

Elevated concentrations of CCR7 ligands in patients with eosinophilic pneumonia

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Abstract

Background: Previous studies suggest that dendritic cells and macrophages play an important role in inflammation of eosinophilic pneumonia. The mechanism of dendritic cell and macrophage accumulation into the lung, however, is unknown. Here, we hypothesized that CCR7 ligands, CCL19 and CCL21, contribute to the accumulation of dendritic cells and alveolar macrophages in the inflamed lung of patients with eosinophilic pneumonia.

Methods: Concentrations of the CCR7 ligands as well as CCL16, CCL17 and CCL22 in the bronchoalveolar lavage fluid of 53 patients with eosinophilic pneumonia, 29 patients with sarcoidosis, 18 patients with idiopathic pulmonary fibrosis and 12 healthy volunteers were measured by enzyme-linked immunosorbent assay. Cell sources of CCR7 ligands and CCR7-expressing cells in the bronchoalveolar lavage fluid were evaluated by immunocytochemistry.

Results: CCL19 and CCL21 levels in the bronchoalveolar lavage fluid were significantly higher in patients with eosinophilic pneumonia than in controls. Levels of CCL19, but not CCL21, were statistically correlated with the levels of CCL16, CCL17 and CCL22 in patients with eosinophilic pneumonia. Immunocytochemistry revealed CCL19 expression in dendritic cells, macrophages and T-lymphocytes harvested from patients with eosinophilic pneumonia, and CCR7 expression in dendritic cells and macrophages. Levels of CCL19, but not CCL21, were significantly decreased after remission in patients with eosinophilic pneumonia. After provocation tests, CCL19 levels were elevated in all patients with eosinophilic pneumonia.

Conclusions: These findings indicate that CCL19 rather than CCL21 may contribute to the accumulation of dendritic cells and macrophages in the inflamed lungs of patients with eosinophilic pneumonia.

Eosinophilic pneumonia (EP) is characterized by a prominent accumulation of eosinophils in the lung parenchyma (1–3). Several molecules, including cytokines and chemokines, are involved in establishing inflammation in EP (4–9). The mechanism underlying eosinophil accumulation in the lungs, however, is not fully understood.

Th2-specific chemokines, CCL17 and CCL22, are highly expressed in lung specimens, contributing to eosinophilic inflammation through the induction of interleukin (IL)-4, IL-5 and IL-13 (5, 7, 9), which may provide clues to the immunopathogenesis of EP. CCL16 has important roles in the accumulation of H4-expressing eosinophils and CCR1-, 2-,

3-, 4- and 5-expressing lymphocytes in EP (8). We previously demonstrated that dendritic cells (DC) and alveolar macrophages (AM) are the sources of CCL16 and CCL17 in EP (7, 8). Dendritic cells and AM are key cells involved in the pathogenesis of inflammation in EP. To date, however, the mechanism of accumulation of DC and AM in the lung in EP has not been fully elucidated. CCL19 and CCL21 act as chemoattractants for CCR7-expressing DC and macrophages (10–12). We hypothesized that CCR7 ligands have an important role in the accumulation of DC and AM in EP.

In the present study, we measured the CCL19 and CCL21 concentrations in the bronchoalveolar lavage fluid (BALF)

from patients with EP and compared the values with those from patients with sarcoidosis and idiopathic pulmonary fibrosis (IPF), as well as healthy volunteers (HV). We also examined CCL19 and CCL21 concentrations during the disease course of EP. Finally, we used immunohistochemistry to determine the cell sources of CCL19 and CCL21, and CCR7-expressing cells in the BALF cells.

Materials and methods

Patient recruitment and bronchoalveolar lavage

This study was approved by the ethics committee of Oita University Faculty of Medicine. Studies were performed in 53 patients with EP, including 12 patients with acute eosinophilic pneumonia (AEP), 22 patients with chronic eosinophilic pneumonia (CEP) and 19 patients with drug-induced eosinophilic pneumonia (drug-EP). Acute eosinophilic pneumonia was diagnosed according to the criteria proposed by Allen et al. (1, 13). Chronic eosinophilic pneumonia was diagnosed by the criteria proposed by Carrington et al. (2). Patients with drug-EP satisfied the diagnostic criteria proposed by Solomon et al. (3). 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 (14). The diagnosis of IPF was based on the international statement on IPF (15). As a control, 12 HV participated in this study. None of the HV had a history of respiratory or allergic disease.

Bronchoalveolar lavage (BAL) was performed after obtaining written informed consent from all subjects, including HV. None of the patients or controls received corticosteroid therapy at the time of sample retrieval. The BAL procedure and cell analysis were performed as described previously (7, 9). Briefly, BAL was performed by injection of 150 ml of saline into the middle lobe or left lingula. Cells were counted in a hemocytometer. A 100- μ l aliquot of cells (1×10^6 /ml) was subjected to cytocentrifugation and air-dried to obtain differential cell counts after staining with May–Grünwald–Giemsa stain. The cell-free supernatants were stored at -80°C until further analysis. Bronchoalveolar lavage was performed during the active state in nine patients with EP, and during the remission state, in four patients with AEP, three patients with drug-EP and two patients with CEP. Among them, spontaneous remission occurred upon removal of the causative agent, such as cigarette smoke or drugs, in one AEP patient and two drug-EP patients. The remaining six patients (three AEP patients, one drug-EP patient and two CEP patients) were treated with corticosteroids. We measured the CCR7 ligands in the BALF obtained in the remission state.

