Adult T-cell leukemia/lymphoma cells from blood and skin tumors express cytotoxic T lymphocyte-associated antigen-4 and Foxp3 but lack suppressor activity toward autologous CD8+ T cells

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Adult T cell leukemia/lymphoma (ATL) cells share the CD4+CD25+ phenotype with regulatory T (Treg) cells. However, it is still controversial whether ATL cells are Treg cells. The aim of the present study was to investigate the Treg nature of ATL cells obtained from peripheral blood and skin tumors in terms of their phenotype and function. By flow cytometry and immunohistochemistry, the expression of the Treg-associated molecule cytotoxic T lymphocyte-associated antigen (CTLA)-4 and Foxp3 was examined in freshly isolated circulating and skin-infiltrating tumor cells from 21 ATL patients with skin eruptions. The expression of CTLA-4 on freshly isolated circulating tumor cells was elevated in two of 15 patients, and Foxp3 was expressed intracytoplasmically at high levels in three of nine patients. In five of the patients examined, skin-infiltrating tumor cells bore variously elevated CTLA-4 with high Foxp3 expression. The potentiality of ATL cells as Treg cells was further addressed by stimulating ATL cells with anti-CD3/CD28 monoclonal antibodies and monitoring CTLA-4 expression. With the stimulation, even CTLA-4-low ATL cells expressed higher levels of CTLA-4 than normal CD4+CD25+ cells. To study function, ATL cells isolated from blood and skin tumors were tested for their ability to suppress the proliferation of autologous CD8+ T cells stimulated with allogeneic lymphocytes. Despite the expression of CTLA-4 and Foxp3, these tumors were incapable of suppressing the proliferation of autologous CD8+ T cells. ATL cells are phenotypically Treg cells in at least some patients, but lack immunoregulatory functions, at least toward CD8+ T cells. (*Cancer Sci* 2008; 99: 98–106)

Adult T cell leukemia/lymphoma (ATL) is a malignancy of mature CD4+ T cells caused by human T-cell leukemia virus type I (HTLV-I).[1,2] This endemic hematological neoplasm develops in 1–5% of individuals infected with HTLV-I after more than two decades of viral persistence.[3,4] Based on organ involvement and severity, ATL is divided into four clinical categories: acute, chronic, lymphoma, and smoldering types.[5] Frequent involvement of the skin is another feature of ATL. The malignant T cells are usually positive for CD3, CD4, CD25, and CD45RO, but negative for CD7, CD8, CD19, and CD20.[5] More recently, ATL cells have been documented to express the chemokine receptor CCR4[6–8] and to share the CD4+CD25+ phenotype with regulatory T (Treg) cells.[9]

Because Treg cells have a suppressive activity in immune responses, they protect against autoimmune diseases and lead to the development of some malignant neoplasms.[10,11] Foxp3, a forkhead/winged helix transcription factor, is a master regulatory gene for the development and function of Treg cells.[12] In addition, both glucocorticoid-induced tumor necrosis factor receptor family related protein (GITR) and cytotoxic T lymphocyte-associated antigen (CTLA)-4 are specific molecules and are expressed on the surface of Treg cells.[13,14]

T-cell activation requires the coordinated engagement of multiple receptors expressed on both T cells and antigen-presenting cells (APC). Binding of the T-cell receptor (TCR) to specific peptide–major histocompatibility complex (MHC) complexes and co-stimulation of the T cell through the CD28 receptor are important for the activation of T cells. CD28 is stimulated by two ligands on the APC, CD80 and CD86, which provide signals that enhance T-cell proliferation, cytokine production, and survival.[15] However, CTLA-4 also interacts with CD80 and CD86 but has inhibitory effects on T-cell function and cell-cycle progression.[16,17] The loss of CTLA-4 causes a lymphoproliferative disease in mice, suggesting that CTLA-4 has an important role in maintaining tolerance to self tissue.[18]

Several groups of investigators have postulated the association of ATL tumor cells with Treg cells, because of the marked immunodeficient state of the patients. With regard to chemokine receptors, both ATL and Treg cells highly express the Th2 chemokine receptor CCR4.[6–8] The expression of Foxp3 in leukemic ATL cells freshly isolated from the patient's peripheral blood mononuclear cells (PBMC) was detected to various degrees.[19–24] The expression of specific surface molecules, GITR and CTLA-4, on ATL cells were also found and correlated with Foxp3 expression.[21,22,25] Most recently, functional suppressive activity in a cell–cell contact-dependent manner has been demonstrated in both circulating and lymph node-infiltrating ATL cells and a HTLV-I-infected cell line.[23,26] However, it remains controversial whether ATL cells serve as Treg, as the cells from only a limited number of patients have been shown to be functional, and no data have been reported on the function of tumor-forming ATL cells.

In the present study, we investigated the relationship between the expression of CTLA-4 and Foxp3 on ATL cells, Treg function, and clinical significance. For this purpose, we used skin tumor-infiltrating cells, because the tumor-forming cells are clinically relevant and phenotypically remarkable, as represented by high expression of CCR4.[25] Our results suggest that ATL cells have phenotypic Treg properties but lack CD8+ T cell-inhibitory activity.

