

Original article

Accumulation of CCR4-expressing CD4⁺ T cells and high concentration of its ligands (TARC and MDC) in bronchoalveolar lavage fluid of patients with eosinophilic pneumonia

Background: Th2 cells are thought to be involved in eosinophilic inflammation of the lung. CC chemokine receptor 4 (CCR4) has been identified as a specific receptor for both thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), and is preferentially expressed on Th2 cells.

Objective: Our aim was to evaluate the role of Th2 cells in the lung of patients with eosinophilic pneumonia (EP).

Methods: The concentrations of TARC, MDC, and interleukin (IL)-5 were measured in bronchoalveolar lavage fluid (BALF) by ELISA. Proportion of CCR4-expressing CD4⁺ T cells (CCR4⁺ CD4⁺ T cells) was determined by flow cytometry.

Results: TARC and MDC concentrations in BALF were higher in patients with EP than in normal subjects. The proportion of CCR4-expressing cells among CD4⁺ T cells was higher in BALF than in peripheral blood of patients with EP. There was a significant correlation between the number of CCR4⁺ CD4⁺ T cells and the levels of TARC, MDC, and IL-5 in BALF of patients with EP.

Conclusions: Our data suggest that Th2 cells, which express CCR4 and its ligands (TARC and MDC), contribute to the pathogenesis of EP in the lung.

S. Katoh¹, K. Fukushima²,
N. Matsumoto¹, K. Matsumoto¹,
K. Abe¹, N. Onai³, K. Matsushima³,
S. Matsukura¹

¹Third Department of Internal Medicine, Miyazaki Medical College, Miyazaki; ²Nagasaki Prefecture Tarami Hospital, Nagasaki; ³Department of Molecular Preventive Medicine, The University of Tokyo, Graduate School of Medicine, Tokyo, Japan

Key words: bronchoalveolar lavage fluid; CC chemokine receptor 4; eosinophilic pneumonia; macrophage-derived chemokine; Th2 cells; thymus and activation-regulated chemokine.

Shigeki Katoh, MD, PhD
Third Department of Internal Medicine
Miyazaki Medical College
5200 Kihara, Kiyotake
Miyazaki, 889-1692
Japan

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Eosinophilic pneumonia (EP) is an inflammatory lung disease characterized by eosinophil accumulation (1–3). Previous studies including those from our laboratory have suggested that interleukin (IL)-5 might be involved in the pathogenesis of EP (4–6). Furthermore, we have recently reported that eotaxin, a specific chemokine for eosinophil, might be important in patients with EP (7).

Thymus and activation-regulated chemokine (TARC)/CCL17 is a recently identified CC chemokine that is expressed constitutively in the thymus and transiently in stimulated peripheral blood mononuclear cells. TARC functions as a selective chemoattractant for T cells (8). Macrophage-derived chemokine (MDC)/CCL22 is a newly discovered CC chemokine that has selective chemotactic activity for activated T cells and monocytes (9, 10). Recently, CC chemokine receptor 4 (CCR4) has been identified as a specific receptor for both TARC and MDC

(11, 12). On chronic stimulation, T lymphocytes develop into Th1 or Th2 cells, based on their profiles of cytokine production (13). Th2 cells release cytokines such as IL-4 and IL-5 to promote humoral immunity and allergic responses (14). CCR4 is preferentially expressed on Th2 cells (15, 16), although a small subset of CCR4⁺ memory CD4 lymphocytes could produce IFN- γ *in vitro* (17, 18). High-affinity ligands for CCR4, TARC and MDC induce selective migration of Th2 cells (19). Both TARC and MDC could play important roles in Th2-type disease conditions such as atopic dermatitis and bronchial asthma (20–22).

