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Original

BAL Fluid Concentrations of Cytokines in Patients with Nonspecific Interstitial Pneumonia, Usual Interstitial Pneumonia, Collagen Vascular Disease Associated with Interstitial Pneumonia, and Sarcoidosis

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SUMMARY

Background : Inflammatory cytokines have been reported to play important roles in the pathogenesis of interstitial lung diseases. However, their individual roles in idiopathic interstitial pneumonitis (IIP) and in the other types of interstitial pneumonitis (IP), including collagen vascular disease associated interstitial pneumonitis (CVD-IP), remain unknown. In this study, we measured the bronchoalveolar lavage (BAL) fluid levels of several cytokines in patients with IIP and CVD-IP.

Methods : Cell subpopulations in BAL fluid were counted, and BAL fluid levels of interleukin (IL)-2, -6, -7, -8, -17, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β1 were measured using a bead suspension array or an enzyme-linked immunosorbent assay (ELISA) kit in 16 patients (8 men, 8 women ; mean age, 60.0±9.9 years) with idiopathic nonspecific interstitial pneumonitis (NSIP), 5 patients (3 men, 2 women ; mean age, 69.0±4.8 years) with idiopathic usual interstitial pneumonitis (UIP), 5 patients (3 men, 2 woman ; mean age, 66.3±5.5 years) with rheumatoid arthritis in CVD-IP (RA), and 5 patients (3 man, 2 women ; mean age, 52.3±14.5 years) with dermatomyositis in CVD-IP (DM), and 13 patients (3 men, 10 women ; mean age, 51.8±17.2 years) with sarcoidosis.

Results : BAL cell subpopulations had high amounts of lymphocytes in NSIP and sarcoidosis, and neutrophils in RA. Levels of IL-7 were significantly ($P<0.05$) higher in DM (9.2±2.2 pg/ml) than in RA (4.5±1.7 pg/ml). IL-7 in DM was significantly ($P<0.05$) correlated with lymphocytes. The levels of TNF-α were highest for RA (18.6±29.5 pg/ml), compared with other IPs (1.6±0.7 pg/ml in UIP, 1.8±2.7 pg/ml in NSIP, 4.4±4.5 pg/ml in DM), and sarcoidosis (5.8±6.1 pg/ml). In addition, the levels of IL-17 were highly detectable in RA (2.5±2.5 pg/ml), but not in NSIP, UIP, or sarcoidosis.

Conclusions : Differences in the cell types of BAL fluid and the level of each cytokine between patients with IIP and CVD-IP might reflect pathogenesis and be useful for diagnosis.

Key Words : BAL fluid, cytokine, NSIP, UIP, CVD-IP, sarcoidosis

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Abbreviations used：

IIP：idiopathic interstitial pneumonia, IP：interstitial pneumonia, NSIP：nonspecific interstitial pneumonia, UIP：Usual interstitial pneumonia, CVD-IP：collagen vascular disease-associated with interstitial pneumonia, RA：rheumatoid arthritis, DM：dermatomyositis, BAL：bronchoalveolar lavage, ELISA：enzyme linked immune assay, TGF-β：transforming growth factor-β, TNF：tumor necrosis factor

INTRODUCTION

The classification of idiopathic interstitial pneumonia (IIP) includes seven clinico-radiologic-pathologic entities. Usual interstitial pneumonia (UIP) and nonspecific interstitial pneumonia (NSIP) are the largest subsets of IIP[1~3]. NSIP is distinguished histologically from UIP by a temporal uniformity of interstitial inflammation and/or fibrosis[1~3]. The distinction between NSIP and UIP is important for clinical decision making because prognosis is generally good and the response to corticosteroids and immunosuppressants is also good in patients with NSIP compared with UIP[1~3]. It is well known that NSIP has a relative degree of lymphocytosis with a predominance of CD8 T cells in bronchoalveolar lavage (BAL) fluid compared with UIP[4~6]. An association between lymphocytosis and levels of interleukin (IL)-6 in BAL fluid of patients with NSIP has also been reported[5]. In addition, the level of regulated upon activation, normal T-cell expressed, and secreted (RANTES) in BAL fluid was higher in patients with NSIP than in healthy volunteers and in those with UIP or sarcoidosis. These findings suggest that NSIP has a different pathogenesis from that of UIP but remains definitively unclear. Furthermore, although correlations are well known to exist between collagen diseases, such as rheumatoid arthritis, systemic sclerosis, dermatomyositis, and lung lesions including interstitial pneumonia[7~9], the pathogenesis of these conditions remains unclear. Several studies have reported that inflammatory cytokines, such as IL-1β, IL-2, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-8, are involved in collagen vascular disease-associated with interstitial pneumonia (CVD-IP) as well as in IIP[10~13]. However, the association between cytokine pattern and disease features of interstitial pneumonia remains unknown.

In this study, we measured the BAL fluid levels of IL-2, -6, -7, -8, -17, IFN-γ, TNF-α, and transforming growth factor (TGF)-β1 in patients with NSIP, UIP in IIP, and CVD-IP in patients with rheumatoid arthritis and dermatomyositis, with sarcoidosis, to determine the likelihood of a distinct cytokine profile for these diseases and to evaluate the correlations between BAL cell populations and the investigated cytokines.

METHODS

Patients

Subjects were patients at the Dokkyo Medical University School of Medicine Hospital and consisted of 5 patients with idiopathic UIP (3 males and 2 females；mean age, 69.0±4.8 years；age range 61–76 years), 16 with idiopathic NSIP (8 males and 8 females；mean age, 60.0±9.9 years；age range 45–71 years), 5 with CVD-IP (rheumatoid arthritis (RA)) (3 males and 2 females；mean age, 676.3±5.5 years；age range 60–70 years), 5 with CVD-IP (dermatomyositis (DM)) (3 males and 2 females；mean age, 52.3±14.5 years；age range 43–69 years), and 13 with pulmonary sarcoidosis (3 males and 10 females；mean age, 51.8±17.2 years；age range 23–70 years) (Table 1).

None of the patients had received steroid therapy at the time of clinical sample collection. Patients with cancer in any organ and those suspected to have malignancy were excluded from the study, and no malignancies were detected in any patient during the study. Diagnosis was confirmed pathologically by lung biopsy in patients with IIP and CVD-IP, and with sarcoidosis as a disease control. Patients with IP associated with collagen vascular diseases diagnosed clinically were included as RA and DM. Diagnosis of RA was clinically established based on the American College of Rheumatology/European League Against Rheumatism[14]. Muscle biopsy samples from patients newly diagnosed with active DM, according to the classification system of Bohan and Peter[15,16], were evaluated. Patients with sarcoidosis with pulmonary lesions were also enrolled. Diagnosis was clinically established based on the path-

Table 1 Clinical characteristics of patients with UIP, NSIP, CVD-IP (RA), CVD-IP (DM), and sarcoidosis. Data are presented as mean ± SD values.

