

Clinical Significance of CCR4 Expression in Adult T-Cell Leukemia/Lymphoma: Its Close Association with Skin Involvement and Unfavorable Outcome¹

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ABSTRACT

Adult T-cell leukemia/lymphoma (ATLL) is a distinct clinical entity among mature T-cell neoplasms, and its causative agent has been confirmed to be long-term infection by human T-lymphotropic virus type 1. A recent study demonstrated frequent expression of a chemokine receptor, CC chemokine receptor (CCR)4, which is known as a Th2 marker but not CXC chemokine receptor (CXCR)3, which is known as a Th1 marker, among both ATLL- and human T-lymphotropic virus type 1-immortalized T cells. In this study, immunostaining analysis for CCR4 and CXCR3 expression in ATLL cells obtained from 103 patients with ATLL was performed, and the clinical parameters and overall survival of the CCR4-positive and -negative cases were compared. Ninety-one (88.3%) of the 103 cases were positive for CCR4 staining, whereas only 5 (4.9%) were positive for CXCR3 staining. Positivity for CCR4 was significantly associated with skin involvement ($P < 0.05$), although there were no significant differences in clinical characteristics between the CCR4-positive and -negative cases at the time of initial diagnosis. CCR4⁺ ATLL cells may accumulate in the

skin because of the expression of a CCR4 ligand, thymus and activation-regulated chemokine (TARC), on normal and inflamed cutaneous endothelia. As for survival analysis, positivity for CCR4 expression was extracted as an unfavorable prognostic factor as well as other factors, including the presence of B symptoms and extranodal involvement of more than one site. Multivariate analysis confirmed that CCR4 expression was an independent and significant prognostic factor ($P < 0.05$). Thus, our finding may provide a novel insight into not only the biological but also the clinical features of ATLL.

INTRODUCTION

Chemokines belong to a superfamily of small, cytokine-like proteins that induce cytoskeletal rearrangement, firm adhesion to endothelial cells, and directional migration of the leukocytes by interacting with G protein-coupled receptors (1–3). This leukocyte trafficking, which is critically regulated by chemokines and their receptors, shares many similarities with tumor cell migration and metastasis. For example, CXCR4, a chemokine receptor, is expressed more abundantly in breast cancer tissues than in normal breast tissues; its ligand, CXCL12, is expressed in various organs such as the lymph nodes, bone marrow, and lungs, where breast cancer cells tend to metastasize (4).

ATLL³ is a peripheral T-cell neoplasm. ATLL is most often composed of highly pleomorphic lymphoid cells and is caused by HTLV-1 (5). In ATLL, leukemic cells frequently infiltrate into organs such as the lymph nodes, spleen, liver, and skin. Various HTLV-1-associated inflammatory diseases are also commonly characterized by the infiltration of HTLV-1⁺ T cells into target organs (6). It is thus conceivable that chemokines and their receptors play important roles in tissue infiltration of ATLL cells and HTLV-1⁺ T cells, analogous to the assumed mechanism in metastatic breast cancer. In addition, it has generally become accepted that the expression profile of chemokine receptors in normal T-cell subsets is associated with the profile of cytokine secretion. Specifically, CCR4, CCR3, and CCR8 are selective markers of the Th2 phenotype, whereas CXCR3 is a selective marker of the Th1 phenotype (7–12),

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³ The abbreviations used are: ATLL, adult T-cell leukemia/lymphoma; HTLV-1, human T-lymphotropic virus type 1; MF, mycosis fungoides; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; RT-PCR, reverse transcription-PCR; PS, performance status; LDH, lactic dehydrogenase; Hb, hemoglobin level; Plt, platelet count; CNS, central nervous system; OS, overall survival; HR, hazard ratio; CI, confidence interval; TARC, thymus and activation-regulated chemokine; MCP, monocyte chemoattractant protein-1; Mig, monokine induced by IFN γ ; CLA, cutaneous lymphocyte antigen; MDC, macrophage derived chemokine; CXCL, CXC chemokine ligand; CCR, CC chemokine receptor.

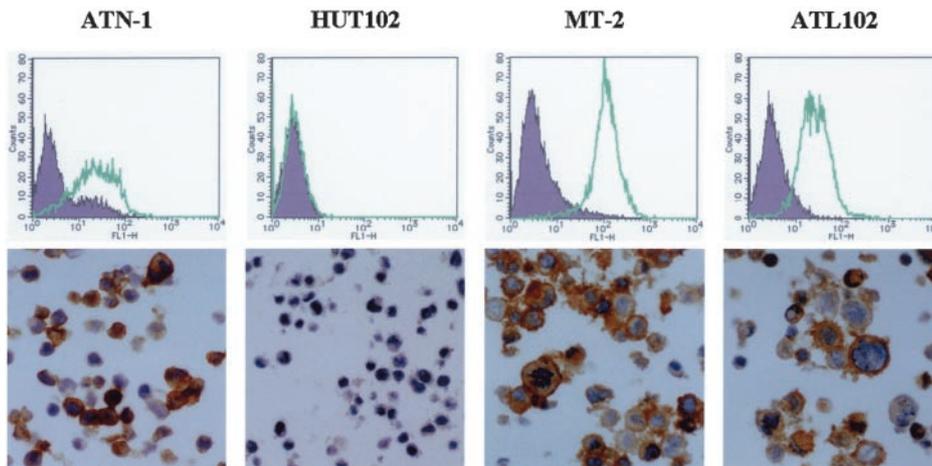


Fig. 1 Flow cytometric and immunostaining analyses of CCR4 expression in ATLL cell lines. ATN-1, MT-2, and ATL102 cells show membrane staining and cytoplasmic granular staining on formalin-fixed, paraffin-embedded cell block sections by immunostaining using the mAb KM2160 (bottom panels). These results paralleled the data obtained by flow cytometric analysis (top panels). HUT102 cells are negative for CCR4 expression on both analyses.

although some controversy exists. As in the case of normal T-cell subsets, there is differential expression of chemokine receptors in T-cell-derived tumors. The pattern of chemokine receptor expression in T-cell-derived non-Hodgkin's lymphoma was characterized previously (13). In ATLL, CXCR3 was not expressed (zero of two), whereas the expression of CCR4, CCR5, Mig, TARC, and MCP-2 was not studied. Interestingly, CCR4 expression was seen in all (5 of 5) patients with large-cell-transformed MFs but in only 1 of the 7 patients with nontransformed MFs (13). This suggests that CCR4-expressing T-cell tumors may acquire a certain growth advantage similar to tumor progression in MF. ATLL develops as a result of multi-step tumorigenesis and results in an unfavorable outcome because of rapid deterioration of the disease. We investigated 103 cases with ATLL to analyze the biological and clinical significance of CCR4 and CXCR3 expression by an immunostaining procedure.

MATERIALS AND METHODS

mAbs against Chemokine Receptors and CLA. We used murine mAbs against CCR4 (KM2160; kindly provided by Kyowa Hakko Kogyo, Inc., Tokyo, Japan) and CXCR3 (1C6; BD PharMingen, San Diego, CA; Refs. 7, 14, 15) and rat mAb against CLA (HECA452; BD PharMingen; Ref. 16). The KM2160 mAb recognizes the NH₂-terminal portion (amino acids 12–29) of the CCR4 molecule.

