

Originals

CD4⁺-Central Memory and Effector Memory T Cells in Patients with Asthma

Bunpei Yamaguchi, MD*, Hirokuni Hirata, MD*, Kyoko Honda, MD*,
Tatsuo Yukawa, MD* and Masafumi Arima, MD**

*Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine,

**Department of Developmental Genetics, Chiba University Graduate School of Medicine,

SUMMARY

Asthma is associated with chronic airway inflammation, suggesting that its pathogenesis is driven by type 2 helper T (Th2) cells among memory/effector CD4⁺T cells. CCR4, a chemokine receptor, is considered a preferential marker for Th2 cells. Another chemokine receptor, CCR7, is regarded to be a suitable molecule for T-cell homing to lymph nodes. Recent studies have demonstrated that memory T cells are subdivided into central memory T cells (TCMs) and effector memory T cell (TEMs), designated as CCR7⁺ CD62L⁺ CD45RA⁻ and CCR7⁻ CD62L⁻ CD45RA⁻, respectively. Nevertheless, the properties of TCMs and TEMs in allergic diseases remains unknown. This study focused on the cytokine production and the populations and survival of CD4⁺ TCMs and CD4⁺ TEMs in patients with asthma (n = 3 - 5), as compared with those in healthy controls (n = 4 - 5). We found that the population of TEMs in asthma was greater than that in healthy control. IL-4-producing cells among both activated TCMs and TEMs and IFN- γ -producing cells among TEMs were more abundant in asthma than in healthy control. Apoptotic cells stained with annexin V and propidium iodide (PI) were more numerous among both TCMs and TEMs in asthma than in healthy control after stimulation with both phorbol myristate acetate and ionomycin. Although CCR4⁺ cell populations among TCMs and TEMs were similar in patients with asthma and healthy controls, cytokine-production profiles differed significantly. Namely, CCR4⁺ (but not CCR4⁻) TCMs and TEMs produced IL-4 and CCR4⁺ (but not CCR4⁻) TCMs produced IFN- γ in both asthma and healthy control. In contrast, both CCR4⁺ and CCR4⁻ TEMs produced IFN- γ . The production levels of IL-4 and IFN- γ by each subpopulation were greater in asthma than in healthy control. Our results suggest that increased CCR4⁺-TEMs in peripheral blood accumulate in the lung and to play an important role in the development and maintenance of airway inflammation in asthma. To our knowledge, this is the first study to investigate CCR4⁺ TCMs and TEMs in bronchial asthma and healthy controls.

Key Words : bronchial asthma, CCR4, CCR7, central memory T cells (TCMs), effector memory T cells (TEMs)

INTRODUCTION

Airway inflammation plays a central role in the pathogenesis of asthma. The large and medium airways of patients with asthma show evidence of chronic inflammation, including leukocyte infiltrates in bronchial tissue, excessive mucus production, epithelial damage, basement membrane thickening, and smooth mus-

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Reprint requests to : Hirokuni Hirata, M.D

Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Mibu, Tochigi, 321-0293, Japan

Table 1 Clinical features of asthmatic patients. M : male, F : female, FEV₁ : forced expiratory volume in one second, PC20 : concentration of histamine required to decrease the FEV₁ by 20 %.

Subjects	Age(years)	Sex	FEV ₁ %	PC20(mg/ml)	IgE(KU/L)	Df-IgE(UA/ml)
1	42	M	97.2	3.8	236	85.1
2	36	M	95.6	2.9	255	45.2
3	25	M	96.6	4.3	366	42.3
4	35	F	98.3	2.1	452	80.7
5	26	F	95.6	3.6	223	71.3

cle hypertrophy^{1, 2)}. The inflammatory infiltrates characteristically contain a substantial population of T cells, as well as eosinophils, monocytes, and neutrophils. The degree of T cell infiltration correlates with the level of airway hyperresponsiveness in mice^{3~5)}. Bronchial asthma is associated with recruitment of eosinophils to the airways, suggesting that its pathogenesis is driven by helper type 2 T (Th2) cells among memory/effector CD4⁺ T cells exposed to inhaled antigens^{4, 6)}. Th2-type immunoregulatory cytokines produced by such memory/effector CD4⁺ T cells play an important role in orchestrating immune and inflammatory processes^{7~10)}.

Previous studies have reported that CCR4, a chemokine receptor for thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC), was a preferential marker for Th2 cells^{11, 12)}. However, a recent study has demonstrated that type 1 helper T (Th1) cells express CCR5 as well as CCR4¹³⁾. CCR4⁺CD4⁺ memory T cells are thought to migrate principally to sites of inflammation, including skin and bronchial tissue where TARC and MDC are produced^{5, 14~16)}. Thus, regulation of CCR4 expression on CD4⁺ T cells appears to have a crucial role in the pathogenesis of bronchial asthma¹⁵⁾.

