45.1 \pm 4.5^*$ (30.6–65.0)	50.1 ± 4.3
Chronic ($n = 17$)	$10.2 \pm 2.4^*$	$25.0 \pm 4.6^*$	20.0 ± 5.0	9.8 ± 3.0	$45.0 \pm 5.9^*$ (26.1–91.0)	50.0 ± 3.8
Sarcoidosis ($n = 15$)	1.9 ± 0.3	$60.3 \pm 5.7^*$	$34.0 \pm 5.1^*$	4.8 ± 2.6	0.4 ± 0.1 (0–1.8)	55.2 ± 3.0

* $P < 0.01$ compared with healthy volunteer subjects.

HV and sarcoidosis ($P < 0.01$ each, data not shown). The percentage of lymphocytes was significantly higher in patients with sarcoidosis and AEP than in healthy subjects ($P < 0.01$ each, Table 1). In addition to the eosinophilia, the absolute number of lymphocytes was higher in patients with AEP and CEP compared with HV ($P < 0.01$ each, data not shown).

IL-5, IL-10, and IL-13 concentrations in BALF

Typical Th2 cytokines (IL-5, IL-10, and IL-13) were assessed in BALF of patients with EP. Interestingly, the concentrations of the above three Th2 cytokines were elevated in patients with EP, but were not detected in the normal control and sarcoidosis. The mean BALF concentrations of IL-5, IL-10, and IL-13 in patients with AEP (1788.7 ± 551.8 , 1.02 ± 0.28 , and 109.7 ± 81.3 pg/ml, respectively) were higher than those of patients with CEP (216.0 ± 122.9 , 0.53 ± 0.17 , and 8.6 ± 4.2 pg/ml, respectively). The differences between AEP and CEP were significant for IL-5 ($P = 0.001$) and IL-13 ($P = 0.005$), but not for IL-10 ($P = 0.13$) (Fig. 1). As a typical Th1 cytokine, INF- γ concentration was measured in BALF. The INF- γ was not detected in any BALF sample (data not shown). These results suggest that Th2 cytokines could play an important role in the pathogenesis of EP.

IL-2, IL-12, and IL-18 concentrations in BALF

The combination of IL-2, IL-12, and IL-18 has been suggested to influence the differentiation of Th1 and Th2 cells (13–16). In the next step, we measured the level of IL-2, IL-12 (p40 + p70), IL-12 (p70), and IL-18 in BALF of the four groups. The mean concentration of IL-2 in BALF of EP (AEP: 7.2 ± 4.8 , CEP: 0.5 ± 0.2 pg/ml) was significantly higher than that of sarcoidosis ($P = 0.0003$ and $P = 0.0061$, respectively) and healthy subjects ($P = 0.001$ and $P = 0.013$, respectively, Fig. 2). The IL-2 concentration in BALF of AEP was significantly higher than in CEP ($P = 0.0329$, Fig. 2). Interestingly, IL-18 levels were elevated in patients with sarcoidosis (36.6 ± 9.6 pg/ml), as well as in those with EP (AEP: 101.2 ± 40.5 , CEP: 194.2 ± 58.7 pg/ml), compared with the healthy subjects (5.2 ± 2.4 pg/ml). Surprisingly, IL-18 levels were higher in patients with EP compared with those of sarcoidosis (AEP: $P = 0.0092$, CEP: $P = 0.0024$) (Fig. 2). The IL-12 (p40 + p70) level was higher in patients with sarcoidosis (18.9 ± 7.1 pg/ml) compared with the healthy subjects (1.5 ± 0.7 pg/ml). On the contrary, IL-12 (p40 + p70) levels were significantly higher in patients with EP (AEP: 39.6 ± 12.7 , CEP: 51.9 ± 15.7) than sarcoidosis ($P = 0.0025$, $P = 0.0003$, respectively). The IL-12 (p70) concentrations were below the detection limit in patients with EP and healthy subjects, while small levels were detected in some patients with sarcoidosis (Fig. 2).