Provocation tests were performed in three patients with EP, and positive results were obtained. We defined the provocation test as positive when the BALF eosinophil number increased again (more than 50%) and symptoms (fever and cough) reappeared. One patient with AEP was exposed to cigarette smoke, and two drug-EP patients were challenged with the causal drug, minocycline, which resulted in the re-appearance of low-grade fever and mild coughing. A third BALF

examination was performed to verify that cigarette smoke or the suspected drug induced the eosinophilic inflammation. The BALF eosinophil numbers were significantly increased, and thus, the provocation tests were interpreted to be positive in these cases. We also measured the CCR7 ligand concentrations in the BALF obtained after the provocation test.

Measurement of CCL16, CCL17, CCL19, CCL21 and CCL22

Concentrations of CCL16, CCL17, CCL19, CCL21 and CCL22 in the BALF were measured by enzyme-linked immunosorbent assay according to the manufacturer's protocol. The minimum detectable dose in the enzyme-linked immunosorbent assay system was 2 pg/ml CCL16, 7 pg/ml CCL17, 3 pg/ml CCL19, 8 pg/ml CCL21 and 62.5 pg/ml CCL22.

Immunocytochemistry

Expression of CCL19, CCL21 and CCR7 was detected in cytocentrifuge preparations of BALF cells using the streptavidin–biotin/horseradish peroxidase method with mouse anti-CCL19 monoclonal antibody (MoAb; R&D Systems, Abingdon, UK), anti-CCL21 MoAb (R&D Systems) and anti-CCR7 MoAb (R&D Systems). Peroxidase activity was visualized with amino-3, 9-ethylcarbazole. For double-staining, the slides were incubated with a second primary antibody, goat anti-CD68 MoAb (R&D Systems) as a cell marker for AM, goat anti-CD3 polyclonal antibody (PoAb; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a cell marker for T-lymphocytes, rat anti-CD1a MoAb (Lifespan Biosciences Inc, Seattle, WA, USA) as a cell marker for immature DC, rabbit anti-CD83 PoAb (Santa Cruz Biotechnology) as a cell marker for immature DC and rabbit anti-S100 PoAb (Nichirei, Tokyo, Japan) and rabbit anti-Langerin PoAb (Santa Cruz Biotechnology) as a cell marker for Langerhans cells. Alkaline phosphatase activity was evaluated using the Vectastain ABC-alkaline phosphatase substrate kit III (Vector Laboratories, Inc., Burlingame, CA, USA).

Statistical analysis

Results are expressed as median and interquartile range. Data were analyzed using the Kruskal–Wallis test for multiple group comparison, followed by the Mann–Whitney *U*-test for between-group comparisons. Differences in the characteristics of the study populations were assessed using the chi-square test. Correlation coefficients were determined using Spearman's rank correlation test. Changes in CCR7 ligands levels during evolution of the disease within the same individual were assessed using the Wilcoxon signed rank test. A *P*-value of $P < 0.05$ was considered significant.

Results

Patient characteristics and BALF findings

Fifty-three patients with EP were enrolled in the study. The control group comprised 29 sarcoidosis patients, 18 IPF

patients and 12 HV. Detailed characteristics of these patients and BALF findings are described in Table 1. More women than men tended to have sarcoidosis. The median age of patients with EP and IPF was greater than that of HV. Patients with sarcoidosis tended to be nonsmokers compared with the other groups. Cell concentrations in the BALF were significantly higher in patients with EP, sarcoidosis and IPF than in HV. The percentage of AM in the BALF was significantly lower in patients with EP, sarcoidosis and IPF compared with HV. The percentage of lymphocytes in the BALF was significantly greater in patients with EP and sarcoidosis than in HV. The percentage of neutrophils in the BALF was significantly greater in patients with IPF than in HV. The percentage of eosinophils in the BALF was significantly greater in patients with EP and IPF than in HV.

CCR7 ligand concentration in EP

The CCR7 ligand concentrations in the BALF from patients with EP, sarcoidosis and IPF, as well as HV are shown in Fig. 1. CCL19 concentrations were significantly higher in patients with EP (median, 87 pg/ml; range, 0–12,492 pg/ml) than in patients with sarcoidosis (median, 10 pg/ml; range, 0–196 pg/ml) and IPF (median, 2 pg/ml; range, 0–620 pg/ml) and in HV (median, 0 pg/ml; range, 0–99 pg/ml). CCL21 concentrations were significantly higher in patients with EP (median, 99 pg/ml; range, 9–418 pg/ml) than in patients with sarcoidosis (median, 49 pg/ml; range, 0–123 pg/ml) or IPF (median, 58 pg/ml; range, 0–173 pg/ml). The BALF CCL19 concentrations were higher in AEP (median 3002 pg/ml, range 45–12,492 pg/mL) than in CEP (median 21 pg/ml, range 0–8,140 pg/ml, $P = 0.0009$), or drug-EP (median 24 pg/ml, range 0–11 465 pg/ml, $P = 0.0039$). On the other hand, the BALF CCL21 levels were not different between the three EP groups. When we divided the EP patients according to smoking status (current/ex/never), the concentrations of

BALF CCL19 were significantly higher in current smokers (median 224 pg/ml, range 0–12 492 pg/ml) than in ex-smokers (median 23 pg/ml, range 0–11,465 pg/ml, $P = 0.0285$) and those who had never smoked (median 21 pg/ml, range 0–538 pg/ml, $P = 0.0248$). On the other hand, the BALF CCL21 levels were not significantly different between the three EP groups regardless of smoking status. In addition, the BALF CCL19 and CCL21 levels were not significantly different between the three EP groups according to smoking status in patients with IPF and sarcoidosis, as well as in HV.