Accumulation and activation of T lymphocytes has been described in patients with EP (4, 23). However, the mechanisms that lead to the migration of T lymphocytes into the lung are not clear at present. We hypothesized that selective recruitment of Th2 cells into the lung in patients with EP might reflect the local production of chemokines. For this purpose, we determined, in the present study, the levels of TARC and MDC in bronchoalveolar lavage fluid (BALF) samples from EP patients by using ELISA. To characterize the immunological

Abbreviations: BALF, bronchoalveolar lavage fluid; CCR4, CC chemokine receptor 4; EP, eosinophilic pneumonia; IL, interleukin; MDC, macrophage-derived chemokine; TARC, thymus and activation-regulated chemokine.

mechanism of EP, results were compared with healthy volunteers and patients with sarcoidosis, which is thought to be a Th1-type disease. Furthermore, we investigated the expression of CCR4 on CD4⁺ T cells in BALF and peripheral blood of EP patients. Finally, we examined the relationship between the number of CCR4-expressing CD4⁺ T cells (CCR4⁺ CD4⁺ T cells) and the levels of TARC, MDC as well as IL-5 in BALF.

Material and methods

Patient population

The study population consisted of 27 untreated patients with EP (13 women and 14 men; age, 39.9 ± 4.1 years), 10 with sarcoidosis (nine women and one man; age, 52.8 ± 4.1 years; chest X-ray staging, four patients had Stage I and six patients had stage II disease), and eight healthy adults (three women and five men; age, 39.6 ± 4.5 years). None of the patients in this study was treated with corticosteroid at the time of the investigation. Eleven patients with EP, one with sarcoidosis, and two of the volunteers were smokers. Statistically, smoking status had no influence on BALF chemokine levels (data not shown).

The diagnosis of EP was based on clinical criteria; patients with no known cause of EP (drugs, parasitic infection and fungal disease), with acute or chronic dyspnea, interstitial infiltrates on chest radiographs, pulmonary eosinophilia, prompt resolution of clinical and radiographic abnormalities with or without corticosteroid therapy, and histological evidence based on examination of trans-bronchial lung biopsy specimens. The diagnosis of sarcoidosis was based on examination of biopsy specimens obtained from the lungs, lymph nodes, or skin showing noncaseating epithelioid cell granulomas, with no evidence of inorganic material known to cause granulomatous diseases.

Bronchoalveolar lavage

After obtaining informed consent from the subject, bronchoalveolar lavage was performed by using a flexible fiber-optic bronchoscope under local anesthesia of the upper airway with 2% lidocaine, as described previously (24). Briefly, the bronchoscope was wedged into the subsegmental bronchus of the right middle lobe or, in patients with EP, into areas of lung parenchyma otherwise normal on chest roentgenography, and 150 ml of normal saline was instilled in three 50-ml aliquots. Harvested BALF was filtered through sterile nylon mesh and centrifuged at 160 *g* for 10 min to obtain the cell preparation. The cells were later stained by the May-Giemsa method, and a differential count was performed on 200 cells. The remaining fluid was centrifuged at 500 *g* for 5 min, and the supernatant was stored at -80°C until use.

Flow cytometry

After lysis of red blood cells, peripheral blood leukocytes and BALF cells were stained with FITC-conjugated anti-CCR4 mAb (mouse IgG1) (19), biotinylated anti-CD4 mAb (Becton Dickinson, Mountain View, CA, USA), followed by phycoerythrin-conjugated streptavidin (Becton Dickinson) for two-color analysis, and finally analyzed by EPICS (Beckman Coulter, Tokyo, Japan). Lymphocytes were gated by flow cytometry based on forward and side light scatter. The percentages of CD3⁺, CD4⁺, and CD8⁺ cells in this gate are shown in Table 1. The frequency of CCR4⁺ cells was assessed by gating on CD4⁺ cells. FITC-conjugated-mouse IgG1 (Beckman Coulter) was used as an isotype-matched control.

Measurement of TARC, MDC, and IL-5 concentrations in BALF

BALF concentrations of TARC and MDC were measured using the respective ELISA kits (R & D Systems Inc., Minneapolis, MN, USA). The concentration of IL-5 in BALF was measured by ELISA, as described previously (25). The detection limits were 7, 62.5, and 5.0 pg/ml for TARC, MDC, and IL-5, respectively. The upper limits of the assays were 1000, 2000, and 320 pg/ml for TARC, MDC, and IL-5, respectively. The harvested BALF was diluted in some cases with saline so as to read the measured value on the standard curve. Concentrations below the lower limit of detection were assumed to be zero for the purpose of statistical analysis. The absolute levels of cytokines in BALF rather than those relative to proteins were reported in this study since cytokine levels in BALF are not influenced by the concentration of albumin, as demonstrated previously by Kadota et al. (24). The mean percentages of recovered fluid are summarized in Table 1. There were no differences in the percentage recovery of BALF among the groups.