Cell Lines and Viable Patient Samples. HUT102, ATL102, and ATN-1 are human T-cell lines established from patients with ATLL (17–19). They have clonal integration of HTLV-1, as determined by Southern blot analysis. MT-2 is a human T-cell line transformed by infection of HTLV-1 (18–20). PBMCs were isolated from two patients diagnosed as having acute-type ATLL and three healthy adult volunteers upon informed consent. Lesional skin, lymph node, and bone marrow tissues were obtained from four patients with acute-type ATLL upon informed consent and used for RNA extraction in addition to histological examination.

Flow Cytometric Analysis. After blocking surface Fc receptors by murine normal serum (Dako, Kyoto, Japan), 1×10^6 cells were incubated at 4°C with biotin-conjugated anti-

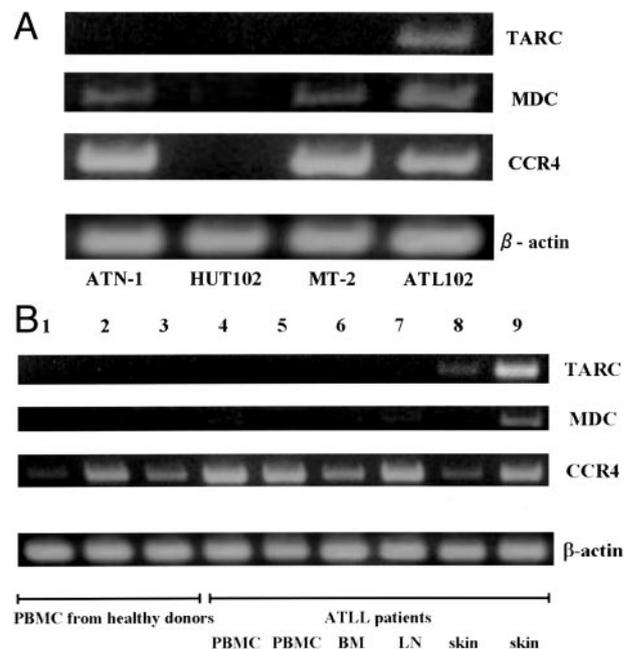
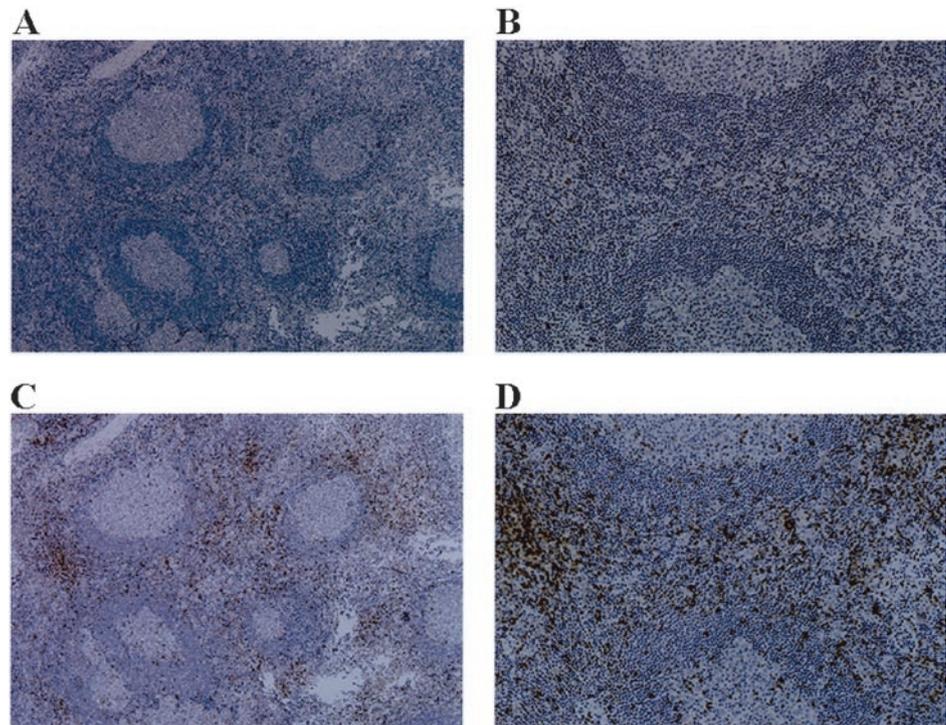


Fig. 2 RT-PCR analysis of the mRNA expression of CCR4 and its ligands, TARC and MDC. **A**, mRNA expression of CCR4, TARC, and MDC in ATLL-derived cell lines. RT-PCR was carried out to amplify CCR4, TARC, MDC, and β -actin from total RNAs prepared from each cell line. **B**, mRNA expression of CCR4, TARC, and MDC in various tissues obtained from ATLL patients, as detected by RT-PCR analysis. Total RNAs were prepared from the PBMC of 3 healthy volunteers and from various tissues with infiltration of atypical lymphoid cells obtained from 6 ATLL patients.

CCR4 or isotype control mAb (MOPC-21; BD PharMingen) for 30 min, washed, and resuspended in PBS. Then, the cells were incubated with streptavidin-FITC conjugate (BD PharMingen) for 30 min, washed, resuspended in PBS, and analyzed by FACScan with the aid of CELLQuest software (Becton Dickinson, San Jose, CA). Intracellular cytokines of ATLL cell lines were analyzed after stimulation with phorbol 12-myristate 13-

Fig. 3 Immunostaining for CCR4 and CXCR3 in normal reactive lymph nodes. Immunostaining was performed on formalin-fixed, paraffin-embedded hyperplastic lymph node sections. *A* and *B*, immunostaining for CCR4 using KM2160 mAb. A small percentage of the T cells in the paracortex area show positivity for CCR4. *C* and *D*, immunostaining for CXCR3 using 1C6 mAb. Nearly half of the interfollicular T cells were stained. *A* and *C*, magnification, $\times 40$; *B* and *D*, magnification, $\times 100$.



acetate, ionomycin, and Breferdin A for 4 h followed by fixation and permeabilization. Double staining for the intracellular cytokine IFN- γ , with FITC and interleukin 4 with phycoerythrin, was performed (21).

RT-PCR. Total RNA was prepared from the cells or tissues. After incubation with DNaseI, 1 μ g of the total RNA was reverse transcribed and dissolved in a total volume of 42 μ l as a first-strand cDNA solution. Two μ l of this solution was used to amplify CCR4, TARC, MDC, or β -actin mRNA (22). The oligonucleotide primers were as follows: for CCR4, sense, 5'-CCCTCGATGAAAGCATATAC-3' and antisense, 5'-GTGAACAAAAGCCAGAGTTT-3'; for TARC, sense, 5'-AGTACTTCAAGGGAGCCATT-3' and antisense, 5'-AGACTT-TAATCTGGGCCCTT-3'; for MDC, sense, 5'-AACACTTC-TACTGGACCTCA-3' and antisense, 5'-TAGGGTTATTAA-GAGGGGAC-3'.