Concentrations of CCL16, CCL17 and CCL22 in EP

The BALF CCL16 levels were significantly higher in patients with EP (median, 29 pg/ml; range, 0–795 pg/ml) than in patients with sarcoidosis (median, 5 pg/ml; range, 0–17 pg/ml; $P < 0.0004$), IPF (median, 12 pg/ml; range, 0–224 pg/ml; $P = 0.0221$) and HV (median, 7 pg/ml; range, 0–31 pg/ml; $P < 0.0001$). The BALF CCL17 levels were significantly higher in patients with EP (median, 30 pg/ml; range, 0–23 225 pg/ml) than in those with sarcoidosis (median, 0 pg/ml; range, 0–10 pg/ml, $P < 0.0001$) and IPF (median, 0 pg/ml; range, 0–81 pg/ml; $P < 0.0001$) and in HV (median, 0 pg/ml; range, 0–36 pg/ml; $P = 0.0004$). The BALF CCL22 levels were significantly higher in patients with EP (median, 136 pg/ml; range, 0–15 955 pg/ml) than in those with sarcoidosis (median, 0 pg/ml; range, 0–187 pg/ml; $P < 0.0001$) and IPF (median, 0 pg/ml; range, 0–150 pg/ml; $P = 0.0069$) and in HV (median, 0 pg/ml; range, 0–140 pg/ml; $P = 0.0426$).

Relationship between CCR7 ligands and CCL16, CCL17, CCL22 in EP

We examined whether the CCR7 ligand concentration in the BALF correlated with the CCL16, CCL17 and CCL22 concentrations in patients with EP. CCL19 levels were

Table 1 Characteristics of the study population

	EP	SAR	IPF	HV
Number	53	29	18	12
Age (yr, median, range)	57 (25, 86)**	56 (25, 83)	69 (52, 81)*	25 (21, 31)
Sex (women/men)	24/29	20/9	6/12	2/10
Smoking habit (current/ex/non)	23/12/18	7/2/20	5/9/4	6/3/3
BALF findings				
TCC ($\times 10^5$ /ml, median, range)	5.1 (0.3, 86.6)##	2.6 (0.7, 5.6)#	2.5 (1.4, 6.0)#	1.5 (0.8, 3.9)
AM (% , median, range)	29.9 (2.7, 79.0)##	77.0 (23.0, 96.3)##	85.1 (14.4, 97.1)¶	95.5 (82.9, 98.0)
Lym (% , median, range)	14.2 (1.4, 56.3)#	20.0 (2.7, 69.0)##	4.7 (0.6, 82.9)	3.5 (1.3, 9.5)
Neut (% , median, range)	1.7 (0.0, 44.0)	0.5 (0.0, 4.6)	3.0 (0.0, 57.0)*	1.0 (0.0, 2.8)
Eo (% , median, range)	40.7 (11.7, 93.0)##	0.0 (0.0, 14.0)	1.0 (0.0, 5.6)*	0.3 (0.0, 2.8)

EP, eosinophilic pneumonia ($n = 53$); SAR, sarcoidosis ($n = 29$); IPF, idiopathic pulmonary fibrosis ($n = 18$); HV, healthy volunteer ($n = 12$); yr, years; current, current smoker; ex, ex-smoker; non, nonsmoker; BALF, bronchoalveolar lavage fluid; TCC, total cell concentration; AM, alveolar macrophage; Lym, lymphocyte; Neut, neutrophil; Eo, eosinophil.

Differences between groups were assessed using the Kruskal-Wallis test, the Mann-Whitney U -test, and the chi-square test.

* $P < 0.05$, compared with HV, ** $P < 0.01$, compared with HV, ¶ $P < 0.001$, compared with HV, # $P < 0.0005$, compared with HV, ## $P < 0.0001$, compared with HV.

