

Characterization of chemokine receptor expression and cytokine production in circulating CD4⁺ T cells from patients with atopic dermatitis: up-regulation of C-C chemokine receptor 4 in atopic dermatitis

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Summary

Background Th2 and Th1 cells have been suggested to express CCR3/CCR4 and CCR5/CXCR3, respectively.

Objective We examined CCR3, CCR4, CCR5 and CXCR3 expression and cytokine production in peripheral blood CD4⁺ T cells from patients with atopic dermatitis (AD), which has been postulated to be a Th2-type cell-mediated disease, and then analysed the possible correlation between these values and the levels of several clinical parameters.

Methods Intracellular cytokine production and chemokine receptor expression in peripheral blood CD4⁺ T cells from 40 AD patients and 20 sex- and age-matched healthy control subjects were studied by flow cytometry.

Results The frequencies of IL-4- and IL-13-producing CD4⁺ T cells from patients with AD were significantly higher than those from healthy control subjects (IL-4: $3.9 \pm 2.1\%$ vs. $1.6 \pm 0.7\%$, $P = 0.0005$, IL-13: $4.0 \pm 2.1\%$ vs. $1.8 \pm 0.8\%$, $P = 0.0023$), whereas the frequencies of IL-2- and IFN- γ -producing CD4⁺ T cells were significantly decreased in AD patients (IL-2: $38.1 \pm 10.3\%$ vs. $51.3 \pm 6.3\%$, $P = 0.0003$, IFN- γ : $9.9 \pm 3.5\%$ vs. $26.4 \pm 4.6\%$, $P < 0.0001$). The percentage of CCR4⁺ cells in CD4⁺ CD45RO⁺ T cells in AD patients was significantly higher than that in healthy control subjects ($24.4 \pm 8.0\%$ vs. $10.9 \pm 2.3\%$, $P < 0.0001$) and was correlated positively with the total serum IgE, serum lactic dehydrogenase (LDH) level, eosinophil number, eruption score, and IL-4 and IL-13 secretion in CD4⁺ T cells, and inversely with IL-2 and IFN- γ secretion in CD4⁺ T cells. In contrast, CCR3 was not detected on circulating CD4⁺ T cells even in AD patients. On the other hand, the percentage of CCR5⁺ or CXCR3⁺ cells in CD4⁺ CD45RO⁺ T cells in AD patients was significantly decreased (CCR5: $23.2 \pm 7.0\%$ vs. $28.4 \pm 5.4\%$, $P = 0.023$, CXCR3: $29.9 \pm 11.4\%$ vs. $38.5 \pm 6.7\%$, $P = 0.028$) and was positively correlated with eruption score ($P < 0.05$). Multiple regression analyses showed that the percentage of CCR4 expression highly correlated with serum IgE, LDH, eosinophil number and eruption in AD patients.

Conclusion CCR4⁺ cells might be involved in the aetiopathogenesis of AD.

Keywords atopic dermatitis, CCR4, CCR5, IL-4, IL-13, IL-2, IFN- γ , Th1/Th2

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Introduction

Th1 and Th2 cells represent two polarized forms of specific CD4⁺ T helper cell-mediated immune responses based on their profiles of cytokine secretion and related functions [1]. In humans, Th1 cells produce IL-2, IFN- γ and lymphotoxin (LT), whereas Th2 cells produce IL-4 and IL-5. Th1 cells participate mainly in cell-mediated immunity and are involved in chronic inflammatory diseases, such as multiple sclerosis and

rheumatoid arthritis, while Th2 cells regulate immune responses, facilitate IgE production and are involved in allergic diseases, including atopic dermatitis (AD) [2,3].

Surface molecules that preferentially associate with human Th1 or Th2 cells have been described previously. Th1-associated molecules include CD26 [4] and lymphocyte activation gene-3 [5], whereas CD62L [6], T1/ST2 [7] and CD30 [8] are preferentially expressed on Th2 cells. Recently, chemokine receptors have been shown to express on human Th1/Th2 cell lines derived *in vitro*, suggesting their possible role in the selective attraction of each of the two subsets, as well as in regulating their migration and/or homing into inflammatory sites. Thus, CXC chemokine receptor 3 (CXCR3) and CC chemokine receptor 5 (CCR5) are selectively expressed on Th1 cells derived

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in vitro, while CCR4 and CCR3 are expressed on Th2 cells [9–12]. However, the clinical significance of the expression of these chemokine receptors on Th cell subsets still remains undetermined. More recently, differential expression of the chemokine receptors among Th cell subsets has been reported [13,14], although there have also been some conflicting reports regarding this point [15–17].

