Targeting of $CD4^+CD25^{high}$ cells while preserving $CD4^+CD25^{low}$ cells with low-dose chimeric anti-CD25 antibody in adoptive immunotherapy of cancer

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Abstract. The $CD4^+CD25^{high}$ regulatory T (Treg) cells have been demonstrated to negatively modulate anti-tumor immune responses in cancer patients. In this study, effects of low dose anti-CD25 antibody (Ab) to attenuate Treg cells were investigated in cancer patients *in vitro* and *in vivo*. Peripheral blood mononuclear cells (PBMCs) from cancer patients were cultivated *in vitro* in the presence of a high-affinity chimeric anti-CD25 Ab (basiliximab). The $CD4^+CD25^{high}$ population, interferon-gamma (IFN-γ) production and *FOXP3* expression were analyzed using flow cytometry (FCM), enzyme-linked immunosorbent assay and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, respectively. During *in vivo* studies, basiliximab was administered intravenously on day 1, followed by AIT using autologous activated lymphocytes on day 8, and the treatment cycle was repeated. Subjective and objective effects were observed, and patients' PBMCs were subjected to FCM and RT-PCR analysis. *In vitro* analysis revealed that a low concentration of 0.01 μg/ml basiliximab reduced almost all of $CD4^+CD25^{high}$ cells, but less of the $CD4^+CD25^{low}$ cells, and augmented IFN-γ production of activated PBMCs. *FOXP3* mRNA expression of PBMCs was not affected with or without basiliximab. An *in vivo* study of 9 metastatic cancer patients (7 colorectal and 2 esophageal) demonstrated no subjective or objective adverse effects, even under repeated administration of basiliximab. The results suggested that low-dose basiliximab can safely be administered repeatedly, and can target $CD4^+CD25^{high}$ Treg cells whilst relatively preserving $CD4^+CD25^{low}$ activated T cells. The host conditioning with low-dose basiliximab may augment the efficacy of AIT for cancer using activated autologous lymphocytes.

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Abbreviations: Ab, antibody; AIT, adoptive immunotherapy; CD, cluster of differentiation; $CD^{25high/low}$, high/low expression of CD25 molecule; CEA, carcinoembryonic antigen; CTCAE, Common Terminology Criteria for Adverse Events; CTLs, cytotoxic T lymphocytes; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; DCs, dendritic cells; ECOG, Eastern Cooperative Oncology Group; FU, fluorouracil; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GITR, glucocorticoid-induced tumor necrosis factor-related protein; IL, interleukin; LAK, lymphokine-activated killer; PBMCs, peripheral blood mononuclear cells; RECIST, Response Evaluation Criteria in Solid Tumors; RPMI, Roswell Park Memorial Institute; RT-PCR, reverse transcriptase-polymerase chain reaction; TILs, tumor-infiltrating lymphocytes; TNF, tumor necrosis factor; Treg, regulatory T

Key words: anti-CD25 antibody, basiliximab, regulatory T cell, adoptive immunotherapy, cancer immunotherapy

Introduction

A crucial T cell growth factor, interleukin-2 (IL-2) makes it possible to manipulate and propagate natural killer cells and T lymphocytes to be effector cells with highly cytotoxic activity against a wide spectrum of tumor cell types (1,2). The effector cells, known as lymphokine-activated killer (LAK) cells (3), tumor-infiltrating lymphocytes (TILs)(4), and cytotoxic T lymphocytes (CTLs) sensitized with tumor cells (5), have been introduced into adoptive immunotherapy (AIT) trials for patients with incurable cancer. However, tumor responses have been limited in renal cell carcinoma, malignant melanoma, and non-Hodgkin lymphoma (6,7). Identification of tumor antigen genes (8) and professional antigen presenting cells, dendritic cells (DCs), have provided a precise understanding of antigen presentation and recognition machinery (9). Although this understanding has enabled researchers to introduce the tumor antigen/DC system into the generation of tumor-specific effector cells, the clinical benefits of AIT using these tumor-specific effector cells alone have remained limited (10). This failure of AIT may be due, in part, to the escape mechanisms of the tumor from the host immune system, which are known to be present in tumor-bearing hosts (11,12).

Regulatory T (Treg) cells have recently been highlighted as one of the mechanisms by which tumor cells escape from the host immune attack (13,14). It has been demonstrated that naturally arising Treg cells express the $CD4^+CD25^{high}$ phenotype and play an essential role in maintaining immunological self-tolerance, which can contribute not only to the inhibition of the development of autoimmune disease, but also to impeding effective immunosurveillance against autologous tumor cells (14). The attenuation of Treg cell-mediated immunosuppression may therefore evoke effective tumor immunity in otherwise non-responsive hosts (15-19). It has been demonstrated that AIT using tumor-antigen-specific lymphocytes under host conditioning with lymphodepleting chemotherapy has brought about enhanced tumor responses in patients with malignant melanoma who had otherwise been resistant to immunotherapy (17). In that study, a preferential decrease of $CD4^+$ T cells compared to $CD8^+$ T cells by lymphodepleting chemotherapy was demonstrated, suggesting the contribution of such chemotherapy to Treg cell depletion. Dannull *et al* (18) reported that the direct elimination of $CD4^+CD25^{high}$ Treg cells using the recombinant IL-2-diphtheria toxin conjugate DAB389IL-2, demonstrating that the DAB389IL-2-mediated elimination of Treg cells followed by vaccination with tumor RNA-transfected DCs significantly improves the stimulation of tumor-specific T cell responses in patients with renal cell cancer when compared with vaccination alone. Moreover, Phan *et al* (15) and Ribas *et al* (19) conducted clinical trials using anti-CTLA4 Ab for Treg cell attenuation and demonstrated objective tumor responses as well as unfavorable autoimmune diseases.