Patient Selection. This study included 103 patients with ATLL diagnosed between 1989 and 2002 at four independent hospitals (Imamura Bun-in Hospital, Nagoya City University Hospital, Shizuoka Saiseikai General Hospital, and Ensyu General Hospital). The diagnosis and classification of clinical subtypes of the ATLL were made according to the criteria proposed by Japan Lymphoma Study Group (23). Clinical variables analyzed in this study included age, sex, clinical subtype, presence or absence of B symptoms and extranodal involvement, PS, serum LDH level, serum calcium level, WBC count, abnormal lymphocyte count, Hb and Plt of the peripheral blood, surface phenotypic markers (CD4, CD8, CD30, and CLA), involved organs (lymph node, bone marrow, peripheral blood, liver, spleen, gastrointestinal tract, lung, CNS, bone, skin, ascites, pericardial effusion, pleural effusion, nasopharynx, Waldeyer's

tonsillar ring, kidney, eye, muscle, and mammary gland tissues), and clonal integration of HTLV-1 provirus in the genomic DNA of the tumor cells as determined by Southern blot analysis. We used the clinical data at the time, when the initial biopsy was performed. Although the treatment for ATLL cases enrolled in this study were variable, combination chemotherapy containing doxorubicin such as RCM protocol (24), LSG15 protocol (25), and cyclophosphamide-doxorubicin-vincristine-prednisone regimen were applied for most patients.

Histopathology. Tissue biopsy of the lesional organs was performed to obtain sufficient amount of materials after informed consent was obtained from the patients. Specimens were fixed in 10% buffered formalin and embedded in paraffin. The morphological classification was performed according to the WHO Classification of Tumors (5) with slight modification. We classified the 103 ATLL samples into the following five groups: (a) pleomorphic large cell type; (b) pleomorphic medium-sized cell type; (c) pleomorphic small-sized cell type; (d) anaplastic large cell lymphoma-like type; and (e) Hodgkin lymphoma-like type.

Immunostaining. The immunostaining analysis was performed with the aid of a SS MultiLink Detection kit, horseradish peroxidase/3,3'-diaminobenzidine (BioGenex Laboratories, San Ramon, CA) using formalin-fixed, paraffin-embedded sections. We immunostained for both CCR4 and CXCR3 in several nearly normal lymph nodes and in the lesional tissues of 103 patients with ATLL. Immunoabsorption with the CCR4 antigen peptide of the CCR4-positive specimens resulted in negative stains (data not shown). Cell pellets that had been prepared from 1×10^7 ATLL cells were also fixed with formalin, embedded in paraffin, and used for immunostaining. We defined a specimen

Table 1 Patient characteristics

Total number		103
Age (yr), median (range)		61 (37–88)
Sex (male/female)		63/40
Clinical subtype ^a	Acute	77 (75.5%)
	Lymphoma	17 (16.7%)
	Chronic	3 (2.9%)
	Smoldering	5 (4.9%)
Histological classification	Pleomorphic large	55 (53.4%)
	Pleomorphic medium	33 (32.0%)
	Pleomorphic small	9 (8.7%)
	ALCL-like ^b	5 (4.9%)
	HL-like	1 (1.0%)
B symptoms ^a	Present	34 (33.3%)
	Absent	68 (66.7%)
PS ^a	0	18 (17.6%)
	1	41 (40.2%)
	2	20 (19.6%)
	3	19 (18.6%)
	4	4 (3.9%)
LDH (/upper limit of normal), median (range) ^c		1.86 (0.59–24.65)
Ca (mg/dl), median (range) ^d		9.6 (8.0–19.4)
WBC ($\times 10^3/\mu\text{l}$), median (range) ^a		8.20 (1.6–208.6)
Hb (g/dl), median (range) ^d		13.2 (6.3–16.9)
Plt ($\times 10^3/\mu\text{l}$), median (range) ^d		202.5 (19.5–443)
Surface-phenotypic markers	CD4 ⁺ CD8 ⁻	91 (88.3%)
	CD4 ⁺ CD8 ⁺	8 (7.8%)
	CD4 ⁻ CD8 ⁻	3 (2.9%)
	CD4 ⁻ CD8 ⁺	1 (1.0%)
CD30 ^e	+	25 (26.0%)
	-	71 (74.0%)
CCR4	+	91 (88.3%)
	-	12 (11.7%)
CXCR3	+	5 (4.9%)
	-	98 (95.1%)
CLA ^f	+	17 (17.3%)
	-	81 (82.7%)
Clonal integration of HTLV-1	+	35 (34.0%)
	-	0
	Not tested or unknown	68 (66.0%)

^a 1 case was not evaluated.

^b ALCL, anaplastic large-cell lymphoma; HL, Hodgkin lymphoma.

^c 2 cases were not evaluated.

^d 3 cases were not evaluated.

^e 7 cases were not evaluated.

^f 5 cases were not evaluated.

as being positive for CCR4 or CXCR3 if >10% of the apparent tumor cells were stained with the respective antibody.

Statistical Analysis. The significance of differences between the CCR4-positive and -negative groups was examined with the Fisher's exact test or the Mann-Whitney *U* test. Patients' survival data were analyzed with the Kaplan-Meier method and were compared using the log-rank and Breslow-Gehan-Wilcoxon tests. Univariate and multivariate analyses were performed with the Cox proportional hazard regression model, and variables used in the latter analysis were selected with the stepwise method. Data were analyzed with the aid of Statview software (version 5.0; SAS Institute, Cary, NC). In this study, *P* < 0.05 was considered as significant.

RESULTS

Frequent Expression of Surface CCR4 among ATLL-derived Cell Lines. We evaluated the surface expression of CCR4 on ATLL cell lines (HUT102, ATN-1, and ATL102) and

on a HTLV-1-transformed cell line (MT-2) by both flow cytometric and immunostaining analyses (Fig. 1). Three (75%) of the four cell lines (ATL102, MT-2, and ATN-1) showed membrane staining and granular cytoplasmic staining, and these findings were consistent with the data obtained by flow cytometric analysis. We next examined to see whether these ATLL cells secrete Th2 cytokines. However, there was no fundamental difference in the profile of cytokine production among these four ATLL cell lines, *i.e.*, all four lines were of the Th0 subtype (data not shown) as previously reported by other investigators (26, 27).

mRNA Expression of CCR4 and Its Ligands, TARC and MDC, in ATLL Cells. The mRNA expression of CCR4 and its ligands, TARC and MDC, in four cell lines was investigated by RT-PCR analysis. On RT-PCR, the ATN-1, MT-2, and ATL102 cell lines showed strong bands for CCR4 mRNA, whereas the HUT102 line showed no visible band at our sensitivity as shown in Fig. 2A. The ATL102 line also showed strong