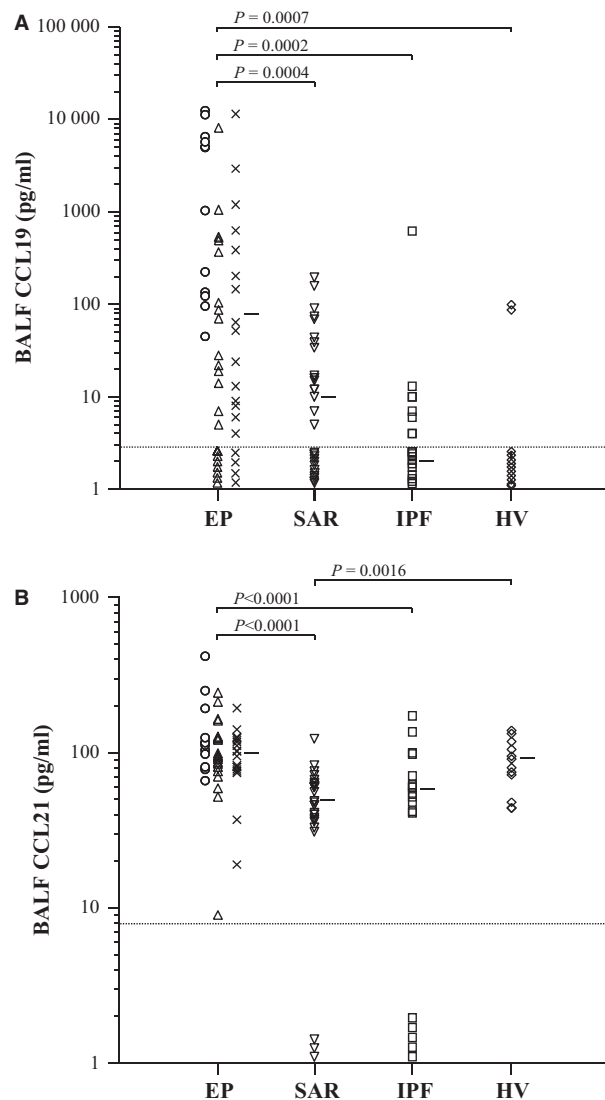


Figure 1 Concentrations of CCL19 and CCL21 in bronchoalveolar lavage fluid obtained from patients with eosinophilic pneumonia ($n = 53$), sarcoidosis ($n = 29$) and idiopathic pulmonary fibrosis ($n = 18$) and from healthy volunteers ($n = 12$). Each concentration is shown in log scale. Median values are represented by short horizontal lines. Detectable limits of CCL19 and CCL21 are represented by horizontal dotted lines. Paired comparisons were evaluated by the Mann-Whitney U -test. BALF, bronchoalveolar lavage fluid; EP, eosinophilic pneumonia; SAR, sarcoidosis; IPF, idiopathic pulmonary fibrosis; HV, healthy volunteer; $\circ$, acute eosinophilic pneumonia; Δ , drug-induced eosinophilic pneumonia; $\times$, chronic eosinophilic pneumonia.

significantly positively correlated with the CCL16 ($r = 0.560$, $P < 0.0001$), CCL17 ($r = 0.643$, $P < 0.0001$) and CCL22 ($r = 0.427$, $P = 0.0017$) levels in the BALF obtained from patients with EP (Fig. 2). In contrast, no correlation was observed between the BALF CCL21 and CCL16, CCL17 or CCL22 levels.

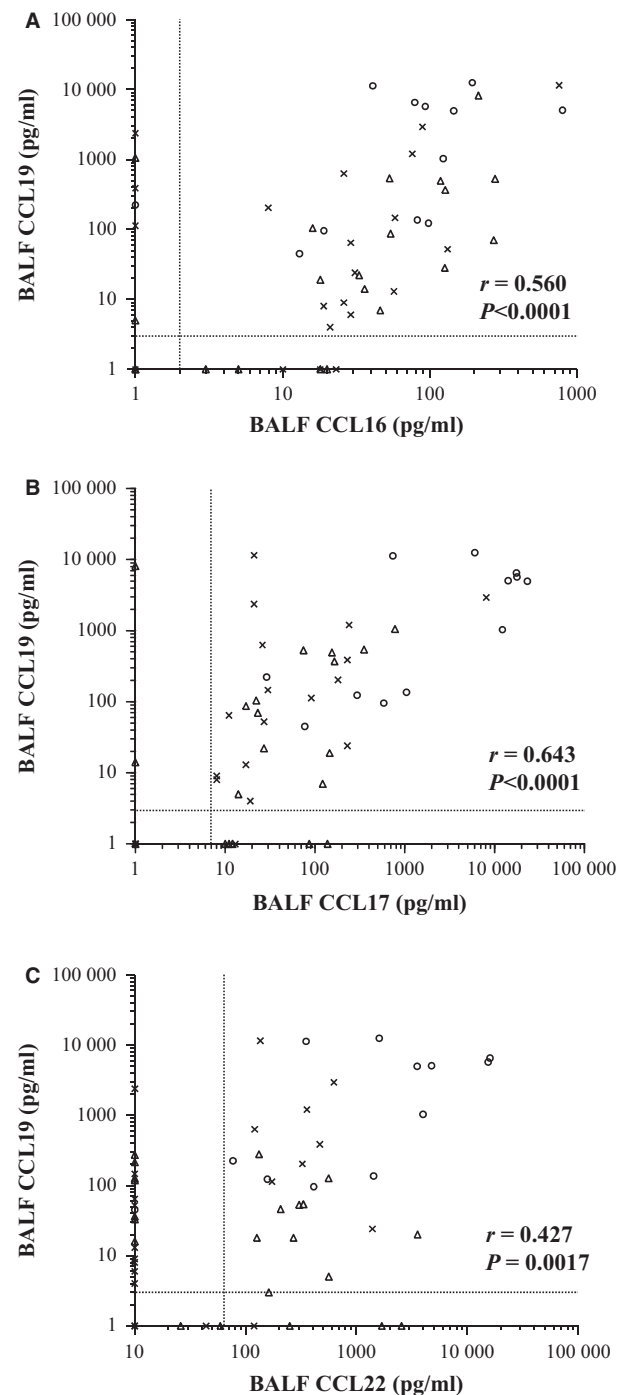


Figure 2 Relation between the concentrations of CCL19 and CCL16 (A), CCL17 (B) and CCL22 (C) in the bronchoalveolar lavage fluid from patients with eosinophilic pneumonia. Each concentration is shown in log scale. Detectable limits of each concentration are represented by the horizontal dotted lines. Correlation coefficients were determined using Spearman's rank correlation test. BALF, bronchoalveolar lavage fluid. $\circ$, acute eosinophilic pneumonia; Δ , drug-induced eosinophilic pneumonia; $\times$, chronic eosinophilic pneumonia.