In the present study, we focused on the use of anti-CD25 antibody as an alternative to lymphodepleting chemotherapy, recombinant IL-2-toxin conjugate and anti-CTLA4 Ab in order to target $CD4^+CD25^{high}$ Treg cells. Basiliximab, a human-mouse chimeric antibody specific for human CD25 antigen with high affinity, has been introduced into clinics to resolve acute rejection reactions in recipients of organ transplantation, where $CD25^+$ effector T cells that are specific for allogeneic antigens play a central role (20,21). Here, we investigated the effects of low-dose and repeated administration of basiliximab for possible targeting of $CD4^+CD25^{high}$ Treg cells *in vitro* and *in vivo*. Treatment with basiliximab in combination with AIT using autologous activated lymphocytes was found to be safe and successfully targeted $CD4^+CD25^{high}$ Treg cells while relatively preserving $CD4^+CD25^{low}$ activated T cells and encouraging anti-tumor activity.

Materials and methods

Collection and activation of lymphocytes. Heparinized venous blood was collected from cancer patients after sufficient informed consent was obtained, and peripheral blood mononuclear cells (PBMCs) were separated by standard density centrifugation. For *in vitro* experiments, PBMCs were cultured in RPMI-1640 medium containing 100 U/ml IL-2 (Sionogi Pharmaceutical Company, Osaka, Japan) plus 1 μM zoledronic acid (Novartis Pharmaceutical Company Tokyo, Japan) at 37˚C for 7-14 days in the presence or absence of human-mouse chimeric anti-CD25 antibody, basiliximab (Novartis Pharmaceutical Company). Activated lymphocytes and culture supernatants were subjected to cytotoxicity assay and IFN-γ assay. For AIT, PBMCs were stimulated with peptide-pulsed autologous dendritic cells and further expanded with an IL-2/anti-CD3 antibody culture system in RPMI-1640 medium supplemented with 2% heat-inactivated autologous plasma, as previously described (10).

Flow cytometry. PBMCs were stained with 10 μg/ml monoclonal antibodies at 4˚C for 30 min, washed, and analyzed using FACScalibur. The antibodies used were anti-CD3, -CD4, -CD8, -CD25, -CD56, and -γδT cell receptor (Becton Dickinson, Mountain View, CA). Data acquisition was stopped at 100,000 events and positive cell populations were analyzed using the attached program after being gated on $CD3^+$ T-cells (22). The percentage of the reduction of $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ cells by basiliximab was calculated using the following formula: percent reduction = (1-positive cells after administration/positive cells on day 0) x100.

Cytotoxicity assay. The cytotoxic activity of activated lymphocytes was determined by a standard ^{51}Cr-releasing assay. The target cells, esophageal cancer cell lines TE12 and TE13 (23), were labeled with ^{51}Cr for 2 h and admixed with effector lymphocytes in 96-well round-bottomed microtiter plates in triplicate at effector-to-target (E/T) ratios of 10 and 20 in a volume of 200 μl. After overnight incubation, the radioactivity of the supernatants was measured using an auto-gamma scintillation counter (Packard, USA). Spontaneous release was determined in wells containing the target cells alone, and maximum release was obtained by adding 100 μl of 1% Triton X-100 solution over the target cells instead of the effector cells. Cytotoxic activity was calculated from triplicate samples using the following formula: cytotoxic activity (percent) = [experimental release (cpm) - spontaneous release (cpm)] /[maximal release (cpm) - spontaneous release (cpm)] x100.

Interferon-γ measurement. PBMCs were cultured with 100 U/ml IL-2 plus 1 μM zoledronic acid at 37˚C for 7 days in the presence or absence of basiliximab. The culture supernatant was subjected to ELISA specific for IFN-γ (R&D Systems Inc., MN, USA) in triplicate according to the manufacturer's instructions.

RT-PCR analysis. Total RNA was extracted from the $5x10^5$ cells of PBMCs using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The prepared total RNA served as the template in first-strand cDNA synthesis using Ready-To-Go™ You-Prime-First-Strand Beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) with 0.5 μg of Oligo(dT)15 primer (Novagen, Darmstadt, Germany). Aliquots of the cDNA were amplified by PCR using primer pairs specific for *FOXP3*, *T-bet*, *GATA3*, *IFN-γ*, *IL-10* and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*(Search-LC GmbH, Heidelberg, Germany) on a LightCycler (Roche Molecular, Mannheim, Germany). The PCR conditions were 95˚C for 10 min for the initial denaturation, followed by 35 cycles of 1 sec at 95˚C, 10 sec at 60˚C, 10 sec at 72˚C, and finally a melting program (60-95˚C) to confirm the correct melting temperature of the product. All clinical samples from patients

were analyzed in parallel with standard samples from Search-LC, calculating the copy numbers on LightCycler software version 3.5 (24).