AD is a chronic inflammatory skin disease observed in individuals with a genetic predisposition to enhanced IgE synthesis and IgE-mediated allergic reactions, such as allergic rhinitis and asthma [18,19]. Th1/Th2 imbalance to environmental allergens is considered to be a critical pathogenic mechanism of the disease. Predominance of Th2 cells from the blood and skin lesions of patients with AD has been demonstrated [20,21]. Indeed, increased frequencies of CCR4⁺ CD4⁺ T cells in the blood have been reported in patients with AD [13,14]. However, little information is available on the possible correlation between Th1/Th2 imbalance in terms of chemokine receptors on CD4⁺ T cells in the circulation and clinical parameters of AD.

In this study, we examined the expressions of CCR3, CCR4, CCR5 and CXCR3 and cytokine production in the peripheral blood CD4⁺ T cells from patients with AD, and compared them with clinical parameters of AD.

Materials and methods

Patients and controls

A total of 40 patients with atopic dermatitis (mean age 25.7 years old, range 10–42) and 20 healthy control subjects (mean age 27.5, range 18–38) were enrolled in this study. AD was diagnosed according to the criteria described by Hanifin and Rajka [22]. AD patients were treated with conventional therapy including systemic antihistamine and topical steroids. None of the patients had been treated with systemic steroids or immunosuppressive agents. The activity of AD was estimated using the SASSAD severity score of Berth-Jones [23]. Informed consent was obtained from all the subjects or from their parents prior to participation in this study.

Antibodies and reagents

Generation of a monoclonal antibody (mAb) against CCR4 (KM2160, mouse IgG1) was described previously [12]. FITC-conjugated anti-CCR3 (61828.111, rat IgG2a) and anti-CXCR3 (49801.111, mouse IgG1) mAbs were purchased from Genzyme/Techne (Minneapolis, MN, USA). Peridin chlorophyll protein (PerCP)-conjugated anti-CD4 and phycoerythrin (PE)-conjugated anti-IL-2, anti-IL-4, anti-IL-13 and anti-IFN- γ mAbs were obtained from Becton Dickinson & Co. (Mountain View, CA, USA). The following mAbs were purchased from PharMingen (San Diego, CA, USA): purified anti-CD3 mAb, FITC-conjugated anti-CD4, anti-CD8 and anti-CCR5 (2D7, mouse IgG2a), PE-conjugated anti-CD45RO mAbs, and all the conjugated isotype-matched control Abs. Brefeldin A was purchased from Sigma (St Louis, MO, USA).

Cell cultures

Cells were cultured in RPMI 1640 with 10% heat-inactivated fetal bovine serum supplemented with antibiotics and 2-mercaptoethanol (50 mM) (RPMI complete) and maintained in a humidified chamber with 95% air and 5% CO₂.

Cytofluorimetric analysis of cell surface markers and chemokine receptors

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by centrifugation on Ficoll-Hypaque gradients. PBMC were resuspended in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin and 0.02% sodium azide and, after saturation of non-specific binding sites with rabbit IgG, incubated for 20 min on ice with fluorochrome-conjugated specific or control mAbs. Stained samples were analysed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), using Lysis II software (Becton Dickinson).

Intracytofluorimetric analysis of cytokine production

For immunostaining of cytoplasmic IL-2, IL-4, IL-13 and IFN- γ , 1×10^6 freshly isolated PBMC in RPMI complete were stimulated with immobilized anti-CD3 mAbs (10 μ g/mL) for 4 h at 37 °C. Then, brefeldin A was added to the culture at a concentration of 1 μ g/mL and the cells were incubated further for 2 h. The cells were washed twice in PBS, and FITC-conjugated anti-CD4 mAb was added and incubated for additional 20 min on ice. They were washed, fixed, made permeable using Cytofix/Cytoperm solution (PharMingen) and stained with PE-conjugated isotype control Abs or mAbs to IL-2, IL-4, IL-13 and IFN- γ . Data were analysed on a FACScan flow cytometer using Lysis II software.