Another chemokine receptor, CCR7, is considered essential for T-cell homing to lymph nodes. Ligands of the receptor, SLC (secondary lymphoid-tissue chemokine) and ELC (Epstein-Barr virus-induced molecule 1 ligand chemokine) are constitutively expressed in lymphoid tissue. Recent studies have demonstrated that memory T cells are subdivided into central memory T cells (TCMs) and effector memory T cell (TEMs), designated as CCR7⁺ CD62L⁺ CD45RA⁻ and CCR7⁻ CD62L⁻ CD45RA⁻ respectively¹⁷⁾. These subpopulations have distinct functional properties^{17, 18)}. TCMs enter lymph nodes through high endothelial ve-

nules and recirculate primarily between blood and lymph, whereas TEMs migrate preferentially from the blood into peripheral tissues, such as the lung and intestinal mucosa^{19, 20)}. Another difference is that TEMs express cytolytic activity or secrete cytokines such as interferon (IFN)- γ , interleukin (IL)-4, and IL-5 more rapidly than do TCMs on restimulation with antigens^{17, 20, 21)}. Although, the behavior of these cells in allergic diseases remains unknown.

This study was designed to compare the properties of CD4⁺ TCMs and TEMs between patients with asthma and healthy controls. To accomplish this goal, we analyzed the subpopulations, survival, and cytokine production of TCMs and TEMs purified from peripheral blood mononuclear cells (PBMCs).

METHODS

Subjects

Five patients with severe asthma (mean age, 32.8 \pm 7.2 years : 2 women and 3 men : mean forced expiratory volume in one second [FEV₁], 96.7 % \pm 1.1 % of predicted value : geometric mean concentration of histamine required to decrease the FEV₁ by 20 percent [PC20], 3.3 mg/ml histamine : geometric mean IgE, 306.4 kU/L) and 5 healthy controls (mean age, 36.3 \pm 2.6 years : 1 woman and 4 men : mean FEV₁, 103.6 % \pm 5.6 % of predicted value : geometric mean PC20, 13.9 mg/mL histamine : geometric mean IgE, 12.6 kU/L) were studied (Table 1). All patients had severe allergic asthma as defined in the consensus report²²⁾. They had a history of intermittent wheeze with reversible airflow obstruction. Asthma had been diagnosed previously by an independent physician. Most of the patients had seasonal asthma (n = 4) and were tested out of their season. Each patient had a positive skin-prick test response, defined as a wheal greater than 4 mm in diameter in response to *Dermatophagoi-*

des farinae (Df), and elevated Df-specific IgE levels (geometric mean 64.9 UA/ml) in sera on testing by CAP-RAST (Pharmacia, Uppsala, Sweden). The patients were not given oral corticosteroids for at least 2 weeks before vein puncture. The 5 healthy subjects had negative skin prick test responses, normal IgE levels (≤ 100 IU/ml), normal Df-specific IgE levels (≤ 0.34 UA/ml), normal lung function test results, no bronchial hyperresponsiveness ($PC_{20} > 8$ mg/mL), and no history of allergic or other diseases. All subjects were nonsmokers, and none had had an episode of acute bronchitis during the 4 weeks before the study.

Ethics

Written informed consent was obtained from all volunteers.

Cell preparation

Peripheral blood was obtained from the subjects by vein puncture, with heparin as an anticoagulant. PBMCs were separated by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden). Cells were washed three times with phosphate-buffered saline (PBS) supplemented with 2% fetal calf serum (FCS) (Gibco Life Technologies) and were resuspended at a concentration of 1×10^6 /ml. CD45RA⁻CCR7⁺CD62L⁺CD4⁺ T cells (TCMs), CD45RA⁻CCR7⁻CD62L⁻CD4⁺ T cells (TEMs), and CCR4⁺ or CCR4⁻ TCMs and TEMs were purified from PBMCs by negative and positive selection with specific antibody complexes directed against cell surface antigens, including CD45RA, CCR7, CD62L, CCR4, and CD4. These cells were used with Stemsep TM Magnetic Colloid (Stem Cell Technologies Inc, Vancouver, Canada) in magnetic cell-separation studies. The purity of the cell preparations was checked by immunofluorescence with anti-CD4, -CD45RA, -CCR4, -CCR7, and -CD62L antibodies (Pharmingen, San Diego, CA). The purity of each cell preparation was $> 95\%$.