Clinical study design. Adult patients under 80 years old were eligible to participate in the present study if they had histologically-confirmed cancer for which standard therapy did not exist or was no longer effective. They had Eastern Cooperative Oncology Group (ECOG) performance status 0-2 (25), and adequate bone marrow, hepatic and renal function. Exclusion criteria included uncontrolled infection, uncontrolled diabetes mellitus, history of or evidence of risk for autoimmune disease, concomitant use of corticosteroids, and history of interstitial pneumonia or pulmonary fibrosis. Patients who had received anti-tumor drugs within the preceding 4 weeks were also ineligible.

The study was an open-label, non-randomized study performed at Hiroshima University Hospital, beginning in March 2006. The protocol was approved by the hospital's institutional review board (approval No. 564), and all subjects provided their written informed consent to participate. The patients received intravenous administration of basiliximab (0.01 or 0.005 mg/kg) in 100 ml saline for 20 min on day 0, and autologous activated lymphocytes (1-5 x 10^9) in 100 ml saline containing 100,000 U/ml IL-2 for 20 min on day 7. The following treatment cycle proceeded on day 14 and cycles were repeated every 2 weeks for 3 cycles. Adverse effects and tumor responses were carefully evaluated after each treatment cycle. Toxicity was assessed using the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. All patients were monitored clinically using imaging analysis such as computed tomographic examination, and clinical efficacy was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) (26). Hemato-chemistry examination and immunological analysis including flow cytometry and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were performed every week.

Statistical analysis. Statistical analysis was conducted by paired and unpaired Student's t-tests using StatView software (Version 5) on a Macintosh computer. All values are presented as a mean ± standard deviation, and $p<0.05$ was defined as statistically significant.

Results

Targeting of $CD4^+CD25^{high}$ cells by basiliximab in vitro. Prior to the present clinical study, *in vitro* investigation was carried out in order to estimate an appropriate starting dose of basiliximab. Patient's PBMCs were incubated with decreasing concentrations of basiliximab (1, 0.25, 0.06, 0.01, 0.001 μg/ml), followed by flow cytometric analysis for detecting $CD3^+CD4^+CD25^{high}$ and $CD3^+CD4^+CD25^{low}$ cells (Table I). Basiliximab, at a concentration of 1 μg/ml, showed almost complete reduction of $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ cell populations in case Nos. 1-3. Reduction of $CD4^+CD25^{high}$ cells while relatively preserving $CD4^+CD25^{low}$ cells was observed at a concentration of 0.06 μg/ml basiliximab in case No. 4, at 0.01 μg/ml in case Nos. 1 and 2, and at 0.001 μg/ml in case No. 2.

We then evaluated *FOXP3* mRNA expression of patients' PBMCs, which were cultured in the medium alone for 3 or 7 days in the presence or absence of basiliximab, using RT-PCR analysis. *FOXP3* mRNA expression was detectable in all patients examined, and there was no significant difference in the *FOXP3* mRNA expression with or without basiliximab (data not shown).

Enhancing effects of basiliximab on IFN-γ production by PBMCs. PBMCs were activated *in vitro* in the presence or absence of basiliximab, and IFN-γ production in the supernatant was evaluated (Table II). The presence of basiliximab affected IFN-γ production of PBMCs, which was reproducibly enhanced with 0.01 μg/ml basiliximab ($p<0.01$) in 3 of 4 independent experiments examined. Basiliximab concentrations larger than 0.1 μg/ml were not effective and were rather inhibitory to IFN-γ production of PBMCs.

Inhibition of cytotoxic activity of effector cells by basiliximab in vitro. Cytotoxicity assays were performed to determine whether basiliximab at an effector phase is able to inhibit the cytotoxic activity of effector lymphocytes *in vitro* (Fig. 1). Concentrations of 1 and 0.3 μg/ml basiliximab resulted in a significant reduction in the cytotoxic activity of effector lymphocytes against TE12 or TE13 tumor cells, when the basiliximab was added at the effector phase of the cytotoxicity assay. However, ≤0.1 μg/ml basiliximab did not inhibit the cytotoxic activity of effector lymphocytes.

Patient characteristics. Nine consecutive patients (5 males, 4 females; median age, 63 years; age range, 42-72 years) with unresectable metastases were enrolled in the present study (Table III). Their ECOG performance status scores were 0 (n=2), 1 (n=5), 2 (n=1), and 3 (n=1). The subjects were 7 colorectal and 2 esophageal cancer patients, 2 of whom showed lung metastasis alone and 7 of whom showed multiple metastases, including metastases to the lung, liver, lymph node, pleuroperitoneal, skin and brain. All patients had undergone surgical operations to remove the primary tumor, and 4 patients had undergone additional operations for metastasis. Chemotherapy had been previously performed in all patients, and 2 patients had received radiotherapy. AIT using autologous activated lymphocytes had been introduced for 2 patients. All previous treatments had failed. The total number of transferred cells in the present study ranged from $2x10^9$ to $24x10^9$ cells.

Adverse events. Adverse events related to the systemic administration of basiliximab followed by AIT were carefully observed in an outpatient clinic; hemato-chemistry examinations were also performed. No adverse events were noted either in subjective symptoms or in objective findings, including gastrointestinal symptoms, body temperature, infusion reactions and allergic reactions. Hemato-chemistry examination also found no adverse events in bone marrow, hepatic, or renal function (data not shown).

Targeting of $CD4^+CD25^{high}$ cells with basiliximab in vivo. Targeting of $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ cells with basiliximab administration was analyzed using flow cytometry.

Table I. Detection of CD4+CD25high andCD4+CD25low cell populations in the presence of basiliximab *in vitro*.