Immunocytochemistry results of CCR7 and CCR7 ligand expression in BALF cells

Immunocytochemistry was performed on BALF cells from patients with EP and HV. Negative controls, which were run by replacing the primary antibodies with an unrelated isotype-matched immunoglobulin, were uniformly nonreactive. We observed weakly CCL19-positive large mononuclear cells on the slides from HV (Fig. 3A). In contrast, slides from patients with EP contained strongly CCL19-positive immunoreactive cells for CCL19. Among them, we observed two types of CCL19-positive cells: a large mononuclear cell and a smaller mononuclear cell (Fig. 3B). CCL21-positive cells were not detected on the slides from patients with HV or EP. CCR7-positive cells were observed in large mononuclear cells in HV as well as in patients with EP. A few small mononuclear cells from patients with EP were strongly positive for CCR7 (Fig. 3C, D).

Determination of CCL19- and CCR7-positive phenotypes

To determine the phenotype of CCL19-positive and CCR7-positive BALF cells, double-staining was performed for cells obtained from nine patients with EP and five HV. For cell markers, we used CD3 for T-lymphocytes, CD68 for AM, CD1a for immature DC, CD83 for mature DC, and S100 and Langerin for Langerhans cells. Large mononuclear cells stained with anti-CCL19 PoAb were

CD68-positive from patient with EP (Fig. 4). Most small CCL19-positive cells were also CD3 positive (Fig. 4B). A few small CCL19-positive cells were also CD1a positive (Fig. 4C). Large CCR7-positive mononuclear cells were positive for CD68 (Fig. 4D). A few small CCR7-positive cells were immunoreactive to CD1a (Fig. 4E). Immunoreactivity for CD83, S100 and Langerin was negative in the slides of BALF obtained from EP patients and HV. CCL19-positive cells were also positive for CD68 (EP: median 87%; range 67–95%, HV: median 100%; range 99–100%, $P = 0.0041$), CD1a (EP: median 5%; range 2–6%, HV: median 0%; range 0–2%) or CD3 (EP: median 9%; range 3–29%, HV: median 0%; range 0–0%, $P = 0.0027$). CCR7-positive cells were also positive for CD68 (EP: median 95%; range 88–97%, HV: median 100%; range 99–100%, $P = 0.0027$) or CD1a (median 7%; range 4–12%, HV: median 0%; range 0–1%, $P = 0.0027$), but not for CD3 (Fig. S1).

Serial changes in the CCR7 ligand levels in patients with EP

To examine whether CCR7 ligands reflect the degree of disease activity in patients with EP, we compared the CCR7 ligand concentration data between BALF obtained during the active phase and the remission phase. A second BAL was performed during the remission phase in nine patients. The elevated CCL19 levels observed in the acute phase were significantly decreased after remission (Fig. 5A). On the other

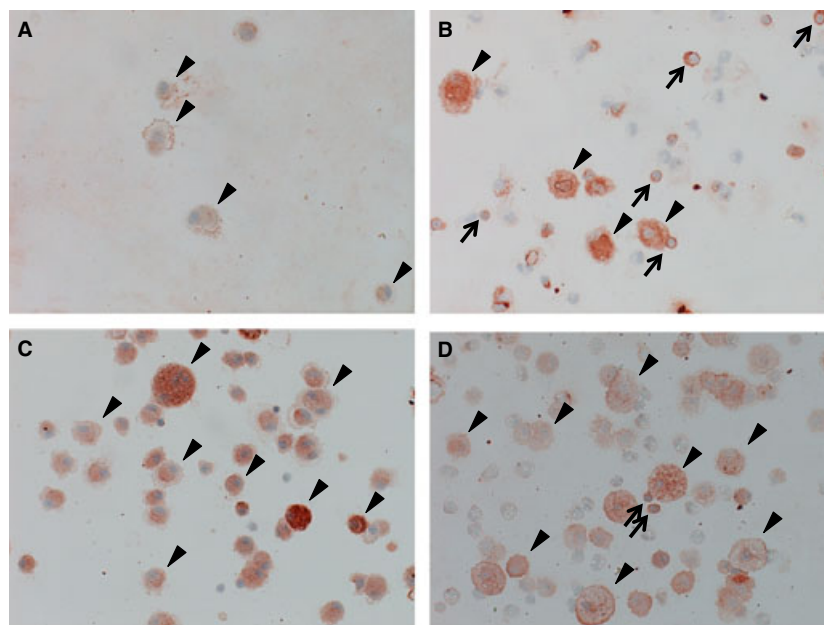


Figure 3 Immunocytochemical staining of bronchoalveolar lavage fluid cells with specific antibodies for CCL19 and CCL21. (A) Large mononuclear cells from healthy volunteers were faintly positive for CCL19 (red, arrowhead). (B) Large mononuclear cells from patients with eosinophilic pneumonia were strongly positive for CCL19 (red, arrowhead), and small mononuclear cells were strongly positive for

CCL19 (red, arrow). (C) Large mononuclear cells from healthy volunteers were strongly positive for CCR7 (red, arrowhead). (D) Large mononuclear cells from patients with eosinophilic pneumonia were strongly positive for CCR7 (red, arrowhead), and a few small mononuclear cells were strongly positive for CCR7 (red, arrow). A through D, Original magnification: $\times 200$.