A CCR4

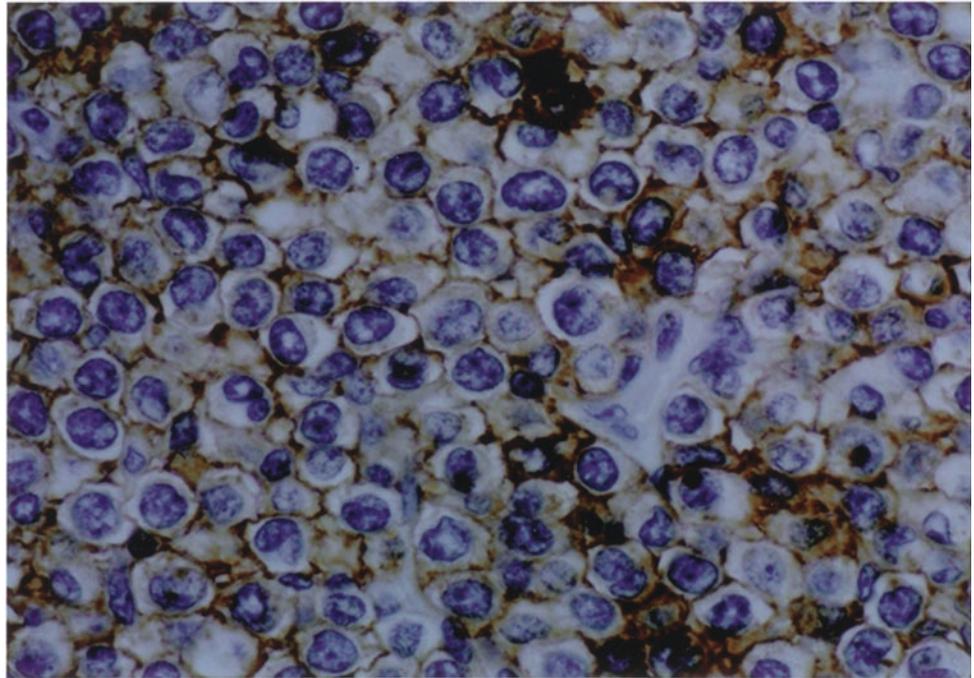
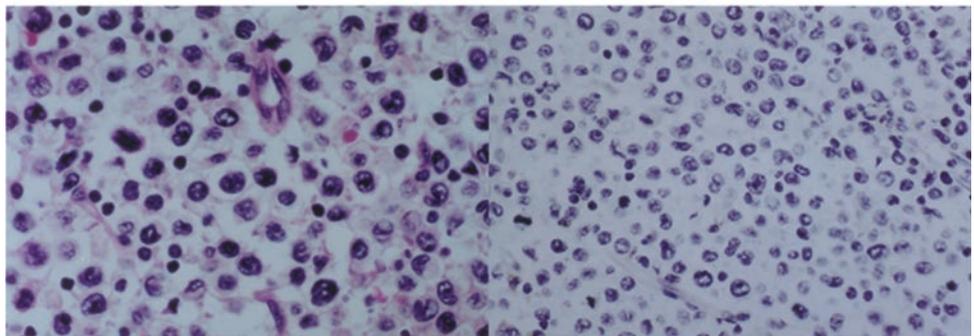


Fig. 4 A representative lymph node finding of a patient with lymphoma type ATLL. *A*, the majority of tumor cells stained for CCR4. Most cells show membrane staining and granular cytoplasmic staining (CCR4 immunostaining, $\times 400$). *B*, proliferation of large atypical cells with irregular and pleomorphic nuclei. This case was classified as pleomorphic large-cell type (H&E staining, $\times 400$). *C*, the same tumor cells of this case are negative for CXCR3 (CXCR3 immunostaining, $\times 400$).

B HE



C CXCR3

bands for TARC and MDC mRNA, whereas the ATN-1 and MT-2 lines showed a weak band for MDC mRNA. Accordingly, all ATLL-related cell lines expressing CCR4 were positive for MDC expression at the mRNA level but were not always positive for TARC expression. We next carried out RT-PCR analysis of CCR4 expression in PBMCs derived from healthy adult volunteers and from ATLL patients and in tissues where leukemic cells had infiltrated. All PBMCs derived from the healthy adult volunteers expressed the mRNA for CCR4, whereas they did not express the mRNA for MDC nor TARC. Similarly, all PBMC samples derived from the ATLL patients expressed the mRNA for CCR4. However, among organs infiltrated by ATLL cells, only the skin showed signals for expression of its ligands. Sample number 8 showed weak TARC mRNA expression, and sample number 9 showed strong TARC and weak MDC expression as shown in Fig. 2*B*.

CCR4 and CXCR3 Expression in Nearly Normal Lymph Node.

In reactive lymph nodes, a few interfollicular T cells were noted to be positive for CCR4 by immunostaining analysis, comprising 3–7% of all cells in the paracortex area (Fig. 3, *A* and *B*). On the contrary, many interfollicular T cells were positive for CXCR3, comprising 30–60% of all cells in the paracortex area (Fig. 3, *C* and *D*). B cells localized in the follicles, mantle zones, and marginal zones were negative for CCR4 and CXCR3. These results are consistent with the data reported previously (7, 10).

Clinical Features of the 103 ATLL Patients and Immunostaining of Their Specimens for CCR4 and CXCR3.

Among the 103 ATLL patients, there were 63 males and 40 females with an age range of 37–88 years (median age, 61 years). The patients' clinical characteristics are summarized in Table 1. Specimens derived from 91 (88.3%) of the 103 cases