Case No.	Cell population	Concentration of basiliximab (μg/ml)					
		0	1	0.25	0.06	0.01	0.001
1	CD4+CD25high	1.3	0	0	0 (100)	0 (100)	-
	CD4+CD25low	16.2	0	0	0 (100)	3.0 (81)	-
2	CD4+CD25high	3.9	0	0	0.1 (97)	0.2 (95)	1.7 (56)
	CD4+CD25low	46.9	0	0	1.6 (97)	3.4 (93)	45.7 (3)
3	CD4+CD25high	3.2	0.2	-	0.2 (94)	1.9 (41)	2.6 (19)
	CD4+CD25low	11.3	0.1	-	0.9 (92)	13.2 (0)	11.3 (0)
4	CD4+CD25high	16.1	-	-	1.6 (90)	14.7 (9)	14.2 (12)
	CD4+CD25low	37.5	-	-	29.6 (21)	42.3 (0)	39.3 (5)

PBMCs were stained with fluorescence dye-conjugated CD4 and CD25 Abs in the presence of varied concentrations of basiliximab *in vitro*, washed, and subjected to flow cytometry. Numbers in parenthesis indicate % down-modulation of cell population by basiliximab.

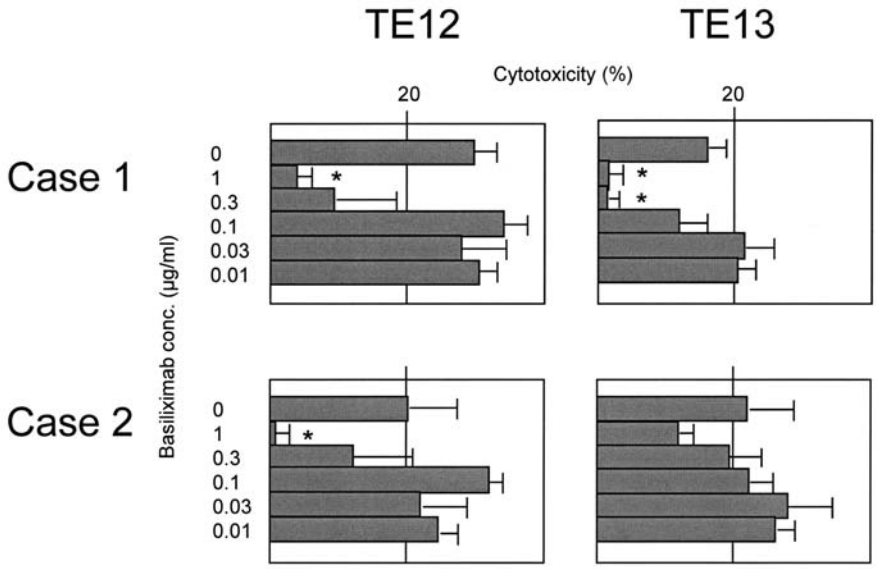

Figure 1. Inhibition of the cytotoxic activity of effector cells by basiliximab *in vitro*. Effector lymphocytes were induced *in vitro* and the cytotoxic activity was determined against TE12 and TE13 cancer cell lines in the presence of varied concentrations of basiliximab at an effector phase of the assay. Significant difference, $^{*}p<0.05$.

Representative profiles of 0.01 mg/kg basiliximab administration are shown in Fig. 2, and changes of the populations are summarized in Table IV. In the present treatment, the first 3 subjects received 0.01 mg/kg basiliximab every 2 weeks, the following 3 subjects received 0.005 mg/kg basiliximab, in addition, and the last 3 subjects received 0.01 mg/kg basiliximab as the initial dose followed by 0.005 mg/kg basiliximab as the repeating dose. When 0.01 mg/kg basiliximab was administered, CD4+CD25high cells were almost completely reduced on day 1, showing a 95% decrease; on day 7, an 80% decrease was still maintained; and on day 14, the cells restored to the level of a 21% decrease (Table IV). The targeting of CD4+CD25low cells by 0.01 mg/kg basiliximab was less than that of CD4+CD25high cells, showing a 60% decrease on day 1, a 38% decrease on day 7, and a 12% decrease on day 14. In contrast, the administration of 0.005 mg/kg basiliximab induced only a 56% decrease in CD4+CD25high cells on day 7. There was a significant difference in the decrease in CD4+CD25high cells on day 7 between basiliximab doses of 0.01 and 0.005 mg/kg (p=0.0351). We therefore omitted further dose reduction of basiliximab in this study. The decrease in CD4+CD25low cells on day 7 with 0.005 mg/kg basiliximab was 31%, and there was no difference in the decrease in CD4+CD25low cells between basiliximab doses of 0.01 and 0.005 mg/kg.

The targeting of CD4+CD25high and CD4+CD25low cells was further analyzed along with the repeated bi-weekly administration of basiliximab (Fig. 3). The dose of 0.01 mg/kg basiliximab showed efficient reduction of CD4+CD25high cells, but also reduced CD4+CD25low cells, which showed no recovery on day 42, even when 14 days had passed since the last administration of basiliximab. The dose of 0.005 mg/kg

Table II. Enhancing effects of basiliximab on IFN-γ production by PBMCs.