Statistical analysis

All data were expressed as mean $\pm$ SEM. The Mann-Whitney *U*-test or paired Student's *t*-test was used to test for differences between the groups. Correlations between two variables were examined using Pearson's correlation coefficient. Probability values of less than 0.05 were regarded as significant.

Results

General characterization of BALF

The total and differential cell counts in BALF are summarized in Table 1. The total number of cells per milliliter of BALF in each patient group was higher than in healthy subjects. The percentage of eosinophils was significantly higher in EP, but not sarcoidosis, than

Table 1. Characteristics of bronchoalveolar lavage fluid cells

	Recovery (%)	Total cells (10^5 ml)	Macrophages (%)	Lymphocytes (%)	Neutrophils (%)	Eosinophils (%)	CD3 (%)	CD4 (%)	CD8 (%)
Healthy volunteers (<i>n</i> = 8)	54.6 ± 3.1	1.5 ± 0.3	88.4 ± 1.8	8.3 ± 1.5	2.8 ± 1.6	0.6 ± 0.5	59.1 ± 8.0	32.7 ± 5.0	24.8 ± 4.7
Eosinophilic pneumonia (<i>n</i> = 27)	49.4 ± 2.8	$9.3 \pm 1.6^*$	$24.8 \pm 3.2^*$	$20.8 \pm 3.4^*$	7.7 ± 2.3	$46.6 \pm 4.2^*$	69.7 ± 3.2	$45.3 \pm 2.8^*$	23.4 ± 2.5
Sarcoidosis (<i>n</i> = 10)	53.6 ± 4.2	$2.6 \pm 0.4^*$	$57.3 \pm 6.4^*$	$36.6 \pm 5.5^*$	5.8 ± 3.9	0.3 ± 0.2	70.2 ± 6.3	$55.8 \pm 5.7^*$	$12.0 \pm 2.7^*$

**P* < 0.05, compared with healthy volunteer subjects. The proportion of CD3, CD4, and CD8 positive cells in alveolar lymphocytes was evaluated by flow cytometry. Alveolar lymphocytes were gated by flow cytometry based on forward and side light scatter.

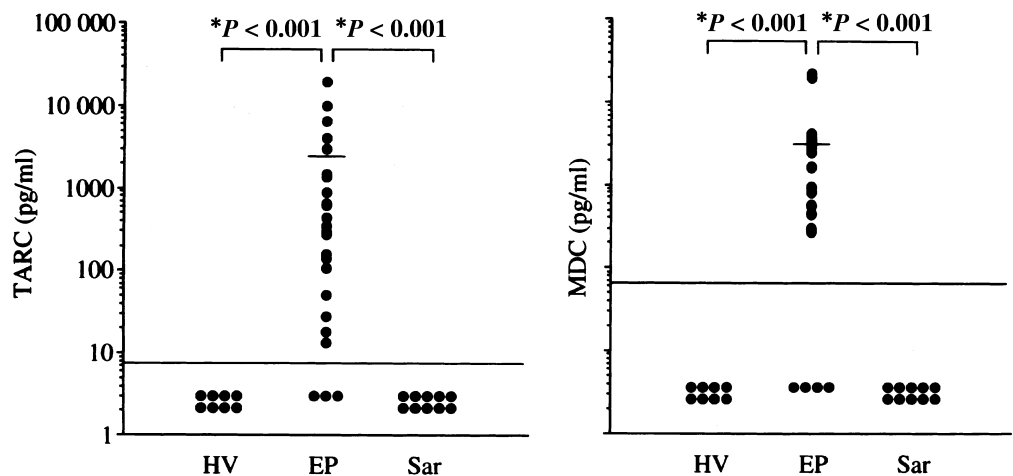


Figure 1. Concentrations of TARC and MDC in BALF. Short horizontal lines represent the mean values. Horizontal lines represent the detection limits of each chemokine. HV, healthy volunteers; Sar, sarcoidosis.