Cell culture and cytokine assays

To measure IL-4 and IFN- γ production by each T cell population, the cells were cultured in RPMI 1640 (Gibco Life Technologies, New York, N.Y.) supplemented with 10% FCS in the presence of both phorbol myristate acetate (PMA) (Sigma, St. Louis, Mo.) and

ionomycin (Sigma), concentrated to 25 ng/ml and 1 μ g/ml, respectively. The cells were then incubated in 96-well plates in culture media, at 37°C in a 5% CO₂ environment. To analyze protein, the supernatants were harvested after 48 hr of culture. The supernatants were stored at -80°C until analysis. The remaining cells were harvested and analyzed for viability by the trypan blue method.

IL-4 and IFN- γ concentrations in culture supernatants were assessed by enzyme-linked immunosorbent assay (ELISA) with Cytoscreen Immunoassay kits (Biosource, Inc., Camarillo, Calif.). The sensitivities of all assays were higher than 7.8 and 15.6 pg/ml for IL-4 and IFN- γ , respectively.

Determination of surface marker expression, intracellular cytokines, and survival/apoptosis by flow cytometry

Surface phenotyping of CD45RA⁻CD4⁺ T cells was performed by staining 1×10^6 cells with marker-specific monoclonal antibodies (mAb) for 15 min at 4°C. The antibodies used were phycoerythrin (PE)-conjugated anti-CCR7 and fluorescein isothiocyanate (FITC)-conjugated anti-CCR4 (Pharmingen, San Diego, CA). The cells were resuspended in PBS supplemented with 2% FCS and 0.7% NaN₃.

Methods for flow-cytometric intracellular-staining have recently been described in detail^{23, 24}. The isolated TCMs and TEMs were counted, and cell cultures were set up with 1×10^6 cells/ml of RPMI 1640 supplemented with 10% FCS, 10 μ g/ml of brefeldin A (Sigma, St. Louis, Mo.), 25 ng/ml of PMA and 1 μ g/ml of ionomycin. The cells were cultured for 4 h (37°C : 5% CO₂) and then washed with PBS supplemented with 0.5% FCS.

Cells were fixed for subsequent staining of intracellular cytokines in the presence of Permeabilizing solution 2 (Becton Dickinson, San Jose CA), as described previously^{23, 24}. The cells were incubated for 30 min at room temperature with the respective PE-labeled IL-4 antibodies and allophycocyanin (APC)-labeled IFN- γ antibodies (Pharmingen, San Diego, CA). After washing twice with PBS supplemented with 0.5% FCS, the cells were resuspended in 1% paraformaldehyde.

Both annexin V- and propidium iodide (PI)-positive cells in TCMs and TEMs were measured with the use

of an annexin V-FITC/PI apoptosis assay kit (BD Biosciences Pharmingen, San Diego, CA). The experiment was performed by following the manufacturer's instructions, with minor changes²⁵. Briefly, TCMs and TEMs cultured in the presence of PMA (25 ng/ml) and ionomycin (1 μ g/ml) for 24 hr were washed twice with ice-cold PBS and then resuspended in binding buffer. Annexin V-FITC and PI were added to the culture tube. TCMs and TEMs were analyzed within 1 h of annexin V-PI labeling. Viable TCMs and TEMs were defined as being negative for annexin V-FITC and PI staining : cells positive for both annexin V and PI staining were considered apoptotic TCMs and TEMs^{25, 26}. The cells were analyzed on a FACScan (Becton Dickinson) equipped with Cell Quest software.

Statistical analysis

Data are expressed as means \pm SD. The statistical significance of differences between groups was examined by analysis of variance with Bonferroni's test. *P* values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Populations of TCMs and TEMs among CD4⁺ T cells in patients with asthma and healthy controls

The number of CD4⁺ memory T cells (CD45RA⁻ CD4⁺ T cells) in peripheral blood obtained by vein puncture was similar in patients with asthma ($3.50 \pm 0.31 \times 10^5$ /mL) and healthy controls ($3.31 \pm 0.35 \times 10^5$ /mL). The population of CD4⁺ memory T cells among PBMCs in patients with asthma ($58.6 \pm 6.9\%$) also did not differ from that in healthy controls ($56.8 \pm 5.7\%$) (individual data not shown). The population of TEMs among CD4⁺ memory T cells in asthma ($85.5 \pm 8.1\%$) was significantly greater than that in healthy control ($68.6 \pm 2.8\%$) ($p < 0.05$). The population of TCMs among CD4⁺ memory T cells in asthma ($11.4 \pm 7.5\%$) was significantly less than that in healthy control ($33.6 \pm 2.7\%$) ($p < 0.05$) (Fig. 1).