Experiment	Basiliximab (μg/ml)	IFN-γ production (pg/ml)	
		Stimulation (-)	(+)
1	0	14	5740±101
	0.003	14	7560±75[a]
	0.01	11	9107±60[a]
	0.03	13	7243±100[a]
	0.1	10	2847±131[a]
	0.3	10	573±12[a]
2	0	40	7580±125
	0.01	37	8210±120[a]
3	0	39	4257±145
	0.01	47	6857±145[a]
4	0	41	2283±105
	0.01	37	2083±110

PBMCs were cultured with the IL-2-containing medium in the presence of basiliximab indicated. The culture supernatant on day 7 was subjected to ELISA specific for IFN-γ. Significant difference, [a]$p<0.01$.

Table III. Patients enrolled in the study.

Patient number	9
Male/Female	5/4
Age (range, median)	42-72, 63
Performance status (0/1/2/3)	2/5/1/1
Primary lesion	
Colorectal	7
Esophageal	2
Metastatic sites	
Lung	3
Liver	2
Lymph node	4
Pleuro-peritoneum	2
Skin	1
Brain	1
Previous treatment	
Surgery (primary/meta.)	9/4
Chemotherapy	9
Radiotherapy	2
AIT	2
Number of transferred lymphocytes (x10^9)	2-24

basiliximab demonstrated, every 14 days, a repeated reduction on day 7 and restoration on day 14 of $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ cells after basiliximab administration, lasting for at least the 42 days of the study period.

FOXP3 mRNA expression after low-dose basiliximab administration in vivo. We analyzed the *FOXP3* mRNA expression levels prior to and after the administration of basiliximab (Table V). *FOXP3* mRNA expression levels were decreased by basiliximab in 5 patients, and increased in 3 others; no change was seen in 1 subject. There was no significant difference in *FOXP3* mRNA expression levels before and after the administration of basiliximab. We also analyzed *T-bet* and *GATA3* mRNA expression during the treatment. There were no significant differences in these expression before and after the administration of basiliximab

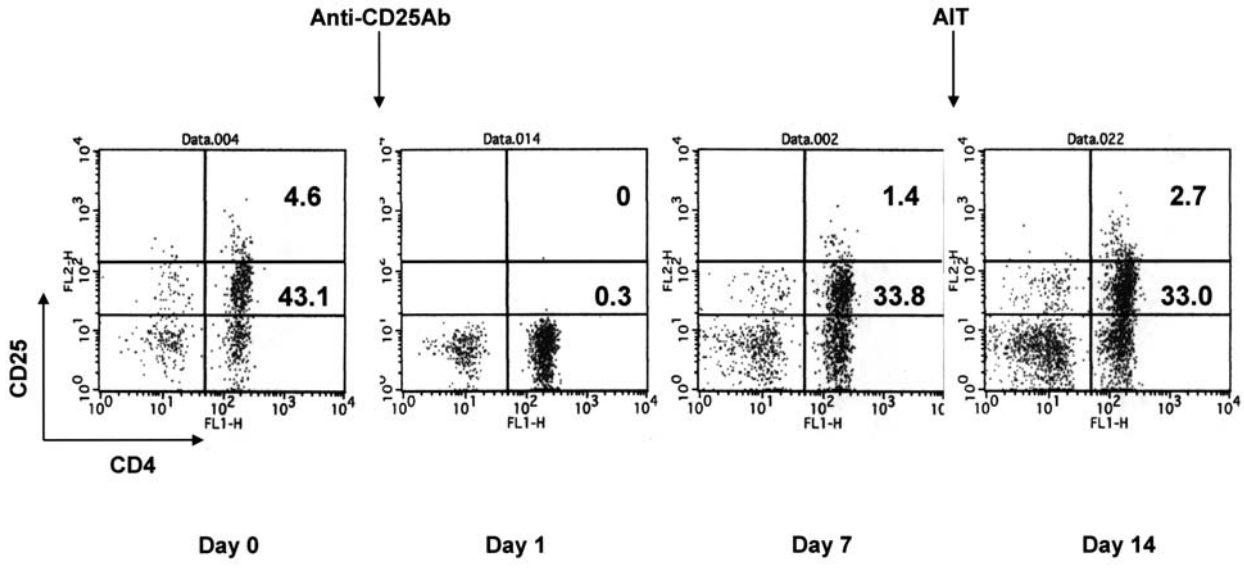

Figure 2. Targeting of $CD4^+CD25^{high}$ cells and $CD4^+CD25^{low}$ cells by basiliximab administration. Basiliximab was administered intravenously. PBMCs were collected before and after the administration, stained with dye-conjugated CD4 and CD25 Abs, and subjected to flow cytometry. A representative series of flow cytometric profiles (0.01 μg/kg basiliximab) is shown.