	Sex Male/Female Age (mean ± SD)	BAL Total cells ($\times 10^5$/ml)	Eo (%)	Ly (%)	Neu (%)	MΦ (%)	Serological analysis LDH (IU/L) (200-400)	KL-6 (U/mL) (<500)	SPD (ng/mL) (<110)
NSIP	8/8 60.0 ± 9.9 years	3.8 ± 2.0	5.6 ± 9.6	25.5 ± 21.4	10.0 ± 10.0	56.4 ± 27.6	372.5 ± 159.8	2175.4 ± 1460.9	308.5 ± 188.8
UIP	3/2 69.0 ± 4.8	4.5 ± 2.5	5.8 ± 7.1	12.0 ± 8.9	11.3 ± 13.7	70.2 ± 18.5	389.8 ± 185.0	1442.1 ± 697.2	295.2 ± 131.6
CVD-IP (RA)	3/2 66.3 ± 5.5	3.3 ± 2.0	3.9 ± 3.1	18.9 ± 9.4	26.6[b] ± 21.3	49.8 ± 20.8	480.5 ± 102.2	1347.8 ± 1058.2	236.5 ± 140.8
CVD-IP (DM)	3/2 52.3 ± 14.5	3.3 ± 1.1	0.7 ± 1.2	14.9 ± 5.5	5.2 ± 5.2	77.9 ± 2.9	1145.3[c] ± 408.8	2289.3 ± 2784.7	178.9 ± 161.2
Sarcoidosis	3/10 51.8 ± 17.2	3.8 ± 1.9	1.5 ± 2.1	48.9[a] ± 14.8	1.5 ± 3.8	47.6 ± 14.9	301.8 ± 86.4	754.6 ± 741.9	175.0 ± 118.8

[a]P<0.05, compared with UIP and CVD-IP. [b]P<0.05, compared with CVD-IP (DM) and sarcoidosis. [c]P<0.05, compared with the other IPs. Eo : eosinophils ; Ly : lymphocytes ; Neu : neutrophils ; MΦ : macrophages ; LDH : lactate dehydrogenase ; KL-6 : Krebs von den lungen ; SPD : surfactant protein D.

ological findings of noncaseous epithelioid cell granulomas in lung biopsy samples[17].

The study protocol was approved by the Human Ethics Review Committee of Dokkyo Medical University, and a signed consent form was obtained from each subject.

Bronchoalveolar lavage

BAL fluid samples were obtained from all subjects using a flexible fiberoptic bronchoscope (Olympus 1T-200, Olympus, Tokyo, Japan) after local anesthesia of the upper airway with 4% lidocaine as described previously[18]. Briefly, the bronchoscope was wedged for lavage into one of the subsegmental bronchi of the right middle lobe or, in patients with peripheral opacities, into areas of lung parenchyma otherwise abnormal on chest radiograph. 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 centrifuged at 500×g for 10 min at 4℃. The BAL fluid was centrifuged at 500×g for 5 min, and the supernatant was stored at −80℃ for further quantification of noncellular components. After washing twice with phosphate buffered saline solution, cells were suspended with 10% heat-inactivated fetal calf serum and counted using a haemocytometer. Differential cell counts were determined from cell suspensions displayed on slides using a cytocentrifuge (Cytospin 2 ; Shandon Instruments ; Sewickley, PA). Cells were dried, fixed on the slide, and stained by the May-Grünwald-Giemsa method. Two hundred cells were identified under a photomicroscope.

Measurement of IL-2, -6, -7, -8, -17, IFN-γ, TNF-α, and TGF-β1 in BAL fluid

BAL fluid samples were concentrated by 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 by the ratio of protein consistency in nonconcentrated BAL fluid to that in concentrated BAL fluid, which was measured by assay (DC protein Assay ; Bio-Rad Laboratories ; Hercules, CA), and the original levels of cytokines were corrected according to this ratio[19]. Since BAL has a dilutional effect on the recovery of cytokines, measurements are occasionally standardized to albumin. A good correlation with IL-6 ($R=0.78$, $P<0.05$) and TNF-α ($R=0.76$, $P<0.05$) was observed between the nonstandardized and standardized values by albumin concentrations in BAL fluid of 5 patients with CVD-IP (RA)

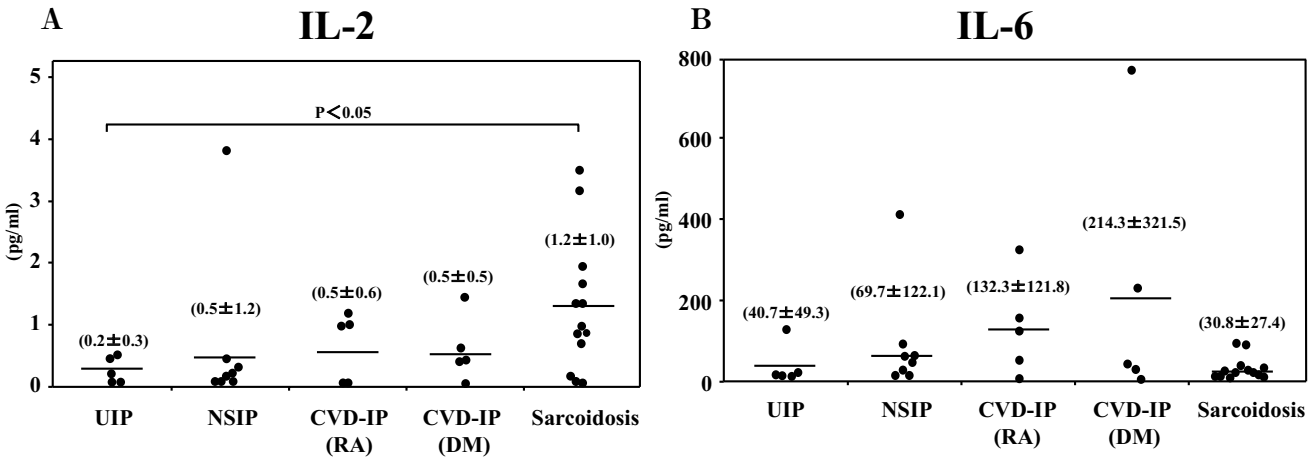

Figure 1 A, B

(data not shown), so the cytokine levels reported in the text are those of measured concentrations rather than those relative to albumin concentrations.

