

Elevated Concentrations of Liver-Expressed Chemokine/CC Chemokine Ligand 16 in Bronchoalveolar Lavage Fluid from Patients with Eosinophilic Pneumonia

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Key Words

Bronchoalveolar lavage fluid • Eosinophilic pneumonia • Immunocytochemistry • Liver-expressed chemokine/CC chemokine ligand 16

Abstract

Background: Eosinophilic pneumonia is characterized by the prominent accumulation of eosinophils and lymphocytes in the lung parenchyma. Liver-expressed chemokine (LEC)/CC chemokine ligand 16 (CCL16) is a novel functional ligand for H4 which is expressed on eosinophils and also an affinity ligand for CCR1, CCR2, CCR5 and CCR8 which are expressed on T lymphocytes and monocytes. The purpose of this study is to clarify the role of LEC/CCL16 in eosinophilic pneumonia. **Methods:** The LEC/CCL16 level was measured using an enzyme-linked immunosorbent assay in the bronchoalveolar lavage fluid (BALF) of 33 patients with eosinophilic pneumonia, 26 patients with sarcoidosis and 10 healthy volunteers. The cell sources of LEC/CCL16 in BALF were evaluated by immunocytochemistry. **Results:** The LEC/CCL16 levels in BALF from patients with eosinophilic pneumonia were significantly higher than those from patients with sar-

coidosis and healthy volunteers. The BALF LEC/CCL16 levels correlated with the numbers of BALF eosinophils and lymphocytes, respectively. The BALF LEC/CCL16 levels were significantly decreased after remission in eosinophilic pneumonia. In immunocytochemistry, the LEC/CCL16 expression was clearly observed in CD1a-positive dendritic cells as well as in CD68-positive macrophages harvested from patients with eosinophilic pneumonia, but not from the controls. **Conclusions:** These results suggest that LEC/CCL16 produced by dendritic cells as well as by alveolar macrophages contributes to the accumulation of eosinophils and lymphocytes into the inflamed lungs of patients with eosinophilic pneumonia.

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Introduction

Eosinophilic pneumonia is characterized by the prominent accumulation of eosinophils in the lung parenchyma [1–3]. Previous studies have focused on several molecules including chemokines as factors involved in the establishment of eosinophilic inflammation in eosino-

philic pneumonia. Eotaxin/CC chemokine ligand 11 (CCL11) and regulated upon activation, normal T-cell expressed and secreted (RANTES)/CCL5 contribute to eosinophil recruitment via CCR3 which is expressed on eosinophils [4, 5]. However, neutralization with a specific antibody against RANTES failed to achieve a complete inhibition of eosinophil chemotactic activity in bronchoalveolar lavage fluid (BALF) [6], thus possibly indicating the synergistic effect of several kinds of chemoattractants in the tissue accumulation of eosinophils. Alternative pathways leading to eosinophil accumulation might be present. In this context, a recent paper suggests that the CXCR3 ligands, interferon-inducible protein 10/CXC chemokine ligand 10 (CXCL10) and monokines induced by interferon- γ /CXCL9, accumulate CXCR3-bearing eosinophils [7]. Besides eosinophil accumulation, T-lymphocyte recruitment and activation may be additional critical events [8]. Previous studies have shown that RANTES, monocyte chemoattractant protein 1/CCL2 and macrophage inflammatory chemokine- β /CCL4 are elevated in BALF in patients with eosinophilic pneumonia [4, 5]. More importantly, highly expressed T helper type 2 (Th2)-specific chemokines in the lung specimens contribute to eosinophilic inflammation through the induction of interleukin (IL)-4, IL-5 and IL-13 [9, 10], which could explain the immunopathogenesis of eosinophilic pneumonia. Hence, chemoattractants not only for eosinophils but also for other inflammatory cells should be extensively examined.

Liver-expressed chemokine (LEC)/CCL16 is a human CC chemokine that is constitutively expressed by the liver parenchymal cells and present in normal plasma at high concentrations [11]. LEC/CCL16 is a low-affinity ligand for CCR1, CCR2, CCR5 and CCR8 and thus attracts monocytes and T lymphocytes [11–14]. A recent report also showed that LEC/CCL16 is a novel functional ligand for H4 and induces an efficient migration of human and mouse eosinophils via H4 [15]. However, to date, there have been no studies elucidating the role of LEC/CCL16 in human allergic lung diseases.

This study measured the LEC/CCL16 concentration in BALF from patients with eosinophilic pneumonia. The values were compared with those from sarcoidosis patients and healthy volunteers. In addition, the role of LEC/CCL16 in the selective accumulation of eosinophils and lymphocytes was examined by measuring the correlation between the LEC/CCL16 concentration and the BALF cell fraction. Finally, experiments were conducted to determine the cell sources of LEC/CCL16 in the BALF cells by immunocytochemistry.