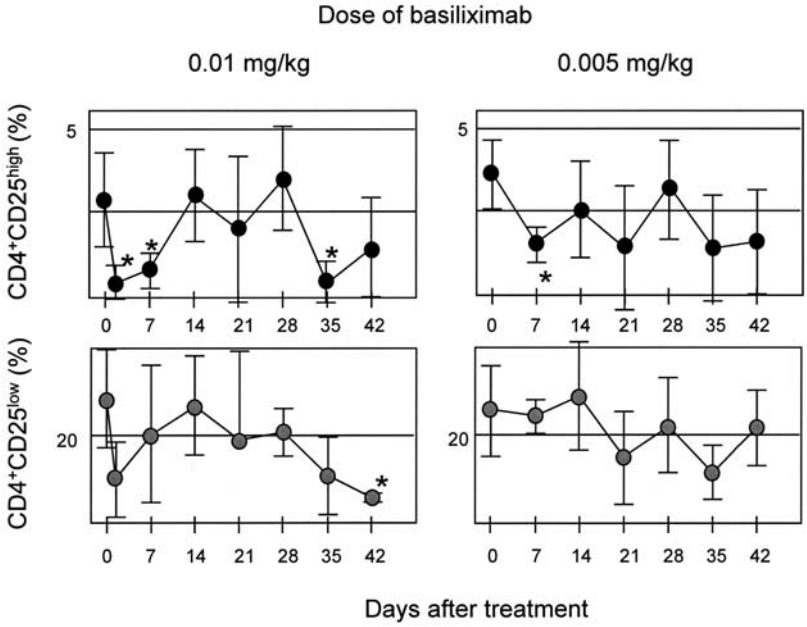

Figure 3. Targeting of $CD4^+CD25^{high}$ cells and $CD4^+CD25^{low}$ cells with repeated administration of basiliximab. The patients received repeated doses of 0.01 or 0.005 μg/kg basiliximab every 2 weeks for 3 cycles. PBMCs were subjected to flow cytometry to analyze $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ cells. Significant difference, $^*p<0.05$.

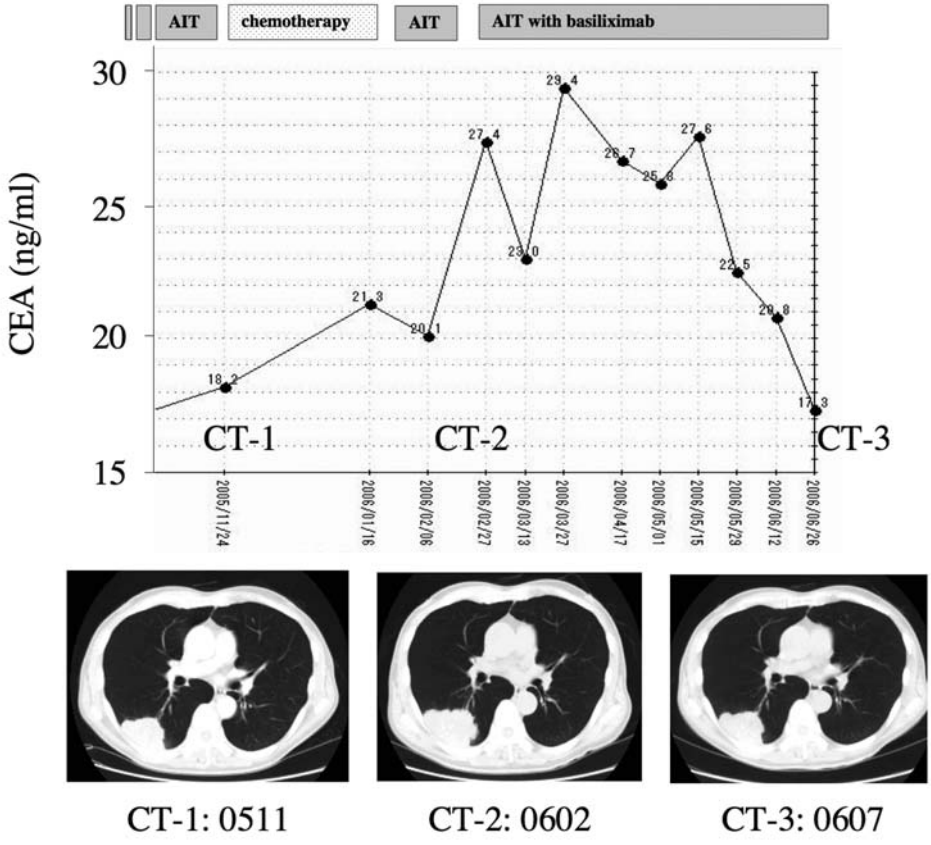

Figure 4. Case report of a rectal cancer patient treated with AIT combined with low-dose basiliximab. A 72-year-old male rectal cancer patient with lung metastasis had previously been treated with AIT followed by 5-FU-based chemotherapy, both of which had failed. The patient received the present AIT combined with low-dose basiliximab. His serum level of CEA decreased immediately following this treatment.

(Table V). Moreover, we did not observe any significant differences in *IFN-γ* and *IL-10* mRNA expression before and after the administration of basiliximab (data not shown).

Changes of lymphocyte phenotypes with low-dose basiliximab administration. Phenotype analysis was performed for $CD3^+$, $CD4^+$, $CD8^+$, and $CD56^+$ lymphocytes during the administration

Table IV. Down-modulation of $CD4^+CD25^{high}$ cells due to low-dose basiliximab.

Basiliximab	Target T cells	% Reduction of positive cells		
		Day 1	Day 7	Day 14
0.01 mg/kg (n=6)	$CD4^+CD25^{high}$	95.3±8.1	80.2±15.8[a]	21.3±38.2
	$CD4^+CD25^{low}$	59.5±48.3	37.9±35.1	12.4±35.5
0.005 mg/kg (n=3)	$CD4^+CD25^{high}$	N.D.	55.8±5.6[a]	41.4±14.1
	$CD4^+CD25^{low}$	N.D.	31.1±6.6	15.3±53.1

PBMCs were collected prior to and after treatment, and $CD4^+CD25^{high}$ and $CD4^+CD25^{low}$ cells were analyzed by flow cytometry. The percentage of reduction from the value prior to treatment was calculated. N.D., not done. Significant difference, [a]$p<0.05$.