BAL fluid levels of IL-2, -6, -7, -8, -17, IFN-γ, and TNF-α were measured using a bead suspension array, and the detection limits were 0.2 pg/ml. In addition, concentrations of TGF-β1 were measured using ELISA kits (R&D, Minneapolis, MN), as per manufacturer instructions. The detection limit was 31.2 pg/ml.

Statistical analysis

Data are expressed as mean ± SD values. Differences between multiple groups were compared by one-way analysis of variance. Fisher's PLSD test was used as the post hoc test. Spearman's rank correlation analysis was used to examine relationships. Statistical analysis was performed using JMP software (SAS Institute, Inc., Cary, NC). Statistical significance was defined by a P value of less than 0.05.

RESULTS

Differential serological results and cell count of BAL fluid

Table 1 shows serological results and the subpopulations of cells in BAL fluid in all subjects. In serological analysis, lactate dehydrogenase (LDH) levels were significantly ($P<0.05$) higher in CVD-IP (DM) than in the other IPs and in sarcoidosis. Levels of Krebs von den Lungen (KL)-6 tended to be high for NSIP and CVD-IP (DM), compared with UIP, CVD-IP (RA), and sarcoidosis. Levels of surfactant protein D (SPD) tended to be high for NSIP and UIP, compared with CVD-IP (DM), and sarcoidosis. Increased LDH levels in CVD-IP (DM) were thought to include muscle enzyme induced by the disease activity.

In the subpopulations of BAL fluid cells, the percentage of lymphocytes was significantly ($P<0.05$) higher in sarcoidosis than in UIP and CVD-IP (DM). The percentage of lymphocytes tended to be high for NSIP compared with UIP. The percentage of neutrophils was significantly ($P<0.05$) higher in CVD-IP (RA) than in CVD-IP (DM), and sarcoidosis. On the other hand, subpopulations of eosinophils were not significantly increased among these diseases.

BAL fluid levels of IL-2, -6, -7, -8, -17, IFN-γ, TNF-α, and TGF-β1

We measured the levels of IL-2, -6, -7, -8, -17, IFN-γ, TNF-α, and TGF-β1 (Figure 1 A, B, C, D, E, F, G, H, respectively) in the BAL fluid of patients with UIP, NSIP, CVD-IP (RA), CVD-IP (DM), and sarcoidosis. IL-2 was significantly ($P<0.05$) higher in sarcoidosis than in UIP. Furthermore, there were no significant differences between NSIP, CVD-IP (RA), and CVD-IP (DM). Levels of IL-6 tended to be high for CVD-IP (RA) and DM, compared with UIP, NSIP, and sarcoidosis. Levels of IL-7 were significantly ($P<0.05$) higher in CVD-IP (DM) than in CVD-IP (RA). In addition, there were no significant differences between UIP, NSIP, and sarcoidosis. Levels of IL-8 tended to be high for CVD-IP (RA), compared with UIP, NSIP, CVD-IP (DM), and sarcoidosis. IL-17 was detected in