Materials and Methods

Patient Recruitment and Bronchoalveolar Lavage

Studies were performed in 33 patients with eosinophilic pneumonia including 10 patients with acute eosinophilic pneumonia (AEP), 12 patients with chronic eosinophilic pneumonia (CEP) and 11 patients with drug-induced eosinophilic pneumonia (drug-EP). AEP was principally diagnosed by previously described criteria by Allen et al. [1, 16]: acute onset, febrile illness, bilateral infiltrates seen on chest radiograph, dyspnea or hypoxemic respiratory failure, eosinophilia $>25\%$ in BALF, no history of hypersensitivity to drugs and no evidence of infection. Each patient with CEP met the previously described criteria by Carrington et al. [2]: typical findings of clinical features consistent with CEP, dense and multiple foci of consolidation in the peripheral lung fields on chest X-ray films, increased percentage of eosinophils in BALF, absence of possible causes and favorable response to corticosteroid therapy. Patients with drug-EP satisfied the following diagnostic criteria [3]: prompt improvement after cessation of the causative drug, absence of other possible causes, a positive reaction with lymphocyte stimulation tests or recurrence of the symptoms with the drug challenge, and eosinophilia $>25\%$ in BALF. The diagnosis of sarcoidosis was based on the typical clinical features together with epithelioid cell granulomas of the lung or lymph node biopsies according to the criteria in the International Statement on Sarcoidosis [17]. As a control, 10 healthy volunteers participated in this study. None of the healthy volunteers had a past history of respiratory or allergic diseases.

Bronchoalveolar lavage (BAL) was performed principally to diagnose each disease after obtaining informed consent. None of the patients and controls received corticosteroid therapy at the time of sample retrieval. The BAL procedure and a cell analysis were performed as described previously [18]. BAL was performed by injection of 150 ml of saline into the middle lobe or left lingula. The cells were counted in a hemocytometer. A 100- μ l aliquot of cells (1×10^6 /ml) was subjected to cytocentrifugation (Cytospin, Shandon Southern, Runcorn, UK) and air dried to obtain differential cell counts after staining with May-Grünwald-Giemsa stain. To determine the phenotype of the lymphocytes in BALF, a fluorescence-activated cell sorter scan (EPICS-MCL, Beckman Coulter, Villepinte, France) was performed by the direct immunofluorescence method using anti-CD3-phycoerythrin-cyanine 5, anti-CD4-phycoerythrin and anti-CD8-fluorescein isothiocyanate monoclonal antibodies (MoAbs) (Beckman Coulter). The cell-free supernatants were stored at -80°C until further analysis.

Measurement of LEC/CCL16

The LEC/CCL16 concentrations in BALF were measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems, Abingdon, UK). The minimum detectable dose in the ELISA system was 2 pg/ml.

Antibodies

The antibodies used for immunocytochemistry and their corresponding dilutions or concentrations were as follows: goat anti-human LEC/CCL16 polyclonal antibody (PoAb) (R&D Systems, Abingdon, UK) at 0.4 μ g/ml, mouse anti-human CD68 MoAb (Dako, Kyoto, Japan) at 0.9 μ g/ml as the

cell marker for macrophages, and mouse anti-human CD1a MoAb (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at 2 µg/ml as the cell marker for dendritic cells, biotin-labeled horse anti-goat IgG (H + L; Vector Laboratories, Inc., Burlingame, Calif., USA), alkaline phosphatase (AP)-labeled goat anti-mouse IgG (H + L) and AP-labeled horse anti-mouse IgG (H + L; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md., USA) at 1:200. As negative controls, the same species and isotype-matched immunoglobulins were used: goat IgG and mouse IgG1 (Dako).

Immunocytochemistry

Aliquots of BALF cells (1×10^6 cells) were pelleted onto glass slides and fixed in cold acetone for 10 min. After incubation in a solution containing 1% bovine serum albumin and 10% normal goat serum or normal horse serum, the slides were incubated with goat anti-human LEC/CCL16 PoAb at room temperature for 2 h. Negative controls were run by replacing the primary antibody with goat IgG at the concentration of each primary antibody. After blocking endogenous peroxidase activity by incubation in 0.3% H₂O₂ and 0.1% NaN₃ for 30 min, the bound antibodies were labeled with biotin-labeled horse anti-goat IgG (H + L). The avidin-biotin-peroxidase complex (ABC) solution (Vector Laboratories) was then applied sequentially for 30 min. The peroxidase activity was visualized with 3-amino-9-ethylcarbazole (AEC); the working AEC solution was applied and incubated for 10 min. The sections were counterstained with Mayer's hematoxylin and mounted with Geltol (Lipshaw Immunon, Pittsburg, Pa., USA).