Statistical analyses

Data are presented as mean $\pm$ SD of the mean unless otherwise indicated. The Mann–Whitney *U*-tests were performed for comparison between patients with AD and healthy control subjects. Linear regression analyses were carried out to examine correlations between the percentages of CCR4⁺ or CCR5⁺ cells in CD4⁺ CD45RO⁺ T cells and several clinical parameters such as the SASSAD severity score, total serum IgE levels, LDH levels and peripheral blood eosinophil counts using log-transformed values except for the SASSAD severity score. Multiple regression analyses were performed to determine whether the frequency of CCR4⁺ or CCR5⁺ cells accounts for the levels of clinical parameters. All statistical analyses were performed using StatView Version 5 (SAS Institute Inc., Cary, NC, USA) on a Macintosh computer, and *P* < 0.05 was considered significant.

Results

Comparison of the chemokine receptor expression between AD patients and healthy control subjects (Table 1)

We compared the percentages of peripheral memory CD4⁺ T cells expressing CCR3, CCR4, CCR5 or CXCR3 in AD patients with those in healthy control subjects. There were no differences in the frequencies of both CD4⁺ and CD4⁺ CD45RO⁺ T cells between AD patients and controls. The percentages of CD8⁺ T cells were significantly decreased in AD patients compared with controls ($19.8 \pm 7.9\%$ vs. $28.9 \pm 5.8\%$, *P* < 0.05), as reported previously [19]. The percentage of CCR4⁺ cells among CD4⁺ CD45RO⁺ T cells was found to be significantly higher ($24.4 \pm 8.0\%$ vs. $10.9 \pm 2.3\%$, *P* < 0.0001), while those of CCR5⁺ and CXCR3⁺ cells were significantly lower in

Table 1. Mean percentage values of peripheral blood T cell sub-populations in normal control subjects and atopic dermatitis (AD) patients

	Control subjects (<i>n</i> = 20)	AD patients (<i>n</i> = 40)	<i>P</i>
CD4 ⁺ T cells (%)	32.2 ± 5.1	35.1 ± 10.2	0.409
CD8 ⁺ T cells (%)	28.9 ± 5.8	19.8 ± 7.9	0.002
CD4 ⁺ CD45RO ⁺ T cells (%)	18.5 ± 2.7	15.3 ± 5.3	0.064
CCR3 ⁺ in CD4 ⁺ CD45RO ⁺ T cells (%)	0.2 ± 0.1	0.3 ± 0.2	0.412
CCR4 ⁺ in CD4 ⁺ CD45RO ⁺ T cells (%)	10.9 ± 2.3	24.4 ± 8.0	< 0.0001
CCR5 ⁺ in CD4 ⁺ CD45RO ⁺ T cells (%)	28.4 ± 5.4	23.2 ± 7.0	0.023
CXCR3 ⁺ in CD4 ⁺ CD45RO ⁺ T cells (%)	38.5 ± 6.7	29.9 ± 11.4	0.028

Data are presented as means ± SD. Statistical method: Mann-Whitney unpaired non-parametric test.