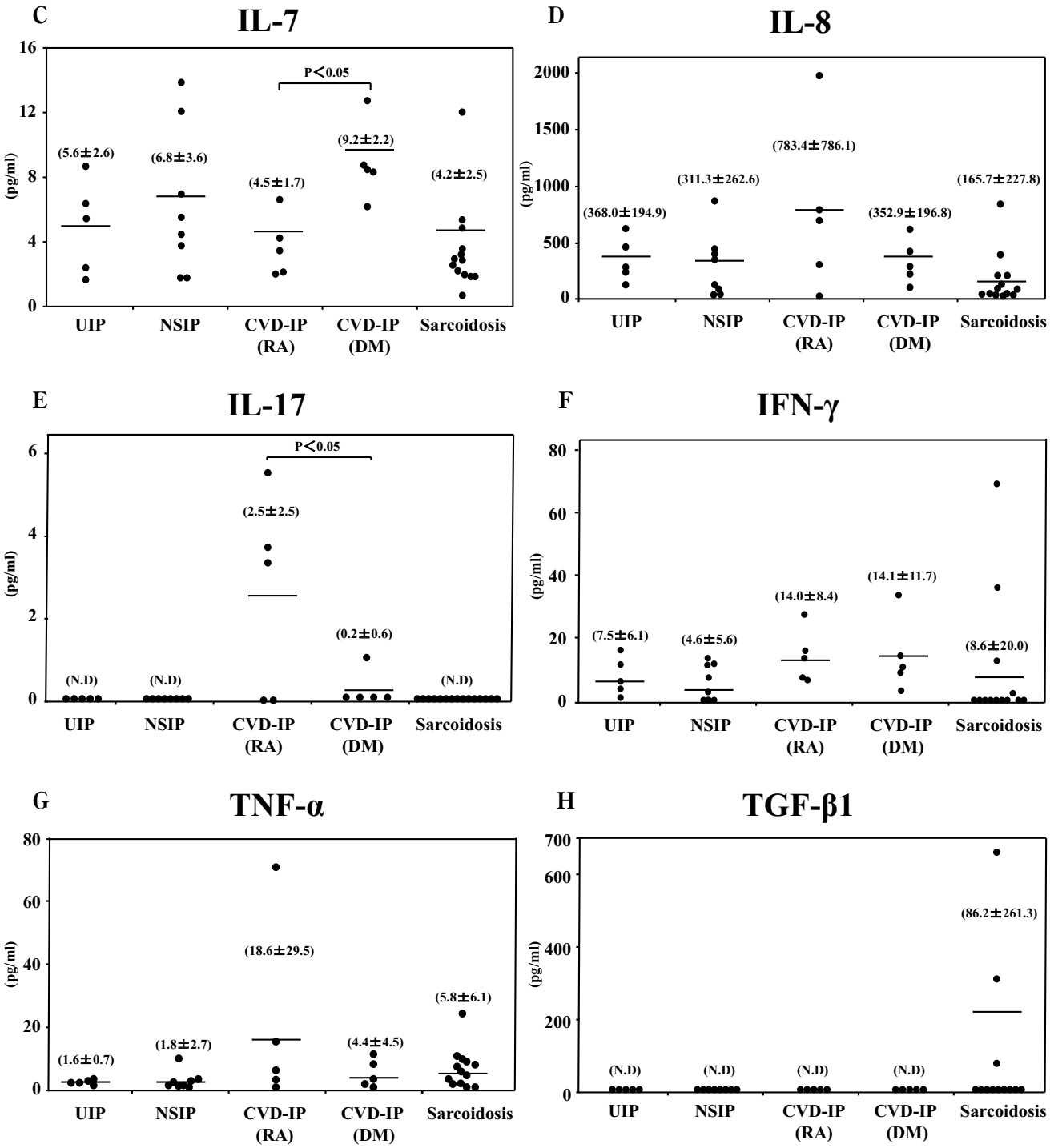

Figure 1 BAL fluid levels of IL-2, -6, -7, -8, -17, IFN-γ, TNF-α, and TGF-β1 (Figure 1 **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H**, respectively) in patients with UIP, NSIP, CVD-IP (RA), CVD-IP (DM), and sarcoidosis. IL-2, -6, -7, -8, -17, IFN-γ, and TNF-α were measured using a bead suspension array. Detection limits were 0.2 pg/ml, respectively. Concentrations of TGF-β1 were measured using ELISA kits, and the detection limit was 31.2 pg/ml. Data are presented as mean ± SD values. $P<0.05$, compared with each IPs.CVD-IP : collagen vascular disease associated with interstitial pneumonia ; NSIP : nonspecific interstitial pneumonia ; UIP : usual interstitial pneumonia ; RA : rheumatoid arthritis ; DM : dermatomyositis.

Table 2 Correlation between cytokines and differential cell counts.

Sperman'srank correlation									
		IL-2	IL-6	IL-7	IL-8	IL-17	IFN-γ	TNF-α	TGF-β
UIP	Macrophages (%)	−0.5643	−0.5000	0.3000	−0.1000	N.D	−0.9000*	−1.0000*	N.D
	Lymphocytes (%)	0.5643	0.1000	0.1000	−0.1000*	N.D	−0.3000	−0.1000	N.D
	Neutrophils (%)	0.6669	0.1000	−0.1000	0.2000	N.D	0.7000	0.9000*	N.D
	Eosinophils (%)	0.1539	−0.4000	0.1000	0.7000	N.D	0.3000	0.4000	N.D
NSIP	Macrophages (%)	−0.0920	−0.1030	0.2492	0.2000	N.D	0.2392	−0.2485	N.D
	Lymphocytes (%)	0.0552	0.0182	−0.3769	−0.1515	N.D	−0.5836	0.1394	N.D
	Neutrophils (%)	−0.0309	0.4024	0.1132	0.3293	N.D	−0.1626	0.7195*	N.D
	Eosinophils (%)	0.0615	0.1228	−0.3696	−0.2004	N.D	−0.0713	0.3951	N.D
CVD-IP (RA)	Macrophages (%)	−0.1316	0.6669	0.4104	0.1539	−0.5000	−0.1026	−0.3591	N.D
	Lymphocytes (%)	0.2052	−0.8000	−0.5000	−0.4000	−0.1539	0.1000	−0.4000	N.D
	Neutrophils (%)	−0.6669	−0.5000	−0.7000	−0.3000	0.4104	−0.3000	0.8000	N.D
	Eosinophils (%)	0.6669	0.3000	0.6000	0.1000	−0.0531	0.6000	−0.6000	N.D
CVD-IP (DM)	Macrophages (%)	−0.1000	−0.3000	0.1000	0.2000	0.7071	−0.3000	−0.3000	N.D
	Lymphocytes (%)	−0.1000	0.5000	0.9000*	0.3000	−0.3536	0.5000	0.7000	N.D
	Neutrophils (%)	−0.4000	−0.8000	−0.6000	−0.7000	0.0000	−0.8000	−0.7000	N.D
	Eosinophils (%)	−0.2000	−0.6000	−1.0000	−0.5000	N.D	−0.6000	−0.6000	N.D
Sarcoi-dosis	Macrophages (%)	0.1628	0.0198	−0.0022	0.0330	N.D	−0.1236	−0.1868	−0.3272
	Lymphocytes (%)	−0.2706	−0.1209	−0.0946	0.0198	N.D	0.0000	0.0769	0.3762
	Neutrophils (%)	0.3334	0.4054	0.1483	0.3742	N.D	0.4370	0.3567	−0.2975
	Eosinophils (%)	−0.1943	0.2580	−0.3488	−0.4212	N.D	−0.0629	0.1117	0.2393

*$P<0.05$. N.D ; not detect.

CVD-IP (RA) and in a part of CVD-IP (DM). Levels of IL-17 were significantly ($P<0.05$) higher in CVD-IP (RA) than in CVD-IP (DM). Levels of IFN-γ showed no significant differences among IPs and sarcoidosis. Levels of TNF-α tended to be high for CVD-IP (RA) compared with UIP, NSIP, CVD-IP (DM), and sarcoidosis. Levels of TGF-β1 were detected in 3 patients with sarcoidosis but not with UIP, NSIP, CVD-IP (RA), or CVD-IP (DM).

Correlation between cytokine levels and cell type in BAL fluid

IL-2, -6, -7, -8, -17, IFN-γ, TNF-α, and TGF-β1 are produced by macrophages, lymphocytes, and neutrophils in the lung, and by other cells such as fibroblasts and epithelial cells. We analyzed the correlation between cytokine levels and cell type in BAL fluid using Spearman's rank correlation (Table 2). TNF-α in NSIP and UIP was significantly ($P<0.05$) correlated with neutrophils (Table 2). In addition, the correlation between cell type and IL-7 in CVD-IP (DM), IL-17 in CVD-IP (RA), or IL-2 in sarcoidosis are shown in Figure 2. There were no correlations between each cell type and IL-17 in CVD-IP (RA) (Figure 2A), or IL-2 in sarcoidosis (Figure 2C). And IL-7 in CVD-IP (DM) was significantly ($P<0.05$) correlated with lymphocytes (Table 2 and Figure 2B). No correlations were noted between serological results (LDH, KL-6, and SPD) and cell type and cytokines (data not shown).