Double Staining

Immunocytochemical double staining was also performed using a combination of the ABC method and the AP technique. After incubation in a solution containing 1% bovine serum albumin and 10% normal goat serum, the slides were incubated at 4°C overnight with goat anti-human LEC/CCL16 PoAb. After blocking endogenous peroxidase activity in 0.3% H₂O₂ and 0.1% NaN₃ for 30 min, the slides were incubated for 30 min with biotin-labeled horse anti-goat IgG, and then ABC solution was applied sequentially for 10 min. The peroxidase activity was visualized with AEC. Next, the slides were incubated with a second primary antibody, mouse anti-human CD68 MoAb, or mouse anti-human CD1a MoAb followed by AP-labeled horse anti-mouse IgG. AP activity was represented with the Vectastain ABC-AP substrate kit III (Vector Laboratories). Endogenous AP activity was inhibited by the addition of 1 mM levamisole to the reaction mixture. Negative controls were run by replacing primary antibodies with an irrelevant isotype-matched immunoglobulin. The cross-reactivity of anti-LEC/CCL16 antibody with the second-step primary antibody was examined processing 4 pairs: anti-LEC/CCL16 antibody (+)/second-step primary antibody (+), (+)/(-), (-)/(+) and (-)/(-).

Statistical Analysis

The results were expressed as the mean $\pm$ standard error of the mean, or the median and interquartile range. The Kruskal-Wallis test was used to compare values of the different groups. In case of a significant difference between groups, the intergroup comparisons were assessed by a nonparametric method using the Mann-Whitney U test. The correlation coefficients

Table 1. Characteristics of the study population

Characteristics	EP	SAR	HV
Subjects	33	27	10
Age, years	47 $\pm$ 4	55 $\pm$ 3	26 $\pm$ 1
Sex, female/male	17/16	19/8	2/8
BALF findings			
TCC, $\times 10^5$ /ml	9.3 $\pm$ 2.6**	2.5 $\pm$ 0.2*	1.6 $\pm$ 0.3
AM, %	29.4 $\pm$ 3.1**	72.3 $\pm$ 3.9**	94.1 $\pm$ 1.4
Lymphocytes, %	22.2 $\pm$ 2.9***	26.4 $\pm$ 3.7**	4.0 $\pm$ 0.9
Neutrophils, %	6.8 $\pm$ 2.1	8.4 $\pm$ 2.8	1.2 $\pm$ 0.3
Eosinophils, %	42.3 $\pm$ 4.1*	0.6 $\pm$ 0.5	0.5 $\pm$ 0.3
CD4/CD8 ratio	2.33 $\pm$ 0.29	7.57 $\pm$ 1.31***	2.06 $\pm$ 0.45

Data are the number of subjects, or the mean $\pm$ standard error of the mean. EP = Eosinophilic pneumonia (n = 33); SAR = sarcoidosis (n = 27); HV = healthy volunteer (n = 10); TCC = total cell concentration; AM = alveolar macrophage. The intergroup comparisons were assessed by a nonparametric method using the Mann-Whitney U test. * p < 0.05, compared with HV; ** p < 0.0005, compared with HV; *** p < 0.0001, compared with HV.

were determined using Spearman's rank correlation test. Serial changes in BALF analysis between the active and remission phase were performed using the Wilcoxon signed-rank test. A p value < 0.05 was considered significant.

Results

Patient Characteristics and BALF Findings

Thirty-three patients with eosinophilic pneumonia were enrolled in this study. The detailed characteristics of these patients are described in table 1. The control group was made up of 27 sarcoidosis patients and 10 healthy volunteers. The cell concentration and the percentage of lymphocytes in BALF from patients with eosinophilic pneumonia and patients with sarcoidosis were significantly higher than those in BALF from healthy volunteers. Patients with eosinophilic pneumonia had significantly increased percentages of eosinophils in BALF in comparison with healthy volunteers. The CD4/CD8 ratios in BALF from patients with sarcoidosis were higher than those in BALF from healthy volunteers.

The LEC/CCL16 Concentration in Eosinophilic Pneumonia

The LEC/CCL16 concentrations in BALF from patients with eosinophilic pneumonia, patients with sarcoidosis and healthy volunteers are shown in figure 1.