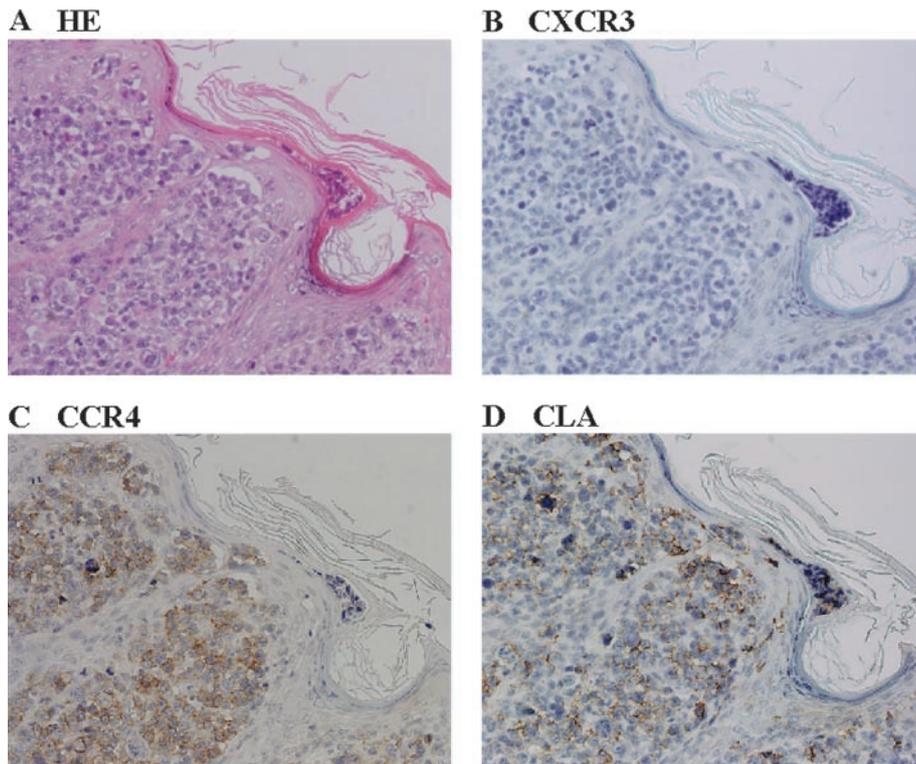
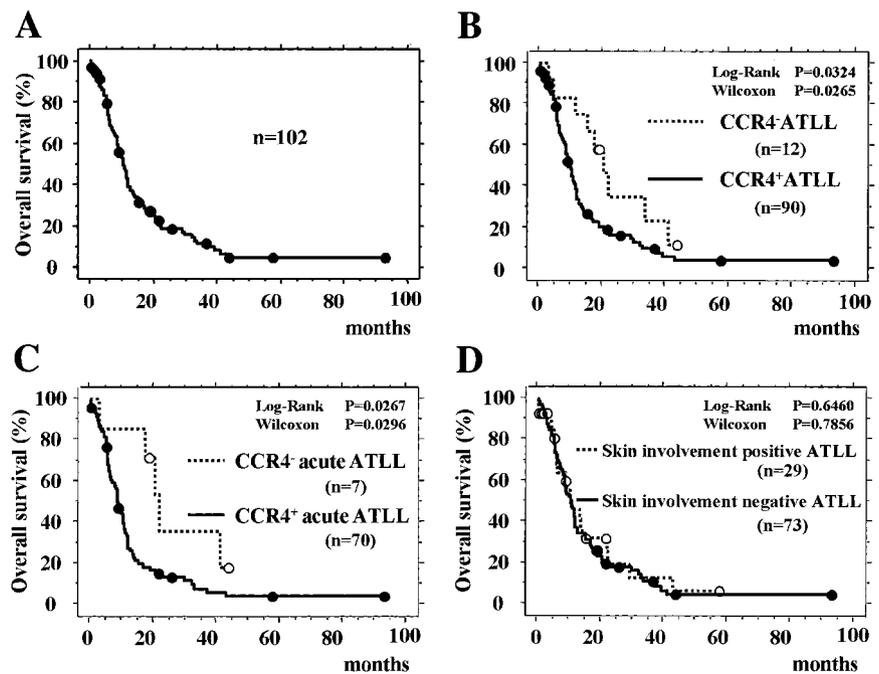


Fig. 5 Representative pictures of the skin involvement in a patient with acute type ATLL. Remarkable infiltration of atypical lymphocytes is seen in the epidermis and dermis. (H&E, $\times 200$; **A**). The tumor cells are negative for CXCR3 (**B**) but positive for CCR4 expression (**C**). Infiltrated large cells are immunostained for CLA on their surface membrane.

Fig. 6 OS of the patients with ATLL enrolled in this study. **A**, OS of the 103 patients with ATLL. The 50% survival time is 10.5 months, which is nearly in agreement with the figures reported in previous clinical trials in Japan. **B**, OS of the ATLL patients based on positivity or negativity for CCR4 expression. The CCR4-negative ATLL patients show significantly better prognosis. **C**, OS of patients with acute-type ATLL based on positivity or negativity for CCR4 expression. The CCR4-negative acute ATLL patients show significantly better prognosis in subgroup analysis. **D**, OS of the ATLL patients based on the presence or absence of skin involvement. No significant difference is seen between the two groups.



were positive for CCR4, and specimens derived from only 5 cases (4.9%) were positive for CXCR3. Immunostaining pictures of lesional tissues from two representative CCR4⁺ patients are shown in Figs. 4 and 5. In most specimens positive for CCR4, nearly all neoplastic cells showed positive staining by

immunohistochemistry as shown in Figs. 4A and 5C. Moreover, the CCR4 expression in skin tumor cells did not vary irrespective of their localization in the skin. The OS curve of all ATLL cases enrolled in this study is shown in Fig. 6A. The 50% survival \pm SE was 10.5 ± 0.6 months.

Table 2 Patient characteristics according to CCR4 expression

		CCR4 ⁺ ATLL	CCR4 ⁻ ATLL	P
Total number		91 (88.3%)	12 (11.7%)	
Age (yr)	≥60	52 (57.1%)	7 (58.3%)	>0.9999
	≤59	39 (42.9%)	5 (41.7%)	
Sex	Male	55 (60.4%)	8 (66.7%)	0.7619
	Female	36 (39.6%)	4 (33.3%)	
PS	0, 1	50 (55.6%)	9 (75.0%)	0.2316
	2, 3, 4	40 (44.4%)	3 (25.0%)	
Clinical subtype	Acute, lymphoma	83 (92.2%)	11 (91.7%)	>0.9999
	Chronic, smoldering	7 (7.8%)	1 (8.3%)	
Histological classification	Non-small	83 (91.2%)	11 (91.7%)	>0.9999
	Small	8 (8.8%)	1 (8.3%)	
B symptoms	+	30 (33.3%)	4 (33.3%)	>0.9999
	-	60 (66.7%)	8 (66.7%)	
Ca (mg/dl)	≥11.0	17 (19.3%)	0 (0.0%)	0.2111
	<11.0	71 (80.7%)	12 (100.0%)	
LDH	>Normal	79 (88.8%)	9 (75.0%)	0.1833
	≤Normal	10 (11.2%)	3 (25.0%)	
Surface-phenotypic markers	CD4 ⁺ CD8 ⁻	81 (89.0%)	10 (83.3%)	0.6286
	Not CD4 ⁺ CD8 ⁻	10 (11.0%)	2 (16.7%)	
CD30	+	19 (22.6%)	6 (50.0%)	0.0730
	-	65 (77.4%)	6 (50.0%)	
CXCR3	+	4 (4.4%)	1 (8.3%)	0.4688
	-	87 (95.6%)	11 (91.7%)	
CLA	+	17 (19.8%)	0 (0%)	0.1192
	-	69 (80.2%)	12 (100%)	
Clonal integration of HTLV-1	+	30 (33.0%)	5 (41.7%)	0.5360
	Not tested or unknown	61 (67.0%)	7 (58.3%)	
WBC (×10 ³ /μl)	median (range), (number)	8.2 (1.6–171.8), (90)	7.6 (3.8–208.6), (12)	0.7357
Hb (g/dl)	median (range), (number)	13.2 (6.3–16.9), (88)	13.4 (10.6–14.6), (12)	0.5523
Plt (×10 ³ /μl)	median (range), (number)	204 (20–443), (88)	191 (59–342), (12)	0.5885