DISCUSSION

Here, we analyzed the correlation between cell subpopulations and cytokines in BAL fluid of patients with UIP, NSIP, CVD-IP (RA), CVD-IP (DM), and sarcoidosis. Occasionally, it is difficult for clinical discrimination between UIP and NSIP. In our results and several reports[20,21], the subpopulations of lymphocytes in BAL may play an important clinically discrimination between UIP and NSIP but the pattern of cytokines could not be unclear. In addition, the involvement of any cytokines, such as IL-8, IL-17, and TNF-α has indicated in RA[22,23]. And also, the cytokines pattern of BAL was similar in CVD-IP (RA), but not in CVD-IP (DM). Thus, we clearly showed the different patho-

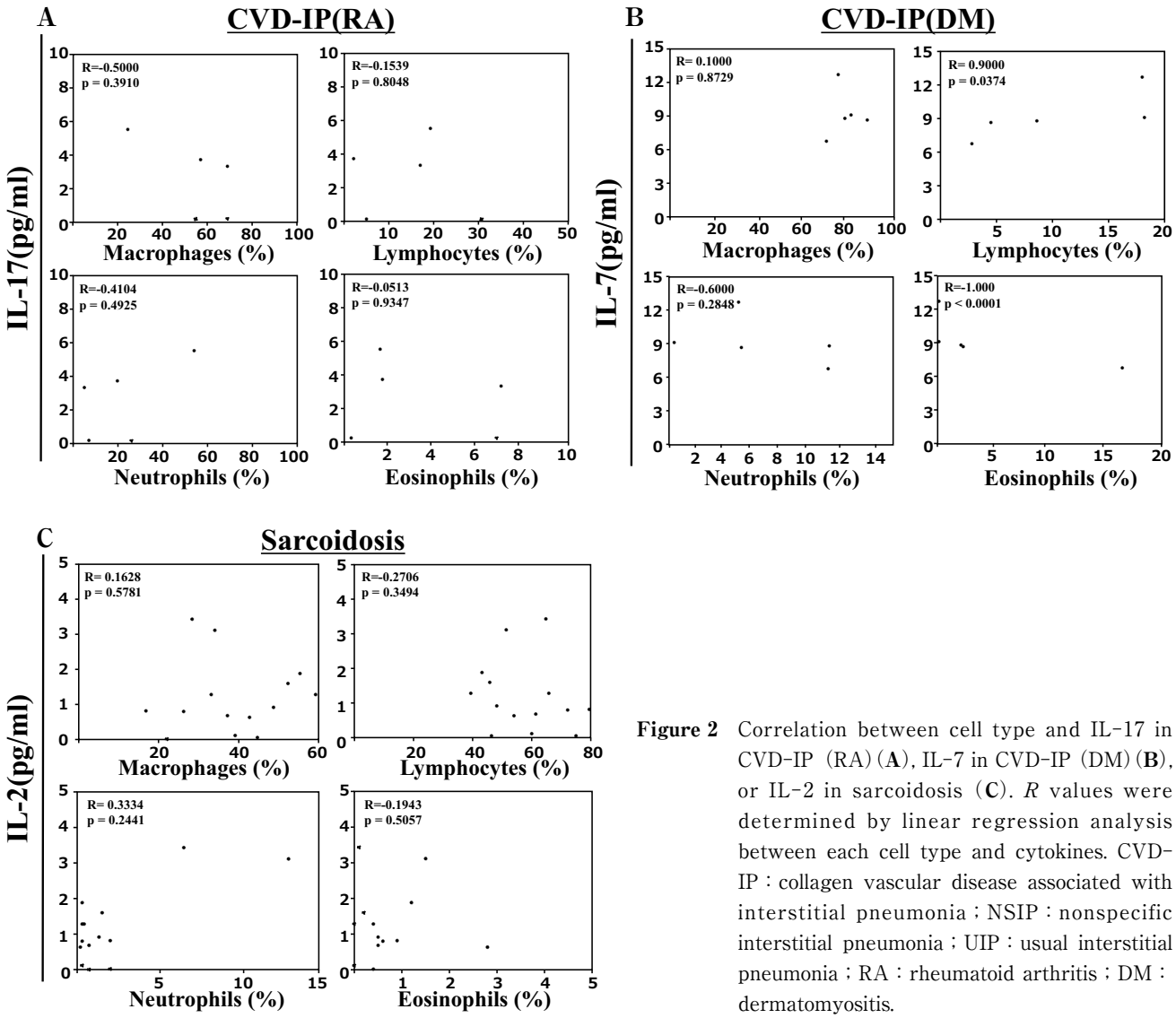

Figure 2 Correlation between cell type and IL-17 in CVD-IP (RA) (**A**), IL-7 in CVD-IP (DM) (**B**), or IL-2 in sarcoidosis (**C**). *R* values were determined by linear regression analysis between each cell type and cytokines. CVD-IP : collagen vascular disease associated with interstitial pneumonia ; NSIP : nonspecific interstitial pneumonia ; UIP : usual interstitial pneumonia ; RA : rheumatoid arthritis ; DM : dermatomyositis.

genesis of lung lesions between RA and DM.

IIP are a heterogeneous group of diffuse parenchymal lung diseases of unknown etiology[24]. Because of differences in clinical manifestation, radiographic features, and prognosis, different types of IIP are classified into seven histopathologic entities that include UIP and NSIP[24]. Separating NSIP from UIP is important because NSIP has better prognosis than UIP[25]. BAL is a non-invasive diagnostic procedure in interstitial lung diseases (ILD)[26,27]. In patients suspected to have UIP or NSIP, analysis of differential white blood cell counts in BAL fluid can be helpful. In the past, BAL lymphocytosis was considered a good prognostic factor while BAL neutrophilia or eosinophilia denoted poor clinical outcome in patients with UIP[20]. Since NSIP was first described in 1994, BAL lymphocytosis is more likely suggestive of NSIP rather than UIP[21]. Similarly, we also found that the percentage of lymphocytes tended to be high for NSIP compared with UIP.

These findings indicate that evaluation of lymphocytes in BAL fluid is useful for distinguishing between UIP and NSIP. In some collagen diseases, RA is a systemic disease process characterized by inflammation of the synovial tissues lining joints. Proliferation of synovial lining cells and infiltration of inflammatory cells, including monocytes and activated leukocytes, such as lymphocytes and neutrophils, into joint tissues results in proinflammatory factors that lead to destruction of both cartilage and bone matrix[28]. Moreover, in DM, inflammatory infiltrates are composed primarily of mac-

Table 3 The expression of each cytokines in UIP, NSIP, CVD-IP (RA), CVD-IP (DM), and sarcoidosis

	IL-2	IL-6	IL-7	IL-8	IL-17	IFN-γ	TNF-α	TGF-β
UIP	+	+	++	+	−	+	±	−
NSIP	+	+	++	+	−	+	±	−
CVD-IP (RA)	++	++	+	++	+++	++	++	−
CVD-IP (DM)	++	++	+++	+	+	++	+	−
Sarcoi-dosis	+++	+	+	±	−	+	+	+

rophages, B cells, and CD4+ cells in the perivascular and perimysial regions[29,30]. Infiltration of these inflammatory cells into skin and muscle develops in the pathogenesis of DM. However, the pathogenesis of interstitial pneumonia in patients with RA and DM remains poorly understood. We found that the population of neutrophils was highest for CVD-IP (RA), compared with other IPs, CVD-IP (DM), and sarcoidosis. On the other hand, there were no significant differences of BAL cells in CVD-IP (DM) compared with BAL findings of healthy subjects[31]. These results suggest that neutrophils in CVD-IP (RA) and lymphocytes and macrophages in CVD-IP (DM) may play an important role in the development of interstitial pneumonia.