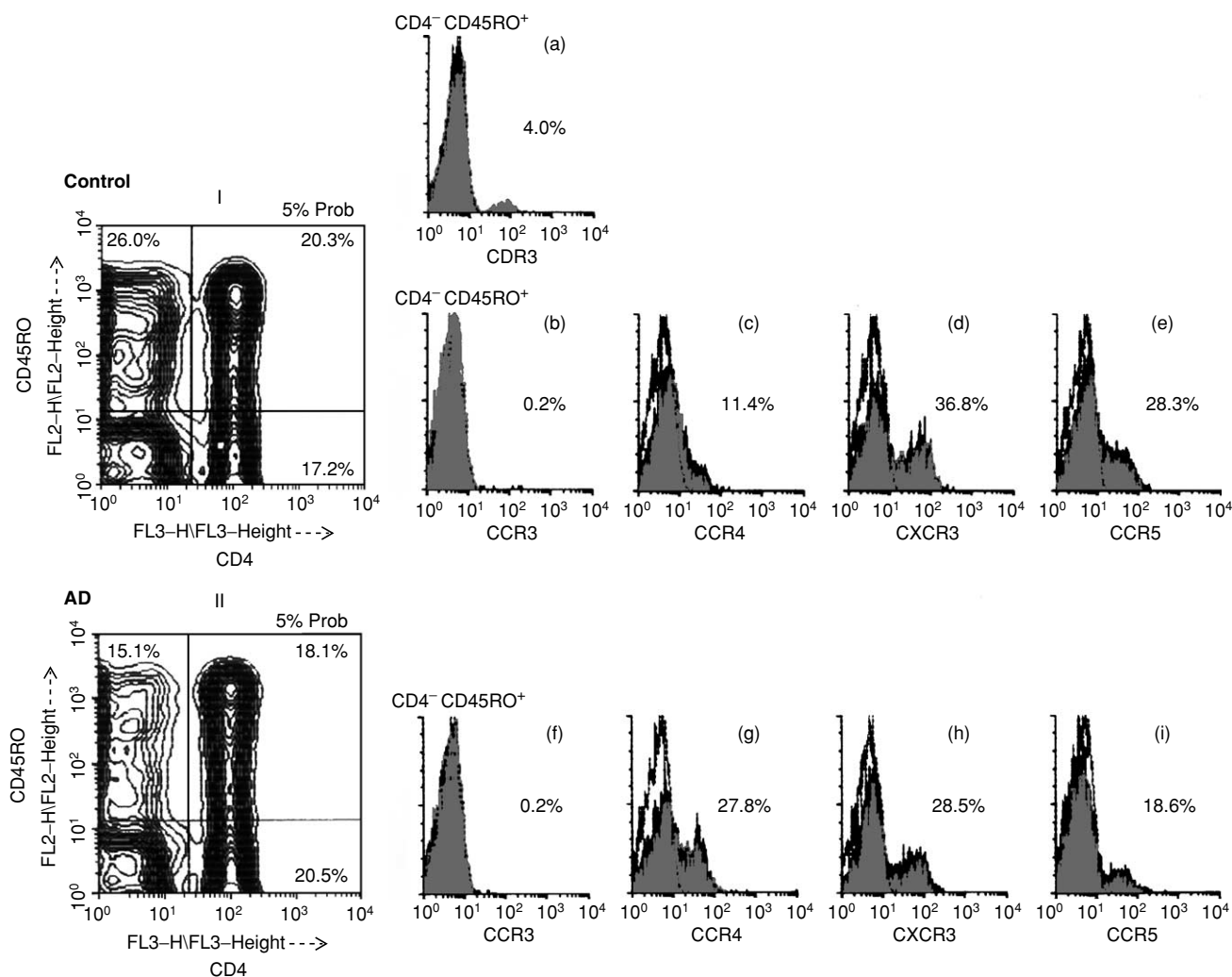


Fig. 1. Representative expression of CCR3, CCR4, CXCR3 and CCR5 on CD4⁺ CD45RO⁺ T cells in the peripheral blood from patients with AD and healthy control subjects. All samples were stained in parallel by three-colour immunofluorescent staining of PBMCs and analysed sequentially by flow cytometry with identical instrumental settings. Contour plots I and II show the CD4 vs. CD45RO staining from a control subject and an AD patient, respectively. The percentages in each quadrant indicate the frequency of the respective sub-populations. Histogram (a) represents the staining of CCR3 after gating CD4⁺ CD45RO⁺ cells from a control subject. Histograms b–e and f–i show the staining of each chemokine receptor after gating CD4⁺ CD45RO⁺ cells from a control subject and an AD patient, respectively. Each histogram shows the staining intensity for the relevant mAbs (shaded area) and the background staining with the control Abs (open area) vs. the relative cell number. The percentage of cells in the shaded area after subtraction of the overlapping open area is given in each histogram.

AD patients in comparison with controls (CCR5: 23.2 ± 7.0% vs. 28.4 ± 5.4%, *P* < 0.05; CXCR3: 29.9 ± 11.4% vs. 38.5 ± 6.7%, *P* < 0.05). However, the percentages of CCR3⁺ among CD4⁺ CD45RO⁺ T cells were negligible in both AD

patients and controls. These results were essentially similar to those of the study by Yamamoto et al. [13]. Figure 1 shows representative results of three-colour immunofluorescent analysis in a healthy control subject and an AD patient. Regarding

the reactivity of anti-CCR3 mAb used here, basophils (in CD4⁺CD45RO⁺ cells), which are known to express highly CCR3 [11], were clearly stained with this antibody (Fig. 1a).