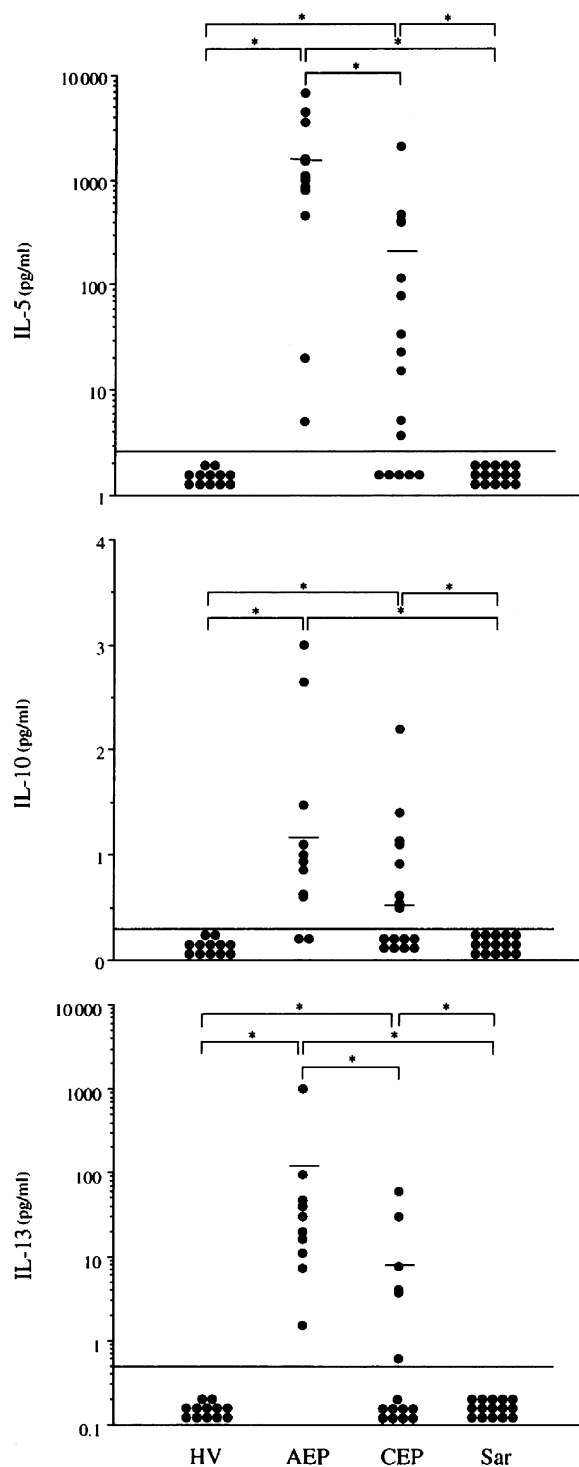


Figure 1. Concentrations of interleukin (IL)-5, IL-10, and IL-13 in bronchoalveolar lavage fluid (BALF) of patients with acute eosinophilic pneumonia (AEP), chronic eosinophilic pneumonia (CEP), sarcoidosis (Sar) and healthy volunteers (HV). Short horizontal lines represent the mean values. The horizontal lines represent the detection limit of each enzyme-linked immunosorbent assay (ELISA) for the cytokine. * $P < 0.01$ compared with each of the other groups. Symbols represent individual subjects.