Materials and Methods

Patients. All patients and normal healthy volunteers were obtained from the Department of Dermatology, University of Occupational and Environmental Health, Kitakyusyu, Japan, and the following study was carried out with informed consent. Twenty-one patients with ATL (16 men and five women; median

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Table 1. Clinical summary of the patients

Patient no.	Clinical type	Type of eruption	Age (years)	Sex	Circulating lymphocytes (10^9/L)	Atypical cells (%)	LDH	sIL-2R	CD4⁺CD25⁺ cells (%)
1	S	Tumors	82	Male	1.00	1.0	226	488	16.2
2	S	Papules	70	Male	2.55	17.0	198	1 668	84.4
3	S	Papules	62	Male	3.97	18.0	212	4 561	73.0
4	S	Tumors	76	Male	0.97	7.0	238	2 752	73.0
5	S	Erythroderma	87	Female	1.2	1.0	211	1 602	29.3
6	A	Purpuras	41	Male	5.04	5.0	176	918	71.4
7	S	Tumors	80	Male	1.53	5.0	222	700	51.2
8	S	Tumors	63	Male	2.18	5.0	191	432	30.0
9	A	Tumors	62	Male	15.3	56.0	659	7 2382	91.5
10	S	Purpuras	68	Female	2.12	11.0	236	ND	37.6
11	S	Plaques	43	Female	0.95	3.0	415	1 654	47.8
12	A	Tumors	80	Female	0.54	4.0	410	11 637	ND
13	S	Papules	76	Male	1.42	0.0	265	8 420	ND
14	S	Tumors	68	Male	1.7	8.0	240	6 250	ND
15	S	Tumors	75	Male	0.75	1.0	157	472	ND
16	S	Granulomas	54	Female	1.91	33.0	336	4 228	44.4
17	S	Tumors	76	Male	1.41	1.0	241	1 015.4	29.2
18	S	Plaques	58	Male	2.58	3.0	216	970	44.2
19	S	Tumors	70	Male	0.5	2.0	193	ND	29.2
20	S	Tumors	88	Male	2.42	12.0	194	678.9	ND
21	A	Erythroderma	69	Male	11.3	41.0	234	12 493.5	56.6

The percentage of CD4⁺CD25⁺ cells in peripheral blood mononuclear cells was determined by flow cytometry. A, acute; LDH, lactate dehydrogenase; ND, not determined; S, smoldering; sIL-2R, soluble interleukin-2 receptor.

age 68.9 years, ranging from 41 to 88 years) listed in Table 1 and eight healthy donors as controls were enrolled in the present study. ATL was diagnosed on the basis of clinical features and laboratory findings according to the criteria.[5] All of the patients had skin eruptions such as red papules, erythematous plaques, nodules, or tumors, and had monoclonal integration of HTLV-I proviral DNA as assessed by standard Southern blotting analysis[2] in PBMC or biopsy specimens from skin lesions.

Cell purification. Peripheral blood was obtained by vein puncture from the patients and normal control subjects, with heparin as an anticoagulant. PBMC were isolated by the standard Ficoll-Paque method (Pharmacia, Uppsala, Sweden). CD4⁺CD25⁺ T cells were isolated from PBMC using the AutoMACS magnetic separation system with a human CD4⁺CD25⁺ isolation kit (Miltenyi Biotec, Auburn, CA, USA). CD8⁺ cells were isolated from PBMC using the BD IMAG cell separation system with the antihuman CD8 Particles-DM (BD Biosciences PharMingen, San Diego, CA, USA) according to the manufacturer's directions. The purity of both CD4⁺CD25⁺ T cells and CD8⁺ T cells was consistently higher than 90%, as assessed by flow cytometry. Single-cell suspensions of skin-infiltrating tumor cells were prepared by teasing of skin biopsy specimens of cases 1, 3, 17, 19, and 20, and used after Ficoll-Paque separation. In addition, CD4⁺CD25⁺ ATL tumor cells were isolated from skin-infiltrating cells in case 17 by using the magnetic separation system.

Antibodies and flow cytometric analysis. Peridinin chlorophyll protein-labeled monoclonal antibodies (mAb) to CD4 (Leu-3a), allophycocyanin-labeled mAb to CD4 (RDA-T4), fluorescein isothiocyanate (FITC)-labeled mAb to CD4 (SK3), CD25 (2A3), IgG_1 (X40), and phycoerythrin (PE)-labeled mAb to CD25 (2A3), CCR4 (1G1), CTLA-4 (BNI3), and IgG_1 (X40) were purchased from BD Bioscience. FITC-labeled mouse IgG_1 or PE-labeled mouse IgG_1 was used as an isotype-matched control. To analyze intracytoplasmic Foxp3 expression, PE-labeled antihuman Foxp3 mAb (PCH101), rat IgG2b (KLH/G2b-1-2), and staining kit were used (all from eBioscience, San Diego, CA, USA). Crude or cultured PBMC and skin tumor-infiltrating cells were washed with phosphate-buffered saline (PBS; pH 7.4). Hanks' balanced salt solution containing 0.1% NaN_3 and 1% fetal calf serum (FCS) was used as the staining buffer. After incubation for 30 min with mAb or isotype-matched controls, 10 000-labeled cells were analyzed on a FACSCalibur or FACSCanto (BD Bioscience) in each sample.

Cell culture. Cells were cultured in RPMI-1640 (Gibco BRL Life Technology, Grand Island, NY, USA) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10^{-5} M sodium pyruvate, 25 mM HEPES, 1% non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Gibco BRL Life Technology). To test the expression of CTLA-4 by PBMC, the cells were cultured in 24-well plates (Corning, New York, NY, USA; 1×10^6 cells/mL culture medium) at 37°C in 5% CO_2 in air in the presence of anti-CD3 mAb (soluble form stimulatory for T cells; PharMingen, San Diego, CA, USA) at 5 µg/mL and anti-CD28 mAb (Immunotech, Marseille, France) at 5 µg/mL. After 12, 24, and 48 h culture, the cells were collected and analyzed on FACS.

Immunohistochemistry. Biopsy samples were taken from the lesional skin of 11 patients with ATL and normal skin of a patient with lipoma. The skin samples were embedded in Tissue-Tek OCT compound (Miles, Elkhart, ID, USA) and were stored at –80°C. These frozen samples were cut with a cryostat set to a thickness of 5 µm and fixed in –20°C cold acetone. After washing twice in PBS and blocking endogeneous peroxidase activity by incubating in 0.3% H_2O_2 solution in PBS for 10 min, the cells were incubated for 1 h with mouse antihuman CTLA-4 mAb (clone BNI3; BD Bioscience PharMingen) or mouse IgG2aκ (clone C1.18.4; BD Bioscience PharMingen) at room temperature in a humidified chamber. The signal was detected using antimouse Ig streptavidin–horseradish peroxidase detection kits (BD Bioscience PharMingen), and counterstained with Mayer's hematoxylin, according to the manufacturer's instructions. These primary antibodies were diluted 1:40, and the biotinylated anti-Ig secondary antibody was 1:50 in the antibody diluent buffer included in the kits.

