

Dose-Finding Study of Anti-CD25 Antibody for Targeting Regulatory T Cells in Locoregional Immunotherapy of Malignant Effusion

Makoto OKAWAKI, Yoshiyuki YAMAGUCHI^{*}, Riki OKITA,
 Masahiro OHARA and Morihito OKADA

Department of Surgical Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan

ABSTRACT

Effects of low-dose anti-CD25 antibody on targeting regulatory T (Treg) cells *in vitro* and *in vivo* were investigated. Human-mouse chimeric anti-CD25 monoclonal antibody basiliximab was administered into the effusion cavity, followed by locoregional immunotherapy using OK-432 on day 7. Peripheral blood mononuclear cells and effusion lymphocytes (ELs) were collected before and after the basiliximab administration and subjected to further investigations. Surface phenotypes, IFN- γ production, cytotoxic activity and foxp3 expression of ELs were assessed by flow cytometry, ELISA, 51Cr-releasing assay, and RT-PCR analysis, respectively. We observed that a low concentration of 0.01 $\mu\text{g/ml}$ basiliximab effectively targeted CD4⁺CD25^{bri} Treg cells while preserving CD4⁺CD25^{dim} activated T cells *in vitro*. This concentration of basiliximab significantly augmented interferon (IFN)- γ production of ELs when interleukin (IL)-2 was added on day 0 or on day 1 after basiliximab. In the clinical study, intracavitary administration of basiliximab on day 0 followed by OK-432 on day 7 was as safe, well-tolerated, and effective as using OK-432 alone, and a low-dose of 0.002-0.005 mg/kg basiliximab could target CD4⁺CD25^{bri} cells for at least 3 days while relatively preserving CD4⁺CD25^{dim} cells. Foxp3 expression of ELs was not changed definitely by the intracavitary basiliximab. These results suggest that low-dose basiliximab can target Treg cells *in vitro* and *in vivo*, and subsequently augment the activation of ELs. Locoregional immunotherapy of malignant effusion using the Treg cell-conditioning regimen with low-dose basiliximab followed by OK-432 administration on day 0 or on day 1 should be evaluated for clinical efficacy in the next phase II trial.

Key words: Regulatory T cells, CD25, Basiliximab, Malignant effusion, OK-432

The action of regulatory T (Treg) cells has recently been highlighted as one of the mechanisms by which tumor cells escape from the host immune attack^{20,22}. It has been demonstrated that naturally arising Treg cells express the CD4⁺CD25^{bri} 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 cells²². The attenuation of Treg cell-mediated immunosuppression may therefore evoke effective tumor immunity in otherwise non-responsive hosts^{2,4,6,18,19}. Adoptive immuno-

Abbreviations: CD, cluster of differentiation; CD25^{bri/dim} cells, CD25-brightly/dimly positive cells; CTLs, cytotoxic T lymphocytes; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; CTCAE, Common Terminology Criteria for Adverse Events; DNA, deoxyribonucleic acid; ECOG, Eastern Cooperative Oncology Group; ELISA, enzyme-linked immunosorbent assay; ELs, effusion lymphocytes; GITR, glucocorticoid-induced tumor necrosis factor-related protein; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; IL-2R, IL-2 receptor; LFA, lymphocyte function-associated antigen; mRNA, messenger ribonucleic acid; PBMCs, peripheral blood mononuclear cells; RPMI, Roswell Park Memorial Institute; RT-PCR, reverse transcription polymerase chain reaction; Treg, regulatory T

^{*} Corresponding author: Dr. Y. YAMAGUCHI; Present address, Department of Clinical Oncology, Kawasaki University School, Matsushima 577, Kurashiki-city, Okayama 701-0192, Japan; Phone, +81-86-462-1111; Fax, +81-86-464-1134; E-mail, shogo@med.kawasaki-m.ac.jp

therapy using tumor-antigen-specific lymphocytes under host conditioning with lymphodepleting chemotherapy has brought about enhanced tumor responses in patients with malignant melanoma who had been resistant to immunotherapy⁶. 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 al⁴) reported the direct elimination of CD4⁺CD25^{br} Treg cells using the recombinant interleukin (IL)-2-diphtheria toxin conjugate DAB389IL-2, demonstrating that the DAB389IL-2-mediated elimination of Treg cells followed by vaccination with tumor RNA-transfected dendritic cells (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 al¹⁸) and Ribas et al¹⁹) conducted clinical trials using anti-CTLA4 antibody for Treg cell attenuation and demonstrated objective tumor responses, indicating the importance of targeting Treg cells for the success of cancer immunotherapy trials.

We have been engaged in locoregional immunotherapy of malignant effusion, and we previously reported the clinical efficacy of locoregional administration of the streptococcal preparation OK-432^{23,24}), molecular mechanisms of effector cell generation by OK-432²³), and T-helper type-1 dysfunction in malignant effusion²⁴). Recently, DeLong et al⁵) reported the existence of Treg cells in malignant pleural effusions secondary to mesothelioma and carcinoma. This encouraged us to conduct a clinical study to address the question of whether the targeting of Treg cells in malignant effusion may contribute to enhancing the efficacy of locoregional immunotherapy using OK-432.

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 antibody in order to target CD4⁺CD25^{br} 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^{1,12}). Here, we investigated the effects of low-dose administration of basiliximab for possible attenuation of Treg cells *in vitro* and *in vivo*. We conducted a clinical trial to examine the safety of basiliximab in locoregional immunotherapy of malignant effusion using OK-432, and we determined a recommended dose of basiliximab for targeting Treg cells in malignant effusion.