Frequency of IL-4-, IL-13-, IL-2-, or IFN- γ -producing CD4⁺ T cells in patients with AD (Table 2)

The frequencies of CD4⁺ T cells producing IL-4, IL-13, IL-2 and IFN- γ were compared between AD patients and controls. After 6 h of stimulation with solid-phase anti-CD3 mAb in the presence of brefeldin A, the frequencies of both IL-4- and IL-13-producing CD4⁺ T cells from AD patients were significantly higher than those in controls. In contrast, the frequencies of both IL-2- and IFN- γ -producing CD4⁺ T cells in AD patients were significantly lower than those in controls. Figure 2 shows representative results of two-colour immunofluorescent analysis (CD4 vs. IL-4, IL-13, IL-2 or IFN- γ) in a healthy control subject and an AD patient. These findings confirmed previous reports [2, 19, 20, 24, 25], suggesting that the immune status in AD patients might be biased towards the Th2-dominant state.

Table 2. Proportion of cytokine-producing CD4⁺ T cells in normal control subjects and AD patients

Cytokines	Control subjects (n = 20)	AD patients (n = 40)	P
IL-4 (%)	1.6 $\pm$ 0.7	3.9 $\pm$ 2.1	0.0005
IL-13 (%)	1.8 $\pm$ 0.8	4.0 $\pm$ 2.1	0.0023
IL-2 (%)	51.3 $\pm$ 6.3	38.1 $\pm$ 10.3	0.0003
IFN- γ (%)	26.4 $\pm$ 4.6	9.9 $\pm$ 3.5	< 0.0001

Data are presented as means $\pm$ SD. Statistical method: Mann-Whitney unpaired non-parametric test.

Correlation between the frequencies of chemokine receptors and parameters of disease activity and cytokine production in AD (Tables 3 and 4)

We next examined the levels of CCR4 or CCR5 expression, several clinical parameters and cytokine production in AD patients to search for a correlation between them (Table 3). With regard to cytokine production, the frequency of CCR4⁺ cells was correlated positively with IL-4 and IL-13 and inversely with IL-2 and IFN- γ . In contrast, the frequency of CCR5⁺ cells was correlated positively only with IFN- γ production. Serum IgE (log IgE), serum LDH (log LDH) and peripheral eosinophil counts (log Eos) in logarithmic scale showed a positive correlation with CCR4 expression but not with those of CCR5 expression. The eruption score was correlated significantly with both CCR4 and CCR5 expression. The correlation plots between the frequency of CCR4⁺ cells and serum IgE (log IgE), eruption score, IL-4 or IFN- γ are shown in Fig. 3. Multiple regression analyses were then performed to evaluate whether CCR4 or CCR5 expression accounts for the clinical parameters in AD (Table 4). Contributions of the levels of CCR4 and CCR5 expression to log LDH were small. Log IgE, Log Eos and the eruption score were explained by the levels of CCR4 and CCR5 expression. The regression coefficients of the levels of CCR4 expression were higher than those of CCR5 expression for all the clinical parameters, suggesting that the frequency of CCR4⁺ cells was more intimately linked to disease activity in AD than that of CCR5⁺ cells.

Discussion

Previous studies have demonstrated the association of some chemokine receptors with human Th1 or Th2 cells polarized *in vitro*. Th1 cells express CXCR3 and CCR5 [9, 10, 26, 27],