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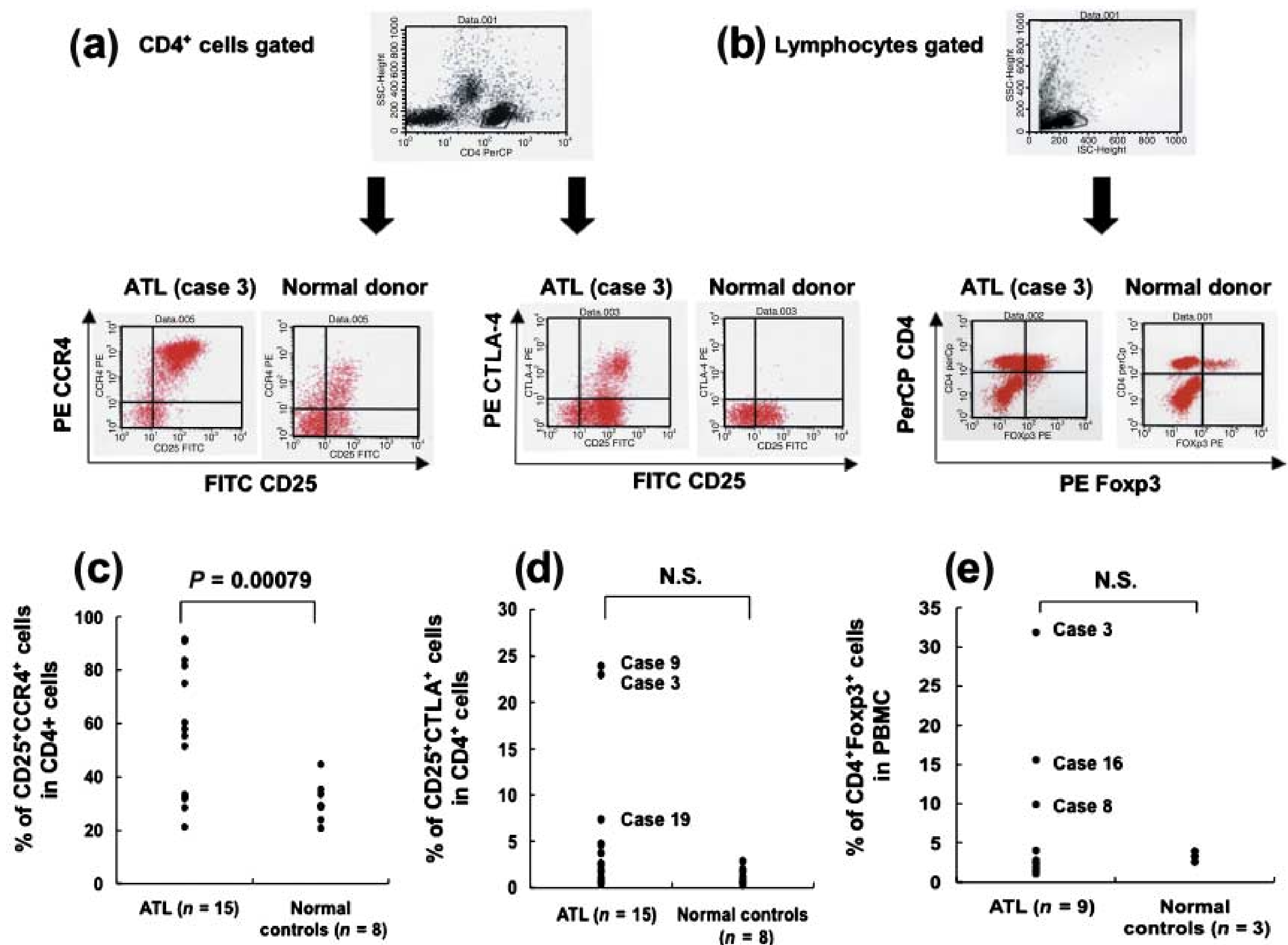

Fig. 1. Flow cytometric analyses of surface CCR4 or cytotoxic T lymphocyte-associated antigen (CTLA)-4 expression in $CD4^+CD25^+$ cells and intracytoplasmic Foxp3 expression in $CD4^+$ cells. Peripheral blood mononuclear cells freshly isolated from adult T cell leukemia/lymphoma (ATL) patients and normal volunteers were stained with peridinin chlorophyll protein-anti-CD4, fluorescein isothiocyanate-anti-CD25, and phycoerythrin-anti-CCR4, CTLA-4 or Foxp3. (a) As represented by case 3, $CD4^+$ cells were gated and the percentages of dual-positive cells for CD25 and CCR4 or CTLA-4 were counted. (b) In the gated lymphocytes, the percentage of dual-positive cells for CD4 and Foxp3 was counted. (c) The percentages of cells expressing both CCR4 and CD25 in circulating $CD4^+$ cells from ATL patients (n = 15) and normal subjects (n = 8). (d) The percentages of cells expressing both CTLA-4 and CD25 in circulating $CD4^+$ cells from ATL patients (n = 15) and normal subjects (n = 8). (e) The percentages of cells expressing both Foxp3 and CD4 in circulating lymphocytes from ATL patients (n = 9) and normal subjects (n = 3). Welch's t-test was used for the significance of data comparison. NS, not significant.

Functional assays of Treg. Allogeneic PBMC were preincubated with mitomycin C (Sigma Aldrich, St Louis, MO, USA) at a concentration of 50 µg/mL in PBS at 37°C and 5% CO_2 in air for 30 min, and used after three washes in PBS. A total of 5×10^4 purified autologous $CD8^+$ T cells (responder cells) were cultured in triplicate with 5×10^4 allogeneic mitomycin C-treated PBMC (stimulator cells) in 96-well round-bottom plates (Becton Dickinson, Franklin Lakes, NJ, USA). In this allo-mixed lymphocyte reaction (MLR) system,[11] purified autologous $CD4^+CD25^+$ T cells, freshly isolated skin-infiltrating tumor cells, or purified $CD4^+CD25^+$ skin-infiltrating tumor cells were added at different numbers (0.17–1.5×10^5 cells/well). Cells were cultured for 6 days in medium, and their proliferative response was tested by the addition of 7.4 KBq/well of [^{3}H]thymidine 16 h before harvest. The amount of incorporated [^{3}H]thymidine was determined by liquid scintillation spectroscopy.