MATERIALS AND METHODS

Collection of lymphocytes

Heparinized venous blood and citrated effusion were obtained from cancer patients by venipuncture and paracentesis, respectively, after written informed consent was obtained. Peripheral blood mononuclear cells (PBMCs) and effusion lymphocytes (ELs) were separated by standard density centrifugation, washed twice, and resuspended in RPMI-1640 medium (10⁷/ml)^{22,23}).

Flow cytometry

PBMCs and ELs (5 × 10⁵) were stained with 10 µg/ml monoclonal antibodies at 4°C for 30 min, washed, and analyzed by FACSCalibur. The antibodies used were anti-CD3, -CD4, -CD8, -CD25 and -CD56 antibodies (Becton Dickinson, Mountain View, CA). When the down-modulation of Treg cells by human-mouse chimeric anti-CD25 antibody was analyzed, lymphocytes were incubated with varied concentrations of basiliximab (Novartis Pharmaceutical Company Tokyo, Japan), washed, and then subjected to surface staining with anti-CD3, -CD4, and -CD25 antibodies. Data acquisition was stopped at 10,000 events and positive cell populations were analyzed by the attached program after being gated on CD3⁺ T-cells¹⁶).

Interferon-γ measurement

ELs (10⁶/ml) were resuspended in RPMI-1640 medium supplemented with 2% heat-inactivated autologous plasma, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, and stimulated with 100 U/ml IL-2 (Sionogi Pharmaceutical Company, Osaka, Japan) for 4-14 days in the presence or absence of basiliximab in a humidified incubator with 5% CO₂ at 37°C. The culture supernatant was collected and subjected to ELISA specific for IFN-γ (R&D Systems Inc., Minneapolis, MN, USA) in triplicate according to the manufacturer's instruction.

Cytotoxicity assay

The cytotoxic activity of IL-2-activated ELs was determined by a standard ⁵¹Cr-releasing assay. Esophageal cancer cell lines TE12 and TE13⁹) were labeled with ⁵¹Cr for 2 hours as target cells and admixed with effector lymphocytes in the presence or absence of basiliximab 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 by the following formula: cytotoxic activity (percent) = (experimental release [cpm] - spontaneous release [cpm]) / (maximal release [cpm] - spontaneous release [cpm]) X 100.

RT-PCR analysis

Total RNA was extracted from the 5×10^5 ELs 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*, interferon- 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 to 95°C) to check the proper 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 3.5¹⁵.

Clinical study design

Adult patients under 80 years old were eligible to participate in the present study if they had a cytologically-confirmed malignant effusion for which systemic standard chemotherapy did not exist or was no longer effective. They had Eastern Cooperative Oncology Group (ECOG) performance status 0 to 3¹⁰, 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.

The study was an open-label, nonrandomized study performed at Hiroshima University Hospital beginning in March 2006. The protocol was approved by the hospital's institutional review board (approval No. 579), and all subjects provided their written informed consent to participate. The patients received intracavitary administration of basiliximab (0.01, 0.005 or 0.002 mg/kg) in 10 ml saline after removal of effusion (up to 1000 ml) on day 0, followed by intracavitary administration of 1-5 KE OK-432 in 10 ml saline on day 7^{23,24}. Adverse events were 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 and ultrasonographic examinations, and effusion responses were carefully evaluated as disappeared or decreased, lasting for more than 1 month or no response. Hemato-chemistry examination and immunological analysis including flow cytometry and RT-PCR analysis were performed on days 0, 1, 3, and 7 after intracavitary basiliximab administration.

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 mean +/- standard deviation, and $p < 0.05$ was defined as statistically significant.

RESULTS

Targeting of CD4⁺CD25^{br}i Treg cells by basiliximab *in vitro*

Prior to the present clinical study, *in vitro* investigation was carried out to ensure that anti-CD25 antibody could target Treg cells while preserving activated lymphocytes. PBMCs were incubated with decreasing concentrations of basiliximab (1, 0.25, 0.06, 0.03, 0.01 µg/ml), followed by flow cytometric analysis for detecting CD3⁺CD4⁺CD25^{br}i cells (Fig. 1). Flow cytometric profiling showed 1.3% and 16.2% positive cells for CD4⁺CD25^{br}i cells and CD4⁺CD25^{dim} cells, respectively, in the absence of basiliximab. When PBMCs were preincubated with 0.1 µg/ml or higher concentration of basiliximab, both CD4⁺CD25^{br}i and CD4⁺CD25^{dim} cells were completely targeted. A lower concentration of basiliximab at 0.01 µg/ml, however, also realized the targeting of CD4⁺CD25^{br}i cells (0%) while relatively preserving CD4⁺CD25^{dim} cells (3%). Similar results were obtained in the other 3 cases investigated. Still lower concentrations of basiliximab were not effective in the targeting of CD4⁺CD25^{br}i cells.

We next evaluated *foxp3* mRNA expression of patients' PBMCs, which were cultured 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 ELs

ELs were activated *in vitro* with IL-2 stimulation in the presence or absence of basiliximab, and IFN- γ production in the supernatant on day 7 was evaluated (Table 1). Varied concentrations of basiliximab affected IFN- γ production of ELs, which was enhanced significantly with 0.01 µg/ml basiliximab ($p < 0.05$). Basiliximab concentrations

larger than 0.1 µg/ml were not effective and were rather inhibitory for IFN-γ production of ELs. This was also observed in experiment 2, showing significantly higher IFN-γ production in ELs with 0.01 µg/ml basiliximab than in ELs without basiliximab (p<0.05). Similar results were obtained in 2 other independent experiments.