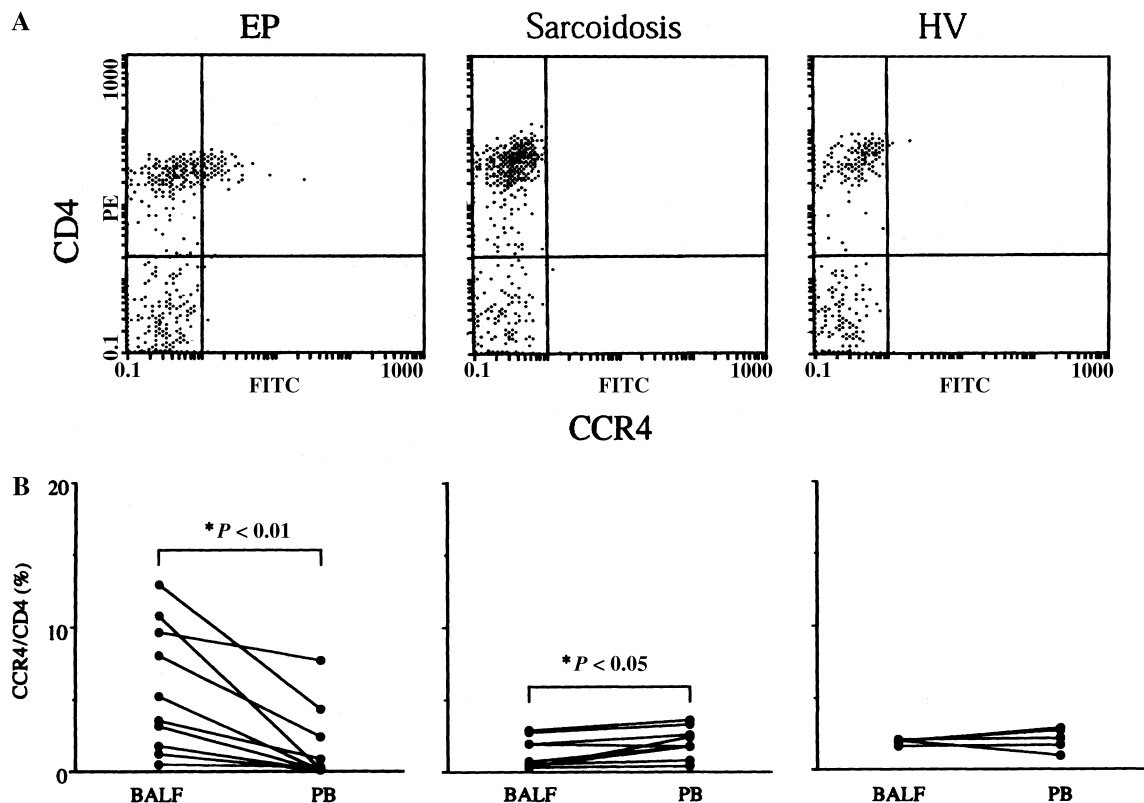


Figure 2. Representative expression of CCR4 on CD4+ T cells in BALF of patients with EP, sarcoidosis, and healthy volunteers (HV) (A). The dot plots represent expression CD4 and CCR4 for alveolar lymphocytes as described in material and methods and Table 1. Quadrants were set according to the staining of control Ab. Comparison of CCR4 expression on CD4+ T cells from BALF and peripheral blood (PB) in patients with EP, sarcoidosis and healthy volunteers (HV) (B). The frequency of CCR4+ cells on CD4+ T cells was assessed by flow cytometry. Data represent values of paired samples obtained from the same patient. Statistical significance of differences between BALF and PB was evaluated by paired Student's *t*-test.

in control subjects ($P < 0.01$). The mean percentage of lymphocytes was significantly higher in patients with EP and sarcoidosis than in healthy subjects ($P < 0.01$).