Comparison of the Clinical Characteristics of the CCR4⁺ and CCR4⁻ Cases. The patient characteristics based on the CCR4 status are summarized in Table 2. Surface CD4, CD8, and CD30 positivity were not associated with CCR4 expression. Regarding CLA expression, although no statistically significant association with CCR4 expression was determined by the Fisher's exact test, it was of interest that all of the CLA-positive cases belonged to the CCR4-positive group. The clinical characteristics of the CCR4⁺ and CCR4⁻ groups did not significantly differ. Involved organs based on the CCR4 status are summarized in Table 3. The CCR4⁺ cases were significantly more likely to have skin involvement than the CCR4⁻ cases ($P = 0.0176$). None of the other involved organs were associated with the CCR4 status. Involved organs based on the CLA expression status were also summarized in Table 4. The CLA⁺ cases significantly favored skin involvement when compared with the CLA⁻ cases ($P = 0.0112$) as previously reported by other investigators (28). With respect to the CD30 expression, 13 of 25 (52.0%) CD30⁺ and 58 of 70 (82.9%) CD30⁻ cases had peripheral blood involvement, indicating that the CD30⁻ cases were significantly more likely to show leukemic status than the CD30⁺ cases ($P = 0.0060$, data not shown), which was consistent with the previously published data (29). The OS was significantly longer in the CCR4⁻ cases than in the CCR4⁺ cases (50% OS; 20.6 versus 9.5 months) as shown in Fig. 6B. Among patients with acute-type ATLL, the CCR4⁻

Table 3 Involvement sites according to CCR4 expression

	CCR4 ⁺ ATLL	CCR4 ⁻ ATLL	P
Total number	90	12	
Involvement sites			
LN ^a	73 (81.1%)	12 (100%)	0.2093
Extranodal involvement			
BM	45 (50.0%)	7 (58.3%)	0.7605
PB	69 (76.7%)	8 (66.7%)	0.4818
Liver	15 (16.7%)	1 (8.3%)	0.6855
Spleen	27 (30.0%)	2 (16.7%)	0.5011
Gastrointestinal tract	23 (25.6%)	1 (8.3%)	0.2853
Lung	5 (5.6%)	0 (0.0%)	>0.9999
CNS	1 (1.1%)	1 (8.3%)	0.2225
Bone	3 (3.3%)	0 (0%)	>0.9999
Skin	29 (32.2%)	0 (0.0%)	0.0176
≥2 sites	69 (76.7%)	6 (50.0%)	0.0771
≥3 sites	49 (54.4%)	5 (41.7%)	0.5409

^a LN, lymph node; BM, bone marrow; PB, peripheral blood; CNS, central nervous system.

cases showed better prognosis than the CCR4⁺ cases (50% OS; 22.2 versus 8.9 months) as shown in Fig. 6C. We then compared the OS between cases who did or did not have skin involvement. No significant difference in OS was recognized between the two groups (Fig. 6D).

Prognostic Factors for ATLL. Univariate Cox proportional hazard analysis identified the following unfavorable prog-

Table 4 Involvement sites according to CLA expression

	CLA ⁺ ATLL	CLA ⁻ ATLL	P
Total number	17	81	
Involvement sites			
LN ^a	13 (76.5%)	72 (88.9%)	0.2317
Extranodal involvement			
BM	8 (47.1%)	42 (51.9%)	0.7932
PB	13 (76.5%)	62 (76.5%)	>0.9999
Liver	0 (0%)	16 (19.8%)	0.0662
Spleen	5 (29.4%)	24 (29.6%)	>0.9999
Gastrointestinal tract	4 (23.5%)	20 (24.7%)	>0.9999
Lung	1 (5.9%)	3 (3.7%)	0.5394
CNS	0 (0%)	2 (2.5%)	>0.9999
Bone	1 (5.9%)	2 (2.5%)	0.439
Skin	9 (52.9%)	16 (19.8%)	0.0112
≥2 sites	13 (76.5%)	60 (74.1%)	>0.9999
≥3 sites	7 (41.2%)	45 (55.6%)	0.2997

^a LN, lymph node; BM, bone marrow; PB, peripheral blood; CNS, central nervous system.

nostic factors with respect to the patients' survival: PS \geq 2; presence of B symptoms; extranodal involvement of more than one site; serum LDH level > upper limit of normal; serum Ca level \geq 11.0 mg/dl; and positive CCR4 expression (Table 5). Multivariate analysis demonstrated the presence of B symptoms (HR = 2.425; 95% CI, 1.517–3.878; P = 0.0002), extranodal involvement of more than one site (HR = 2.106; 95% CI, 1.213–3.656; P = 0.0081), and positive CCR4 expression (HR = 2.197; 95% CI, 1.080–4.469; P = 0.0298) to be independent and significant unfavorable prognostic factors (Table 5).

DISCUSSION

In this paper, we reported that most ATLL cells were positive for CCR4 by immunostaining analysis, thus supporting the previous finding that showed its mRNA expression (30). In the normal condition, CCR4 is selectively expressed in CD4⁺ memory T cells (14), and CCR4 is expressed in a few circulating peripheral T cells (13, 15, 31–33); in contrast, CXCR3 is expressed in many circulating peripheral T cells (11, 15, 32). These findings show that there are similarities between circulating T-cell subset and interfollicular T cells in normal lymph nodes with respect to the distribution of CCR4 and CXCR3 positivity. Our data may suggest that the origin of ATLL cells in most patients is CCR4-positive T cells, or ATLL is more likely to develop in those HTLV-1⁺ patients who have altered immune regulation leading to increased numbers of CCR4-positive-infected cells. Interestingly, however, the profile of cytokine production in the CCR4-positive and -negative ATLL cell lines are all Th0 subtype. This indicates that the cytokine profiles of ATLL cells and HTLV-1-immortalized T cells do not simply shift to the Th2 phenotype (26, 27), suggesting that ATLL cells show an aberrant phenotype or they abnormally express CCR4 for unknown reasons probably associated with tumorigenesis.

In our study, the CCR4⁺ ATLL was significantly associated with the skin involvement, and only the involved skin tissues showed expression of the CCR4 ligand, TARC. Recent studies have also confirmed TARC to be expressed on normal and inflamed cutaneous endothelia (33, 34). The skin involve-

ment was also associated with CLA expression in our study. In fact, recent studies have also revealed that most CLA⁺ memory CD4⁺ lymphocytes express high levels of CCR4, whereas mAb against CCR4 also recognized a smaller number of CLA⁻ peripheral blood memory CD4⁺ lymphocytes (34). Because the relationship between CCR4 and CLA expression was not statistically confirmed and some CCR4⁺CLA⁻ ATLL cases obviously had skin lesions in our study, our finding suggests that CCR4 and TARC participate in skin-specific recruitment of ATLL cells and are consistent with a selective role for CCR4 in skin lymphocyte homing (35).