Although the pathogenesis of sarcoidosis has not been elucidated, many observations, including the presence of oligoclonal T cells in BAL fluid, blood, and skin granulomas, are suggestive of an antigen-driven autoimmune disease[32,33]. Similarly, we also found that the population of lymphocytes, but not that of neutrophils and macrophages, was highest for sarcoidosis, compared with other IPs. The results indicate that lymphocytes play an important role in the pathogenesis of sarcoidosis. On the basis of these findings, biological studies of interstitial lung disease have detected several cell types that are differentially expressed in patients with IIPs and CVD-IP.

Inflammatory cytokines and chemokines have been reported to play important roles in the pathogenesis of interstitial lung diseases[34,35]. Table 3 shows the pattern of each cytokines and chemokines among IIPs, CVD-IP, and sarcoidosis. Monocyte chemoattractant protein (MCP)-1 is closely associated with the expression of adhesion molecules and the migration of inflammatory cells into the lung, and Yoshioka et al[35] suggested that MCP-1 in BAL fluid may play an important role in inflammatory cell recruitment to the lung in NSIP compared with UIP. An association between lymphocytosis and IL-6 levels in BAL fluid of patients with NSIP has also been reported[36]. Our results showed that the levels of MCP-1 (data not shown) and IL-6 did not differ between UIP and NSIP. This discrepancy can be attributed to differences in the activity and severity of NSIP, as well as the timing of BAL. It is widely accepted that TGF-β1 might play an important role in the pathogenesis of lung injury such as UIP and NSIP. TGF-β1 is produced by lymphocytes, macrophages, and fibroblasts in the lung and is considered a critical mediator of pathological, fibrotic, and remodeling responses in the lung and other organs, and such responses are thought to be mediated by the ability of TGF-β1 to active fibrogenic and apoptotic injury pathways[37~39]. In our study however, no TGF-β1 was detected in UIP, NSIP, or CVD-IP. This may be due to the low measurement sensitivity for TGF-β1 using the ELISA kit.

In DM, the primary cellular sources of cytokines are immune cells, but endothelial cells and muscle fibers can also express these immune regulators[40]. The important role of TNF as a regulator of chronic inflammation associated with DM has previously been established[41,42]. IL-1β is significantly upregulated in DM[43]. Our results showed no differences in the levels of IL-1β (data not shown) and TNF-α between CVD-IP (DM) and other IPs. The discrepancy was considered to be due to differences in pathogenesis between lung lesions and other organs, such as skin and muscle, and disease activity. Levels of IL-7 were higher in CVD-IP (DM) than in CVD-IP (RA). IL-7 is a growth factor

of not only lymphocytes but also fibroblasts through the receptor IL-7R[44,45]. Therefore, these inflammable cells may be involved in the development of lung inflammation and fibrosis.

In RA, synovial tissue in inflamed joints infiltrated with activated macrophages and leukocytes is a rich source of pro-inflammatory cytokines, including TNF-α, IL-1, IL-6 and IL-17, that impact bone remodeling within the RA bone microenvironment[22]. TNF-α induces the production of other proinflammatory cytokines (IL-1, IL-6, IL-8)[23] and is synthesized mainly by macrophages and activated T cells. IL-6 is a pleiotropic proinflammatory cytokine produced by a variety of cell types in the inflamed RA bone microenvironment including macrophages and fibroblast-like synoviocytes[46]. IL-6 as an important effector of inflammation-induced joint destruction in arthritis is supported by the observation that mice deficient in IL-6 were protected against bone destruction in an antigen-induced arthritis model[47]. IL-8 is a chemokine produced by macrophages and other cell types such as epithelial cells and endothelial cells[48]. The primary function of IL-8 is the induction of chemotaxis in neutrophils[49]. We showed that the levels of IL-6, IL-8, and TNF-α tended to be higher in CVD-IP (RA) than in other IPs. Our results indicate that these cytokines may play an important role in the pathogenesis of lung lesions in RA. In addition, there were no correlations between these cytokines and BAL cell populations, suggesting that these cytokines correlate with lung fibroblasts and/or epithelial cells. 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]. Interestingly, IL-17 levels were detectable only in CVD-IP (RA) and in a part of CVD-IP (DM), but not in UIP, NSIP, or sarcoidosis. Moreover, IL-17 did not correlate with the positive results of rheumatoid factors in patients with other UIP and NSIP (data not shown). These findings suggest that IL-17 may be a specific biomarker of CVD-IP (RA).

Th1 cells, and their products IL-2 and IFN-γ, are thought to be involved in the pathogenesis of sarcoidosis[51]. We found that the levels of IL-2 tended to be higher in sarcoidosis than in other IPs. On the other hand, IFN-γ levels were consistent across these IPs. This could be because IFN-γ also plays an important role in the pathogenesis of not only sarcoidosis but also other IPs. Pulmonary fibrosis develops in approximately 25% of patients with chronic sarcoidosis[52]. Therefore, high levels of TGF-β1 in the 3 patients with sarcoidosis may develop in lung fibrosis in the near future.

We found the correlations between IL-7 and lymphocytes in CVD-IP (DM). IL-7 is secreted by stromal cells in red marrow and the thymus, and it is also produced by keratinocytes, dendritic cells, hepatocytes, neurons, and epithelial cells but is not produced by lymphocytes[53~56]. Accordingly, IL-7 might be involved in the proliferation of lymphocytes and lung fibroblasts through IL-7 R in CVD-IP (DM). In addition, IL-17 plays an important role in pathogenesis of CVD-IP (RA). IL-17 is produced by T helper cells, and recruit neutrophils into various tissues. In our results, there was no correlation between IL-17, lymphocytes, and neutrophils. This discrepancy was thought to be due to the differences between the amount of IL-17 and its responsibility to neutrophils, and between the number of IL-17-producing cells and its amount from a cell in individual patients. IL-2 plays an important role in the pathogenesis of sarcoidosis. IL-2 is produced by Th1 cells and proliferated by lymphocytes through its receptor IL-2 R[57], while IFN-γ is produced by Th1 cells and macrophages and has antiviral, immunoregulatory, and anti-tumor properties[58]. In our results, there was no correlation between IL-2 and lymphocytes, possibly because of the differences between the amount of IL-2 and its responsibility to lymphocytes in individual patients.