Populations of IL-4- and IFN- γ -producing cells in activated TCMs and TEMs

We analyzed intracellular IL-4 and IFN- γ staining in activated TCMs and TEMs at 4 hr (Fig. 2A, B) and in total CD4⁺ memory T cells (Fig. 2C). The popula-

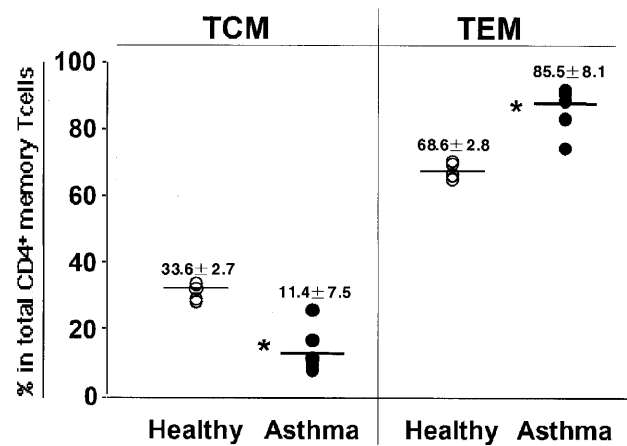


Fig. 1 Populations of TCMs and TEMs among CD45RA⁻ CD4⁺ T cells in patients with asthma ($n = 5$) and healthy controls ($n = 5$). The profiles of these cells were analyzed by flow cytometry. * $p < 0.05$ as compared with the population of TCMs or TEMs in healthy controls.

tions of IL-4-producing cells among activated TCMs ($2.7 \pm 1.5\%$ vs. $0.8 \pm 0.5\%$) and TEMs ($3.1 \pm 1.8\%$ vs. $1.1 \pm 1.0\%$) in asthma were significantly greater than those in healthy control. The population of IFN- γ -producing cells among activated TEMs ($11.9 \pm 4.5\%$) in asthma was also greater than that among activated TEMs ($4.8 \pm 3.1\%$) in healthy control. There was no significant difference in the percentage of IFN- γ -producing cells among activated TCMs between asthma ($5.9 \pm 3.8\%$) and healthy control ($8.0 \pm 6.9\%$) (Fig. 2B). The percentages of cytokine-producing TEMs among total CD4⁺ memory cells were higher in asthma (IL-4 ; $3.5 \pm 2.1\%$, IFN- γ ; $9.8 \pm 5.9\%$) than in healthy control (IL-4 ; $0.6 \pm 0.3\%$, IFN- γ ; $4.1 \pm 3.5\%$) for both IL-4 and IFN- γ . In contrast, the percentages of cytokine-producing TCMs in patients with asthma (IL-4 ; $0.1 \pm 0.1\%$, IFN- γ ; $1.9 \pm 2.3\%$) did not differ from those in healthy controls (IL-4 ; $0.4 \pm 0.2\%$, IFN- γ ; $3.2 \pm 2.6\%$) for either IL-4 or IFN- γ (Fig. 2C). However, there was no significant difference in the mean fluorescence intensity of staining for either cytokine between asthma and healthy control (data not shown). This finding indicated that cytokine production by CD4⁺ memory T cells in asthma is similar to that in healthy control.