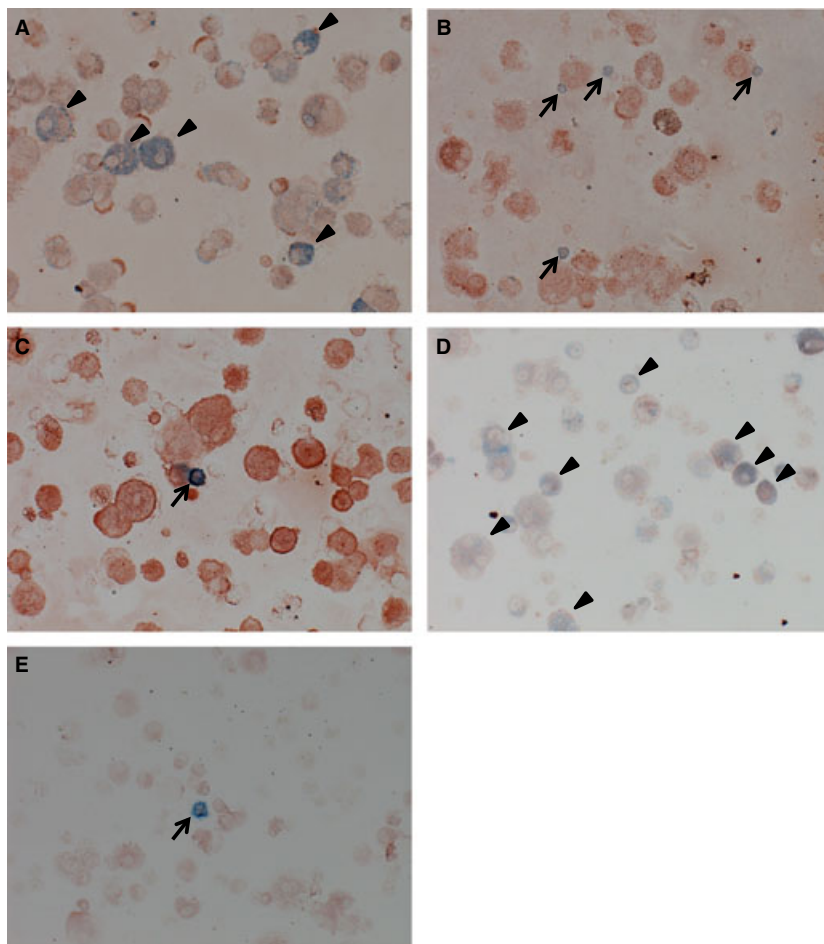


Figure 4 Double-staining of bronchoalveolar lavage fluid cells from a patient with eosinophilic pneumonia. (A) Large mononuclear cells were double-positive for CCL19 and CD68 (red + blue, arrowhead). (B) Most small mononuclear cells were double-positive for CCL19 and CD3 (red + blue, arrow). (C) Small round mononuclear cells

were strongly double-positive for CCL19 and CD1a (dark brown, arrow). (D) Most large mononuclear cells were double-positive for CCR7 and CD68 (red + blue, arrowhead). (E) Small round mononuclear cells were double-positive for CCR7 and CD1a (red + blue, arrow). A through E, Original magnification: $\times 200$.

hand, the CCL21 levels did not change significantly after remission (Fig. 5B).

Changes in the CCR7 ligand levels in patients with EP after provocation tests

We compared the CCR7 ligand level data in BALF harvested during the remission phase and after the provocation test. The provocation test was performed in three patients. No statistically significant difference was detected due to the small sample number. The CCL19 levels increased after remission in all three patients (Fig. 5C). The CCL21 levels increased in two patients and almost same in one patient (Fig. 5D).

Discussion

The findings of the present study demonstrated that CCL19 and CCL21 concentrations were elevated in patients with EP.

Immunocytochemistry of the BALF from patients with EP revealed that DC, AM and T-lymphocytes were CCL19-positive and no CCL21-positive cells were detected. Dendritic cells and AM were also CCR7 positive. BALF CCL19 levels significantly correlated with CCL16, CCL17 and CCL21 levels, whereas BALF CCL21 levels did not correlate with the levels of any of these chemokines. The elevated BALF CCL19 levels observed in the active disease state were significantly decreased after remission, but BALF CCL21 levels were not. Moreover, BALF CCL19 levels in all patients with EP increased after the provocation test. These findings indicate that CCL19, but not CCL21, might contribute to the accumulation of CCR7-expressing DC and AM, which produce CCL16, CCL17 and CCL21 in the pulmonary parenchyma, thereby contributing to the eosinophilic inflammation.

Dendritic cells and AM are key cells involved in the inflammation of EP. We previously demonstrated that