Timing analysis optimal for IL-2 stimulation after basiliximab in vitro

We addressed the timing optimal for IL-2 stimulation following the basiliximab treatment in

vitro. ELs were incubated with or without 0.01 µg/ml basiliximab and subsequently stimulated with IL-2 on days 0, 1 and 3, and then IFN-γ production for 7 days in the supernatant was evaluated (Fig. 2). In 3 ELs examined, IL-2 administration on day 0 (patients 1 and 2) or on day 1 (patients 1-3) induced significantly high IFN-γ production in the presence of basiliximab, when compared with production in the presence of IL-2 alone (p<0.05). IL-2 administration on day 3, however, was less (patient 2) or no longer (patient 1 and 3) stimulat-

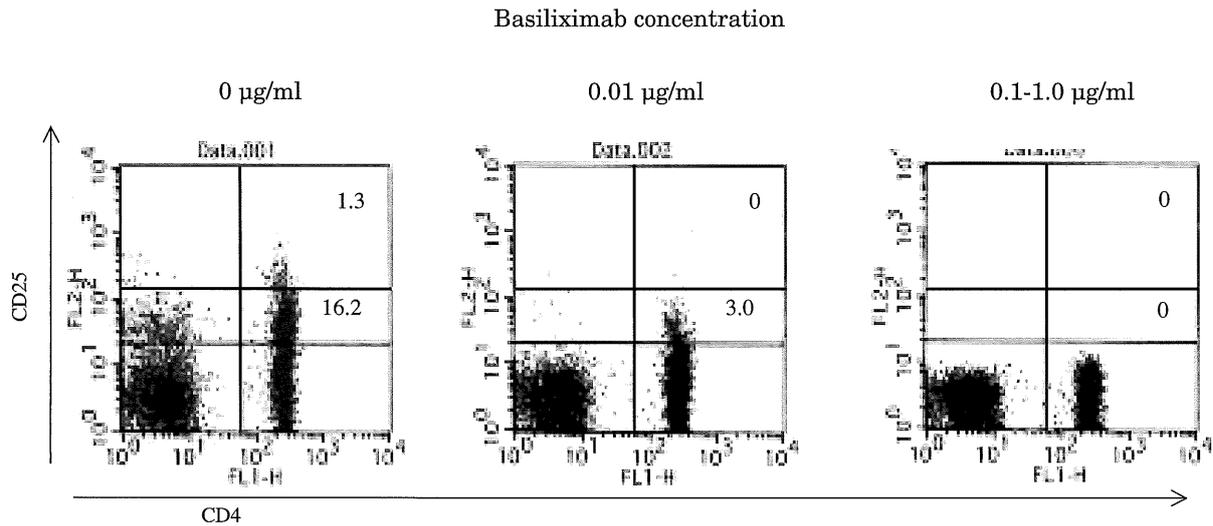


Fig. 1. Targeting of CD4⁺CD25^{bri} Treg cells by basiliximab *in vitro*.

PBMCs were pretreated with varied concentration of basiliximab indicated, washed, and then stained with anti-CD4 and -CD25 antibodies. Flow cytometry for detecting CD4⁺CD25^{bri} Treg cells and CD4⁺CD25^{dim} activated T cells was performed on FACSCalibur.

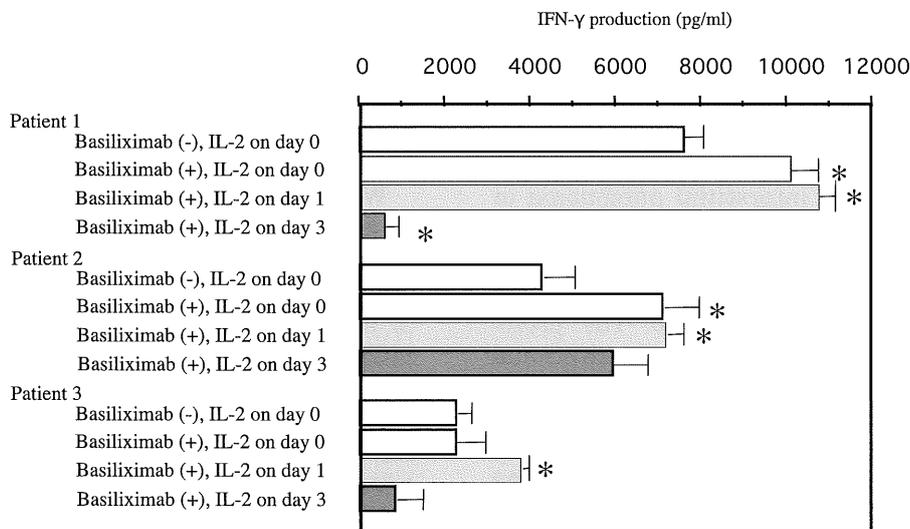


Fig. 2. Timing study of lymphocyte activation after basiliximab *in vitro*.

ELs were incubated with or without 0.01 µg/ml basiliximab. IL-2 stimulation was made on days 0, 1, and 3. IFN-γ production for 7 days in the supernatant of triplicate wells was assessed by ELISA. Results of 3 independent ELs are shown. We found significant differences from the value of IL-2 alone, *p<0.05.

