

Enhancement of lymphokine-activated killer cell induction using anti-CD25 and anti-CTLA-4 monoclonal antibodies

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Abstract. Immunosuppression may contribute to cancer progression, in which regulatory T (T-reg) cells have been demonstrated to play important roles. We investigated whether anti-CD25 (α -CD25) monoclonal antibody (mAb) and anti-cytotoxic T lymphocyte-associated antigen-4 (α -CTLA-4) mAb could augment *in vitro* proliferation and cytotoxic activity against cancer cell lines of lymphokine-activated killer (LAK) cells. Human LAK cells with immobilized α -CD3 Ab plus IL-2 were significantly augmented, including LAK/ α -CD25 (10 μ g/ml, $p=0.045$) and LAK/ α -CTLA-4 (5 μ g/ml, $p=0.025$; 10 μ g/ml, $p=0.019$). LAK/ α -CD25 and LAK/ α -CTLA-4 showed significant cytotoxic activities against gastric cancer cell lines ($p<0.05$). The phenotype of LAK cells showed that α -CD25 and α -CTLA-4 mAb more selectively induced the phenotype of CD8⁺ cells. The secretion of IFN- γ increased significantly in LAK/ α -CTLA-4 ($p=0.032$). α -CD25 mAb reduced intracellular CTLA-4 ($p=0.0069$), and α -CTLA-4 mAb reduced intracellular FOXP3 ($p=0.049$), respectively. These results suggest that LAK cells are highly augmented in the presence of α -CD25 mAb and α -CTLA-4 mAb through the possible mechanism of the suppression of T-reg.

Introduction

Adoptive immunotherapy using lymphokine-activated killer (LAK) cells for human cancer was first introduced in 1985 (1), and limited clinical efficacy was demonstrated against renal cell carcinoma and malignant melanoma. Following LAK therapy studies, tumor-infiltrating lymphocyte (TIL) (2), cytotoxic T lymphocytes (CTLs) sensitized with autologous tumor cells (3), peptide-pulsed dendritic cell (DC)-activated killer cells (4), and tumor RNA-induced DC-activated killer cells (5) were introduced into clinics; however, the clinical results of these adoptive immunotherapy trials showed limited efficacy (6). It has been shown that tumor cells acquire an escape mechanism, including the haplotype loss of HLA expression in many types of clinical tumors (7), and disordered antigen processing (8). CTLs therefore fail to recognize these tumor cells, resulting in treatment failure. In this aspect of antigen presentation and recognition, effector cells that are not restricted HLA expression, such as LAK cells (9), NK cells (9,10), NKT cells (10), and $\gamma\delta$ T cells (11), may have an advantage in treating clinical tumors.

Another mechanism, by which tumor cells escape from the immune system, is immunosuppression in the tumor-bearing host. It is well understood that immunosuppression may contribute to cancer progression. Recent studies have demonstrated that regulatory T (T-reg) cells, which have the phenotype of CD4⁺CD25⁺ cells, contribute to immune dysfunction in cancer patients, and a relative increase in T-reg cells is related to tumor progression in patients with esophageal cancer, gastric cancer and non-small cell lung cancer (12-14). Cell-surface molecules, such as CD25, and intracellular molecules, such as cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), are representative markers on T-reg cells (15,16). T-reg cell attenuation therapy may lead to the establishment of a more effective immunotherapy protocol for cancer patients.

In this study, we aimed to clarify the advantages of applying anti-CD25 (α -CD25) monoclonal antibody (mAb) and α -CTLA-4 mAb to down-modulate T-reg cells in the LAK cell induction system. Our results showed that proliferative responses, secretion of IFN- γ , and cytotoxic activity of LAK cells were augmented in the presence of α -CD25 mAb and, in particular, α -CTLA-4 mAb, indicating the possibility of novel effector cell generation for adoptive immunotherapy for cancer.

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Abbreviations: LAK, lymphokine-activated killer; TIL, tumor-infiltrating lymphocyte; CTL, cytotoxic T lymphocyte; DC, dendritic cell; NK cell, natural killer cell; NKT cell, natural killer T cell; T-reg, regulatory T cell; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; CFSE, carboxyfluorescein diacetate succinimidyl ester; DI, division index; Per-CP, peridin chlorophyll protein; FITC, fluorescein isothiocyanate; PE, phycoerythrin; GTR, glucocorticoid-induced tumor necrosis factor receptor; CBA, cytometric bead array; AIT, adoptive immunotherapy

Key words: FOXP3, regulatory T cell, lymphokine-activated killer cell, cytotoxic T lymphocyte-associated antigen-4, adoptive immunotherapy, CD25, gastric cancer, cell line, carboxyfluorescein diacetate succinimidyl ester

Materials and methods

Blood samples and cell isolation. Peripheral blood was collected from 7 healthy volunteers. Written informed consent under an Institutional Review Board-approved protocol was obtained from all subjects before enrollment in the study. Heparinized peripheral blood was obtained and then centrifuged over Lymphoprep (Nycomed Pharma, Norway) gradients for 15 min at 800 \times g at room temperature. Peripheral blood mononuclear cells (PBMCs) were collected from the interface and washed three times.