The Treg activity of ATL tumor cells against autologous $CD4^+CD25^-$ non-ATL cells in the presence of autologous APC was also examined, as previously reported by Yano *et al*.[26] Autologous $CD4^-$ cells obtained from PBMC in two ATL patients (cases 2 and 21) and a normal healthy control were pretreated with mitomycin C and used as APC. Autologous $CD4^+CD25^-$ T cells were also prepared and used as responder cells. A total of 5×10^4 purified $CD4^+CD25^-$ T cells were cultured with the same number of autologous APC and anti-CD3 mAb at 0.5 µg/mL in triplicate in 96-well round-bottom plates. Purified autologous $CD4^+CD25^+$ T cells were added at a ratio of 1:1. Culture groups of autologous $CD4^+CD25^+$ T cells plus APC or APC only were also tested. Cells were cultured for 3 days and their proliferative responses were determined by [^{3}H]thymidine incorporation.

Statistical analysis. Statistical significance was determined using Welch's t-test or Student's t-test. Differences were considered significant when $P < 0.05$.

Results

Leukemic ATL tumor cells express CTLA-4 and Foxp3. Twenty-one patients suffering from acute- or smoldering-type ATL were enrolled in the present study. All of the patients had skin involvement, such as dome-shaped tumors, nodules, red papules, and purpuric lesions (Table 1). We first examined the Treg

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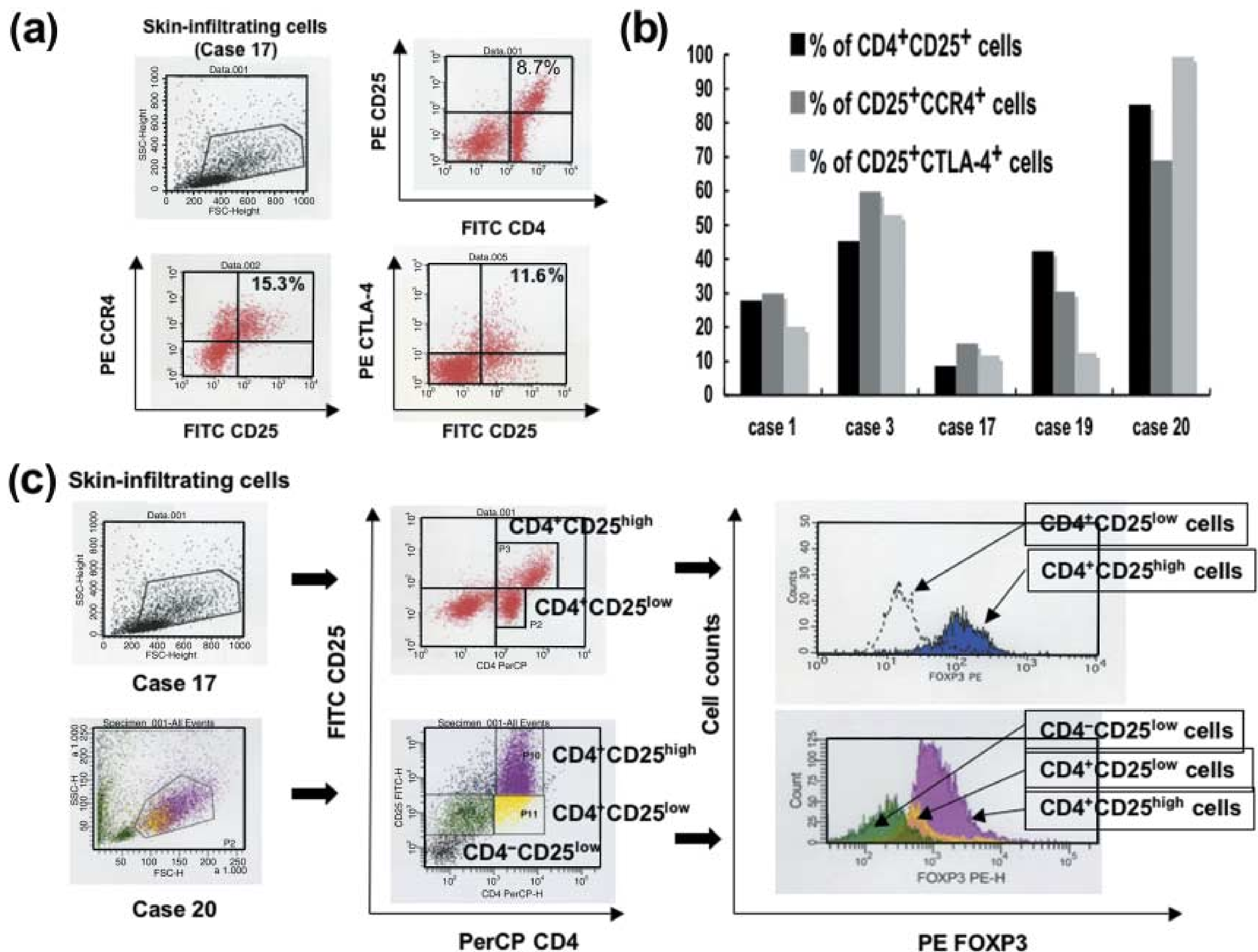