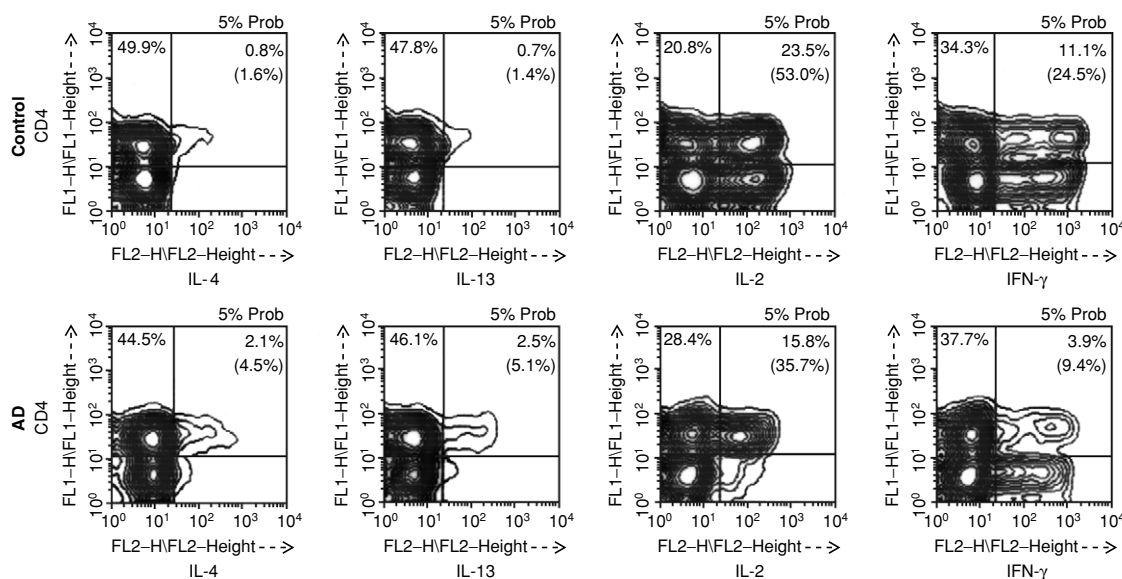


Fig. 2. Representative expression of IL-4, IL-13, IL-2 or IFN- γ in the cytoplasm vs. surface CD4 in the peripheral blood from a patient with AD and a healthy control subject. PBMCs were activated with immobilized anti-CD3 mAbs in the presence of brefeldin A. Intracellular cytokine production was determined by flow cytometry with two-colour analysis. Contour plots show the surface CD4 vs. intracellular cytokine staining from a control subject and an AD patient. The percentages in each quadrant indicate the frequency of the respective sub-populations and parentheses represent the frequency of each of the cytokine-producing cells among CD4⁺ T cells.

Table 3. Correlations between the percentage of CCR4 and CCR5 expression and other predictors of disease activity and cytokine production in AD

	Median value (range)	Correlation analysis CCR4		Correlation analysis CCR5	
		<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Serum IgE (U/mL)*	12218 (34–40413)	0.689	< 0.0001	0.286	0.054
Serum LDH (U/mL) *	568 (320–1029)	0.465	0.006	0.099	0.582
Eosinophil (/μL) *	900 (91–5002)	0.531	0.002	0.140	0.437
Eruption score	34 (8–59)	0.392	0.020	0.349	0.040
IL-4 (%)	3.9 (0.8–7.9)	0.543	0.0002	0.241	0.124
IL-13 (%)	4.0 (1.0–8.2)	0.541	0.0002	0.238	0.129
IL-2 (%)	38.1 (15.7–61.4)	0.517	0.0005	0.054	0.734
IFN-γ (%)	9.9 (3.9–15.1)	0.726	< 0.0001	0.357	0.020

Statistical method: linear regression analyses. *The values were log-transformed for linear regression analyses.

Table 4. Multiple regression analyses to evaluate whether levels of CCR4 and CCR5 expression account for clinical markers in AD*

	Coefficient of determination		Regression coefficient CCR4		Regression coefficient CCR5	
	<i>r</i> ²	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Serum IgE (U/mL)*	0.505	< 0.0001	0.670	< 0.0001	– 0.230	0.034
Serum LDH (U/mL)*	0.165	0.026	0.461	0.009	0.024	0.883
Eosinophil (/μL)*	0.238	0.006	0.522	0.002	0.247	0.173
Eruption score	0.234	0.014	0.341	0.038	0.289	0.753

*Multiple regression analyses were carried out with each of the clinical markers as a dependent variable and levels of CCR4 and CCR5 expression as independent variables.

whereas Th2 cells express CCR3 and CCR4 [11, 12], although one-to-one correspondence between chemokine receptor expression and Th cell subsets *in vivo* remains undetermined. In view of the current concept that an imbalance of Th1 and Th2 cells underlies allergic and autoimmune diseases, this study was designed to examine whether the putative Th1 and Th2 effector cells, which express distinctive chemokine receptors, actually circulate in the peripheral blood of patients with AD. Recent studies have indicated that in multiple sclerosis, a Th1-dominated disorder, the levels of CXCR3-expressing T cells increase in the blood and they infiltrate into the brain lesions [28], and in AD, a Th2-dominated inflammatory disease, CCR4-expressing T cells are predominant in the peripheral blood [13, 14].

In this study, using specific anti-chemokine receptor mAbs, we corroborated and extended the use of chemokine receptors as markers for the detection of circulating Th cell subsets in patients with AD. The major T cell-related pathogenetic process in AD patients has been shown to be mediated by memory CD4⁺ T cells with a Th2-type cytokine profile [21, 29].