The proportion of CD3, CD4, and CD8 positive cells in BALF was evaluated by flow cytometry (Table 1). The proportion of CD4+ T cells was significantly

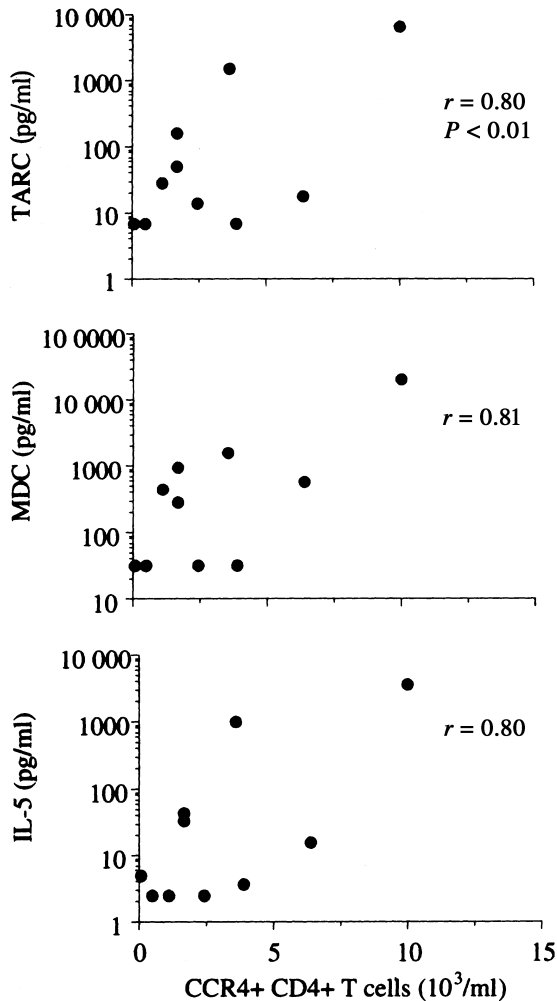


Figure 3. Relationship between the number of CCR4+ CD4+ T cells and the levels of TARC, MDC, and IL-5 in BALF of patients with EP. Correlations between absolute number of CCR4+ CD4+ T cells and concentration of TARC (top), MDC (middle), and IL-5 (bottom) in BALF of patients with EP are shown.

higher in EP and sarcoidosis, than in healthy subjects ($P < 0.05$).

TARC and MDC concentrations in BALF

Accumulation of lymphocytes of the Th2 phenotype is observed at sites of allergic inflammation (26, 27). In the next step, we assessed two specific ligands for CCR4, TARC and MDC, typical CC chemokines chemotactic for Th2 cells, in BALF of patients with EP. Interestingly, a significant amount of TARC was detected only in patients with EP (2310 ± 814 pg/ml, Fig. 1). Furthermore, elevated levels of MDC were observed in BALF of patients with EP (3176 ± 1036 pg/ml, Fig. 1), while these levels were below the detection limit (< 62.5 pg/ml) in BALF of healthy volunteers and patients with

sarcoidosis (Fig. 1). There was a high correlation between concentration of TARC and MDC in BALF of patients with EP ($r = 0.80$, $P < 0.001$, not shown). Measurement of the concentration of TARC in BALF was repeated after treatment with corticosteroid in eight patients with EP. TARC was not detected after treatment in these eight patients with EP (data not shown).

CCR4 expression on blood and BALF CD4+T cells

CCR4 is preferentially expressed in Th2 cells (15, 16). CCR4 expression on CD4+ T cells in BALF was determined using two-color flow cytometry (Fig. 2A). In patients with EP ($n = 10$), the proportion of CCR4+ cells among CD4+ T cells was significantly higher in BALF than in peripheral blood (6.8 ± 1.4 vs $1.7 \pm 0.8\%$, $P < 0.01$, Fig. 2B), while the frequency of CCR4+ cells among CD4+ T cells was significantly lower in BALF than in peripheral blood (1.3 ± 0.4 vs $2.1 \pm 0.4\%$, $P < 0.05$, Fig. 2B) in patients with sarcoidosis ($n = 9$). There was not a significant difference in frequency of CCR4+ cells among CD4+ T cells between BALF and peripheral blood (1.9 ± 0.1 vs $2.1 \pm 0.3\%$, Fig. 2B) in healthy volunteers ($n = 5$). Furthermore, absolute number of CCR4+ CD4+ T lymphocytes was significantly higher in BALF of patients with EP than in that of patients with sarcoidosis (3.8×10^3 vs 0.6×10^3 ml $^{-1}$, $P < 0.01$). There was a significant correlation between the number of CCR4+ CD4+ T lymphocytes and the levels of TARC ($r = 0.80$, $P < 0.01$, Fig. 3) and MDC ($r = 0.81$, $P < 0.01$, Fig. 3) in BALF of patients with EP. As previously reported (6, 25), the level of IL-5 in patients with EP was elevated in BALF (data not shown). The level of IL-5 in BALF also correlated with the number of CCR4+ CD4+ T lymphocytes in BALF ($r = 0.80$, $P < 0.01$, Fig. 3).