Survival and apoptosis of activated TCMs and TEMs

To assess cell survival, we examined annexin V and

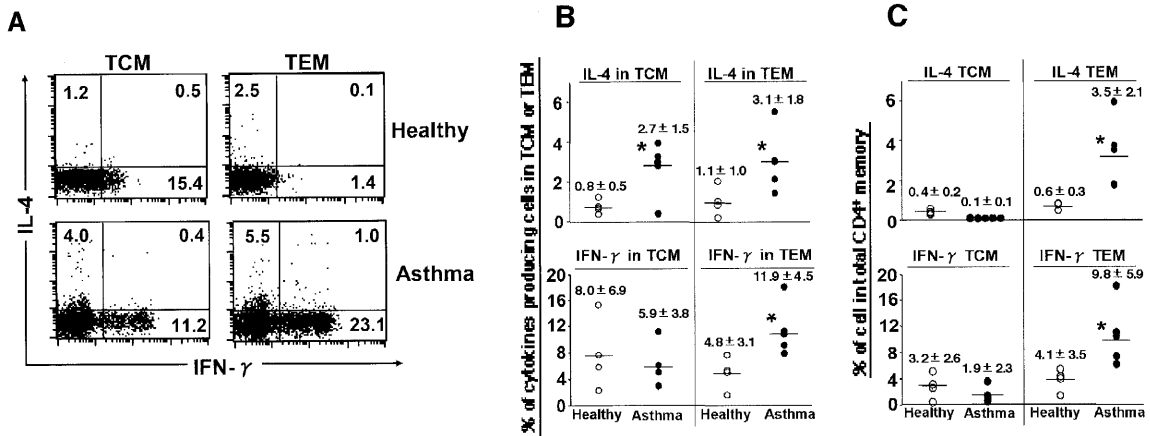


Fig. 2 Populations of IL-4⁻ and IFN- γ -producing cells among TCMs and TEMs (A, B) and among total CD4 memory T cells (C) in patients with asthma and healthy controls. One representative experiment of five in patients with asthma or of four in healthy controls (A). Purified TCMs and TEMs were cultured in the presence of PMA (25 ng/ml) and ionomycin (1 μ g/ml) for 4 hr in patients with asthma and healthy controls. The profiles of these cells were analyzed by flow cytometry. Mean values derived from five or four independent experiments in patients with asthma or healthy controls, respectively, are shown (B, C). *p < 0.05 as compared with mean of the population for IL-4⁻ or IFN- γ -producing cells in healthy controls.

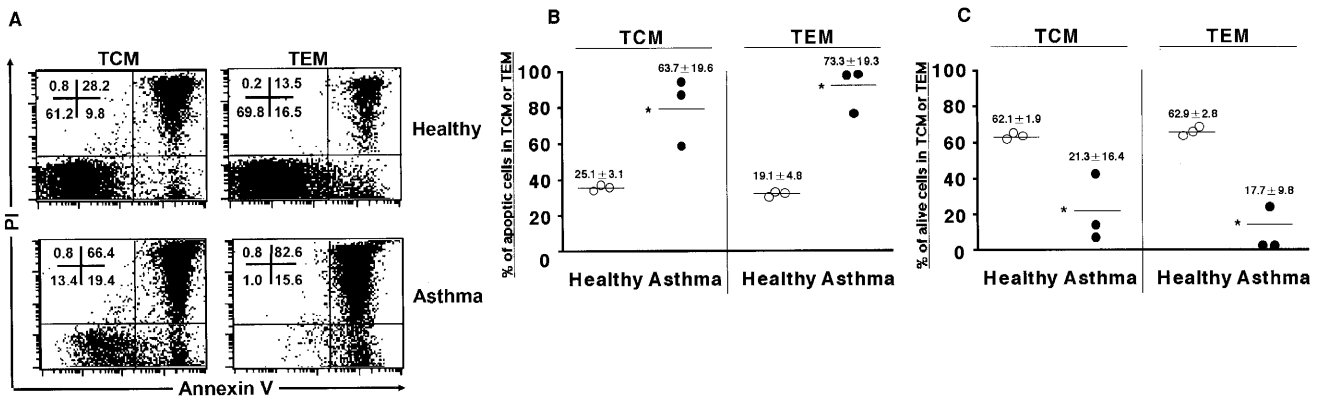


Fig. 3 An analysis of cell survival by annexin V and PI staining for TCMs and TEMs in patients with asthma and healthy controls (A, B, C). Purified TCMs and TEMs were cultured in the presence of PMA (25 ng/ml) and ionomycin (1 μ g/ml) for 24 hr in patients with asthma and healthy controls. The profiles of these cells were analyzed by flow cytometry. One representative experiment of three in patients with asthma or healthy controls (A). A representative result of analysis for annexin V-positive and PI-positive staining, indicating late apoptotic cells (B), and annexin V-negative and PI-negative staining, indicating alive cells (C), among TCMs and TEMs in patients with asthma and healthy controls. *p < 0.05 as compared with the means for the population for apoptotic or alive cells in healthy controls.

PI staining of TCMs and TEMs in asthma and healthy control (Fig. 3). The rates of both annexin V-positive and PI-positive staining (late apoptosis) of activated TCMs (63.7 ± 19.6%) and TEMs (73.3 ± 19.3%) in asthma was significantly greater than the respective rates (TCMs, 25.1 ± 3.1%; TEMs, 19.1 ± 4.8%) in healthy control (Fig 3A, B). Conversely, the rates of both annexin V-negative and PI-negative staining

(alive) of activated TCMs (21.3 ± 16.4%) and TEMs (17.7 ± 9.8%) in asthma were significantly lower than those in healthy controls (TCMs, 62.1 ± 1.9%; TEMs; 62.9 ± 2.8%) (Fig. 3A, C). These results suggested that activation-induced death of memory T cells in asthma might differ from that in healthy controls.