Cell lines. MKN-28 (17) and TMK-1 (17) are moderately and poorly differentiated human gastric adenocarcinoma cell lines, respectively. Cells were grown in RPMI-1640 containing 10% FCS (Gibco, Gaithersburg, MD, USA) in a humidified atmosphere containing 5% CO₂ at 37°C.

Cell proliferation assay. PBMCs were labeled with 5 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE, Dojinkagaku, Kumamoto, Japan) for 8 min at 37°C in a humidified 5% CO₂ incubator and washed extensively. PBMCs labeled with CFSE (1 \times 10⁵/ml) were resuspended in RPMI-1640 (Gibco) medium containing 800 IU/ml IL-2 (TGP-3, Takeda Pharmaceutical Co., Ltd., Osaka, Japan) and 2% autologous serum, and stimulated to generate LAK cells in a round bottom 96-well tissue culture plate, coated with 1 μ g/ml α -CD3 Ab (OKT-3, Kyowahakko, Tokyo, Japan) for more than 48 h (α -CD3/IL-2 system) and rinsed with RPMI-1640 medium more than three times. The cells were incubated at 37°C in a humidified 5% CO₂ incubator for 7 days in the presence of either 5 μ g/ml α -CD25 mAb (Ray Biotech, Norcross, GA) [LAK/ α -CD25(5)], 10 μ g/ml α -CD25 mAb [LAK/ α -CD25(10)], 5 μ g/ml F(ab')₂ α -CTLA-4 Ab (Ancell, Bayport, MN) [LAK/ α -CTLA-4(5)], 10 μ g/ml F(ab')₂ α -CTLA-4 mAb [LAK/ α -CTLA-4(10)] or 10 μ g/ml control mouse Ig (Becton-Dickinson Biosciences, CA, USA) (LAK/Ig). Cells were subjected to flow cytometry, as described below, and proliferative activity was calculated from triplicate samples by the following formula (Fig. 1A) (18):

$$\text{Division index (DI)} = (100 - Y)/Y$$

$$Y = M1 + M2/2 + M3/4 + M4/8 + M5/16 + M6/32 + M7/64 + M8/128$$

Cytotoxic activity of LAK cells against cancer cells. The cytotoxic activity of LAK cells induced either with α -CD25 mAb or α -CTLA-4 mAb was investigated using a mixed LAK cell-tumor cell culture system. Target cells were labeled with 5 μ M CFSE for 8 min at 37°C in a humidified 5% CO₂ incubator and washed extensively. CFSE-labeled target cancer cells (MKN-28 and TMK-1) (10⁴ cells/ml) and various non-labeled LAK cells (10⁵ cells/ml), the effector:target ratio = 1:10, including LAK, LAK/ α -CD25(5), LAK/ α -CD25(10), LAK/ α -CTLA-4(5), LAK/ α -CTLA-4(10) and LAK/Ig cells, were admixed to a final volume of 2 ml in RPMI-1640 medium with 5% heat-inactivated FCS in 6-well plates. The samples were incubated in a humidified 5% CO₂ incubator at 37°C for 7 days (TMK-1 cells) and 14 days (MKN-28 cells). Cytotoxic activities were calculated from DIs of triplicate samples by the following formula (18):