Fig. 2. Flow cytometric analyses of surface CD4, CCR4, or cytotoxic T lymphocyte-associated antigen (CTLA)-4 expression in CD25+ cells in skin-infiltrating tumor cells. Single-cell suspensions of skin-infiltrating tumor cells were prepared and stained with the indicated monoclonal antibodies. (a) As represented by case 17, the percentages of dual-positive cells for CD4 and CD25, CD25 and CCR4, or CD25 and CTLA-4 were counted in the broad range of small to large cells. (b) The skin-infiltrating tumor cells in all five patients tested expressed both CCR4 and CTLA-4 at high levels. (c) The skin tumor-infiltrating cells were stained with peridinin chlorophyll protein-anti-CD4, fluorescein isothiocyanate-anti-CD25, and phycoerythrin-anti-Foxp3, and CD4+CD25low, CD4+CD25high, or CD4–CD25low populations were identified. CD4+CD25high cells highly expressed Foxp3 compared to CD4+CD25low or CD4–CD25low cells.

phenotype of circulating ATL cells. CD4+ cells were gated from the patients' and normal subjects' PBMC and examined for the expression of CD25, CCR4, and CTLA-4 by three-color flow cytometry (Fig. 1a). To analyze Foxp3, lymphocytes were tested for the expression of both surface CD4 and intracytoplasmic Foxp3 protein by two-color flow cytometry (Fig. 1b). In confirmation of previous reports,[16,17] the percentage of circulating CD4+CD25+CCR4+ cells was high in ATL patients compared with normal volunteers (mean ± SD; 58.1 ± 26.0 *vs* 30.7 ± 7.4%) (Fig. 1c). For CTLA-4 expression on CD4+CD25+ cells (Fig. 1d) and Foxp3 expression in CD4+ cells (Fig. 1e), there was no significant difference between ATL patients and normal volunteers. However, we found markedly high levels of CTLA-4 expression in 2 of 15 patients (cases 3 and 9) and high Foxp3 expression in three of nine patients (cases 3, 8, and 16). Case 3 had remarkably high expression of both CTLA-4 and Foxp3 (CD4+CD25+CTLA-4+ T cells, 23.0%; CD4+Foxp3+, 31.9%) as shown in Fig. 1a,b. All of these three cases (cases 3, 9, and 16) had circulating abnormal cells at high percentages (11–56%), suggesting that proliferating or activating ATL cells have a phenotype characteristic of Treg cells.

Skin-infiltrating ATL tumor cells express CTLA-4 highly. Skin-infiltrating cells in the five tumor-forming cases were analyzed by flow cytometry. As shown in Fig. 2a, the percentages of CD4+CD25+, CD25+CCR4+, and CD25+CTLA-4+ cells were counted by gating normal- to large-sized lymphocytes. Eight to 70% of the infiltrating cells were tumor cells as determined by the CD4+CD25+ or CD25+CCR4+ phenotype. These percentages were comparable to those of CD25+CTLA-4+ cells in four of the five cases (cases 1, 3, 17, and 20) (Fig. 2b). Moreover, CD4+CD25high skin-infiltrating tumor cells expressed a high level of Foxp3 compared with CD4+CD25low cells (Fig. 2c). Considering that only 3 of 15 cases had CTLA-4+ circulating ATL cells, the tumor-forming ATL cells tended to express CTLA-4 at high levels.

Immunostaining for CTLA-4 in ATL skin lesions. To exclude the possibility that the flow cytometric data of CTLA-4 in the skin-infiltrating cells included normal Treg cells, skin specimens from 11 patients were stained immunohistochemically for CTLA-4. Clinically, most of the patients had tumorous skin lesions (Fig. 3a–c). The biopsy specimens demonstrated dense and diffuse proliferation of various-sized atypical lymphocytes with irregular and pleomorphic nuclei in the dermis to subcutis (Fig. 3d–f).

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Fig. 3. Cytotoxic T lymphocyte-associated antigen (CTLA) expression of adult T cell leukemia/lymphoma (ATL) cells infiltrating the lesional skin. Immunohistochemical staining was carried out using a monoclonal antibody against CTLA-4 in biopsy specimens of ATL tumors and normal skin. (a–c) Clinical picture of tumorous skin lesions. (d–f) Hematoxylin–eosin staining showing atypical lymphocyte infiltration in the dermis with epidermotropism. (g–j) CTLA-4 staining showing positivity of ATL tumor cells, infiltrating from the upper dermis to the epidermis. In the normal skin, CTLA-4 was negative in skin-homing lymphocytes. (k–n) Control staining with an isotype-matched IgG2α,κ monoclonal antibody. Original magnifications: ×100 or ×400.

These tumor cells showed epidermotropism, forming Pautrier's microabscesses (Fig. 3d,e). As shown representatively in Fig. 3h–j, large tumor cells had apparent immunoreactivity for CTLA-4 compared with the isotype-matched control (Fig. 3l–n) in six patients (positivity, 54.5%). In normal skin, skin-homing lymphocytes were negative for CTLA-4 (Fig. 3g).

Elevated CTLA-4 expression on ATL cells upon stimulation with anti-CD3 and -CD28 mAb. Because CTLA-4 is a surface marker expressed on activated T cells,[27] we monitored CTLA-4 expression on $CD4^+CD25^+$ cells by culturing PBMC with anti-CD3 and -CD28 mAb. There was an increase in the level of CTLA-4 expression on $CD4^+CD25^+$ cells from both ATL patients and normal individuals (Fig. 4). After 12 h culture, however, CTLA-4 expression was significantly higher in the patients compared with the normal individuals (25.6 ± 15.0 *vs* $10.7 \pm 2.1\%$). Therefore, ATL cells were suggested to readily express CTLA-4 upon TCR-mediated activation.