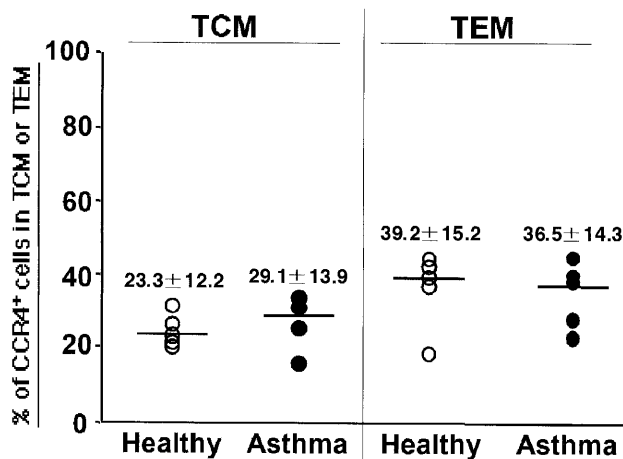


Fig. 4 Subpopulations of $CCR4^+$ cells among TCMs and TEMs in patients with asthma ($n = 5$) and healthy controls ($n = 5$). The profiles of these cells were analyzed by flow cytometry. The means of five independent experiments in patients with asthma or healthy controls are shown.

Subpopulations of $CCR4^+$ cells in TCMs and TEMs

Subpopulations of $CCR4^+$ cells among TCMs and TEMs in patients with asthma and healthy controls was shown in Fig. 4. There was no significant difference in the subpopulations of $CCR4^+$ cells in either TCMs or TEMs between asthma and healthy control. The proportion and mean fluorescence intensity of $CCR4^+$ cells among TCMs and TEMs in asthma were similar to those in healthy control (data not shown). These results indicated that regulation of $CCR4$ expression on memory T cells was intact in asthma.

IL-4 and IFN- γ production by $CCR4^+$ TCMs and TEMs

As shown in Fig. 5, IL-4 production was higher in $CCR4^+$ TCMs and $CCR4^+$ TEMs than in $CCR4^-$ TCMs and $CCR4^-$ TEMs in both asthma ($CCR4^+$ TCMs, 2.4 ± 0.7 ng/ml, $CCR4^-$ TCMs, 0.3 ± 0.1 ng/ml; $CCR4^+$ TEMs, 2.1 ± 0.6 ng/ml, $CCR4^-$ TEMs, 0.3 ± 0.2 ng/ml) and healthy control ($CCR4^+$ TCMs, 1.1 ± 0.4 ng/ml, $CCR4^-$ TCMs, 0.2 ± 0.1 ng/ml; $CCR4^+$ TEMs, 1.4 ± 0.3 ng/ml, $CCR4^-$ TEMs, 0.3 ± 0.1 ng/ml). IL-4 production by $CCR4^+$ TCMs and TEMs in asthma was thus significantly greater than that by $CCR4^+$ TCMs and TEMs in healthy control. In contrast, IFN- γ production by $CCR4^+$ (4.1 ± 0.9 ng/ml) and $CCR4^-$ (2.1 ± 0.7 ng/ml) TCMs in asthma was

similar to that by $CCR4^+$ (2.8 ± 1.4 ng/ml) and $CCR4^-$ (0.7 ± 0.3 ng/ml) TEMs in healthy controls. Both $CCR4^+$ and $CCR4^-$ TEMs showed significant production of IFN- γ . IFN- γ production by $CCR4^+$ (8.0 ± 1.8 ng/ml) and $CCR4^-$ (8.1 ± 1.1 ng/ml) TEMs in asthma was significantly higher than that by $CCR4^+$ (5.0 ± 0.1 ng/ml) and $CCR4^-$ (5.1 ± 0.9 ng/ml) TEMs in healthy controls. These results suggested that $CCR4^+$ TCMs and $CCR4^+$ TEMs are generally the main cells producing IL-4 and IFN- γ .

DISCUSSION

We have shown that the population of TEMs among PBMCs in asthma was greater than that in healthy control. The populations of IL-4-producing cells among both TCMs and TEMs and of IFN- γ -producing cells among TEMs in asthma were greater than those in healthy control. In particular, the proportions of Th1 and Th2 type TEMs among total memory $CD4^+$ T cells was increased in asthma. TEMs and TCMs in asthma showed increased susceptibility to activated T cell apoptosis. We also demonstrated that the expression of $CCR4$ on both TCMs and TEMs was similar in asthma and healthy control. Our results also showed that the cellular sources of IL-4 and IFN- γ production in asthma and healthy control were $CCR4^+$ TCMs and $CCR4^+$ TEMs, and that IFN- γ also was produced by $CCR4^-$ TEMs. Cytokine production by the each subpopulation of memory cells in asthma was higher than that in healthy control. Evidence of IFN- γ production in asthma indicates the importance of this cytokine in addition to IL-4 in asthma and supports the findings of a recent study showing that not only Th2 cells^{8, 27)} but also Th1 cells independently induce strong airway hyperresponsiveness in mice^{28, 29)}.