We also demonstrated that CCR4 expression in ATLL cells as detected by immunostaining is an independent and statistically significant prognostic factor. The reason why CCR4 expression in ATLL cells contributes to poorer outcome in ATLL remains unclear. CCR4 positivity was significantly associated with skin involvement, whereas the presence of skin lesions itself did not affect prognosis. In ATLL, multistep oncogenic events are involved in the development of the full malignant phenotype in HTLV-1-infected cells after a long latency. The cumulative incidence of ATLL is estimated to be ~2.5% among HTLV-1 carriers in Japan, and the median age at the time of diagnosis of ATLL is ~55 years. CCR4 expression itself does not seem to be necessary for early development of ATLL because a small percentage of the ATLL cases in our study were negative for CCR4 expression. In patients with chronic or smoldering variants of ATLL, their neoplastic cells are usually small with minimal cytological atypia, and they occasionally develop into acute crisis, resulting in histological and clinical progression. As in the case of MF, it can be hypothesized that ATLL cells acquire the ability to express CCR4 as the tumors expand, resulting in aggressive behavior and poor prognosis. However, there was no significant relationship between the clinical subtype of ATLL and CCR4 expression. As for the histological classification, there was no significant relationship between histological large-cell transformation and CCR4 expression in ATLL in our study. In this regard, ATLL differs from MF in which CCR4 expression is associated with large-cell transformation (13). This suggests that CCR4 expression may represent certain biological behaviors rather than evident clinical and/or histological progression. CCR4 expression did not seem to reflect the tumor burden because CCR4 expression was not associated with a high LDH level. An interesting hypothesis is that an autocrine loop via the CCR4-ligand system with respect to growth stimulation may exist in addition to probable paracrine CCR4 activation through intermixed macrophages and adjacent blood vessels. From this point of view, it is of interesting that all ATLL cell lines expressing CCR4 were positive for MDC expression. As a matter of fact, it has been demonstrated that I-309, a member of the family of CC chemokines, inhibits apoptosis of ATLL cells, and may substantially contribute to their growth through an autocrine loop (36). In small cell lung cancer, a regulation mechanism of cellular proliferation through CXCR4 was reported previously (37). Our novel findings that CCR4 expression on ATLL cells is closely associated with skin involvement and with an unfavorable outcome will provide new insights into understanding the pathogenesis of ATLL, although its underlying mechanism, particularly in terms of biological behavior, remains to be clarified in additional

Table 5 Prognostic factors affecting OS

Variable	Unfavorable	Univariate		Multivariate	
		HR (CI)	P value	HR (CI)	P
Comparison with risk factors					
PS	2,3,4	2.108 (1.364–3.259)	0.0008	–	
B symptom	Present	2.466 (1.571–3.869)	<0.0001	2.425 (1.517–3.878)	0.0002
Extranodal disease	≥2 sites	2.538 (1.486–4.337)	0.0007	2.106 (1.213–3.656)	0.0081
LDH	>Normal	2.121 (1.021–4.404)	0.0437	–	
Ca (mg/dl)	≥11.0	2.485 (1.428–4.322)	0.0013	–	
CCR4	Positive	2.097 (1.045–4.205)	0.0371	2.197 (1.080–4.469)	0.0298

investigations. In addition, specifically targeted therapy against the CCR4 molecule using anti-CCR4 mAb could be developed as an alternative treatment for the patients with ATLL.

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REFERENCES

- Zlotnik, A., and Yoshie, O. Chemokines: a new classification system and their role in immunity. *Immunity*, *12*: 121–127, 2000.
- Campbell, J. J., and Butcher, E. C. Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. *Curr. Opin. Immunol.*, *12*: 336–341, 2000.
- Bucher, E. C., Williams, M., Youngman, K., Rott, L., and Briskin, M. Lymphocyte trafficking and regional immunity. *Adv. Immunol.*, *72*: 209–253, 1999.
- Müller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Brereton, J. L., Mohar, A., Verastegui, E., and Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. *Nature (Lond.)*, *410*: 50–56, 2001.
- Kikuchi, M., Jaffe, E. S., and Ralfkiaer, E. Adult T-cell leukaemia/lymphoma. *In*: E. S. Jaffe, N. L. Harris, H. Stein, and J. W. Vardiman (eds.), *Pathology and Genetics Tumors of Haematopoietic and Lymphoid Tissues*, pp. 200–203, Lyon, France: IARC Press, 2001.
- Uchiyama, T. Human T cell leukemia virus type 1 (HTLV-1) and human diseases. *Annu. Rev. Immunol.*, *15*: 15–37, 1997.
- Qin, S., Rottmann, J. B., Myers, P., Kassam, N., Weinblatt, M., Loetscher, M., Koch, A. E., Moser, B., and Mackay, C. R. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J. Clin. Investig.*, *101*: 746–754, 1998.
- Bonocchi, R., Bianchi, G., Bordignon, P. P., D'Lang, R., Borsatti, A., Sozzani, S., Allavena, P., Gray, P. A., Mantovani, A., and Sinigaglia, F. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.*, *187*: 129–134, 1998.
- D'Ambrosio, D., Iellem, A., Bonocchi, R., Mazzeo, D., Sozzani, S., Mantovani, A., and Sinigaglia, F. Selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. *J. Immunol.*, *161*: 5111–5115, 1998.
- Zingoni, A., Soto, H., Hedrick, J. A., Stoppacciaro, A., Storlazzi, C. T., Sinigaglia, F., D'Ambrosio, D., O'Garra, A., Robinson, D., Rocchi, M., Santoni, A., Zlotnik, A., and Napolitano, M. The chemokine receptor CCR8 is preferentially expressed in Th2 but not Th1 cells. *J. Immunol.*, *161*: 547–551, 1998.
- Yamamoto, J., Adachi, Y., Onoue, Y., Adachi, Y. S., Okabe, Y., Itazawa, T., Toyoda, M., Seki, T., Morihashi, M., Matsushima, K., and Miyawaki, T. Differential expression of the chemokine receptors by the Th1- and Th2 type effector populations within circulating CD4⁺ T cells. *J. Leuk. Biol.*, *68*: 568–574, 2000.
- Andrew, D. P., Ruffing, N., Kim, C. H., Miao, W., Heath, H., Li, Y., Murphy, K., Campbell, J. J., Butcher, E. C., and Wu, L. C-C chemokine receptor 4 expression defines a major subset of circulating nonintestinal memory T cells of both Th1 and Th2 potential. *J. Immunol.*, *166*: 103–111, 2001.
- Jones, D., O'Hara, C., Kraus, M. D., Perez-Atayde, A. R., Shahsafaei, A., Wu, L., and Dorfman, D. M. Expression pattern of T-cell-associated chemokine receptors and their chemokines correlates with specific subtypes of T-cell non-Hodgkin lymphoma. *Blood*, *96*: 685–690, 2000.
- Imai, T., Nagira, M., Takagi, S., Kakizaki, M., Nishimura, M., Wang, J., Gray, P. W., Matsushima, K., and Yoshie, O. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int. Immunol.*, *11*: 81–88, 1999.
- Jones, D., Benjamin, R. J., Shahsafaei, A., and Dorfman, D. M. The chemokine receptor CXCR3 is expressed in a subset of B cells and is a marker for B-cell chronic lymphocytic leukemia. *Blood*, *95*: 627–632, 2000.
- Duijvestijn, A. M., Horst, E., Pals, S. T., Rouse, B. N., Steere, A. C., Picker, L. J., Meijer, C. J., and Butcher, E. C. High endothelial differentiation in human lymphoid and inflammatory tissues defined by monoclonal antibody HECA-452. *Am. J. Pathol.*, *130*: 147–155, 1988.
- Naoe, T., Akao, Y., Yamada, K., Utsumi, K. R., Koike, K., Shamoto, M., Koike, K., Kurosawa, Y., Kato, K., Abe, M., and Shiku, H. Cytogenetic characterization of a T-cell line, ATN-1, derived from adult T-cell leukemia cells. *Cancer Genet. Cytogenet.*, *34*: 77–88, 1988.
- Gazdar, A. F., Carney, D., Bunn, P. A., Russell, E. K., Jaffe, E. S., Schechter, G. P., and Guccion, J. G. Mitogen requirements for the *in vitro* propagation of cutaneous T-cell lymphomas. *Blood*, *55*: 409–417, 1980.
- Bunn, P. A., and Foss, F. M. T-Cell lymphoma cell lines (HUT102 and HUT78) established at the National Cancer Institute: history and importance to understanding the biology, clinical features, and therapy of cutaneous T-cell lymphomas (CTCL) and adult T-cell leukemia-lymphomas (ATLL). *J. Cell. Biochem. Suppl.*, *24*: 12–23, 1996.
- Miyoshi, I., Kubonishi, I., Yoshimoto, S., Akagi, T., Ohtsuki, Y., Shiraishi, Y., Nagata, K., and Hinuma, Y. Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells. *Nature (Lond.)*, *294*: 770–771, 1981.
- Schauer, U., Jung, T., Krug, N., and Frew, A. Measurement of intracellular cytokines. *Immunol. Today*, *17*: 305–306, 1996.
- Ponte, P., Ng, S. Y., Engle, J., Gunning, P., and Kedes, L. Evolutionary conservation in the untranslated regions of actin mRNAs: DNA