Table 1. IFN- γ production of ELs stimulated with IL-2 in the presence of basiliximab

Basiliximab concentration($\mu\text{g/ml}$)	IFN- γ production (pg/ml)
Expt. 1	
0	5831 +/- 48
0.003	7710 +/- 68*
0.01	9213 +/- 106*
0.03	7128 +/- 65*
0.1	2892 +/- 20*
0.3	699 +/- 14*
Expt. 2	
0	4010 +/- 91
0.01	7011 +/- 108*

ELs were collected from patients with malignant effusion, and stimulated with IL-2 in the presence of varied concentrations of basiliximab. IFN- γ production of ELs in the supernatant of triplicate wells was determined by ELISA. Results from 2 of 4 independent experiments (Expt.) were shown. Significant differences from the value of IL-2 alone were observed, * $p < 0.05$.

Inhibition of cytotoxic activity of effector cells by basiliximab *in vitro*

Cytotoxicity assays were performed to determine whether basiliximab is able to inhibit the cytotoxic activity of effector lymphocytes *in vitro* when added at an effector phase of the assay (Table 2). Concentrations of 1 and 0.3 $\mu\text{g/ml}$ basiliximab resulted in a significant reduction in the cytotoxic activity of effector lymphocytes against TE12 or TE13 tumor cells. However, 0.1 $\mu\text{g/ml}$ basiliximab or less did not inhibit the cytotoxic activity of effector lymphocytes. A similar result was obtained in 2 other independent experiments.

Patients' characteristics

Twelve consecutive patients (7 males, 5 females; median age, 68 years; age range, 34-80 years) with malignant effusion were enrolled in the present study (Table 3). Their ECOG performance status scores were 0 ($n=2$), 1 ($n=5$), and 2 ($n=5$). The subjects were 2 lung, 2 esophageal, 3 stomach, and 5 colorectal cancer patients, 4 of whom showed pleural effusion and 8 of whom showed ascites. Seven patients had undergone surgical operation to remove the primary tumor. Systemic chemotherapy had been previously performed in all patients, and all treatments had failed.

Adverse events

Adverse events related to the intracavitary administration of basiliximab followed by OK-432 administration were carefully observed. The intracavitary administration of basiliximab did not induce any adverse events at all. However, when

Table 2. Cytotoxic activity of ELs activated with IL-2 in the presence of basiliximab

Basiliximab concentration ($\mu\text{g/ml}$)	cytotoxicity (%)	
	TE12	TE13
0	29 +/- 4	16 +/- 2
1	4 +/- 2*	3 +/- 1*
0.3	10 +/- 9*	2 +/- 1*
0.1	34 +/- 5	12 +/- 5
0.03	27 +/- 6	22 +/- 4
0.01	29 +/- 3	21 +/- 3

ELs were collected from patients with malignant effusion, and stimulated with IL-2. Cytotoxic activity of ELs against TE12 and TE13 target cells was evaluated using ^{51}Cr -releasing assay in triplicate in the presence or absence of varied concentrations of basiliximab at an effector phase. A representative result of 3 independent experiments was shown. Significant differences from the value without basiliximab were observed, * $p < 0.05$.

Table 3. Characteristics of the patients enrolled

Patient number	12
Male/Female	7/5
Age (range, median)	34-80, 68
Performance status (0/1/2)	2/5/5
Primary lesion	
Lung	2
Esophageal	2
Stomach	3
Colorectal	5
Site of effusion	
Pleural	4
Ascites	8
Previous treatment	
Surgery for primary lesion	7
Chemotherapy	12

OK-432 was added after basiliximab administration, abdominal or chest pain at the site of drug administration at grade 2 was found in 3 patients (25%) and fever elevation at grade 1 was observed in all of the patients treated (100%). Both of these symptoms are usually observed with the intracavitary administration of OK-432 alone²⁴). Hematochemistry examination showed no adverse events in bone marrow, hepatic, or renal function (data not shown).

Effusion responses

Effusion responses with intracavitary administration of basiliximab followed by OK-432 are shown in Table 4. Effective responses were obtained in 7 patients (63%), consisting of 2 patients in whom effusion disappeared (18%) and 5 patients in whom effusion decreased (45%).

Targeting of CD4⁺CD25^{bri} Treg cells with intracavitary administration of basiliximab *in vivo*

Targeting of CD4⁺CD25^{bri} Treg cells and CD4⁺CD25^{dim} activated T cells with intracavitary administration of basiliximab was analyzed by flow cytometry. Representative profiles of 0.01 mg/kg basiliximab administration (ELs from a 34-year-old gastric cancer patient) are shown in Fig. 3, and changes of the populations are summarized in Table 5. When 0.01 mg/kg basiliximab was administered, CD4⁺CD25^{bri} cells were targeted and almost completely reduced from 4.1 ± 2.4% to 0.1 ± 0.2% on days 1-3, and returned to 5.0 ± 1.7% on day 7. There was a significant difference between the values of day 0 and days 1-3 ($p < 0.01$).

Table 4. Efficacy of locoregional immunotherapy of malignant effusion using OK-432 in combination with basiliximab

Effusion	patient numbers (%)
Disappeared	2 (18)
Decreased	5 (45)
No response	4 (36)

Locoregional immunotherapy using basiliximab on day 0 followed by OK-432 on day 7 was performed to treat patients with malignant effusion. Responses were assessed clinically as disappeared effusion, decreased effusion, or no response.