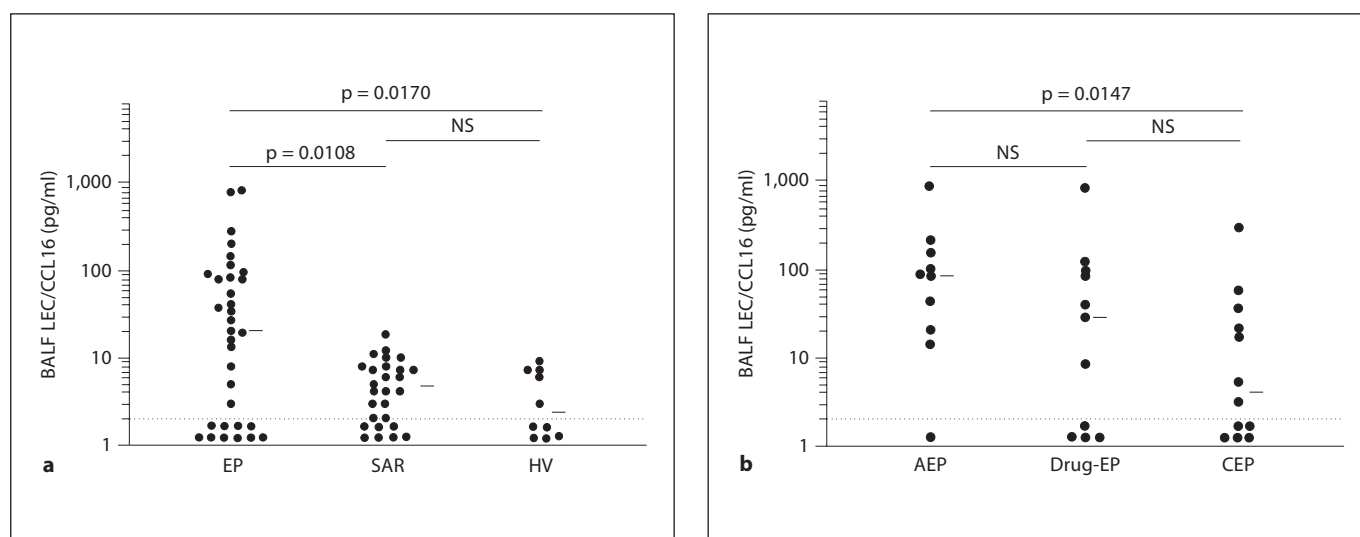


Fig. 1. Concentrations of LEC/CCL16 in BALF obtained from patients with eosinophilic pneumonia (EP; n = 33), sarcoidosis (SAR; n = 27) and healthy volunteers (HV; n = 10) (a), or from patients with AEP (n = 10), drug-EP (n = 11) and CEP (n = 12) (b). Each concentration is represented in the log scale. A short horizontal line represents the median value. The detectable limits of LEC/CCL16 are represented as a horizontal dotted line. The intergroup comparisons were assessed by a nonparametric method using the Mann-Whitney U test. NS = Not significant.

The LEC/CCL16 concentrations in the eosinophilic pneumonia group (median 20 pg/ml, range 0–795) were significantly higher than those in the sarcoidosis group (median 6 pg/ml, range 0–52) and healthy volunteers (median 4 pg/ml, range 0–51). The differences reached a statistical significance. There was no significant difference in the BALF LEC/CCL16 levels between patients with sarcoidosis and healthy volunteers. When the eosinophilic pneumonia population was divided into the following 3 groups, AEP (median 81 pg/ml, range 0–795), CEP (median 4 pg/ml, range 0–277) and drug-EP (median 26 pg/ml, range 0–754), the LEC/CCL16 levels in BALF from patients with AEP were significantly higher than those in BALF from patients with CEP ($p = 0.00147$).

Relationship between the LEC/CCL16 Levels and BAL Cell Profile

The LEC/CCL16 concentration was compared with the number of inflammatory cells in BALF. As shown in figure 2, there was a significant positive correlation between the levels of BALF LEC/CCL16 and the numbers of BALF lymphocytes ($r = 0.466$, $p < 0.0001$) and eosinophils ($r = 0.364$, $p < 0.0014$) in the population studied. In contrast, no correlation was ob-

served within either group of eosinophilic pneumonia or sarcoidosis.

Serial Change of the LEC/CCL16 Level in Eosinophilic Pneumonia

To examine whether LEC/CCL16 may reflect the degree of disease activity in eosinophilic pneumonia, the LEC/CCL16 level was compared with the percentages of eosinophils and lymphocytes in the BALF harvested at the acute phase and the remission phase. Among 33 patients, the second BAL at the remission phase could be performed in 9 patients. The results are shown in figure 3. The elevated levels of LEC/CCL16 at the acute phase significantly decreased after remission (fig. 3a). The percentages of BALF eosinophils and lymphocytes also declined (fig. 3b, c).