$$\text{Cytotoxic activity} = DI/DI_{\text{control}} \times 100$$

Flow cytometry. After 7-day stimulation, CFSE-labeled or non-labeled LAK cells were washed and resuspended in the medium, and subjected to flow cytometry to determine cell division. Three-color flow cytometry was also performed to determine T-cell phenotypes using α -CD3-Per-CP, α -CD4-FITC, α -CD4-PE, α -CD4-Per-CP, α -CD8-FITC, α -CD25-PE and α -CD62L-FITC Abs (Becton-Dickinson Biosciences). Flow cytometry was also performed to determine intracellular CTLA-4 and FOXP3. For intracellular staining of 7-day LAK cells with α -CTLA-4-PE Ab (Becton-Dickinson Biosciences), cells were fixed and permeabilized using a BD Cytofix/Cytoperm (Becton-Dickinson Biosciences). Intracellular staining of 7-day LAK cells with α -FOXP3-FITC Ab was performed using an anti-human FOXP3 staining set (eBioscience, CA, USA) according to the manufacturer's directions. Flow cytometric analysis was performed on FACSCalibur (Becton-Dickinson, NJ, USA) and Cell Quest software (Becton-Dickinson). Lymphocytes were gated on CD3⁺ cells or using light scatter. The relative number of lymphocyte populations was expressed as a percentage (%) of the total number of lymphocytes.

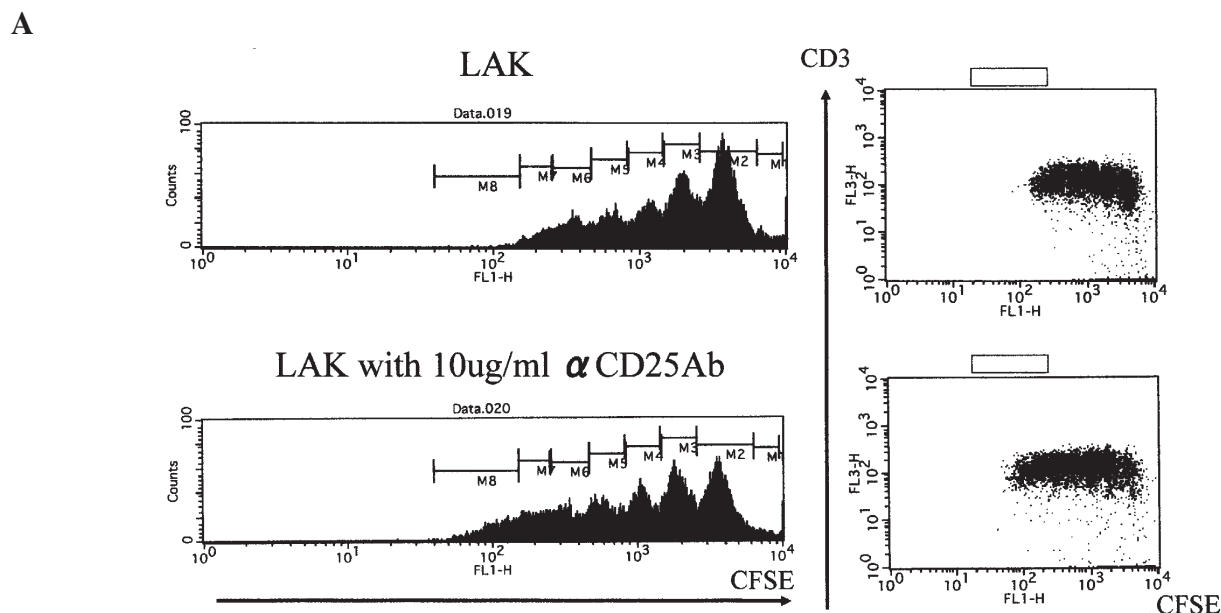
Cytokine assay. Supernatants from the above cultures were harvested and frozen (-20°C) until further use. Cytokine production of IFN- γ , TNF- α , IL-2, IL-4, IL-6 and IL-10 was measured using a CBA (Cytometric Bead Array kit, Becton-Dickinson Biosciences) according to the manufacturer's instructions. The results are expressed as pg/ml.

Statistical methods. Statistical analyses were performed using the paired and unpaired t-test. Significant difference was determined as $p < 0.05$. Results are expressed as the mean \pm SD.

Results

α -CD25 mAb and α -CTLA-4 Ab augment proliferation of LAK cells. The proliferative responses of LAK cells generated with α -CD3/IL-2 system were assessed in the presence of α -CD25 mAb or α -CTLA-4 mAb by using CFSE (Fig. 1A). DI of LAK, LAK/ α -CD25(5), LAK/ α -CD25(10), LAK/ α -CTLA-4(5), LAK/ α -CTLA-4(10) and LAK/Ig cells were 4.1 \pm 1.7, 4.5 \pm 1.6, 4.6 \pm 1.8, 4.4 \pm 1.7, 4.4 \pm 1.5 and 3.9 \pm 1.4, respectively (Fig. 1B). There were significant increases in DI of LAK/ α -CD25(10) ($p=0.045$), LAK/ α -CTLA-4(5) ($p=0.025$) and LAK/ α -CTLA-4(10) cells ($p=0.019$) compared with that of control LAK cells.