Although the pattern of cell types, cytokines, and chemokines tended to differ between IIPs and CVD-IPs, it was difficult to find clearly the disease specific biomarker except IL-17 in CVD-IP (RA). The findings were thought to be because of the small number of the diseases and/or the activity. We comprehensively considered that BAL may be useful for not only diagnosing a variety of specific CVD-IPs but also narrowing the differential diagnosis and predicting prognosis in fibrotic IIP, such as NSIP and UIP. Therefore, BAL should be performed in IIP and CVD-IP whenever possible. A long-term prospective study is needed to define the role of BAL more clearly in IIP

and CVD-IP.

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REFERENCES

1) American Thoracic Society : Idiopathic pulmonary fibrosis : diagnosis and treatment. International consensus statement. Am J Respir Crit Care Med **161** : 646-664, 2000.
2) Ryu JH, Colby TV, Hartman TE : Idiopathic pulmonary fibrosis : current concepts. Mayo Clin Proc **73** : 1085-1101, 1998.
3) Katzenstein AL, Myers JL : Idiopathic pulmonary fibrosis : clinical relevance of pathologic classification. Am J Respir Crit Care Med **157** : 1301-1315, 1998.
4) Nagai S, Kitaichi M, Itoh H, et al : Idiopathic nonspecific interstitial pneumonia/fibrosis : comparison with idiopathic pulmonary fibrosis and BOOP. Eur Respir J **12** : 1010-1019, 1998.
5) Park CS, Chung SW, Ki SY, et al : Increased levels of interleukin-6 are associated with lymphocytosis in bronchoalveolar lavage fluids of idiopathic nonspecific interstitial pneumonia. Am J Respir Crit Care Med **162** : 1162-1168, 2000.
6) Ishii H, Mukae H, Kadota J, et al : High serum concentrations of surfactant protein A in usual interstitial pneumonia compared with non-specific interstitial pneumonia. Thorax **58** : 52-57, 2003.
7) Park IN, Kim DS, Shim TS, et al : Acute exacerbation of interstitial pneumonia other than idiopathic pulmonary fibrosis. Chest **132** : 214-220, 2007.
8) Shimizu S, Yoshinouchi T, Niimi T, et al : Differing distributions of CXCR3- and CCR4-positive cells among types of interstitial pneumonia associated with collagen vascular diseases. Virchows Arch **450** : 51-58, 2007.
9) Tachikawa R, Tomii K, Ueda H, et al. Clinical features and outcome of acute exacerbation of interstitial pneumonia : collagen vascular diseases-related versus idiopathic. Respiration **83** : 20-27, 2012.
10) Ishioka S, Maeda A, Hiyama K, et al : Pulmonary manifestation of collagen vascular diseases : role of cytokines in interstitial pneumonia associated with collagen vascular diseases. Nihon Kyobu Shikkan Gakkai Zasshi **33** : 277-283 1995.
11) Nishi Y, Sano H, Kawashima T, et al : Role of galectin-3 in human pulmonary fibrosis. Allergol Int **56** : 57-65, 2007.
12) Kodama N, Yamaguchi E, Hizawa N, et al : Expression of RANTES by bronchoalveolar lavage cells in nonsmoking patients with interstitial lung diseases. Am J Respir Cell Mol Biol **18** : 526-531 1998.
13) Kotsianidis I, Nakou E, Bouchliou I, et al : Global impairment of CD4+CD25+FOXP3+ regulatory T cells in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med **179** : 1121-1130 2009.
14) Felson DT, Smolen JS, Wells G, et al : American College of Rheumatology ; European League Against Rheumatism. American College of Rheumatology/European League Against Rheumatism provisional definition of remission in rheumatoid arthritis for clinical trials. Arthritis Rheum **63** : 573-586 2011.
15) Bohan A, Peter JB : Polymyositis and dermatomyositis (second of two parts). N Engl J Med **292** : 403-407 1975.
16) Bohan A, Peter JB : Polymyositis and dermatomyositis (first of two parts). N Engl J Med **292** : 344-347 1975.
17) O'Regan A, Berman JS. Sarcoidosis. Ann Intern Med **156** : ITC51, 2012.
18) Mukae H, Iiboshi H, Nakazato M, et al : Raised plasma concentrations of alpha-defensins in patients with idiopathic pulmonary fibrosis. Thorax **57** : 623-628 2002.
19) Kadota J, Mukae H, Tomono K, et al. High concentrations of beta-chemokines in BAL fluid of patients with diffuse panbronchiolitis. Chest **120** : 602-607, 2001.
20) Watters LC, Schwarz MI, Cherniack RM, et al : Idiopathic pulmonary fibrosis : pretreatment bronchoalveolar lavage cellular constituents and their relationships with lung histopathology and clinical response to therapy. Am Rev Respir Dis **135** : 696-704, 1987.
21) American Thoracic Society. Idiopathic pulmonary fibrosis : diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). Am J Respir Crit Care Med **161** : 646-664, 2000.
22) Sougata K, Jonathan K, Ellen MG : Bone Damage in Rheumatoid Arthritis-Mechanistic Insights and Approaches to Prevention. Rheum Dis Clin North Am

36 : 385-404, 2010.

23) Butler DM, Maini RN, Feldmann M, et al : Modulation of proinflammatory cytokine release in rheumatoid synovial membrane cell cultures. Comparison of monoclonal anti TNF-alpha antibody with the interleukin-1 receptor antagonist. Eur Cytokine Netw **6** : 225-230, 1995.

24) American Thoracic Society/European Respiratory Society International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the European Respirato2001 and by the ERS Executive Committee, June 2001. Am J Respir Crit Care Med **165** : 277-304, 2002.

25) Yon Ju Ryua, Man Pyo Chungb, Joungho Hanc, et al : Bronchoalveolar lavage in fibrotic idiopathic interstitial pneumonias. Respiratory Medicine **101** : 655-660, 2007.

26) Clinical guidelines indications for bronchoalveolar lavage (BAL) : Report of the European Society of Pneumology Task Group on BAL. Eur Respir J **3** : 937-976, 1990.

27) Hunninghake GW, Costabel U, Ando M, et al : ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and otherGranulomatous Disorders. Sarcoidosis Vasc Diffuse Lung Dis **16** : 149-173, 1999.

28) Gravallese EM, Monach PA : Rheumatoid synovitis and pannus. In : Hochberg MSA, Smolen J, Weinblatt M, Weisman M, editors. Rheumatology **4** : 841-865, 2008.