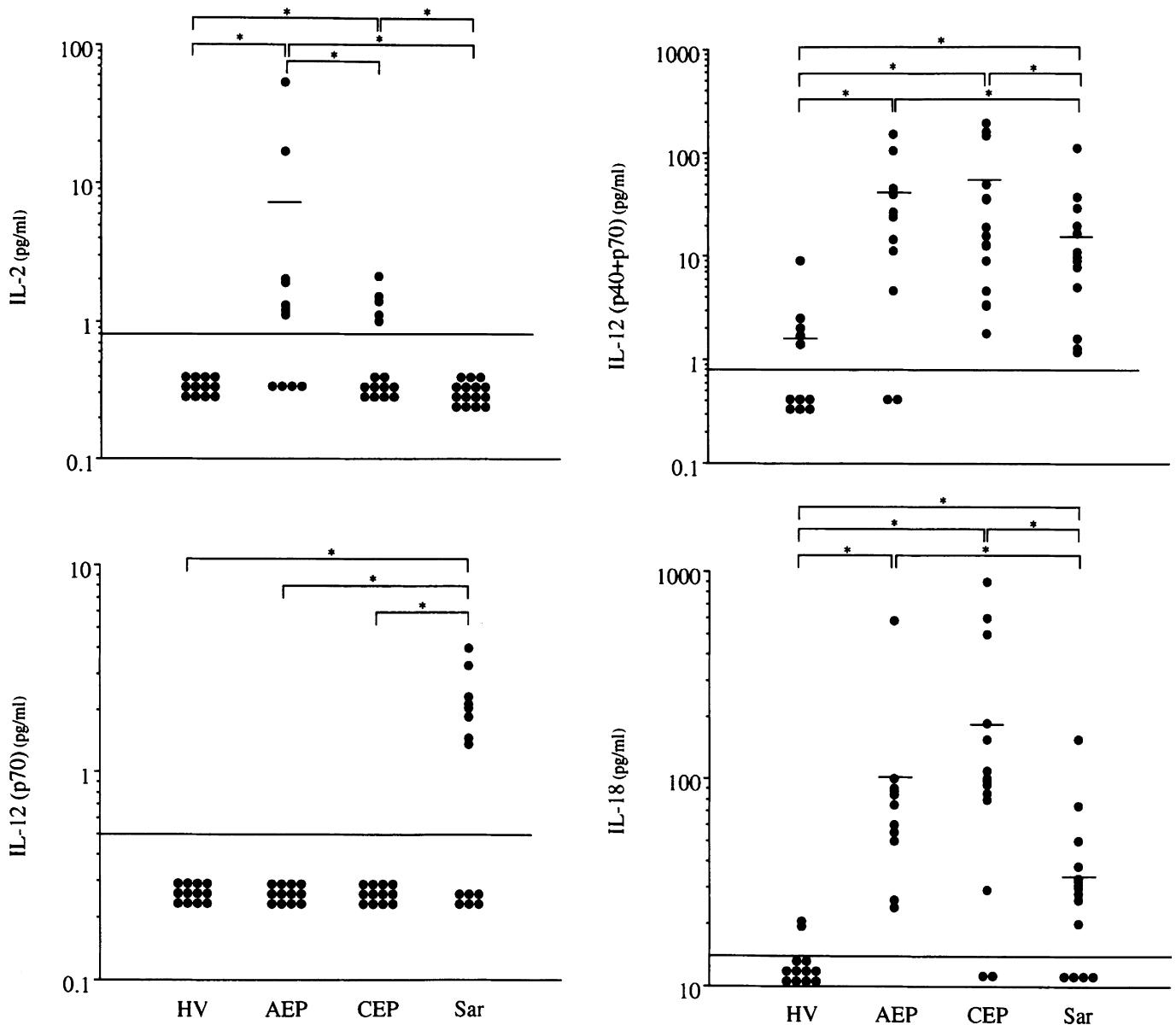


Figure 2. Concentrations of interleukin (IL)-2, IL-12 (p40 + p70), IL-12 (p70), and IL-18 in bronchoalveolar lavage fluid (BALF) of patients with acute eosinophilic pneumonia (AEP), chronic eosinophilic pneumonia (CEP), sarcoidosis (Sar) and healthy volunteers (HV). Short horizontal lines represent the mean values. The horizontal lines represent the detection limit of each enzyme-linked immunosorbent assay (ELISA) for the cytokine. * $P < 0.01$ compared with each of the other groups. Symbols represent individual subjects.

Statistically, smoking status had no influence on BALF cytokine levels in each subject group (data not shown).