With regard to Th2-associated chemokine receptor expression, we hardly detected CCR3-expressing memory CD4⁺ T cells, even in AD patients, as recently reported [13]. Thus, we believe that CCR3 is not a good cell-surface marker for Th2 effector cells in the peripheral blood. In contrast, CCR4 was detected on a substantial proportion of memory CD4⁺ T cells in the blood. Most importantly, the frequency of CCR4⁺ memory CD4⁺ T cells was significantly higher in AD patients than healthy control subjects and was correlated positively with disease severity of AD: serum IgE, serum LDH, eosinophil number and skin eruption score. In addition, the frequency of CCR4⁺ cells was correlated positively with Th2-type cytokines

(IL-4 and IL-13) and negatively with Th1-type cytokines (IL-2 and IFN-γ). With regard to Th1-associated chemokine receptor expression, the frequencies of both CCR5⁺ and CXCR3⁺ memory CD4⁺ T cells were lower in AD patients and were correlated positively with skin eruption score and IFN-γ production. The results suggest that there may be global Th1 dysregulations of memory CD4⁺ T cells in the peripheral blood of AD patients, as reported previously [24]. These observations suggested that CCR4 and CCR5/CXCR3 expression might be associated with Th2 and Th1 cells, respectively, in the peripheral blood from AD patients.

Other investigators [13, 14] have recently reported that significant numbers of freshly isolated circulating CCR4⁺ or CXCR3⁺ memory CD4⁺ T cells readily expressed the Th2-type cytokines IL-4, IL-5 and IL-13 or the Th1-type cytokine IFN-γ, respectively, after short-term stimulation, indicating that CCR4 and CXCR3 could serve as useful markers for Th2 and Th1 effector populations in the circulation. Our results are in accord with them. However, there have been several reports that contradict the dissection of Th cell subsets on the basis of chemokine receptor expression. Andrew et al. [16] reported that CCR4⁺ memory CD4⁺ T cells coexpress Th1-associated CCR5 and CXCR3, and can produce the Th1-type cytokine IFN-γ as well as Th2-type cytokines, suggesting potential problems with regard to usage of these markers for Th cell subsets. Nanki et al. [15] also reported the lack of correlation between chemokine receptor expression and Th2/Th1 cytokine production by individual memory T cells using single-cell RT-PCR. The reasons for these discrepancies are unclear at present but might be related to differences in the regulation of chemokine receptors and cytokine production under normal and chronic inflammatory conditions. Our results indicated that in AD