However, the targeting of CD4⁺CD25^{dim} cells was also found with the administration of 0.01 mg/kg basiliximab, showing a decrease from 20.0 ± 5.9% to 5.0 ± 2.7% on days 1-3 ($p < 0.01$), and a return to 21.5 ± 7.9% on day 7. On the other hand, a reduced dose of 0.005 mg/kg basiliximab could still induce a significant decrease of CD4⁺CD25^{bri} cells from 1.8 ± 0.7% to 1.0 ± 0.9% on days 1-3 ($p < 0.05$), while preserving CD4⁺CD25^{dim} cells (18.7 ± 4.5% to 6.7 ± 4.6%, $p > 0.05$). The administration of 0.002 mg/kg basiliximab also showed similar changes in CD4⁺CD25^{bri} and CD4⁺CD25^{dim} cells.

Table 5. Targeting of CD4⁺CD25^{bri} Treg cells by intracavitary administration of basiliximab

Basiliximab (mg/kg)		CD4 ⁺ CD25 ^{bri} (%)	CD4 ⁺ CD25 ^{dim} (%)
0.01	day 0	4.1 ± 2.4	20.0 ± 5.9
	day 1-3	0.1 ± 0.2**	5.0 ± 2.7**
	day 7	5.0 ± 1.7	21.5 ± 7.9
0.005	day 0	1.8 ± 0.7	18.7 ± 4.5
	day 1-3	1.0 ± 0.9*	6.7 ± 4.6
	day 7	1.9 ± 0.6	16.7 ± 6.5
0.002	day 0	1.6 ± 0.2	25.0 ± 6.0
	day 1-3	0.2 ± 0.3*	17.0 ± 11.8
	day 7	1.8 ± 0.3	25.7 ± 6.0

Decreasing doses of basiliximab (0.01, 0.005, and 0.002 mg/kg for 6, 3, and 3 patients, respectively) were administered into the effusion cavity, and CD4⁺CD25^{bri} cells and CD4⁺CD25^{dim} cells were measured by flow cytometry. Significant differences from the values on day 0 were observed, * $p < 0.05$, ** $p < 0.01$.

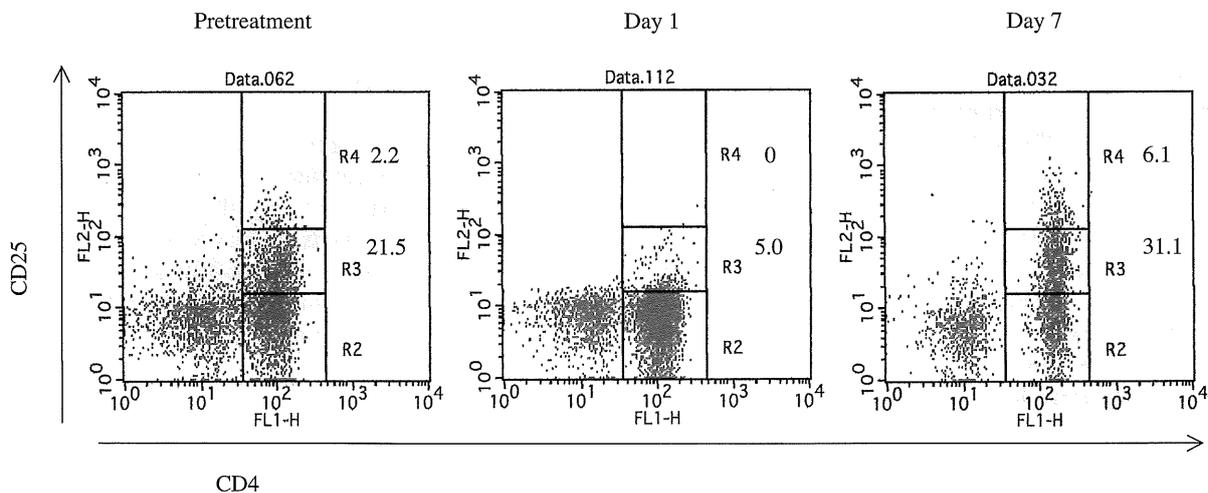


Fig. 3. Targeting of CD4⁺CD25^{bri} Treg cells by basiliximab in malignant effusion.

Basiliximab was administered into the cavity. ELs were collected on days 0, 1, and 7, and then subjected to flow cytometry for detecting CD4⁺CD25^{bri} Treg cells and CD4⁺CD25^{dim} activated T cells. A representative profile of a 34-year-old gastric cancer patient with malignant ascites is displayed.

Foxp3 mRNA expression after intracavitary administration of basiliximab *in vivo*

We analyzed the foxp3 mRNA expression of ELs prior to and after the intracavitary administration of basiliximab (Fig. 4). In 8 ELs investigated, foxp3 mRNA expression levels were decreased by basiliximab in 4 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 intracavitary administration of basiliximab. We also analyzed IFN- γ and IL-10 mRNA expression during the treatment. There were no 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 intracavitary administration of basiliximab

Phenotype analysis was performed for CD3⁺, CD4⁺, CD8⁺, and CD56⁺ lymphocytes in ELs during the intracavitary administration of basiliximab (Table 6). No significant changes at all were observed in the lymphocyte phenotypes of ELs on

Table 6. Phenotypic analysis of ELs after intracavitary administration of basiliximab

Phenotype	day 0	day 3	day 7
CD3	64 +/- 19	66 +/- 15	62 +/- 11
CD4	62 +/- 19	69 +/- 20	63 +/- 18
CD8	20 +/- 14	26 +/- 15	21 +/- 13
CD56	31 +/- 24	30 +/- 29	24 +/- 19

Basiliximab was administered into the effusion cavity, and flow cytometry was performed to detect phenotypes of ELs. Percentages of the positive population were shown as mean +/- standard deviation of 12 patients treated.