Cytotoxic activity of LAK cells induced with α -CD25 mAb and α -CTLA-4 Ab. The cytotoxic activities of LAK cells were investigated (Fig. 2). MKN-28 cells and TMK-1 cells admixed with LAK, LAK/ α -CD25(10) and LAK/Ig cells grew and formed innumerable colonies. In contrast, both cancer cells co-cultured with LAK/ α -CTLA-4(10) cells could not form colonies (Fig. 2A and B). DIs of MKN-28 cancer cells admixed with LAK, LAK/ α -CD25(10), LAK/ α -CTLA-4(10) and LAK/Ig cells were 100%, 73.0 \pm 10.7% ($p=0.049$), 46.1 \pm 1.5%, ($p=0.0003$) and 75.6 \pm 15.6%, respectively (Fig. 2C), and those of TMK-1 cancer cells were 100%, 94.2 \pm 10.9%,



$$\text{Division Index (DI)} = (100 - Y) / Y$$

$$Y = M1 + M2/2 + M3/4 + M4/8 + M5/16 + M6/32 + M7/64 + M8/128$$

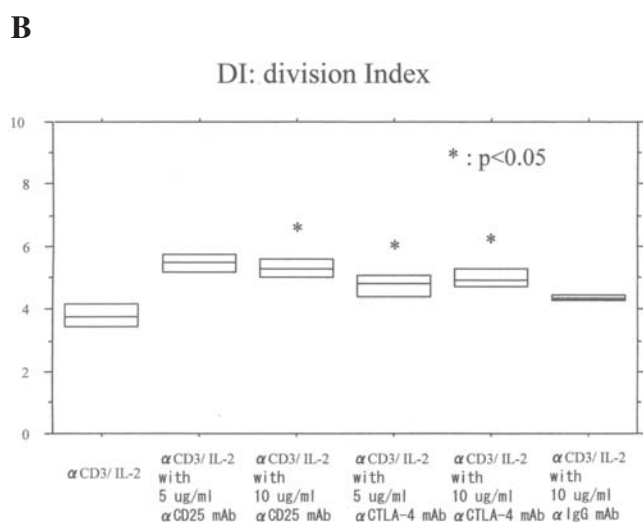


Figure 1. Proliferation analysis of lymphokine-activated killer (LAK) cells using carboxyfluorescein diacetate succinimidyl ester (CFSE). (A) Formula of division index (DI) and histogram of cell division. PBMCs were labeled with CFSE and stimulated with α -CD3/IL-2 system to generate LAK cells in the absence (upper) or presence (lower) of α -CD25 mAb. DIs of LAK cells were assessed on flow cytometry and calculated as a formula. (B) DIs of LAK, LAK/ α -CD25, LAK/ α -CTLA-4 and LAK/Ig cells (n=3). Significant difference, * $p < 0.05$.

53.6 \pm 7.0% ($p = 0.0075$) and 97.4 \pm 11.3%, respectively (Fig. 2D). A significant increase of cytotoxic activity was observed in LAK/ α -CTLA-4(10) cells against both tumor cells and in LAK/ α -CD25(10) cells against MKN-28 cells compared to LAK cells.

Phenotypes of LAK cells stimulated with α -CD25 mAb and α -CTLA-4 Ab. Flow cytometric analysis was performed to determine the phenotypes of LAK cells induced with α -CD25 mAb and α -CTLA-4 mAb (Fig. 3). CD4⁺ cells in LAK, LAK/ α -CD25(10), LAK/ α -CTLA-4(10), and LAK/Ig cells were

33.8, 20.4, 13.6 and 23.2%, respectively. On the other hand, CD8⁺ T cells in LAK, LAK/ α -CD25(10), LAK/ α -CTLA-4(10), and LAK/Ig(10) cells were 59.1, 74.2, 81.5 and 69.0%, respectively (Fig. 3A-D).

We next analyzed CD25⁺ and CD62L⁺ cells in LAK cells (Fig. 3E-H). The phenotypes of CD4⁺CD25⁺ and CD4⁺CD25⁺CD62L⁺ cells in LAK cells were 62.9 and 49.4%, respectively. On the other hand, in LAK/ α -CTLA-4(10) cells, the phenotypes of CD4⁺CD25⁺, and CD4⁺CD25⁺CD62L⁺ cells were 68.7 and 53.8%, respectively.