Asthma is characterized by airway inflammation, airway hyperresponsiveness, and reversible airflow limitation^{3~5)}. Many inflammatory cells participate in the pathogenesis of asthma^{30~35)}. Among these cells, T cells play a pivotal role in orchestrating airway inflammation^{3~6)}. In asthmatic airways, higher numbers of $CD4^-$ and $CD8^-$ positive memory T cells are found in patients with asthma³⁶⁾, whereas the percentage of $CD45RA^-$ (memory/effector) T cells among PBMCs does not significantly differ between patients with asthma and healthy controls³⁷⁾. Memory/effector T

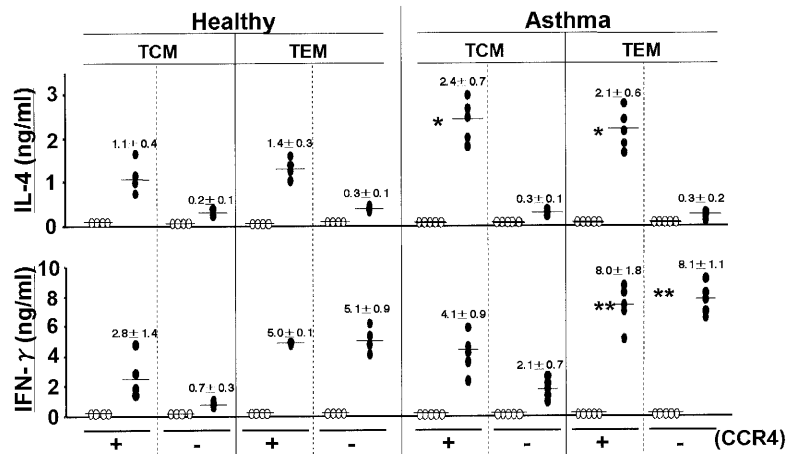


Fig. 5 IL-4 and IFN- γ production by CCR4⁺ or CCR4⁻ TCMs and TEMs in patients with asthma (n = 5) and healthy controls (n = 4). Purified CCR4⁺ or CCR4⁻ TCMs and TEMs were cultured in the presence (dark dots) or the absence (light dots) of PMA and ionomycin for 48 hr in patients with asthma and healthy controls. IL-4 and IFN- γ concentrations in culture supernatants were assessed by enzyme-linked immunosorbent assay. The means of five or four independent experiments are shown for patients with asthma or healthy controls. * p < 0.05 as compared with IL-4 production by CCR4⁺ TCMs or TEMs in healthy controls. ** p < 0.05 as compared with IFN- γ production by CCR4⁺ or CCR4⁻ TEMs in healthy controls.

cells in asthma release IL-4, IL-5, IL-9, and IL-13, a pattern compatible with predominant activation of Th2-type cells⁷⁻¹⁰, suggesting that activated memory/effector T cells in the airway may contribute to inflammation with eosinophil accumulation in asthma^{38, 39}. Recent studies have shown that TCM subsets show hypo-acetylated cytokine genes and have no or low effector functions, but efficiently differentiate into Th1 type or Th2 type TEMs after T cell receptor stimulation in the presence of IL-12 or IL-4, respectively^{17, 20, 21}. In contrast, TEMs show polarized cytokine gene acetylation patterns in vivo and rapidly produce large amounts of IFN- γ or IL-4 on antigenic stimulation^{17, 20, 21}. Rivino et al¹³ reported that not only TEMs but also TCMs can produce some cytokines, such as IL-4 or IFN- γ . Our study showed by FACS analysis that TEMs contained cell populations that rapidly produced IL-4 in both asthma and healthy control, whereas peak cytokine production by TCMs was delayed (data not shown). Therefore, although the functions of TCMs and TEMs and their interrelations during differentiation remain uncertain, regulatory mechanisms for cytokine production might differ between TCM and TEM. We found that the total number of CD45RA⁻CD4⁺ T

cells (CD4⁺ memory T cells) among white blood cells and the percentage of these cells among PBMCs were similar in asthma and healthy control, although the next part of our study demonstrated that the population of TEMs among PBMCs in asthma was greater than that in healthy control. In addition, the populations of IL-4-producing cells among both TCMs and TEMs in patients with asthma were greater than those in healthy control. Moreover, the population of IL-4-producing TEMs among total CD4⁺ memory T cells in patients with asthma was higher than those of IL-4-producing TCMs in patients with asthma as well as in healthy controls. These findings indicated that the population of antigens, including house-dust-mite-specific CD4⁺ memory T cells, in patients with asthma might be higher than that in healthy controls. In contrast, the populations of IFN- γ -producing cells among TEMs as well as among total CD4⁺ memory T cells in patients with asthma were greater than those in healthy controls. Recent studies have shown that Th1 cells induce strong airway hyperresponsiveness in mice^{28, 29}. Taken together, our results suggest that not only Th2 cells but also Th1 cells may be involved in the pathogenesis of asthma. Thus, memory T cells in

asthma may preferentially differentiate into TEMs because of unknown immunological abnormalities.