Immunocytochemistry Results of LEC/CCL16 Expression on BALF Cells

Immunocytochemistry was performed on BALF cells from patients with eosinophilic pneumonia, patients with sarcoidosis and healthy volunteers. Negative controls, which were run by replacing the primary antibodies with an irrelevant isotype-matched immunoglobulin, were uniformly nonreactive. No LEC/CCL16-

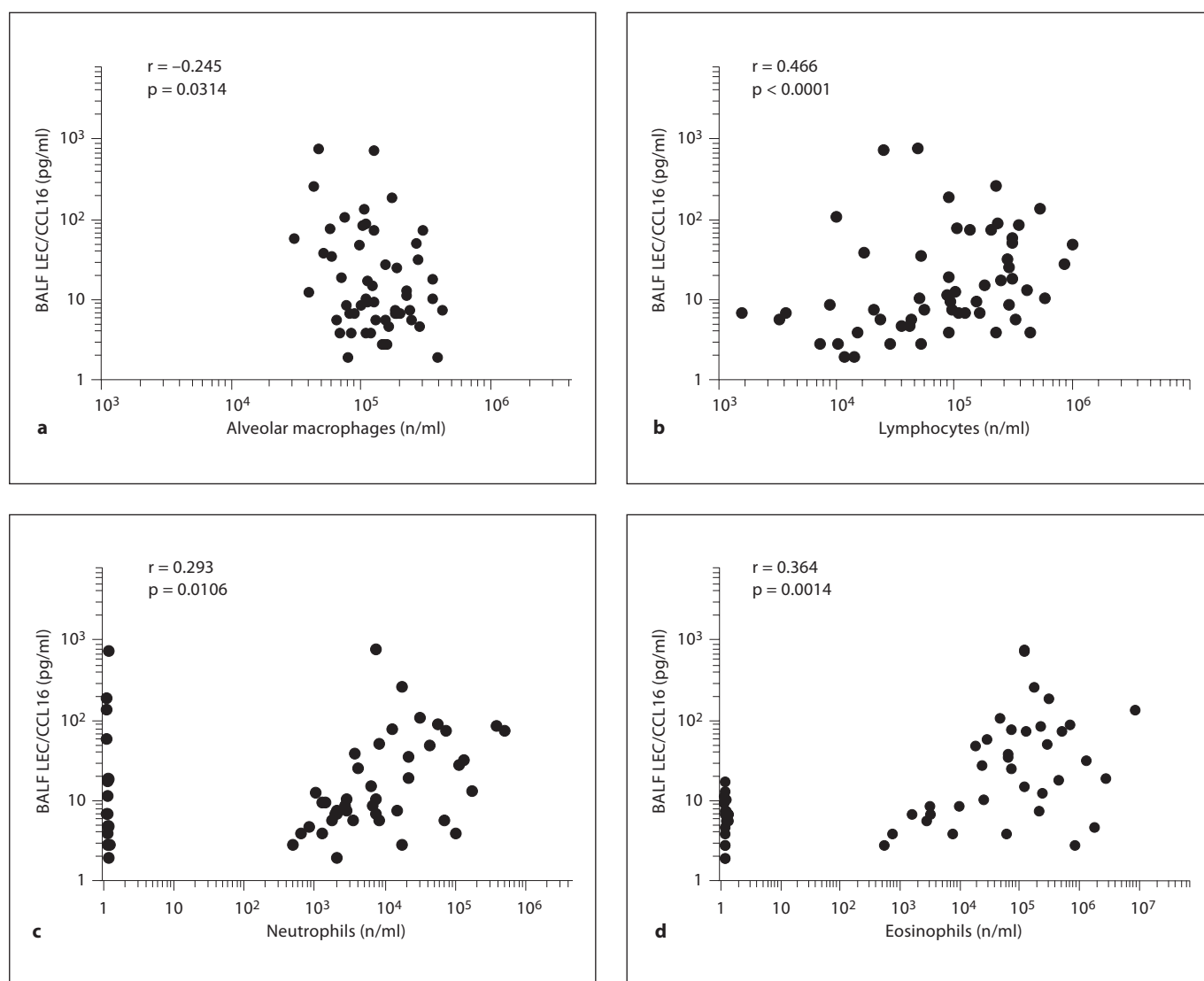
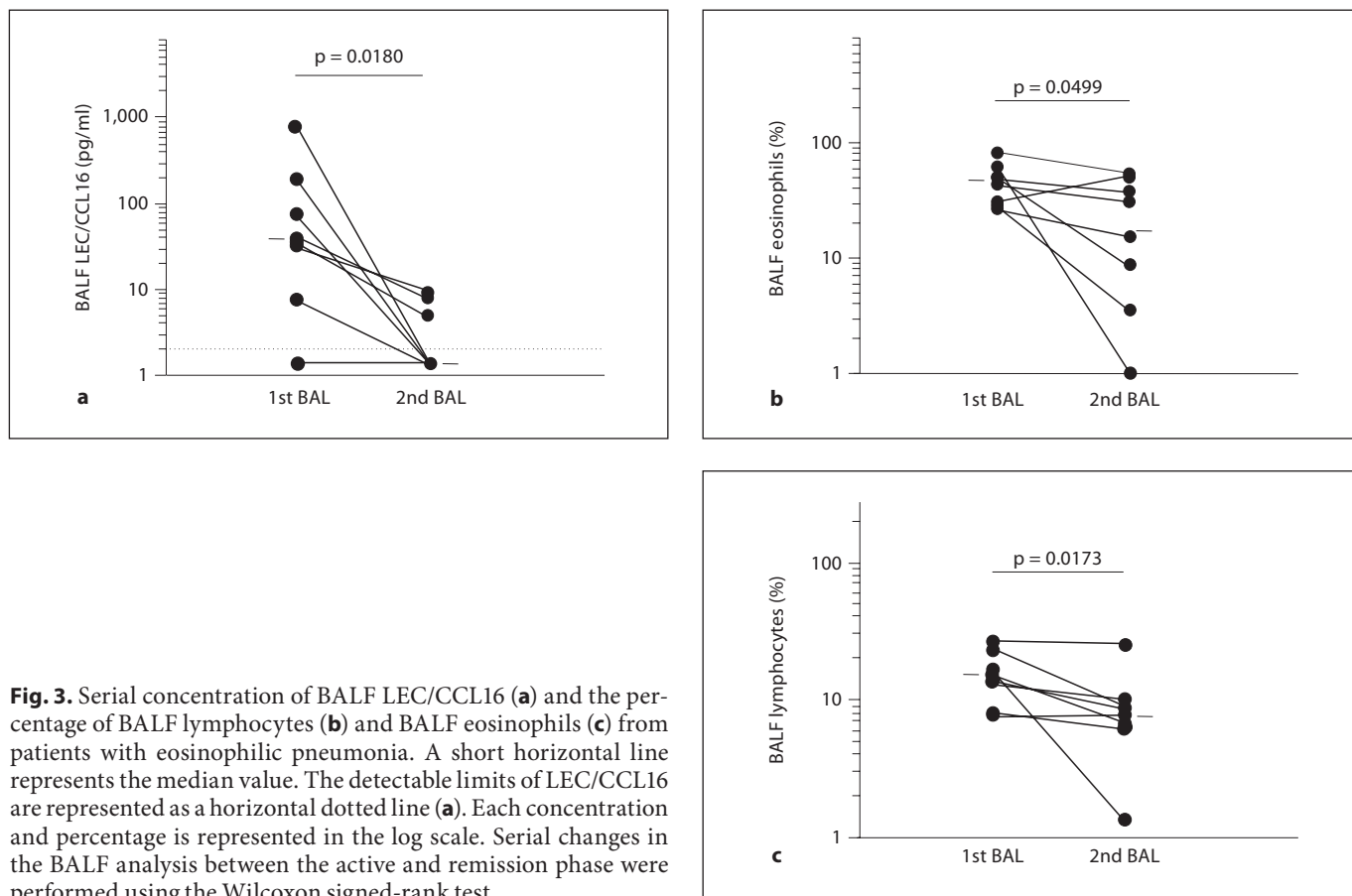