α -CD25 mAb and α -CTLA-4 Ab augment IFN- γ secretion of LAK cells. IFN- γ production of LAK cells was examined in the presence of α -CD25 mAb or α -CTLA-4 Ab using CBA (Fig. 4). The secretion of IFN- γ from LAK, LAK/ α -CD25(5), LAK/ α -CD25(10), LAK/ α -CTLA-4(5), LAK/ α -CTLA-4(10) and LAK/Ig cells was 4664 \pm 1062, 5454 \pm 1240, 5741 \pm 772, 5800 \pm 741, 5893 \pm 439 and 5171 \pm 1780 pg/ml, respectively. Significantly higher levels of IFN- γ production were observed in LAK/ α -CTLA-4(10) cells compared to LAK cells ($p = 0.032$). All other cytokine levels showed no significant change (data not shown) (n=3).

α -CD25 mAb reduced intracellular CTLA-4 and α -CTLA-4 Ab reduced intracellular FOXP3 in 7-day LAK. Flow cytometric analysis was performed to determine the intracellular CTLA-4 and FOXP3 expression of 7-day LAK cells (Fig. 5). Intracellular CTLA-4⁺ cells in LAK, LAK/ α -CD25(10), LAK/ α -CTLA-4(10) and LAK/Ig cells were 31.3 \pm 0.8, 24.8 \pm 8.7, 37 \pm 12.7 and 35.8 \pm 7.0%, respectively. On the other hand, intracellular FOXP3⁺ cells in LAK, LAK/ α -CD25(10), LAK/ α -CTLA-4(10) and LAK/Ig cells were 83.3 \pm 2.5, 84.4 \pm 2.7, 77.1 \pm 4.2 and 83.2 \pm 4.2%, respectively. Intracellular CTLA-4⁺ cells were significantly reduced in LAK/ α -CD25(10) cells