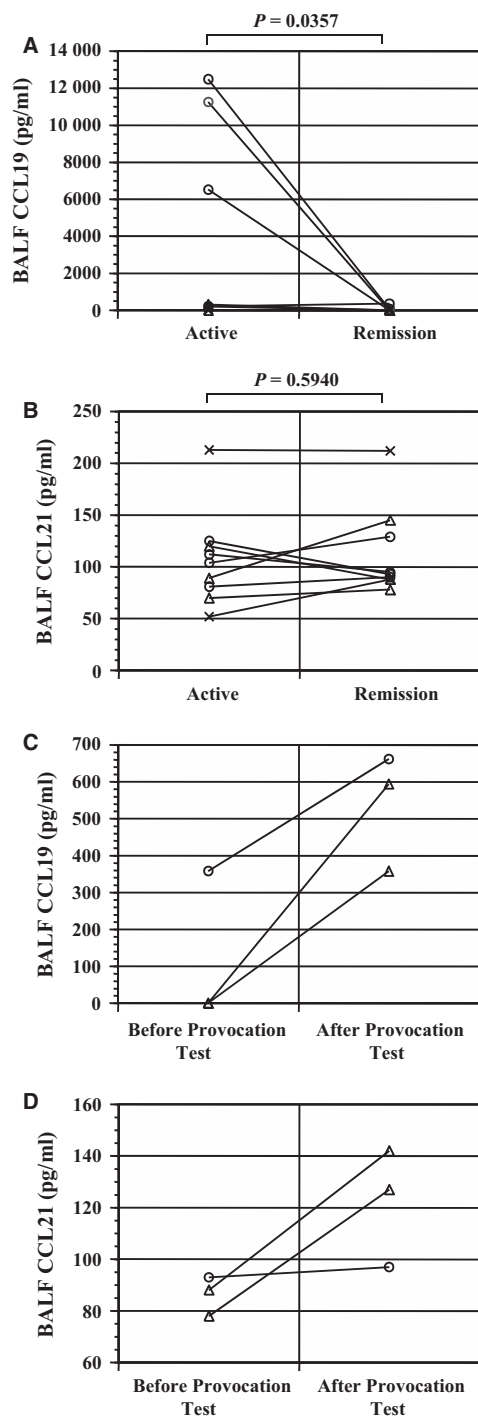


Figure 5 Serial concentrations of CCL19 (A) and CCL21 (B) in bronchoalveolar lavage fluid from patients with eosinophilic pneumonia in active and remission states ($n = 9$). Serial concentrations of CCL19 (C) and CCL21 (D) in the BALF from patients with eosinophilic pneumonia before and after the provocation test ($n = 3$). Changes in each concentration were assessed using the Wilcoxon signed rank test (A, B). BALF, bronchoalveolar lavage fluid. $\circ$, acute eosinophilic pneumonia; Δ , drug-induced eosinophilic pneumonia, $\times$, chronic eosinophilic pneumonia.

CCL17 and CCL22 produced by DC and AM accumulate in CCR4-expressing Th2 cells, leading to eosinophilic inflammation (7). CCL16 produced by DC and AM accumulates in H4-expressing eosinophils in the lung parenchyma (8). In this study, although both BALF CCL19 and CCL21 levels were elevated, only CCL19 levels correlated with the CCL16, CCL17 and CCL22 levels in the BALF from patients with EP. CCL19 is commonly produced by several types of cells, such as stromal cells in T cell-rich lymph nodes, T-lymphocytes, monocytes and macrophages (12, 16–19). CCR7 is an interesting receptor with the potential to influence responses in peripheral tissues and lymph nodes (20). CCR7 is expressed on immune cells, such as DC, T-cells and macrophages (21–23). In our study, CCL19-positive cells were observed in CD1a-positive/CD83-negative immature DC, CD3-positive T-lymphocytes and CD68-positive AM, while CCR7 was expressed in CD1a-positive/CD83-negative immature DC and CD68-positive AM in the BALF from patients with EP. Altogether, CCL19 produced by BALF cells in patients with EP may induce the accumulation of CCR7-expressing immature DC and AM, which produce CCL16, CCL17 and CCL22 in the alveolar spaces.

Although we detected elevated levels of CCL21, no CCL21-positive cells were observed in patients with EP. Immunohistochemistry in small transbronchial lung biopsy samples revealed no CCL21-positive cells in patients with EP, whereas positive staining is observed in inducible bronchus-associated tissue from patients with rheumatoid arthritis (24). CCL21 was originally reported to be a chemokine that is constitutively expressed by stromal cells and high endothelial venules in secondary lymphoid tissues and endothelium of afferent lymphatics, directing CCR7-positive cells (19, 25, 26). It is possible that CCL21 is constitutively generated by stromal cells as well as high endothelial venules, thus contributing to the homing of CCR7-expressing dendritic cells from the alveolar space to the secondary lymph nodes in the pathogenesis of EP.

Both CCL19 and CCL21 levels were elevated in the BALF from patients with EP; however, CCL19, but not CCL21, fluctuated with changes in disease activity. This discrepancy between CCL19 and CCL21 might be due to differences in organ immune systems. Enhanced expression of CCL21 is observed in some chronically inflamed organs, such as liver with chronic hepatitis C (27) and skin with T-cell-autoimmune skin disease (28). On the other hand, CCL19 seems to contribute to inflammation in pulmonary disease. CCL19 is induced locally in the lung in response to *Mycobacterium tuberculosis* and *Mycobacterium bovis bacillus Calmette-Guérin* infection (29, 30). CCL19 expression and CCL21 expression are differentially regulated; CCL19 is increased and CCL21 is decreased in the lung after *Pseudomonas aeruginosa* infection and cigarette smoke exposure (31, 32). Given that the challenge tests with cigarette smoke or drug induced CCL19 production in the BALF of EP patients in the present study, causative agents may promote CCL19 expression from immature DC and AM. Newly produced CCL19 may accelerate further accumulation of CCR7-positive DC and AM to the alveolar space.

Whether the CCR7 ligands worsen or resolve allergic inflammation remains controversial. In animal models of asthma and allergic rhinitis, CCR7 blockade or CCL19/CCL21 deficiency results in worsening of the disease (33, 34). Chemokines CCL21 and CCL19 are critical for the resolution of airway inflammation (35). On the other hand, CCR7 blockade at the ocular surface inhibits the immunopathogenesis of allergic conjunctivitis (36). In our study, the BALF CCL19 levels were elevated in the active phase and then significantly decreased in the remission phase of patients with EP. Moreover, the BALF CCL19 levels were increased after the provocation test. Similar results were observed in the levels of BALF Th2 chemokines (CCL17 and CCL22) (7, 9). Given the positive relationship between the CCL19 and CCL17 levels, the mechanism of DC accumulation may be closely linked to the mechanism of Th2 cell accumulation. It is possible that there may be a positive feedback pathway that CCL19 induces CCR7-bearing DC2 and then the DCs generate CCL19 as well as Th2 chemokines, CCL17 and CCL22. Thus, CCL19 probably acts as a molecule worsening of eosinophilic inflammation by inducing the migration of CCR7-expressing DC and AM.