Fig. 2. Relationship between the BALF concentrations of LEC/CCL16 and the BALF numbers of alveolar macrophages (a), lymphocytes (b), neutrophils (c) and eosinophils (d). Each concentration and number is represented in the log scale. The correlation coefficients were determined using Spearman's rank correlation test.

positive cells were observed on the slides from the patients with sarcoidosis and healthy volunteers (fig. 4a, b). On the contrary, the slides from patients with eosinophilic pneumonia contained immunoreactive cells for LEC/CCL16. Among them, 2 types of LEC/CCL16-positive cells were found: one included the large mononuclear cell faintly stained with the antibody and the other included the smaller-sized mononuclear cells with very strong reactivity (fig. 4c).

Determination of LEC/CCL16-Positive Phenotypes

To determine the phenotype of LEC/CCL16-positive BALF cells, double staining was performed for 5 patients with eosinophilic pneumonia. CD68 and CD1a molecules were used as the cell markers for alveolar macrophage and dendritic cells, respectively. As a result, large mononuclear cells faintly stained with anti-LEC/CCL16 PoAb were found to be CD68 positive (fig 4d). In contrast, the smaller part of LEC/CCL16-positive cells with strong immunoreactivity were CD1a positive (fig. 4e), but CD68 negative (fig. 4d).



Discussion

Several studies have demonstrated that LEC/CCL16 is involved in the pathophysiology of various disease conditions. LEC/CCL16 inhibits mammary tumor growth and prevents metastatic-induced death [19]. In addition, LEC/CCL16 has a powerful inflammatory activity which is up-regulated by IL-10 in active ulcerative colitis [20]. Moreover, LEC/CCL16 acts as a mediator contributing to the inflammatory events associated with endometriosis [21]. The present study is the first to show the role of LEC/CCL16 in allergic lung diseases.