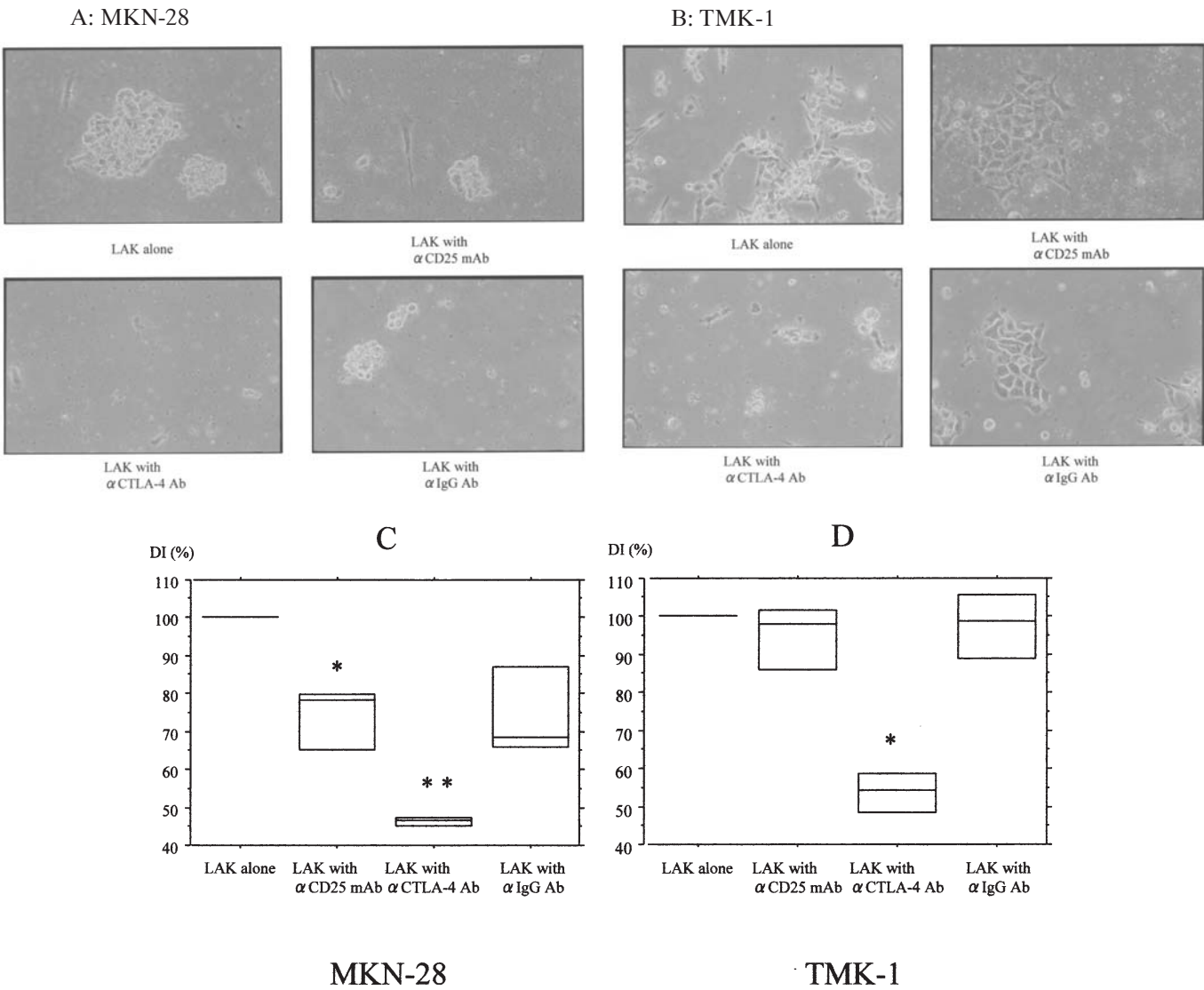


Figure 2. Cytotoxic activity of LAK cells. CFSE-labeled MKN-28 (A) or TMK-1 (B) tumor cells were admixed with LAK, LAK/ α -CD25, LAK/ α -CTLA-4 and LAK/Ig cells, and observed by phase-contrast microscopy (A and B). DIs of MKN-28 (C) or TMK-1 (D) cells were determined on flow cytometry. Analyses were calculated from triplicate samples.

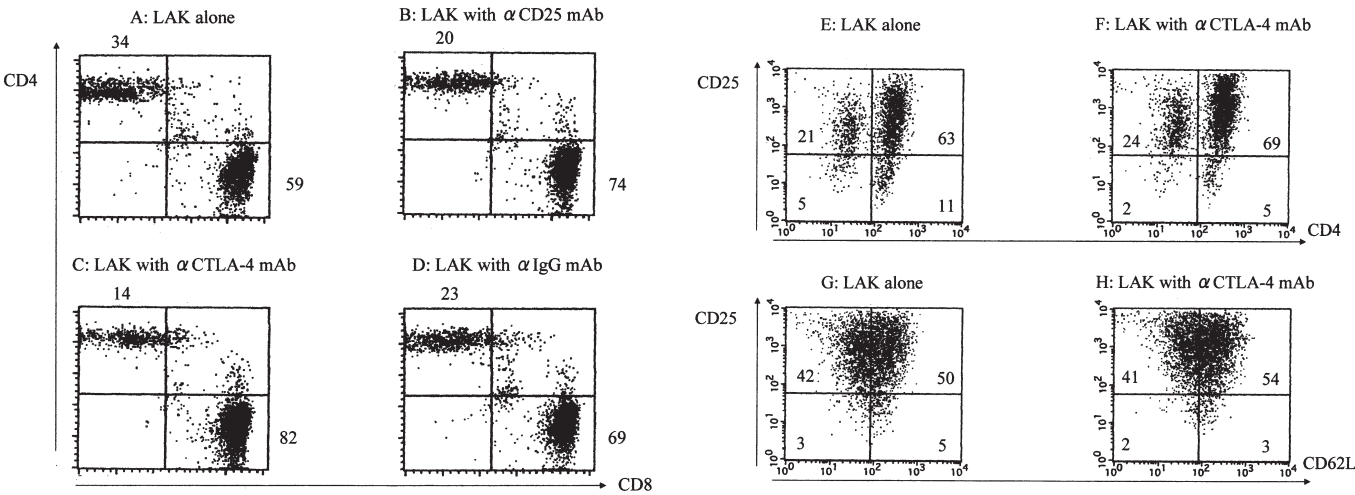


Figure 3. Phenotype analysis on LAK cells. LAK cells were induced with or without α -CD25 mAb, α -CTLA-4 mAb, and LAK/Ig, and stained with Abs as indicated. Flow cytometric analysis was performed on FACScalibur after gating on CD3⁺ cells. CD4 vs CD8 (A-D); CD4 vs CD25 (E, F); and CD62L vs CD25 (G, H). Analyses were calculated from triplicate samples.