Finally, we examined the relationship between the two groups of cytokines. There were not any significant correlations between IL-18 levels and the concentrations of Th2 cytokines, between IL-12 levels and Th2 cytokines levels, in BALF of patients with EP (data not shown). Interestingly, there was a significant correlation between IL-2 levels and both IL-5 ($r = 0.419$, $P = 0.026$) and IL-13 ($r = 0.944$, $P < 0.0001$) concentrations in BALF of patients with EP (Fig. 3).

Discussion

The EP is a group of disorders characterized by eosinophil accumulation (1). Accumulation and activation of T lymphocytes was also described in patients with EP (8, 9). In chronic conditions, T lymphocytes develop into Th1 or Th2 cells, based on their profiles of cytokine production (23). We have recently reported accumulation of CCR4-expressing CD4⁺ T cells in BALF of patients with EP (11). As previously reported (10), high levels of Th2 cytokines (IL-5, IL-10, and IL-13) were also confirmed in

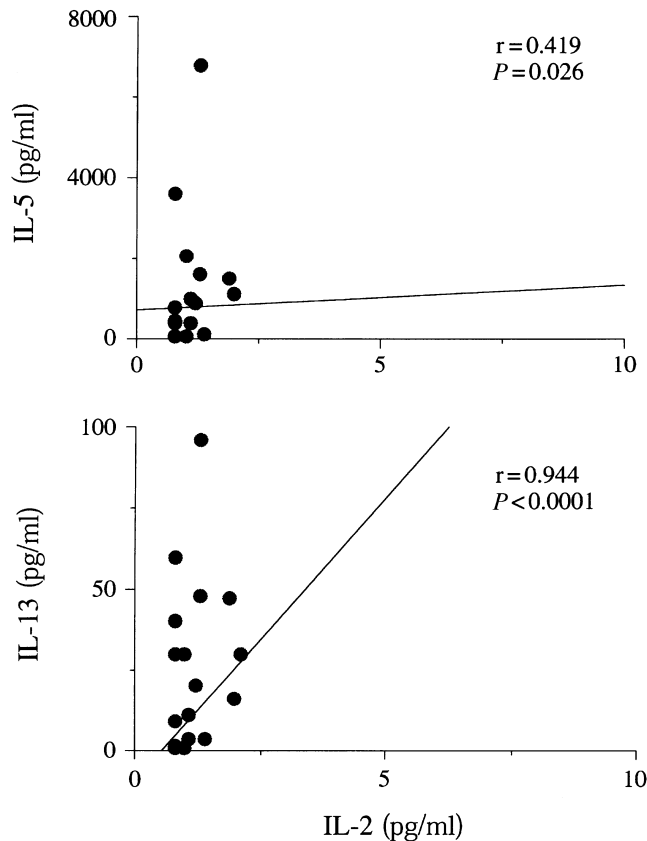


Figure 3. Correlation between interleukin (IL)-2 and IL-5 (top), and between IL-2 and IL-13 (bottom) concentrations in bronchoalveolar lavage fluid (BALF) of patients with eosinophilic pneumonia.

this study (Fig. 1). The accumulation of Th2 cells into the lung is thought to be critical in the pathogenesis of EP.

In addition to Th2 cytokines, high BALF concentrations of IL-2 were reported in patients with EP by Walker et al. (8). We have recently reported the presence of elevated concentrations of IL-2 in BALF of a murine model of eosinophilic inflammation of the lung (24). In the present study, we also found slightly but significantly high concentrations of IL-2 in BALF of patients with EP (Fig. 2). The IL-2 acts as a growth factor for activated T cells (25). It has also been reported that IL-2 induces and enhances IL-5 production by CD4⁺ T cells (26, 27). Furthermore, recent studies suggest that IL-2 acts as a synergistic inducer of the expression of Th2 cytokines in combination with IL-18 (15, 16).