- sequences of a human β -actin cDNA. *Nucleic Acids Res.*, *12*: 1687–1696, 1984.
23. Shimoyama, M., and members of the Lymphoma Study Group (1984–1987). Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. *Br. J. Haematol.*, *79*: 428–437, 1991.
24. Uozumi, K., Hanada, S., Ohno, S., Ishitsuka, K., Shimotakehara, S., Otsuka, M., Chyuman, Y., Nakahara, K., Takeshita, T., Kuwazuru, Y., Saito, T., Makino, T., Iwahashi, M., Utsunomiya, A., and Arima, T. Combination chemotherapy (RCM protocol: response-oriented cyclic multidrug protocol) for the acute or lymphoma type adult T-cell leukemia. *Leuk. Lymphoma*, *18*: 317–323, 1995.
25. Yamada, Y., Tomonaga, M., Fukuda, H., Hanada, S., Utsunomiya, A., Tara, M., Sano, M., Ikeda, S., Takatsuki, K., Kozuru, M., Araki, K., Kawano, F., Niimi, M., Tobinai, K., Hotta, T., and Shimoyama, M. A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukemia-lymphoma: Japan Clinical Oncology Group Study 9303. *Br. J. Haematol.*, *113*: 375–382, 2001.
26. Sagawa, K., Mochizuki, M., Katagiri, K., Tsuboi, I., Sugita, S., Mukaida, N., and Itoh, K. *In vitro* effects of immunosuppressive agent on cytokine production by HTLV-1-infected T cell clones derived from the ocular fluid of patients with HTLV-1 uveitis. *Microbiol. Immunol.*, *40*: 373–379, 1996.
27. Yamada, Y., Ohmoto, Y., Hata, T., Yamamura, M., Murata, K., Tsukasaki, K., Kohno, T., Chen, Y., Kamihira, S., and Tomonaga, M. Features of the cytokines secreted by adult T cell leukemia (ATL) cells. *Leuk. Lymphoma*, *21*: 443–447, 1996.
28. Tanaka, Y., Wake, A., Kevin, J. H., Murakami, S., Aso, M., Saito, K., Oda, S., Morimoto, I., Uno, H., Kikuchi, H., Izumi, Y., and Eto, S. Distinct phenotype of leukemic T cells with various tissues tropisms. *J. Immunol.*, *158*: 3822–3829, 1997.
29. Ohtsuka, E., Kikuchi, H., Nasu, M., Takita-Sonoda, Y., Fujii, H., and Yokoyama, S. Clinicopathological features of adult T-cell leukemia with CD30 antigen expression. *Leuk. Lymphoma*, *15*: 303–310, 1994.
30. Yoshie, O., Fujisawa, R., Nakayama, T., Harasawa, H., Tago, H., Izawa, D., Tatsumi, Y., Matsushima, K., Hasegawa, H., Kanamaru, A., Kamihira, S., and Yamada, Y. Frequent expression of CCR4 in adult T-cell leukemia and human T-cell leukemia virus type 1-transformed T cells. *Blood*, *99*: 1505–1511, 2002.
31. Hasae, K., Tani, K., Shimizu, T., Ohmoto, Y., Matsushima, K., and Sone, S. Increased CCR4 expression in active systemic lupus erythematosus. *J. Leuk. Biol.*, *70*: 749–755, 2001.
32. Nakatani, T., Kaburagi, Y., Shimada, Y., Inaoki, M., Takehara, K., Mukaida, N., and Sato, S. CCR4⁺ memory CD4⁺ T lymphocytes are increased in peripheral blood and lesional skin from patients with atopic dermatitis. *J. Allergy Clin. Immunol.*, *107*: 353–358, 2001.
33. Imai, T., Baba, M., Nishimura, M., Kakizaki, M., Takagi, S., and Yoshie, O. The T-cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. *J. Biol. Chem.*, *272*: 15036–15042, 1997.
34. Campbell, J. J., Haraldsen, G., Pan, J., Rottmann, J., Qin, S., Ponath, P., Andrew, D. P., Warnke, R., Ruffing, N., Kassam, N., Wu, L., and Butcher, E. C. The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature (Lond.)*, *400*: 776–780, 1999.
35. Kunkel, E. J., Boisvert, J., Murphy, K., Vierra, M. A., Genovese, M. C., Wardlaw, A. J., Greenberg, H. B., Hodge, M. R., Wu, L., Butcher, E. C., and Campbell, J. J. Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes. *Am. J. Pathol.*, *160*: 347–355, 2002.
36. Ruckes, T., Saul, D., Snick, J. V., Hermine, O., and Grassman, R. Autocrine antiapoptotic stimulation of cultured adult T-cell leukemia cells by overexpression of the chemokine I-309. *Blood*, *98*: 1150–1159, 2001.
37. Kijima, T., Gautam, M., Patrick, C. M., Elena, V., Tibaldi, R. E., Turner, B. R., Martin, S., Bruce, E. J., and Ravi, S. N. Regulation of cellular proliferation, cytoskeletal function, and signal transduction through CXCR4 and c-kit in small cell lung cancer cells. *Cancer Res.*, *62*: 6304–6311, 2002.