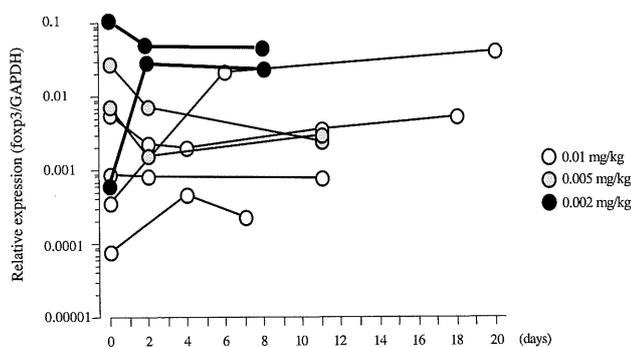


Fig. 4. Changes of foxp3 mRNA expression in ELs after basiliximab administration.

Basiliximab was administered into the cavity. ELs were collected as indicated, mRNA was extracted, and then RT-PCR analysis specific for foxp3 and GAPDH was performed on LightCycler. Ratios of foxp3 over GAPDH mRNA expression were plotted.

days 1-3 or on day 7 after intracavitary administration of basiliximab.

DISCUSSION

When using anti-CD25 antibody in cancer immunotherapy, the targeting only of CD4⁺CD25^{bri} Treg cells while preserving CD4⁺CD25^{dim} activated T cells is ideally required. Ko et al¹⁰ have demonstrated in an animal model that the co-administration of anti-CD25 antibody and anti-glucocorticoid-induced tumor necrosis factor-related protein (GITR) antibody, which is a marker specific for Treg cells, is less effective than anti-GITR treatment alone for tumor treatment by inhibiting Treg cells, because anti-CD25 antibody targets not only CD4⁺CD25^{bri} naturally occurring Treg cells but also CD4⁺CD25^{dim} activated T cells, which include tumor-specific cytotoxic T lymphocytes involved in tumor eradication. However, the dosage of basiliximab currently recommended for treating acute rejection reactions is a high dose (40-60 mg) that results in a long-lasting (more than one month) complete targeting of all CD25⁺ cells^{1,12}. Dannull et al⁴ have reported the effective elimination of Treg cells with the recombinant IL-2-diphtheria toxin conjugate DAB389IL-2 in treating patients with renal cell cancer using tumor RNA-transfected DC vaccines, where low concentration of 5 nM conjugates is optimal. Therefore, we hypothesized that low-dose anti-CD25 antibody might be sufficient to target CD4⁺CD25^{bri} Treg cells without down-modulating CD4⁺CD25^{dim} activated T cells, because CD25^{bri} cells express many more CD25 molecules on the cell surface than do CD25^{dim} cells.

In our *in vitro* experiments, we found expectedly that a low concentration of 0.01 μ g/ml basiliximab achieved preferential targeting of CD4⁺CD25^{bri} cells while preserving CD4⁺CD25^{dim} cells and, importantly, effective IFN- γ production of ELs. This implied the possibility of low-dose basiliximab administration *in vivo*. We therefore designed a clinical study to determine the optimal dose of intracavitary basiliximab that would achieve effective targeting of CD4⁺CD25^{bri} cells while preserving CD4⁺CD25^{dim} cells in malignant effusion. A selected starting dose was calculated of 0.01 mg/kg basiliximab, which could produce an effusion concentration of approximately 0.5 μ g/ml basiliximab, if 1000 ml of effusion was estimated to remain in the cavity after removal. We then conducted a dose-decreasing study design to reach basiliximab concentrations of less than 0.3 μ g/ml in the effusion, because basiliximab concentrations of more than 0.3 μ g/ml directly inhibited cytotoxic activity of ELs *in vitro*.

Surprisingly, this low-dose intracavitary administration of 0.01 mg/kg basiliximab was found to work effectively *in vivo*, resulting in the efficient

targeting of CD4⁺CD25^{bri} cells. However, the undesirable targeting of CD4⁺CD25^{dim} cells was also found with this dose of basiliximab. On the other hand, decreasing doses of 0.005 and 0.002 mg/kg basiliximab were still effective for the targeting of CD4⁺CD25^{bri} cells, while relatively preserving CD4⁺CD25^{dim} cells. We therefore conclude that the recommended dose of intracavitary administration of basiliximab for targeting CD4⁺CD25^{bri} Treg cells while preserving CD4⁺CD25^{dim} cells is 0.002 or 0.005 mg/kg, which may be dependent on residual effusion volumes estimated as less or more than 1000 ml, respectively.

Adverse events observed in this study were locoregional pain (25%) and fever elevation (100%), which were comparable with the locoregional administration of OK-432 alone and were well-tolerated and manageable with anti-inflammatory drugs²⁴). Moreover, the phenotypes of ELs including CD3⁺, CD4⁺, CD8⁺, and CD56⁺ cells were not affected by the basiliximab administration. These results indicate the safety of locoregional immunotherapy of malignant effusion using basiliximab followed by OK-432 administration.