LEC/CCL16 is the functional ligand for H4 that is expressed on eosinophils [15], thus indicating a novel function of this chemokine in eosinophil recruitment. The current study demonstrated high concentrations of LEC/CCL16 in BALF from patients with eosinophilic pneumonia. The LEC/CCL16 levels correlated with the eosinophil number in BALF from the population studied. Moreover, the elevated BALF LEC/CCL16 levels

seen during the active phase subsequently declined after remission. When the LEC/CCL16 levels were compared among clinically distinct types of eosinophilic pneumonia, AEP, CEP and drug-EP, BALF LEC/CCL16 levels in AEP were significantly higher than those in CEP. Collectively, the elevated LEC/CCL16 in BALF of eosinophilic pneumonia could act as eosinophil chemoattractant in the active and acute phase of the disease. In contrast, no correlation was observed between the LEC/CCL16 and the eosinophil number when restricted to the eosinophilic pneumonia group. This may be due to the participation of other chemotactic factors for eosinophils in the eosinophil accumulation, because several chemokines are considered to cooperatively act as eosinophil attractants. Since the current results did not include functional studies, further investigation into the contribution of LEC/CCL16 to eosinophil recruitment in comparison with other eosinophil chemoattractants, such as eotaxin/CCL16 and RANTES/CCL5, would be of great value.