Lack of regulatory function of circulating ATL tumor cells against autologous $CD8^+$ T cells. The regulatory function of ATL cells was evaluated using allo-MLR. $CD4^+CD25^+$ T cells and $CD8^+$ T cells were isolated from PBMC of four ATL patients and a normal healthy donor with immunomagnetic beads. As represented

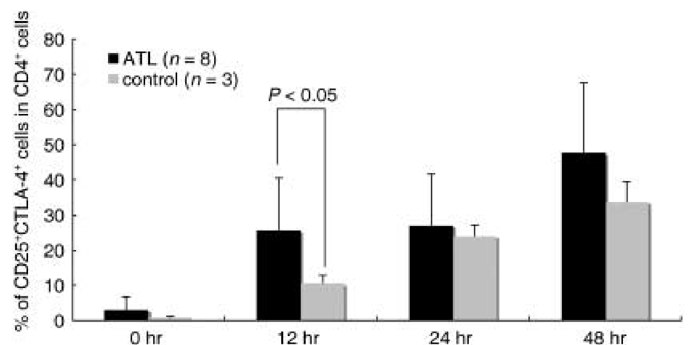

Fig. 4. Increased levels of cytotoxic T lymphocyte-associated antigen (CTLA)-4 by T-cell receptor-mediated stimulation in $CD4^+CD25^+$ cells from adult T cell leukemia/lymphoma (ATL) patients and normal healthy subjects. Peripheral blood mononuclear cells isolated from ATL patients ($n = 8$) and normal volunteers ($n = 3$) were cultured for 12, 24, or 48 h with anti-CD3 and -CD28 monoclonal antibodies, and $CD25^+CTLA\text{-}4^+$ cells in $CD4^+$ cells were counted by flow cytometry. Welch's *t*-test was used for the significance of data comparison.

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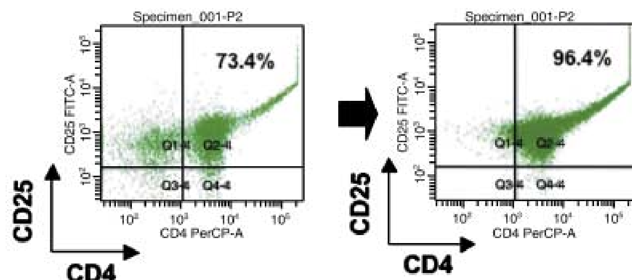

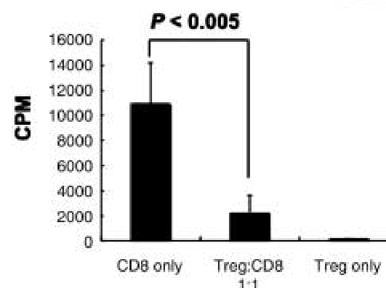

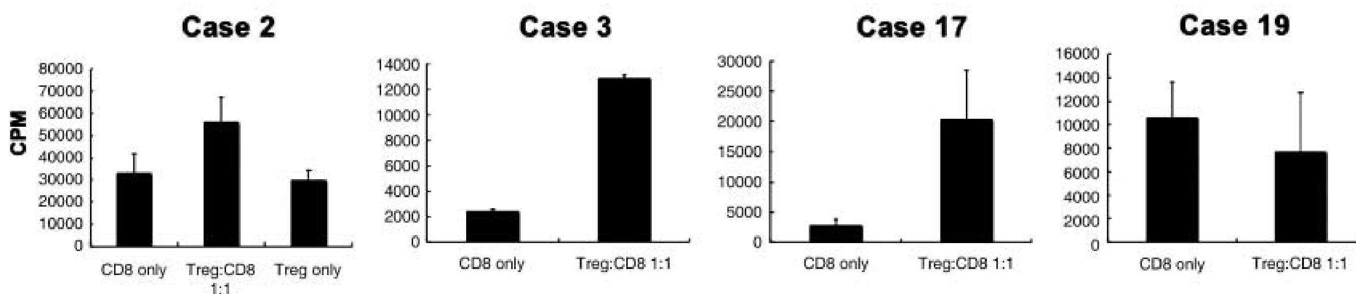

Fig. 5. Lack of regulatory function of circulating adult T cell leukemia/lymphoma (ATL) tumor cells. (a) $CD4^+CD25^+$ T cells were isolated immunomagnetically, and the purity was always higher than 95%, as represented by case 2. A total of 5×10^4 $CD8^+$ T cells from ATL patients and a normal volunteer were cultured with autologous $CD4^+CD25^+$ T cells at a ratio of 1:1 under allogeneic peripheral blood mononuclear cell (5×10^4 cells) stimulation for 6 days. On the last day of culture, the cells were tested for [^{3}H]thymidine incorporation. Error bars represent the mean + SD c.p.m. from triplicate cultures. (b) Normal $CD4^+CD25^+$ T cells suppressed the proliferation of allo-stimulated $CD8^+$ T cells. (c) $CD4^+CD25^+$ T cells failed to suppress the proliferation of allo-stimulated autologous $CD8^+$ T cells in four of the ATL patients tested.

in Fig. 5a, the purity of $CD4^+CD25^+$ T cells was higher than 95% with the immunomagnetic procedure. Notably, case 3 had a high percentage of cells expressing both CTLA-4 and Foxp3. In a healthy control, $CD4^+CD25^+$ T cells were not proliferative in the allo-MLR and strongly inhibited the proliferation of autologous $CD8^+$ T cells, showing the ordinary suppressive activity of Treg (Fig. 5b). However, in all of the ATL patients tested, the proliferation of $CD8^+$ T cells was not suppressed by $CD4^+CD25^+$ T cells (Fig. 5c). Rather, the uptake was increased by the addition of $CD4^+CD25^+$ T cells in cases 2, 3, and 17, presumably as a result of the self-proliferation of $CD4^+CD25^+$ ATL cells.

Lack of regulatory function of skin-infiltrating tumor cells against autologous $CD8^+$ T cells. In addition to the circulating ATL cells, single-cell suspensions of skin-infiltrating tumor cells were prepared in cases 17, 19, and 20, and the flow cytometric profile of these cells is shown in Fig. 2c. Similarly, the skin-infiltrating ATL cells did not inhibit autologous $CD8^+$ T-cell proliferation at any of the ratios (Fig. 6a). Because skin-infiltrating tumor cells contained various inflammatory cells, we purified skin-infiltrating ATL tumor cells by Ficoll-Paque separation and subsequent positive selection for $CD4^+CD25^+$ cells. Again, the $CD4^+CD25^+$ ATL tumor cells did not suppress the proliferation of autologous $CD8^+$ T cells in the allo-MLR system (Fig. 6b).