The optimal timing for the addition of immunostimulating treatment such as OK-432 administration to the conditioning regimen using low-dose basiliximab treatment is an important issue. Kudo et al¹³) 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, indicating apparently that the targeting of Treg cells with anti-CD25 antibody is required at the beginning of inducing antitumor effector cells. Our study showed that the intracavitary administration of basiliximab effectively targeted Treg cells in effusion at least for the initial 3 days but no longer than 7 days, and that IFN- γ production of ELs could be augmented *in vitro* when ELs were stimulated on day 0 or on day 1 in the presence of basiliximab. Taken together, these results suggest that the locoregional immunotherapy of malignant effusion using OK-432 may be combined on day 0 or on day 1 with the Treg cell-conditioning regimen using low-dose basiliximab.

We obtained an effusion response of 63% in this study, which was highly comparable with that of OK-423 alone^{23,24}). In this study, basiliximab was administered on day 0 followed by OK-432 on day 7, the regimen of which may not be optimal. This treatment regimen was chosen because one of the endpoints of the study was to clarify immunological responses in effusion during 7 days after basiliximab administration. The true clinical response may be defined after a phase II trial, in which OK-432 is administered on day 0 or on day 1 in combi-

nation with the Treg cell-conditioning regimen of 0.002 or 0.005 mg/kg basiliximab administration.

It is an interesting question 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⁺CD25^{bri} cells in flow cytometric analysis after basiliximab administration does not indicate a physical depletion of cell numbers but only the masking of the CD25^{bri} cells escaping detection by flow cytometry using the second anti-CD25 antibody¹⁷). Moreover, Game et al⁷) 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, Kohm et al¹¹) have demonstrated an exacerbation of autoimmune encephalomyelitis by the administration of anti-CD25 antibody in an animal model, showing, in those mice, a decrease in the CD25^{bri} cell population but not in foxp3⁺ cells, which suggests that the injection of anti-CD25 antibody results in the functional inactivation, but not physical depletion, of Treg cells. 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 antibody blocks Treg cell function potentially through alteration in the expression of the glucocorticoid-induced tumor necrosis factor receptor-family gene¹¹). Taken together, these results suggest that the basiliximab treatment in the present study may have achieved down-modulation of Treg cell function, but not Treg cell depletion. The targeting of Treg cell function in effusion may nevertheless provide excellent conditions for the locoregional immunotherapy of malignant effusion using OK-432.

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)⁸) and anti-Her2 antibody (Trastuzumab)³) are 375 mg/m² 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 transplantation²¹). In a pig study, Lohse et al¹⁴) 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 the functionality of CD4 and CD8 molecules 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 targeting of Treg cells while preserving CD4⁺CD25^{dim} activated T cells, indicating that the low-dose approach is highly active for modulating the immune system. We believe that it is important to use the lowest possible 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 treatment¹⁴.

ACKNOWLEDGMENTS

The authors would like to thank Miss Y. Nakatani for her valuable assistance with the tissue culture.

(Received November 28, 2007)

(Accepted December 19, 2007)