Discussion

EP is characterized by increased influx of eosinophils into the lung (1–3). IL-5 is thought to play a central role in lung eosinophilia as shown in experimental mice models of the disease (28, 29). IL-5-producing CD4+ Th2 type cells could play a pivotal role in aeroallergen-induced eosinophilia (30). In fact, elevated levels of IL-5 in BALF were reported in patients with EP in previous studies (4–6). Furthermore, accumulation of CD25-bearing activated CD4+ T lymphocytes in the lung was also reported in patients with EP (4, 23). CCR4 is thought to be preferentially expressed on Th2 cells (16). In this study, we found the significant accumulation of CCR4+ CD4+ T cells in the lung of patients with EP compared with sarcoidosis (3.8×10^3 vs 0.6×10^3 ml $^{-1}$, $P < 0.01$). Furthermore, we found a small but significantly higher proportion of CCR4+ cells among CD4+ T cells in BALF than peripheral blood in patients with EP, but not

so in patients with sarcoidosis (Fig. 2B). Taken together with our findings and previous reports, Th2 cells in the lung may be important in the development of EP.

TARC and MDC have been recently identified as specific chemotactic factors for Th2 cells (19) and both were found to be expressed in the lung and up-regulated in allergic inflammation in mice models of asthma (21, 31). It has also been reported that treatment with anti-TARC Ab strikingly attenuates the accumulation of eosinophils as well as lymphocytes in the lung of laboratory mice with asthma (31). Thus, TARC and MDC could play an important role in eosinophilic inflammation of the lung in mice. Furthermore, CCR4 and its ligands (TARC and MDC) may play an important role in pathogenesis of asthma (32, 33). Recently, it has been reported that extremely high level of TARC in BALF is found in patients with acute EP (34). In the present study, we demonstrated extremely high levels of TARC and MDC in BALF of patients with EP (Fig. 1), and TARC could not be detected after treatment with corticosteroid. These results suggest that TARC and MDC play an important role in the pathogenesis of EP. Further studies are required to evaluate the role of TARC and MDC in allergic reactions and parasitic disorders in which eosinophils is also prominent. The expression of TARC and MDC in human bronchial epithelial cells and its upregulation in patients with asthma after antigen-challenge or cytokine stimulation have been reported (32, 33). Previous studies showed that MDCs were produced by macrophage lineage cells (10, 21, 35), while there was no significant correlation between the number of alveolar macrophages and the level of MDC in BALF (data not shown). The

main source of these chemokines in the lung and the regulatory mechanisms of their production remain to be defined. It is, however, likely that a common regulatory mechanism might be involved in the production of TARC and MDC in patients with EP, because these levels in BALF were highly correlated ($r = 0.80$, $P < 0.001$).

CCR4 is a specific receptor for TARC and MDC (19). A positive correlation between TARC or MDC concentrations and the number of CCR4+ CD4+ T cells in BALF of patients with EP suggest that these chemokines could play a role in accumulation of CCR4-bearing (Th2) cells into the lung from peripheral blood (Figs 2 and 3). Further studies are required to clarify the mechanisms of accumulation of CCR4-negative T lymphocytes. It has been reported that CCR4+ and CXCR3+ CD4+ T cells predominantly produced IL-4 and IFN- γ , respectively (36). In the present study, we showed a positive correlation between the number of CCR4+ CD4+ T cells and IL-5 concentration in BALF of patients with EP (Fig. 3). Although we do not have direct evidence that CCR4+ CD4+ T cells in BALF are producing IL-5, our finding suggests that CCR4+ Th2 cells could contribute to the production of IL-5 in the lung of patients with EP (Fig. 3). Our study is the first to shed some light on the possible mechanism of accumulation of Th2 cells in the lung in patients with EP.

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