The IL-18 is a pleiotropic cytokine. A number of animal and *in vitro* studies have implicated IL-18 in the divergence of Th1 and Th2 immune response. The IL-18 was initially discovered as an IFN- γ -inducing factor, and is a novel cytokine that plays an important role in Th1 cell response primarily through its ability to induce IFN- γ production, especially in collaboration with IL-12 (12–14). In addition, IL-18 together with IL-12 inhibits

IgE production by induction of IFN- γ production (13). On the contrary, Hoshino et al. (15) reported that IL-18 can induce IL-10, IL-13, and IFN- γ production by T cells in the presence of IL-2. Furthermore, Yoshimoto et al. (19) demonstrated that IL-18 can increase IgE production in a CD4⁺ T cell-, IL-4- and STAT6-dependent manner. Recent experimental studies also reported that splenic T cells in IL-18 transgenic mice produce higher levels of IFN- γ , IL-4, IL-5, and IL-13 than the control wild-type mice. Aberrant expression of IL-18 *in vivo* results in increased production of both Th1 and Th2 cytokines (28). Furthermore, clinical studies also showed high levels of IL-18 in both serum and BALF of patients with sarcoidosis, which is a Th1 cytokine-dominant disease (20). On the contrary, low levels of IL-18 have been reported in BALF of asthmatics, while other studies demonstrated high serum IL-18 levels in patients with acute exacerbations of asthma (21, 29). Therefore, IL-18 seems to play a distinct role in asthma, depending on the immunologic environment. Elevated IL-18 levels might induce a different immunologic response in Th1 and Th2 cytokine-dominant disease. In the present study, we first demonstrated increased level of IL-18 in BALF of patients with AEP and CEP compared with sarcoidosis and healthy subjects. Our observation suggests that IL-18 could induce Th2 response in the presence of IL-2 in patients with EP, which is thought to be a Th2 cytokine-dominant disease. Indeed, IL-2 levels correlated positively with levels of Th2 cytokines (IL-5 and IL-13) in BALF of patients with EP (Fig. 3).

The IL-12 [IL-12 (p70)] is a heterodimer composed of two disulfide-linked subunits of 35 kDa (p35) and 40 kDa (p40), which are encoded by two separate genes. Association of IL-12 (p35) and IL-12 (p40) subunits forms the bioactive heterodimer of 70–75 kDa [IL-12 (p70)] (30, 31). The biologic activities of IL-12 (p70) require interaction of the IL-12 (p40) subunit with the β 1-chain of the IL-12 receptor, and the interaction of IL-12 (p35) subunit with the β 2-chain of the IL-12 receptor (32). The β 2-chain of the IL-12 receptor was induced upon activation through the T-cell receptor on naive CD4⁺ T cell and its expression was increased during IL-12 induced Th1 development (33, 34). The IL-12 (p70) is a key cytokine for induction of Th1 immune response. In addition to dimerizing with IL-12 (p35) to form the heterodimeric molecule IL-12 (p70), the IL-12 (p40) subunit can also form homodimers. Both *in vitro* and *in vivo* studies have shown that IL-12 (p40) acts as an antagonist of IL-12 (p70) because of its ability to compete with IL-12 (p70) for binding to the IL-12 receptor (35, 36). In the present study, we first demonstrated that the IL-12 (p40 + p70) is overproduced in patients with EP, but not the IL-12 (p70) heterodimer, compared with sarcoidosis. Increased levels of IL-12 (p40) subunit in BALF of EP could inhibit IL-12 (p70) and IL-18 induced Th1 development.

In conclusion, we have demonstrated in the present study the presence of elevated levels of Th2 cytokines in BALF of patients with EP. Furthermore, higher concentrations of IL-18 and IL-2 were found in BALF of patients with EP than those of patients with sarcoidosis. These findings suggest that IL-18 could

induce Th2 cytokine-dominant responses in patients with EP in collaboration with IL-2. Further studies designed to examine the expression of IL-2, IL-12, and IL-18 receptors on inflammatory cells are necessary to clarify the role of these cytokines in eosinophilic lung diseases.

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