Table V. Changes in FOXP3 expression and lymphocyte phenotypes due to basiliximab administration.

Expression	Day 0	Day 7	Day 14
FOXP3 ($x10^3$)	2.24±1.96	1.52±1.82	1.64±1.72
T-bet ($x10^3$)	9.73±10.5	17.35±34.3	6.54±7.98
GATA3 ($x10^3$)	24.85±15.73	25.75±11.42	33.65±32.43
CD3 (%)	60.2±15.1	56.9±16.9	54.1±18.2
CD4 (%)	62.1±16.0	60.0±16.2	57.9±18.5
CD8 (%)	27.9±13.3	29.4±13.6	28.4±16.3
CD56 (%)	24.8±13.6	26.8±8.2	26.7±10.5
$CD3^+CD56^+$ (%)	3.2±1.8	3.1±1.8	2.4 ±1

PBMCs were collected from 9 subjects prior to and after treatment. *FOXP3*, *T-bet* and *GATA3* mRNA expression was analyzed by RT-PCR and the copy numbers were calculated and corrected by *GAPDH* mRNA expression. Lymphocyte phenotypes were analyzed by flow cytometry.

of basiliximab (Table V). No significant changes were observed in the lymphocyte phenotypes on day 7 or 14 after basiliximab administration.

Tumor responses. No definitive objective responses were observed in this study. However, we experienced possible responses strongly related to low-dose basiliximab administration combined with AIT in 2 colorectal cancer patients with lung metastasis who showed reproducible and apparent decreases in tumor marker CEA. A 72-year-old male patient with lung metastasis had been treated with standard chemotherapy based on 5-FU and with AIT, but all previous treatments had failed with increases of CEA. Low-dose basiliximab administration combined with AIT, however, yielded an obvious decrease in CEA immediately following the treatment, with no adverse effects (Fig. 4).

Discussion

When using anti-CD25 antibody in cancer immunotherapy, only the targeting of $CD4^+CD25^{high}$ Treg cells while preserving $CD4^+CD25^{low}$ activated T cells is ideally required. Ko *et al* (26) have demonstrated in an animal model that the co-administration of anti-CD25 Ab and anti-glucocorticoid-induced tumor necrosis factor-related protein (GITR) Ab, is less effective than anti-GITR treatment alone for tumor treatment by inhibiting Treg cells, because anti-CD25 Ab reduces not only $CD4^+CD25^+$ naturally occurring Treg cells but also $CD25^+$ activated T cells, which include tumor-specific effector CTLs involved in tumor eradication. The dosage of basiliximab currently recommended for treating acute rejection reactions to renal transplants is a high dose (40-60 mg) that results in a long-lasting (more than one month) complete disappearance of all $CD25^+$ cells (20,21), suggesting the requirement of modification for basiliximab administration in cancer immunotherapy. Dannull *et al* (18) have reported effective elimination of Treg cells with the recombinant IL-2-diphtheria toxin conjugate DAB(389)IL-2 in treating patients with renal cell cancer using tumor RNA-transfected DC vaccines, where a low concentration of 5 nM conjugates is optimal. Therefore, we hypothesized that low doses of anti-CD25 antibody might be sufficient to target $CD4^+CD25^{high}$ Treg cells without reducing $CD4^+CD25^{low}$ activated T cells, because $CD25^{high}$ cells express many more CD25 molecules on the cell surface than do $CD25^{low}$ cells.

In our *in vitro* experiments, we found that a low concentration of basiliximab at ~0.01 μg/ml achieved preferential targeting of $CD4^+CD25^{high}$ cells while relatively preserving $CD4^+CD25^{low}$ cells and was effective for IFN-γ production of PBMCs, although there were differences among individuals. This implied the possibility of low-dose basiliximab administration *in vivo*, and we therefore designed a clinical study in order to determine the optimal dose of basiliximab. The selected initial dose of 0.01 mg/kg, which may produce a serum concentration of ~0.1 μg/ml basiliximab, was calculated based on a pharmacokinetic study of basiliximab, in which a dose of 60 mg gave a serum concentration of 10 μg/ml immediately following administration (21). As expected, this

low dose administration of 0.01 mg/kg basiliximab *in vivo* was found to work effectively, resulting in the efficient targeting of CD4+CD25high cells while relatively preserving CD4+CD25low cells for at least 7 days. Repeated administration of 0.01 mg/kg basiliximab, however, caused undesirable reduction of CD4+CD25low cells, indicating that, while 0.01 mg/kg basiliximab may be appropriate as an initial dose, it may be excessive for repeated administration. In contrast, a dose of 0.005 mg/kg was less effective for the initial targeting of CD4+CD25high cells but seemed sufficiently effective during repeated administration. We therefore concluded that the recommended dose of basiliximab for regulating CD4+CD25high Treg cells is 0.01 mg/kg as an initial dose followed by 0.005 mg/kg as a maintenance dose in a 2-week treatment cycle combined with AIT.