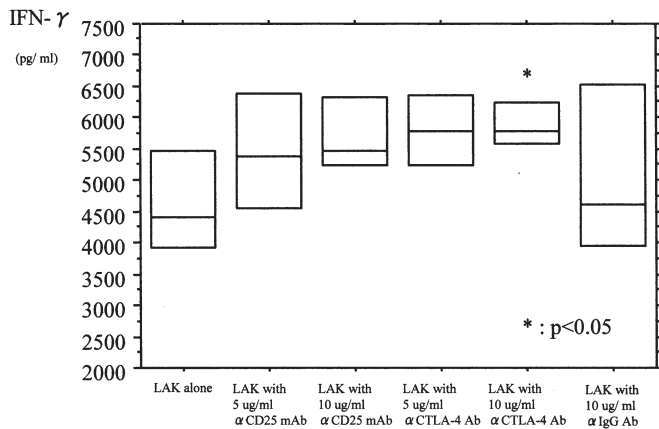


Figure 4. IFN- γ production of LAK cells. LAK cells were induced with or without α -CD25 mAb, α -CTLA-4 mAb, and LAK/Ig, and IFN- γ production in the culture supernatant was measured (n=3). Significant difference, *p<0.05.

(p=0.0069) (Fig. 5A), and intracellular FOXP3⁺ cells were significantly reduced in LAK/ α -CTLA-4(10) cells (p=0.049) compared to LAK/Ig cells (Fig. 5B).

Discussion

After the discovery of T-reg cells (15), increasing attention has focussed on T-reg cell down-modulation combination in cancer immunotherapy. Dudley *et al* reported that adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy can mediate significant tumor regression in patients with metastatic melanoma (19). In that study, the average CD4⁺ cell counts fell and did not recover for almost 6 months, whereas the average CD8⁺ cell counts transiently rose in lymphodepleting chemotherapy, suggesting the possible attenuation of T-reg cells by non-myeloablative lymphodepleting chemotherapy (19). Several molecules, including CD25 (15), CTLA-4 (16), GITR (20) and FOXP3 (21) can be a target of T-reg cell depletion or functional inactivation. Clinical studies of tumor-specific peptide vaccination therapy combined with fully humanized α -CTLA-4 Ab have been

reported and showed both cancer regression and severe autoimmunity (22). Administration of diphtheria toxin-conjugated recombinant IL-2, which selectively eliminates CD25-expressing T-reg cells, in tumor RNA-transfected DC vaccination therapy, showed improved tumor-specific T cell responses (23). In this study, we investigated *ex vivo* stimulation of PBMCs with α -CD3/IL-2 culture system in the presence of α -CD25 mAb and α -CTLA-4 mAb, and demonstrated enhanced cell proliferation and cyto-toxic activity against tumor cells. Although CD25 and CTLA-4 are not specific markers expressed on T-reg cells as they can be also expressed on activated T cells, such as CTLs, the expression of CD25 and CTLA-4 molecules on T-reg cells is relatively and constitutively high (24), indicating the possibility of targeting CD25 and CTLA-4 molecules for down-modulating T-reg cells. Moreover, Kudo *et al* have shown that the administration of α -CD25 mAb at the time of vaccination reduced the number of CD4⁺CD25⁺ cells, but simultaneously enhanced vaccine-induced CD4⁺ cells and CD8⁺ T cell responses, thus apparently not having an inhibitory effect on activated T cell responses (25). This observation also suggests that α -CD25 mAb and α -CTLA-4 mAb may regulate T-reg cells more selectively at the pre-activation phase of lymphocytes compared to the post-activation phase. Therefore, we investigated LAK cell generation combined with T-reg cell modulation by α -CD25 mAb and α -CTLA-4 mAb *ex vivo* at the beginning of LAK cell induction.

Our data showed that α -CD25 mAb therapy enhanced LAK cell proliferation and cytotoxic activity against cancer cell lines. Shimizu *et al* have reported that α -CD25 mAb therapy augments anti-tumor immunity by the depletion of T-reg cells (20); however, a recent study reported that α -CD25 mAb failed to physically deplete T-reg cells but down-regulated and/or induced shedding of CD25 from the surface of T-reg cells (26). It has also been demonstrated that interruption of the IL-2R/IL-2 signaling pathway blocks T-reg cell effector function (27), indicating that α -CD25 mAb induces the functional inactivation of T-reg cells. Moreover, we showed that α -CD25 mAb treatment significantly reduced intracellular CTLA-4 in 7-day LAK cells. The negative immunoregulatory function of CTLA-4 has been definitively demonstrated in