In conclusion, we demonstrated that concentrations of CCR7 ligands are elevated in patients with EP. Our results indicate that CCL19 rather than CCL21 contributes to the accumulation of DC and AM, which may participate in triggering eosinophilic inflammation by producing Th2-attracting chemokines in the inflamed lungs. Further studies are needed

to examine the detailed roles of CCR7 ligands in patients with EP.

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Conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (A), (B), (C) Percentages of CD1a-, CD3-, or CD68-positive cells among CCL19-positive cells in the bronchoalveolar lavage fluid from healthy volunteers (HV; $n = 5$) and patients with eosinophilic pneumonia (EP; $n = 9$).

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Figure S1

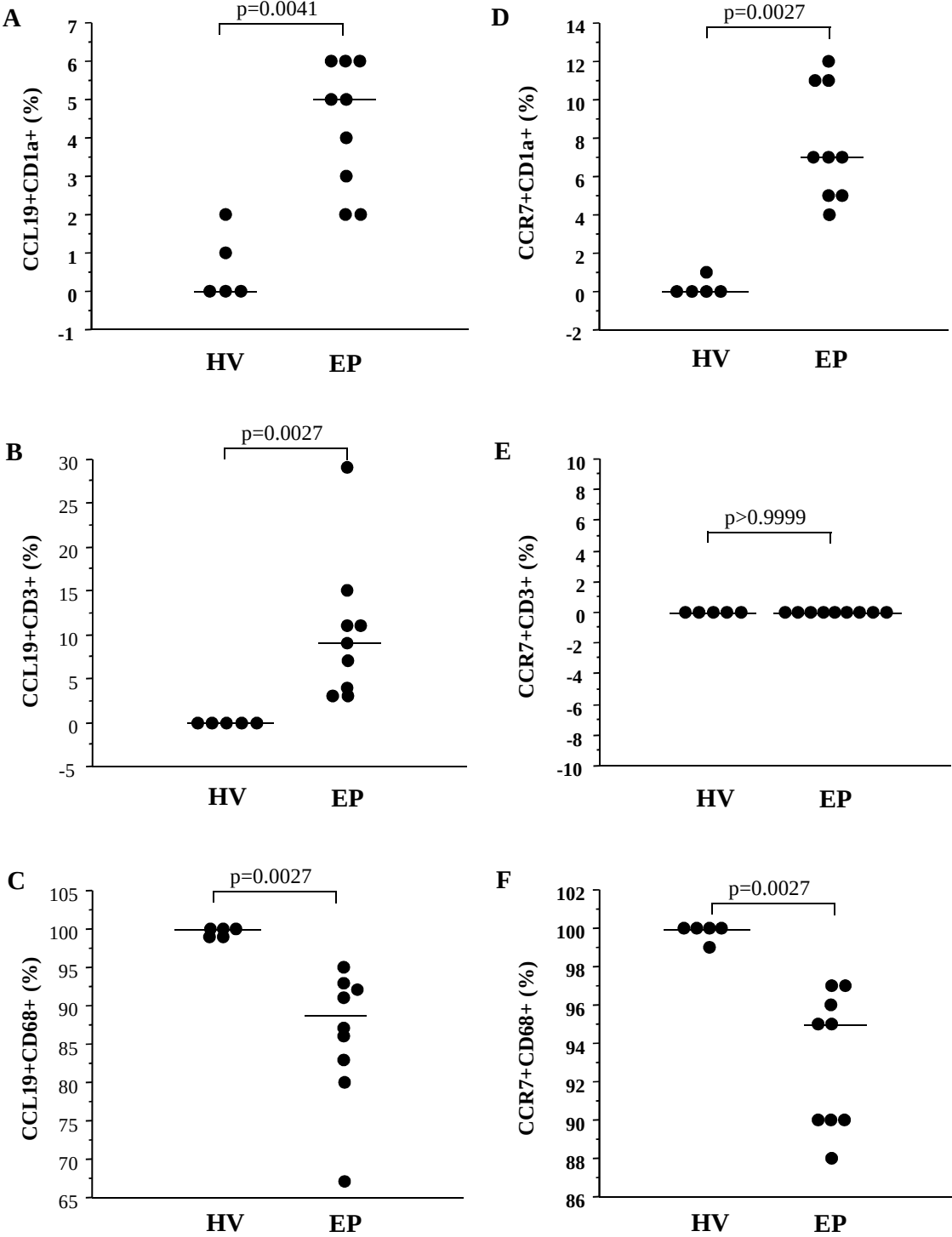


Figure S1. (A), (B), (C) Percentages of CD1a-, CD3-, or CD68-positive cells among CCL19-positive cells in the bronchoalveolar lavage fluid from healthy volunteers (HV; n=5) and patients with eosinophilic pneumonia (EP; n=9). The Y-axis shows the each percentage. The short horizontal line represents the median value. (D), (E), (F) Percentages of CD1a-, CD3-, or CD68-positive cells among CCR7-positive cells in the bronchoalveolar lavage fluid from healthy volunteers (HV; n=5) and patients with eosinophilic pneumonia (EP; n=9). The Y-axis shows the each percentage. The short horizontal line represents the median value. Paired comparisons were evaluated using the Mann-Whitney U-test.