Lack of regulatory function of circulating ATL tumor cells against autologous $CD4^+CD25^-$ T cells. Regulatory T cells suppress autologous $CD4^+CD25^-$ T cells as well as $CD8^+$ T cells under stimulation with autologous APC and anti-CD3 mAb. Thus, we also carried out the Treg functional assay using $CD4^+CD25^+$ ATL cells, $CD4^+CD25^-$ non-ATL cells, and autologous APC. $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were isolated from PBMC of two ATL patients (cases 2 and 21) and a normal healthy donor. In a healthy control, $CD4^+CD25^+$ T cells strongly inhibited the proliferation of autologous $CD4^+CD25^-$ T cells (Fig. 7c). However, the proliferation of $CD4^+CD25^-$ T cells was not suppressed by $CD4^+CD25^+$ ATL cells in ATL patients (Fig. 7a,b).

Discussion

The present study demonstrated that malignant T cells in both PBMC and lesional skin of ATL patients highly expressed CTLA-4, as has been reported recently by other groups in PBMC.[21] Moreover, when freshly isolated PBMC were stimulated via CD3 and CD28, the expression of CTLA-4 on ATL cells was promoted more markedly than that on normal $CD4^+CD25^+$ cells. Accordingly, skin-infiltrating tumor cells, seemingly in an activated state, expressed CTLA-4 at a higher level than circulating ATL cells, as high expression was found in four of five patients tested by flow cytometry, and in six of 11 patients by immunohistochemistry.

A recent study documented that the expression levels of Foxp3 and GITR were significantly higher in ATL cells than in normal Treg cells, and PBMC isolated from ATL patients suppressed the proliferation of stimulated T cells, whereas they did not respond to TCR stimulation.[22] Another group of investigators demonstrated that circulating ATL tumor cells express Foxp3 highly and have an ability to suppress the proliferation of $CD25^-CD4^+$ T cells by cell–cell contact[23] or $CD4^+$ non-ATL cells stimulated with both autologous APC and anti-CD3 mAb.[26] These observations have suggested that some but not all ATL cells are functional Treg cells, depending on the assessment

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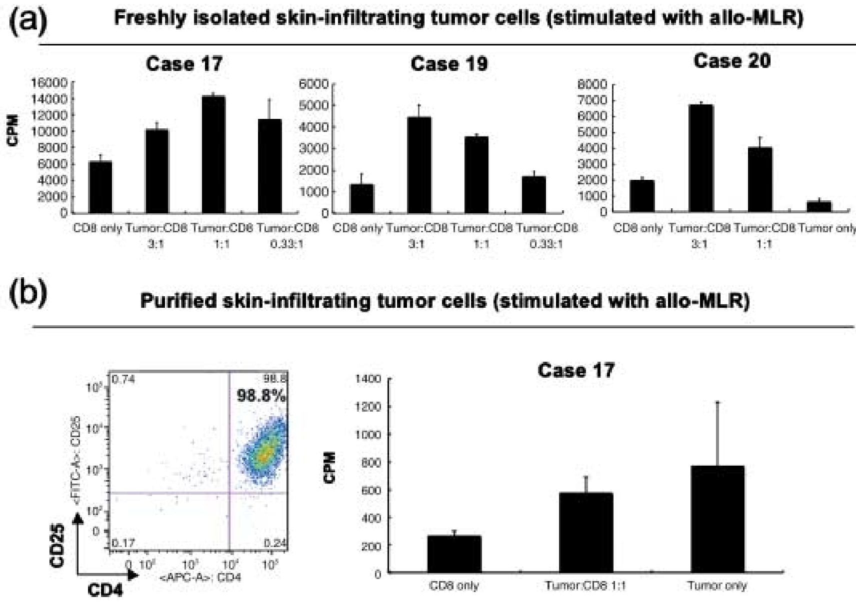

Fig. 6. Lack of regulatory function of skin-infiltrating tumor cells against autologous CD8$^+$ T cells. (a) Skin-infiltrating tumor cells isolated from cases 17, 19, and 20 were cultured with autologous CD8$^+$ T cells at various ratios under allogeneic peripheral blood mononuclear cell stimulation for 6 days. Student's *t*-test was used for the significance of data comparison. (b) CD4$^+$CD25$^+$ adult T cell leukemia/lymphoma (ATL) tumor cells were isolated immunomagnetically from a lesional skin tumor of case 17, and the purity was as high as 98.8%, as shown in the flow cytometry. ATL tumor cells and autologous CD8$^+$ T cells were used for the allo-mixed lymphocyte reaction system.