REFERENCES

- Amlot, P.L., Rawlings, E., Fernando, O.N., Griffin, P.J., Heinrich, G., Schreier, M.H., Castaigne, J.P., Moore, R. and Sweny, P. 1995. Prolonged action of a chimeric interleukin-2 receptor (CD25) monoclonal antibody used in cadaveric renal transplantation. *Transplantation* **60**: 748-756.
- Belardelli, F., Ferrantini, M., Parmiani, G., Schlom, J. and Garaci, E. 2004. International meeting on cancer vaccines: How can we enhance efficacy of therapeutic vaccines? *Cancer Res.* **64**: 6827-6830.
- Cobleigh, M.A., Vogel, C.L., Tripathy, D., Robert, N.J., Scholl, S., Fehrenbacher, L., Wolter, J.M., Paton, V., Shak, S., Lieberman, G. and Slamon, D.J. 1999. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J. Clin. Oncol.* **17**: 2639-2648.
- Dannull, J., Su, Z., Rizzieri, D., Yang, B.K., Coleman, D., Yancey, D., Zhang, A., Dahm, P., Chao, N., Gilboa, E. and Vieweg, J. 2005. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J. Clin. Invest.* **115**: 3623-3633.
- DeLong, P., Carroll, R.G., Henry, A.C., Tanaka, T., Ahmad, S., Leibowitz, M.S., Serman, D.H., June, C.H., Albelda, S.M. and Vonderheide, R.H. 2005. Regulatory T cells and cytokines in malignant pleural effusions secondary to mesothelioma and carcinoma. *Cancer Biol. Ther.* **4**: 342-346.
- Dudley, M.E., Wunderlich, J.R., Yang, J.C., Sherry, R.M., Topalian, S.L., Restifo, N.P., Royal, R.E., Kammula, U., White, D.E., Mavroukakis, S.A., Rogers, L.J., Gracia, G.J., Jones, S.A., Mangiameli, D.P., Pelletier, M.M., Gea-Banacloche, J., Robinson, M.R., Berman, D.M., Filie, A.C., Abati, A. and Rosenberg, S.A. 2005. Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. *J. Clin. Oncol.* **23**: 2346-2357.
- Game, D.S., Hernandez-Fuentes, M.P. and Lechler, R.I. 2005. Everolimus and basiliximab permit suppression by human CD4⁺CD25⁺ cells in vitro. *Am. J. Transplant.* **5**: 454-464.
- Grillo-Lopez, A.J., White, C.A., Varns, C., Shen, D., Wei, A., McClure, A. and Dallaire, B.K. 1999. Overview of the clinical development of rituximab: first monoclonal antibody approved for the treatment of lymphoma. *Semin. Oncol.* **26**: 66-73.
- Katoh, M. 2002. Expression of human SOX7 in normal tissues and tumors. *Int. J. Mol. Med.* **9**: 363-368.
- Ko, K., Yamazaki, S., Nakamura, K., Nishioka, T., Hirota, K., Yamaguchi, T., Shimizu, J., Nomura, T., Chiba, T. and Sakaguchi, S. 2005. Treatment of advanced tumors with agonistic anti-GITR mAb and its effects on tumor-infiltrating Foxp3⁺CD25⁺CD4⁺ regulatory T cells. *J. Exp. Med.* **202**: 885-891.
- Kohm, A.P., Podojil, J.R., Williams, J.S., McMahon, J.S. and Miller, S.D. 2005. CD28 regulates glucocorticoid-induced TNF receptor family-related gene expression on CD4⁺ T cells via IL-2-dependent mechanisms. *Cell Immunol.* **235**: 56-64.
- Kovarik, J., Wolf, P., Cisterne, J.M., Mourad, G., Lebranchu, Y., Lang, P., Bourbigot, B., Cantarovich, D., Girault, D., Gerbeau, C., Schmidt, A.G. and Souillou, J.P. 1997. Disposition of basiliximab, an interleukin-2 receptor monoclonal antibody, in recipients of mismatched cadaver renal allografts. *Transplantation* **64**: 1701-1705.
- Kudo-Saito, C., Schlom, J., Camphausen, K., Coleman, C.N. and Hodge, J.W. 2005. The requirement of multimodal therapy (vaccine, local tumor radiation, and reduction of suppressor cells) to eliminate established tumors. *Clin. Cancer Res.* **11**: 4533-4544.
- Lohse, L., Nielsen, J. and Eriksen, L. 2006. Long-term treatment of pigs with low doses of monoclonal antibodies against porcine CD4 and CD8 antigens. *A.P.M.I.S.* **114**: 23-31.
- Matsuura, M., Yamaguchi, Y., Ueno, H., Osaki, A., Arihiro, K. and Toge, T. 2006. Maturation of dendritic cells and T cell responses in sentinel nodes in breast cancer patients. *Cancer* **106**: 1227-1236.
- Okita, R., Saeki, T., Takashima, S., Yamaguchi, Y. and Toge, T. 2005. CD4⁺CD25⁺ regulatory T cells in the peripheral blood of patients with breast cancer and non-small cell lung cancer. *Oncol. Rep.* **14**: 1269-1273.
- Orentas, R.J., Kohler, M.E. and Johnson, B.D. 2006. Suppression of anti-cancer immunity by regulatory T cells: Back to the future. *Cancer Biol.* **16**: 137-149.
- Phan, G.Q., Yang, J.C., Sherry, R.M., Hwu, P., Topalian, S.L., Schwartzentruber, D.J., Restifo, N.P., Haworth, L.R., Seipp, C.A., Freezer, L.J., Morton, K.E., Mavroukakis, S.A., Duray, P.H., Steinberg, S.M., Allison, J.P., Davis, T.A. and Rosenberg, S.A. 2003. Cancer regression and auto-

- immunity induced by CTLA-4 blockade in patients with metastatic melanoma. *Proc. Natl. Acad. Sci. U.S.A.* **100**: 8372-8377.
19. **Ribas, A., Camacho, L.H., Lopez-Berestein, G., Pavlov, D., Bulanhagui, C.A., Millham, R., Comin-Anduix, B., Reuben, J.M., Seja, E., Parker, C.A., Sharma, A., Glaspy, J.A. and Gomez-Navarro, J.** 2005. Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. *J. Clin. Oncol.* **23**: 8968-8977.
 20. **Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M.** 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of single mechanism of self-tolerance causes various autoimmune disease. *J. Immunol.* **155**: 1151-1164.
 21. **Segovia, J., Rodriguez-Lambert, J.L., Crespo-Leiro, M.G., Almenar, L., Roig, E., Gomez-Sanchez, M.A., Lage, E., Manito, N. and Alonso-Pulpon, L.** 2006. A randomized multicenter comparison of basiliximab and muromonab (OKT3) in heart transplantation: SIMCOR study. *Transplantation* **81**: 1542-1548.
 22. **Yamaguchi, T. and Sakaguchi, S.** 2006. Regulatory T cells in immune surveillance and treatment of cancer. *Seminars in Cancer Biology* **16**: 115-123.
 23. **Yamaguchi, Y., Miyahara, E., Ohshita, A., Kawabuchi, Y., Ohta, K., Shimizu, K., Minami, K., Hihara, J., Sawamura, A. and Toge, T.** 2003. Locoregional immunotherapy of malignant effusion from colorectal cancer using the streptococcal preparation OK-432 plus interleukin-2: Induction of autologous tumor-reactive CD4+ Th1 killer lymphocytes. *Br. J. Cancer* **89**: 1876-1884.
 24. **Yamaguchi, Y., Ohshita, A., Kawabuchi, Y., Hihara, J., Miyahara, E., Noma, K. and Toge, T.** 2004. Locoregional immunotherapy of malignant ascites from gastric cancer using DTH-oriented doses of the streptococcal preparation OK-432: Treatment of Th1 dysfunction in the ascites microenvironment. *Int. J. Oncol.* **24**: 959-966.