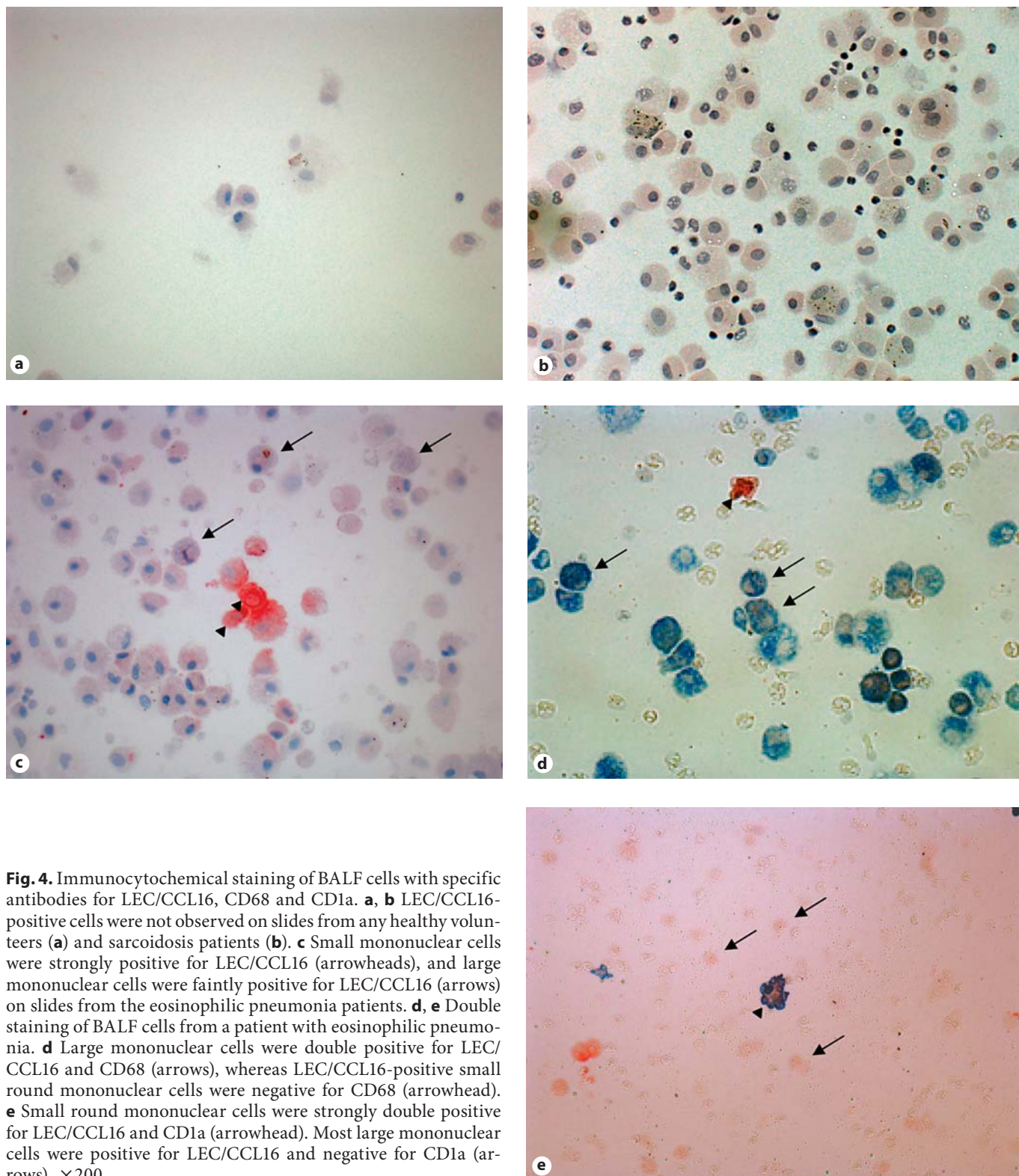


Fig. 4. Immunocytochemical staining of BALF cells with specific antibodies for LEC/CCL16, CD68 and CD1a. **a, b** LEC/CCL16-positive cells were not observed on slides from any healthy volunteers (**a**) and sarcoidosis patients (**b**). **c** Small mononuclear cells were strongly positive for LEC/CCL16 (arrowheads), and large mononuclear cells were faintly positive for LEC/CCL16 (arrows) on slides from the eosinophilic pneumonia patients. **d, e** Double staining of BALF cells from a patient with eosinophilic pneumonia. **d** Large mononuclear cells were double positive for LEC/CCL16 and CD68 (arrows), whereas LEC/CCL16-positive small round mononuclear cells were negative for CD68 (arrowhead). **e** Small round mononuclear cells were strongly double positive for LEC/CCL16 and CD1a (arrowhead). Most large mononuclear cells were positive for LEC/CCL16 and negative for CD1a (arrows). $\times 200$.

Previous studies have shown that different subsets of lymphocytes accumulate into the inflamed lung of patients with eosinophilic pneumonia and sarcoidosis. The Th1 cells preferentially expressing CCR5 and CXCR3 dominate in the granuloma sites of sarcoidosis [22–24]. Th2 cells bearing CCR4 increase in the lungs of patients with eosinophilic pneumonia [25], in which the CCR4 ligands are correspondingly elevated in BALF [9, 10, 25], suggesting the Th2-polarized condition of eosinophilic pneumonia. However, controversy remains, because RANTES and macrophage inflammatory chemokine- β /CCL4, the ligands for CCR5, increase in BALF of eosinophilic pneumonia [4, 6]. Moreover, the levels of interferon-inducible protein 10/CXCL10 and monokines induced by interferon- γ /CXCL9, the ligands for CXCR3, are elevated in BALF from patients with eosinophilic pneumonia [7]. These results suggest that Th1 as well as Th2 lymphocytes may participate in the pathogenesis of eosinophilic pneumonia. According to a previous investigation, the LEC/CCL16 has been shown to be an affinity ligand for CCR1, CCR2, CCR5 and CCR8 which is expressed on lymphocytes [11–14]. The present study demonstrated a correlation between the LEC/CCL16 level and the absolute number of lymphocytes; therefore, it may act as chemoattractant for lymphocytes with no regard to Th1 and Th2. However, eosinophilic pneumonia, but not sarcoidosis, demonstrated an elevation of LEC/CCL16, which may suggest LEC/CCL16 functions only in the pathogenesis of eosinophilic pneumonia.

In the immunocytochemical study, positive staining for LEC/CCL16 protein was detected in the CD68-positive alveolar macrophages harvested from patients with eosinophilic pneumonia but not from sarcoidosis patients and controls. This is consistent with the findings of

previous studies; LEC/CCL16 is induced in monocytes by IL-10 [26], and BALF IL-10 levels are elevated in eosinophilic pneumonia, but not in sarcoidosis [27]. More strikingly, CD1a-positive dendritic cells strongly expressed LEC/CCL16, which is a novel finding. Consistent with the findings of a previous report demonstrating that dendritic cells are chief producers of TARC/CCL17 in the lung [28], a recent study showed that alveolar dendritic cells harvested from AEP patients strongly expressed TARC/CCL17 [10]. Given that dendritic cells are regulators of immunity and tolerance, eosinophilic inflammation may start by activation of dendritic cells and chemokine production. Taken together, the present data, in combination with those of previous studies, may indicate that dendritic cells as well as alveolar macrophages play an important role in the pathogenesis of eosinophilic pneumonia via the production of these chemokines.

In conclusion, elevated levels of LEC/CCL16 were detected in BALF of patients with eosinophilic pneumonia. The LEC/CCL16 levels correlated with the numbers of eosinophils and lymphocytes. The LEC/CCL16 expression was observed in alveolar macrophages and more strongly in dendritic cells. These results suggest that LEC/CCL16 may be part of the pathogenic pathway in eosinophilic pneumonia. Further studies are needed to elucidate the distinct role of LEC/CCL16 in eosinophilic pneumonia.

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