Th1 type and Th2 type TEMs may play an important role in the pathogenesis of asthma because TEMs are known to produce cytokines more rapidly than TCMs. In addition, a recent study has demonstrated that CCR7⁻ T cells accumulate in mouse asthmatic lung, while CCR7⁺ T cells continue to migrate into afferent lymph⁴⁰⁾. Thus, TEMs in patients with asthma may play an important role in chronic airway inflammation. On the other hand, another recent study has reported that TCMs have a higher proliferation rate than TEMs⁴¹⁾ and that TCMs can differentiate into TEMs. Therefore, TCMs may contribute to providing TEMs for maintaining the pathogenesis of asthma. This hypothesis is supported by experimental data showing that TCMs have higher expansion potential than TEMs: TCMs retain flexibility to differentiate into Th2 and Th1 cells on antigenic stimulation: and activated TCMs further differentiate, losing CCR7 to acquire non-lymphoid homing potential and high levels of effector functions⁴²⁾. However, apoptosis of activated TCMs as well as TEMs in asthma was greater than that in healthy control. These findings suggest that memory T cells, including TCMs and TEMs, in asthma have an elevated activation status, produce higher amounts of several cytokines than healthy control, and have increased susceptibility to activation-induced cell death⁴²⁾. This phenomenon may involve the up-regulation of Fas/Fas ligand expression^{43, 44)} and/or the down-regulation of BCL2 expression⁴⁵⁾ in activated T cells after stimulation with antigen in asthma.

Previous studies have reported that CCR4 is a preferential marker for Th2 cells^{11, 12)}. Several other studies have shown that the bronchial epithelium of asthmatic patients expresses TARC and MDC protein and that CCR4⁺ Th2-type CD4⁺ T cells reside on the bronchial epithelium^{5, 14, 15)}. These facts suggest that CCR4 guides Th2 cell recruitment into bronchial tissue. However, we and Rivino et al¹³⁾ have found that Th1 cells express not only CCR5, but also CCR4 (data not shown). Since CCR4 is believed to be expressed on a major subset of both Th1 type and Th2 type memory T cells, we examined Th1 and Th2 cytokine production by CCR4⁺ TCMs and TEMs in asthma and healthy control. We found that IL-4 production is high-

er in CCR4⁺ TEMs and TCMs than in CCR4⁻ TEMs and TCMs in both asthma and healthy control. IFN- γ production by TCMs was particularly high in CCR4⁺ cells. In contrast, IFN- γ production by TEMs was high in both CCR4⁺ and CCR4⁻ cells. These results suggest that CXCR3⁺ TEMs in CCR4⁺ or CCR4⁻ cells can produce IFN- γ ¹³⁾. In addition, cytokine production in each cell population was higher in asthma than in healthy control. Rivino et al¹³⁾ though has reported that CCR4⁺ TCMs in healthy controls could not produce IFN- γ . The discrepancy between their findings and ours may be related to different experimental conditions, including culture conditions and the time course and intensity of stimulation. Such differences may account for the fact that not only IFN- γ production but also IL-4 production by CCR4⁺ TCMs and TEMs in the study by Rivino et al was weaker than that in our study. Perhaps T cells producing IL-4, IFN- γ , or both (especially TEMs) are guided for tissue-homing by interactions between CCR4 and its ligands, such as TARC and MDC. Furthermore, regulation of CCR7 expression might be critical for designating the allocation of CCR4⁺ TEMs and TCMs to lung tissue or lymphoid tissue in asthma⁴⁰⁾.

In summary, our results showed an increased population of CCR4⁺ TEMs in the peripheral blood of patients with asthma as compare with that in healthy controls. We also showed that CCR4⁺ TCMs and TEMs are generally the major cells producing IL-4 and IFN- γ . Furthermore, the production of cytokines by CCR4⁺ TCMs and TEMs was higher in asthma than in healthy control. On the basis of our findings, we propose that CCR4⁺ TCMs after restimulation by antigens differentiate into CCR4⁺ TEMs. Consequently, increased numbers of CCR4⁺ TEMs accumulate in the lung, where they play an important role in the development and maintenance of airway inflammation in asthma.

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