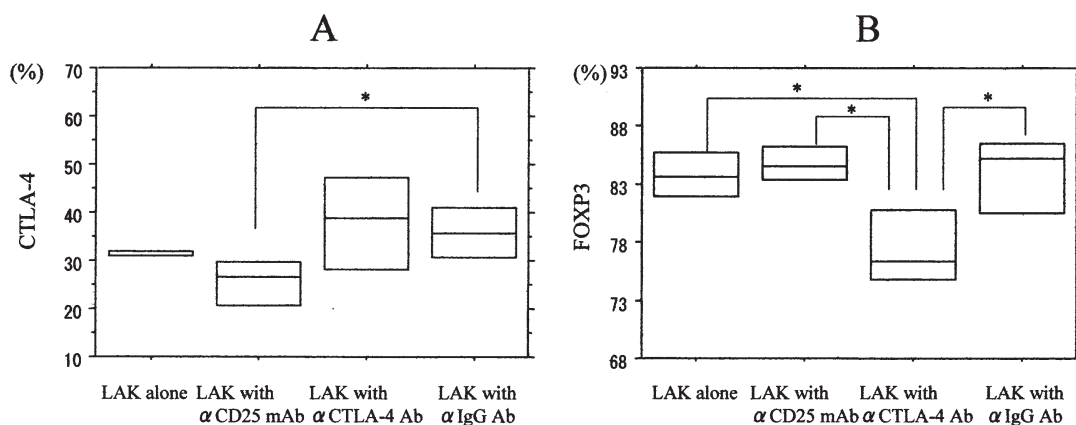


Figure 5. CTLA-4 and FOXP3 expression of LAK cells. LAK cells were induced with or without α -CD25 mAb, α -CTLA-4 mAb, and LAK/Ig, and intracellular expression of CTLA-4 or FOXP3 was stained. Flow cytometric analysis was performed on FACScalibur after gating on lymphocyte (n=3). Significant difference, *p<0.05.

CTLA-4-deficient mice that developed lethal lymphoproliferative disease with infiltration of visceral organs by activated T cells (28). These studies suggest that α -CD25 mAb may augment LAK cell induction by down-modulation of T-reg cell function and CTLA-4 molecules.

On the other hand, our data showed that α -CTLA-4 mAb therapy enhanced not only LAK cell proliferation and cytotoxic activity against cancer cell lines, but also secretion of IFN- γ . It has been demonstrated that most cancer patients do not develop a satisfactory immunological anti-tumor response due to several mechanisms, such as T-cell dysfunction and the marked impairment of IFN- γ secretion (29). Phan *et al* have demonstrated the restoration of T-cell immunity and anti-tumor responses by α -CTLA-4 mAb treatment (22). Our results that IFN- γ production from LAK cells could be enhanced using α -CTLA-4 mAb may explain the mechanisms of the restoration of T-cell dysfunction in cancer patients by blockade of the CTLA-4 molecule. In addition, α -CTLA-4 mAb therapy selectively induced the cell proliferation of CD8⁺ T cells, but reduced CD4⁺ T cells and the induction of CD25⁺ and CD62L⁺ cells. This indicated the enhancement of T-cell activation and chemotaxis *ex vivo* (30). Additionally, α -CTLA-4 mAb treatment significantly reduced intracellular FOXP3, a master gene of T-reg cells, in 7-day LAK cells. Taken together, these findings support an important mechanism of augmenting LAK cell function; that α -CTLA-4 mAb treatment, as well as α -CD25 mAb, may deplete and/or functionally inactivate FOXP3⁺ T-reg cells.

The most important finding was that LAK cell cytotoxic activities against cancer cell lines were modestly augmented with α -CD25 mAb and α -CTLA-4 mAb. LAK/ α -CTLA-4(10) cells particularly showed preferential proliferation of CD8⁺ T cells, CD25⁺ and CD62L⁺-activated T cells, and enhanced IFN- γ production, suggesting the possible clinical use of these 'hyper' LAK cells for AIT of cancer. It remains an important question to identify the molecules that these LAK cells recognize. Phan *et al* (22) have reported that effector cells induced *in vivo* with tumor-specific peptide vaccine in combination with α -CTLA-4 mAb recognize tumor antigens as well as self antigens, resulting in severe autoimmune diseases. Although we did not use antigen-presenting cells and antigens to generate LAK cells *in vitro*, our LAK cells may be reactive with self antigens, but this remains to be elucidated. Regarding the tumor escape mechanism, AIT using HLA-non-restricted effector cells may be indistinguishable in advances in tumor response when orchestrated with HLA-restricted approaches. Clinical trials of AIT using LAK cells are now in progress for the possible treatment of patients with metastatic cancer.

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