29) Arahata K, Engel AG. Monoclonal antibody analysis of mononuclear cells in myopathies. I : Quantitation of subsets according to diagnosis and sites of accumulation and demonstration and counts of muscle fibers invaded by T cells. Ann Neurol **16** : 193-208, 1984.

30) Engel AG, Arahata K. Monoclonal antibody analysis of mononuclear cells in myopathies. II : Phenotypes of autoinvasive cells in polymyositis and inclusion body myositis. Ann Neurol **16** : 209-215, 1984.

31) Domagała-Kulawik J : BAL in the diagnosis of smoking-related interstitial lung diseases : review of literature and analysis of our experience. Diagn Cytopathol **36** : 909-915, 2008.

32) Forman JD, Klein JT, Silver RF, et al : Selective activation and accumulation of oligoclonal v beta-specific T cells in active pulmonary sarcoidosis. J Clin Invest **94** : 1533-1542, 1994.

33) Mempel M, Flageul B, Suarez F, et al : Comparison of the T cell patterns in leprous and cutaneous sarcoid granulomas : presence of valpha24-invariant natural killer T cells in T-cell-reactive leprosy together with a highly biased T cell receptor valpha repertoire. Am J Pathol **157** : 509-523, 2000.

34) Horowitz JC, Thannickal VJ : Idiopathic pulmonary fibrosis : new concepts in pathogenesis and implications for drug therapy. Treat Respir Med **5** : 325-342, 2006.

35) Yoshioka S, Mukae H, Sugiyama K, et al : High-BAL fluid concentrations of RANTES in nonspecific interstitial pneumonia compared with usual interstitial pneumonia. Respir Med **98** : 945-951, 2004.

36) Park CS, Chung SW, Ki SY : Increased levels of interleukin-6 are associated with lymphocytosis in bronchoalveolar lavage fluids of idiopathic nonspecific interstitial pneumonia. Am J Respir Crit Care Med **162** : 1162-1168, 2000.

37) Taylor AW : Review of the activation of TGF-beta in immunity. J Leukoc Biol **85** : 29-33, 2009.

38) Wen FQ, Kohyama T, Sköld CM, et al : Glucocorticoids modulate TGF-beta production by human fetal lung fibroblasts. Inflammation **27** : 9-19, 2003.

39) Toossi Z, Hirsch CS, Hamilton BD, et al : Decreased production of TGF-beta 1 by human alveolar macrophages compared with blood monocytes. J Immunol **15** : 3461-3468, 1996.

40) Hak AE, de Paepe B, de Bleecker JL, et al : Dermatomyositis and polymyositis : new treatment targets on the horizon. J Med **69** : 410-420, 2011.

41) de Bleecker JL, Meire VI, Declercq W, et al : Immunolocalization of tumor necrosis factor-alpha and its receptors in inflammatory myopathies. Neuromuscul Disord **9** : 239-246, 1999.

42) Kuru S, Inukai A, Liang Y, et al : Tumor necrosis factor-alpha expression in muscles of polymyositis and dermatomyositis. Acta Neuropathol **99** : 585-588, 2000.

43) Szodoray P, Alex P, Knowlton N, et al : Idiopathic inflammatory myopathies, signified by distinctive peripheral cytokines, chemokines and the TNF family members B-cell activating factor and a proliferation

inducing ligand. Rheumatology (Oxford) **49**: 1867-1877, 2010.
44) Hartgring SAY, Bijlsma JWJ, Lafeber EPJG, et al: Interleukin-7 induced immunopathology in arthritis. Ann Rheum Dis **65**: iii69-iii74, 2006.
45) Pickens SR, Chamberlain ND, Volin MV, et al: Characterization of interleukin-7 and interleukin-7 receptor in the pathogenesis of rheumatoid arthritis. Arthritis Rheum **63**: 2884-2893, 2011.
46) Okamoto H, Yamamura M, Morita Y, et al: The synovial expression and serum levels of interleukin-6, interleukin-11, leukemia inhibitory factor, and oncostatin M in rheumatoid arthritis. Arthritis Rheum **40**: 1096-1105, 1997.
47) Boe A, Baiocchi M, Carbonatto M, et al: Interleukin 6 knock-out mice are resistant to antigen-induced experimental arthritis. Cytokine **11**: 1057-1064, 1999.
48) Wolff B, Burns AR, Middleton J, Rot A. Endothelial cell memory of inflammatory stimulation: human venular endothelial cells store interleukin 8 in Weibel-Palade bodies. J Exp Med **188**: 1757-1762, 1998.
49) Brullo C, Spisani S, Selvatici R, et al: N-Aryl-2-phenyl-2,3-dihydro-imidazo [1,2-b] pyrazole-1-carboxamides 7-substituted strongly inhibiting both fMLP-OMe- and IL-8-induced human neutrophil chemotaxis. Eur J Med Chem **47**: 573-579, 2012.
50) Poon AH, Eidelman DH, Martin JG, et al: Pathogenesis of severe asthma. Clin Exp Allergy **42**: 625-637, 2012.
51) Zaba LC, Smith GP, Sanchez M, et al: Dendritic cells in the pathogenesis of sarcoidosis. Am J Respir Cell Mol Biol **42**: 32-39, 2010.
52) Kruit A, Grutters JC, Ruven HJ, et al: Transforming growth factor-beta gene polymorphisms in sarcoidosis patients with and without fibrosis. Chest **129**: 1584-1591, 2006.
53) Heufler C, Topar G, Grasseger A, et al: Interleukin 7 is produced by murine and human keratinocytes. J Exp Med **178**: 1109-1114, 1993.
54) Kröncke R, Loppnow H, Flad HD, et al: Human follicular dendritic cells and vascular cells produce interleukin-**7**: a potential role for interleukin-7 in the germinal center reaction. Eur J Immunol **26**: 2541-2544, 1996.
55) Sawa Y, Arima Y, Ogura H, et al: Hepatic interleukin-7 expression regulates T cell responses. Immunity **30**: 447-457, 2009.
56) Watanabe M, Ueno Y, Yajima T, et al: Interleukin 7 is produced by human intestinal epithelial cells and regulates the proliferation of intestinal mucosal lymphocytes. J Clin Invest **95**: 2945-2953, 1995.
57) Boyman O, Sprent J: The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol **12**: 180-190, 2012.
58) Horras CJ, Lamb CL, Mitchell KA. Regulation of hepatocyte fate by interferon-γ. Cytokine Growth Factor Rev **22**: 35-43, 2011.