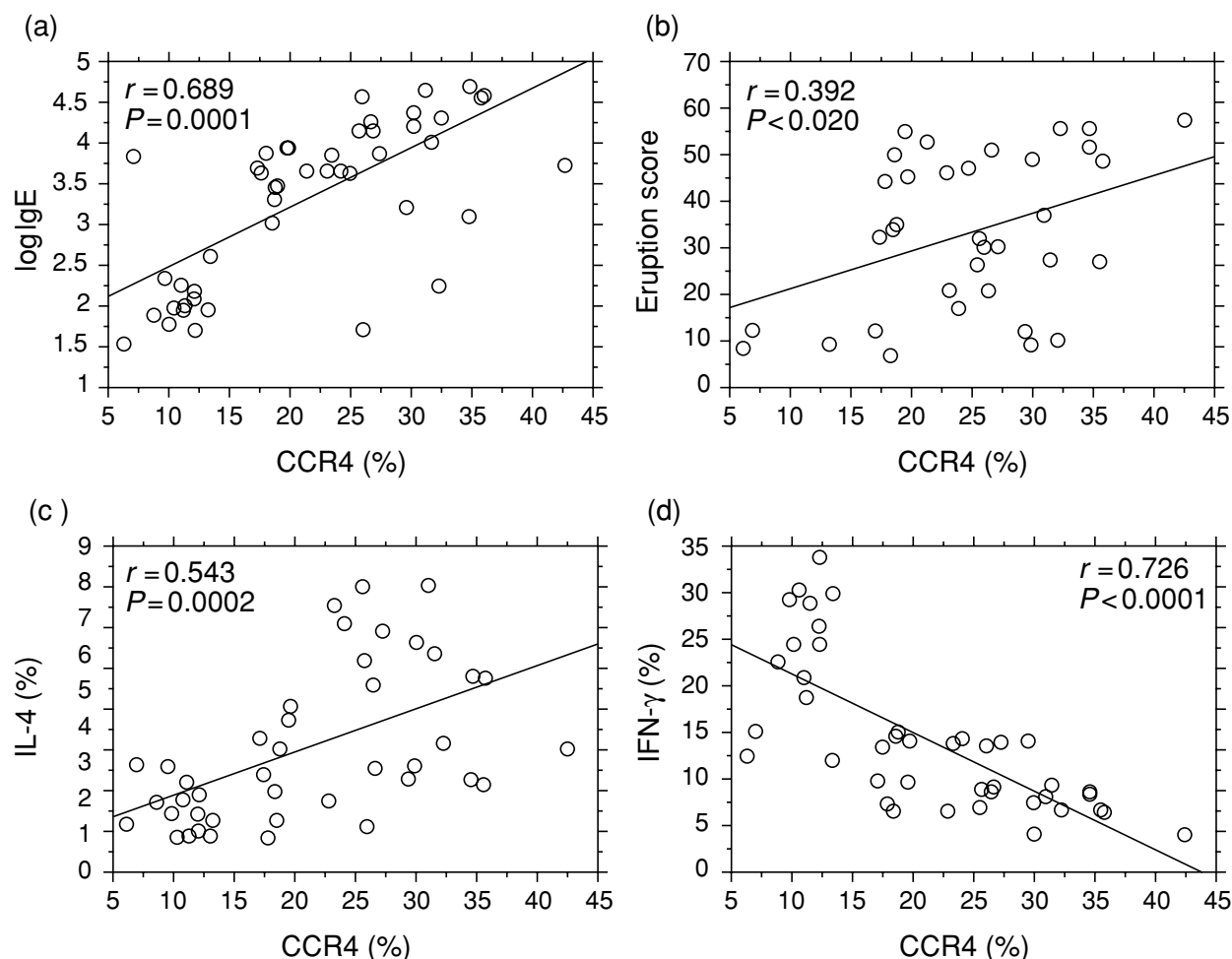


Fig. 3. Correlation of the frequency of CCR4⁺ cells in memory CD4⁺ T cells of the peripheral blood from patients with AD against serum total IgE (log IgE) (a), eruption score (b), and the frequency of IL-4-producing (c) or IFN- γ -producing (d) cells in CD4⁺ T cells.

patients there is a tight linkage between CCR4 and Th2 cell subsets, although the linkage might be compromised under different pathological conditions.

The frequencies of both CCR4⁺ and CCR5⁺ cells showed significant, albeit weak, correlations with skin eruption score. These findings suggested that not only Th2 but also Th1 cells might play a role in producing chronic skin lesions in AD [21]. Recently, Grewe et al. [25] proposed a model of sequential Th cell activation in AD patients. During the initiation phase allergen-specific Th2 cells dominate. Subsequently, infiltrating eosinophils and macrophages produce IL-12, which leads to the activation of allergen-specific and allergen-non-specific Th1 and Th0 cells, and finally, IFN- γ -producing T cells may be responsible for the chronic phase of AD and the severity of the disease. In this study, multiple regression analyses indicated that the percentage of CCR4 expression had a much higher correlation coefficient with serum IgE, LDH levels, eosinophil number and skin eruption score of AD than that of CCR5. An increase in the number of cells with surface CCR4 may predict worsening of AD, although it needs further investigations to confirm this.

It is necessary to clarify the mechanisms leading to an increase in circulating CCR4⁺ memory CD4⁺ T cells in AD patients. The ligands of CCR4 are CCL17 (thymus- and activation-

regulated chemokine: TARC) and CCL22 (macrophage-derived chemokine: MDC) [30]. Some dendritic cells (DCs) express both CCL17 and CCL22 [31, 32], and they might recruit CCR4-bearing T cells and augment preferential expansion of the Th2 arm of the immune response. In this regard, the recent observations that serum CCL17 levels of patients with AD were elevated and associated with disease activity of AD are of particular interest [33]. However, it is also possible that the increase in overall CCR4⁺ T cell number in the circulation reflects an expansion of the T cell population rather than recruitment to the vascular compartment. More detailed information regarding the roles of CCR4⁺ T cells might contribute considerably to a better understanding of the mechanisms that precipitate atopic conditions.

In conclusion, the frequency of CCR4⁺ cells in memory CD4⁺ T cells might be a useful circulating marker of disease activity in AD.

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