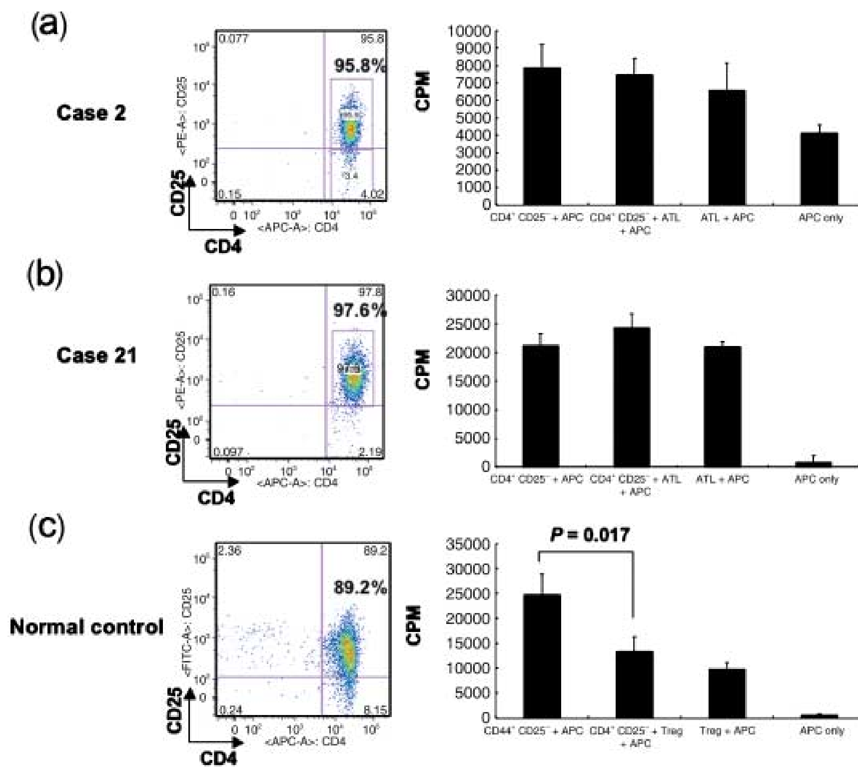

Fig. 7. Lack of regulatory function of circulating adult T cell leukemia/lymphoma (ATL) tumor cells against autologous CD4$^+$CD25$^-$ T cells. CD4$^-$ cells obtained from peripheral blood mononuclear cells in two ATL patients (case 2 and 21) and a normal healthy control were pretreated with mitomycin C and used as antigen-presenting cells (APC). Autologous CD4$^+$CD25$^-$ T cells were also prepared and used as responder cells. A total of 5×10^4 purified CD4$^+$CD25$^-$ T cells were cultured with the same number of autologous APC and anti-CD3 monoclonal antibody at 0.5 µg/mL for 3 days with or without purified autologous CD4$^+$CD25$^+$ T cells at a ratio of 1:1. On the last day of culture, the cells were tested for [^{3}H]thymidine incorporation. Error bars represent the mean + SD cpm from triplicate culture. (a,b) CD4$^+$CD25$^+$ ATL cells did not suppress the proliferation of autologous CD4$^+$CD25$^-$ non-ATL cells. (c) In a normal healthy control, Treg cells suppressed the CD4$^+$CD25$^-$ T cells.

systems. In patients with HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP), the HTLV-I-infected CD4$^+$CD25$^+$ T cells were not functionally suppressive but, rather, stimulative for the HTLV-I tax-specific proliferation of CD8$^+$ T cells.[28] In another study, CD4$^+$CD25$^+$ T cells from HAM/TSP patients expressed decreased levels of both Foxp3 and CTLA-4 and lacked the regulatory function.[29] Finally, CD4$^+$CD25$^+$ T cells derived from normal healthy donors lost their regulatory function after HTLV-I tax transfection.[29]

Our present study showed that despite the high expression of CTLA-4, ATL cells circulating in the peripheral blood and infiltrating into the lesional skin neither suppress nor help CD8$^+$ T cells stimulated with allo-MLR. In some malignancies, tumor-infiltrating CD4$^+$CD25^{high} Treg cells express Foxp3 and inhibit the *in vitro* proliferation and cytokine production of autologous, infiltrating CD4$^+$CD25$^-$ as well as CD8$^+$ T cells in a cell contact-dependent manner.[11] In addition, ATL cells are well known to express Tax in culture, and Tax is an important target of cytotoxic T lymphocytes in HTLV-I-infected patients. Thus, we analyzed the Treg function of ATL tumor cells against the autologous CD8$^+$ T cells. In addition, CD8$^+$ T cells are usually used as target cells for analysis of normal Treg cells.[30] We demonstrated that

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ATL tumor cells could not suppress autologous CD8+ T cells. From the view point of tumor immunology, the results suggest that immunodeficient state in ATL patients is not derived from suppression of CTL activity.

We also evaluated the Treg activity of ATL using CD4+CD25+ ATL cells, CD4+CD25– non-ATL cells (as responder cells), and mitomycin C-treated CD4– cells (as autologous APC). This Treg functional assay system was referred to in a previous report.[26] Although we investigated a limited number of cases, ATL cells purified from PBMC could not suppress autologous CD4+CD25– non-ATL cells under stimulation with both autologous APC and anti-CD3 mAb. Thus, we could no detect any Treg-cell function of ATL cells, differing from the studies reported previously.[23,26] Presumably, there were some differences in the experimental system, or in the patients' characteristics between their study and ours. In this regard, as shown in Table 1, our patients were of smoldering ($n = 17$) or acute ($n = 4$) type, and our functional analysis against CD8+ T cells was done in the patients of smoldering type with skin tumors. Thus, we examined only limited types of ATL, possibly making a difference from the past studies. In addition, for the functional analysis, we used the sorted CD4+CD25+ cells as ATL cells, but the atypical cell ratios were varied.

It should be noted that there was a discrepancy between the percentage of atypical cells and that of CD4+CD25+ cells. This discrepancy happens occasionally because of the normal appearance of malignant T cells. We would consider that the vast majority of CD4+CD25+ cells are ATL cells, and our functional analysis likely aimed at ATL cells. Even if CD4+CD25+ cells contained a high number of normal Treg cells, the functional analysis should have indicated the downregulatory function of CD4+CD25+ cells. Therefore, the lack of regulatory function is thought to be derived from ATL cells.

This notion is clinically relevant. It is known that ATL patients or HTLV-I carriers are prone to develop autoimmune diseases,[31–33] suggesting the dysfunction of CD4+CD25+ T cells. In addition, we reported that chronic actinic dermatitis, a CD8+ T cell-mediated disorder, occurred in an ATL patient presumably as a result of a relief from Treg control.[34] This clinical circumstantial evidence implies that ATL cells do not exert a substantial Treg effect on autoimmune T cells.

It has been shown that CTLA-4 is also expressed on malignant T cells in mycosis fungoides, particularly the leukemic Sézary stage of the disease.[35] Given that the leukemic state is more advanced than the plaque and tumor stage in cutaneous T-cell lymphoma, CTLA-4 expression may be associated with disease progression. In our study, CTLA-4 expression was demonstrated in ATL tumor cells stimulated *in vitro* with anti-CD3 and -CD28 mAb, or those infiltrating the skin. It is likely that the expression of CTLA-4 reflects the activation or proliferation state as well as the possible Treg nature of malignant T cells. In this concept, it is concluded that CTLA-4 is not a predicting molecule for Treg cells but is a useful activation marker in ATL.

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