To the best of our knowledge, the low-dose antibody treatment has not yet been investigated in humans. In cancer treatment, the recommended doses of anti-CD20 antibody (Rituximab) (28) and anti-Her2 antibody (Trastuzumab) (29) are 375 mg/m^2 and 4 mg/kg, respectively, which are much higher doses than those found to be appropriate in the present study. Anti-CD3 antibody is also used at a high dose (5 mg) to create immunosuppressive circumstances in regulating acute rejection reactions in organ transplantation (30). In a study in pigs, Lohse *et al* (31) investigated a low-dose treatment with murine monoclonal antibodies against porcine CD4 and CD8 surface antigens on lymphocytes, demonstrating specific modulation of the peripheral blood T lymphocyte population and providing a novel method of elucidating their functionality in protecting against infectious disease, even though high-dose administration of the antibody has been only partially successful in achieving short-term cell modulation. In the present investigation, the low-dose administration of basiliximab accomplished the desirable decrease of CD4+CD25high cells, even though the low dose of 0.01 mg/kg may be excessive for repeated administration because it reduced CD4+CD25low cells to an undesirable degree, indicating that the low-dose approach is highly active for modulating the immune system. We believe that it is important to use the lowest active doses of therapeutic antibodies in order to minimize the burden of injected xenogeneic human-murine chimeric protein, and to reduce the high costs associated with antibody treatment (31). Moreover, the subjective symptoms or the objective findings, including the hemato-chemistry examinations, showed no adverse effects, indicating the safety of the low-dose administration of basiliximab in combination with AIT. The lymphocyte phenotypes, which were identified using flow cytometry, and the other regulatory T cell populations of T helper type (Th)-1 and Th-2, which were detected by the expression of *IFN-γ* and *T-bet* (32), and *IL-10* and *GATA3* mRNA (33), respectively, were not affected in this study. These biological effects of the low-dose basiliximab administration remained to be ensured in future clinical trials.

The optimal timing for the addition of anti-tumor immunotherapy to the conditioning regimen using low-dose basiliximab treatment is an important issue, which remains to be resolved. Kudo-Saito *et al* (34) have addressed this question by examining tumor-bearing mouse models treated with recombinant vaccinia virus vaccine consisting of tumor antigen, B7-1, ICAM-1 and LFA-3 genes, and have demonstrated that vaccine-induced T-cell immune responses can be optimally augmented when anti-CD25 antibody is administered at the same time as vaccination; this apparently indicates that the administration of anti-CD25 Ab at the same time as vaccination does not have an inhibitory effect on subsequently activated T-cell responses. This may be the case in an active immunotherapy protocol, which may require Treg cell targeting by anti-CD25 antibody at the time of vaccination in order to effectively trigger the induction phase of effector cell generation *in vivo*. However, our case of the AIT protocol using autologous effector lymphocytes activated *ex vivo* does require Treg cell targeting by anti-CD25 Ab at the effector phase *in vivo*. When administered at the same time, anti-CD25 Ab directly inhibits the cytotoxic activity of the adoptively transferred effector lymphocytes. The present *in vitro* experiments showed that the cytotoxic activity of activated effector cells was inhibited at doses of basiliximab of >0.1 μg/ml. Therefore, we added AIT on day 7 after low-dose basiliximab administration, when the basiliximab concentration decreases to <0.1 μg/ml in serum (21), and just before the CD4+CD25high Treg cells targeted by basiliximab begin to recover.

It is useful to know whether Treg cells are physically depleted or functionally modulated by basiliximab treatment. In the present study, *FOXP3* mRNA expression was not definitively decreased after basiliximab administration. There is some discussion in the literature suggesting that a targeted population of CD4+CD25high cells in flow cytometric analysis after basiliximab administration does not indicate the physical depletion of the cell numbers but rather indicates only the masking of the CD25high cells escaping detection by flow cytometry using the second anti-CD25 Ab (35). Moreover, Game *et al* (36) have shown, during *in vitro* experiments, that basiliximab permits the suppression of proliferation and IFN-γ secretion by CD4+CD25- cells responding to allogeneic and other polyclonal stimuli, suggesting that basiliximab does not affect Treg cell function. However, Smithberg *et al* (37) have shown that CD4+CD25+ Treg cells are reduced in cat after anti-CD25 antibody treatment in the circulation (82% decrease), in the thymus (69%), secondary lymphoid tissues (66%), and gut (67%), and that foxp3 levels remain depressed, although CD4+CD25+ cells rebound by day 35 post-treatment. Kohm *et al* (38) have demonstrated an exacerbation of autoimmune encephalomyelitis by the administration of anti-CD25 Ab in an animal model, showing, in those mice, a decrease in the CD25high cell population but not in foxp3+ cells, which suggests that the injection of anti-CD25 Ab results in the functional inactivation, but not physical depletion, of Treg cells; these results are very similar to our own. It has also been reported that CD25 molecules are functionally essential for Treg cells, and that the interruption of the IL-2/IL-2R signaling pathway with anti-CD25 Ab blocks Treg function potentially through alteration in the expression of the glucocorticoid-induced TNFR-family gene (39). Collectively, these results suggest that the basiliximab treatment in the present study may achieve down-modulation of Treg cell function, but not Treg cell depletion. This issue will be resolved by further functional analyses for Treg cell activity (currently in progress).

We were encouraged by the possible, but not yet definitive, clinical responses in this study. The arrest of tumor growth with an obvious decrease in serum tumor markers was observed in 2 of 9 patients enrolled, in whom AIT without basiliximab had failed. A phase II study is thus warranted to ensure the efficacy of AIT using autologous activated lymphocytes in combination with the conditioning treatment of low-dose basiliximab for treating patients with metastatic cancer.

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