Regulatory T-Cells Are Possible Effect Prediction Markers of Immunotherapy for Cancer Patients

JUNJI WADA[1], AKIO YAMASAKI[1], SHUNTARO NAGAI[1], KOUSUKE YANAI[1], KOUTA FUCHINO[2], CHIZU KAMEDA[1], HARUO TANAKA[1], KENICHIRO KOGA[1], HIROSHI NAKASHIMA[1], MASAFUMI NAKAMURA[1], MASAO TANAKA[3], MITSUO KATANO[1] and TAKASHI MORISAKI[2]

Departments of [1]Cancer Therapy and Research, and [3]Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka; [2]Yakuin CA Clinic, Fukuoka, Japan

Abstract. *We previously showed that a combination therapy with tumor cell-pulsed monocyte-derived dendritic cells (DCs) and activated lymphocytes was well tolerated in patients with disseminated carcinomas. Recently, accumulating evidence has indicated that regulatory T-cells (Tregs), a unique population of $CD4^+$ T-cells, are increased in patients with several advanced malignancies and prevent cell-mediated immune responses against tumors. However, reports analyzing the relationship between the Tregs population and the effects of immunotherapy are extremely rare. In the present study, 22 patients received an intravenous injection of DC-activated lymphocytes (DAK) and/or a subcutaneous injection of tumor-pulsed DCs (DC vaccine) every 2 to 4 weeks. The Tregs were defined based on their expression of CD4, CD25 and FOXP3, a transcription factor. Most $CD4^+CD25^{high}$ T-cells expressed FOXP3. Therefore, $CD4^+CD25^{high}$ T-cells were evaluated as Tregs in the present study. As reported previously, the percentage of Tregs (% Tregs) among total $CD4^+$ T-cells in peripheral blood mononuclear cells (PBMCs) was significantly higher for advanced cancer patients than for healthy volunteers. When the patients were divided into three groups according to their survival time, i.e. 12 short-survival patients, 4 medium-survival patients and 6 long-survival patients, the % Tregs of the long-survival patients before the therapy was significantly lower than that of the short-survival patients ($p=0.026$). The % Tregs decreased after the therapy, although the difference did not reach statistical significance. When the patients were divided into a high group (>4.99%: 7 patients) and a low group (<4.99%: 15 patients) according to their % Tregs before the therapy, the survival times of the two groups differed significantly ($p=0.0034$). These data suggest that the % Tregs among the PBMCs might be used as an effect prediction factor of immunotherapy for patients with advanced cancer.*

Recent accumulating evidence has indicated the existence of a unique $CD4^+$ T-cell population, designated regulatory T-cells (Tregs) (1-3). Tregs were originally identified as $CD4^+$ T-cells that constitutively express the interleukin (IL)-2 receptor α-chain (CD25) (4) and account for 5-10% of the total $CD4^+$ T-cell population. More recent studies have shown that the transcription factor forkhead box P3 (FOXP3) is not only a key intracellular marker, but also a crucial developmental and functional factor for $CD4^+CD25^+$ Tregs (5-7). Therefore, it is now generally considered that $CD4^+$ T-cells expressing both CD25 and FOXP3 are Tregs.

The human immune system consists of an elegant balance between immune surveillance and immune ignorance of self-antigens. Tregs have been shown to contribute to the prevention of autoimmune disorders by controlling the activity of autoreactive T-cells (2, 8, 9). In other words, Tregs are considered to act as players in immune tolerance against self-antigens. Tregs are increased in the peripheral blood and cancer tissues in several types of advanced cancer (10-14), and these increases in Tregs have been proposed to play critical roles in immune tolerance against malignancies (15), since most tumor-associated antigens are self-antigens. Importantly, it has been shown that increased numbers of Tregs were associated with increasing tumor burden, and that removal of the Tregs resulted in enhanced antitumor immune responses (16). Based on these investigations, several

Abbreviations: Tregs, regulatory T-cells; Foxp3, forkhead box protein P3; FACS, fluorescence-activated cell sorting; DC, dendritic cells; DAK, dendritic cells-activated lymphocytes; PBMCs; peripheral blood mononuclear cells.

Correspondence to: Mitsuo Katano, Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel: +81 92 6426941, Fax: +81 92 6426221, e-mail: mkatano@tumor.med.kyushu-u.ac.jp

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Table I. *Profiles of cancer patients.*

Case no.	Age (years)/gender	Primary site	Site of metastasis	Surgery	Previous therapy	Immunotherapy
1	59/F	Stomach	Pt, ovary, bone	+	Chemotherapy	DAK
2	56/M	Lung	Lung, bone, LN	–	Radiation	DAK
3	64/F	Pancreas	Pt, liver	+	Chemotherapy	DAK
4	63/F	Skin	Pt, LN	–	Chemotherapy	DAK+DC
5	70/M	Kidney	Lung, bone, LN	+	Chemotherapy	DAK+DC
6	70/M	Brain	LN	+	Chemotherapy	DAK+DC
7	65/F	Pancreas	Pt	–	Chemotherapy	DAK
8	58/M	Bile duct	Liver, bone	+	Chemotherapy	DAK
9	60/M	Bile duct	Pt, liver	+	Chemotherapy	DAK
10	77/M	Stomach	Pt	+	Chemotherapy	DAK
11	49/F	Colon	Liver, lung, LN	+	Chemotherapy	DAK
12	79/M	Lung	Pl	+	Chemotherapy + radiation	DAK
13	73/M	Liver	Pt, liver, bone	+	Chemotherapy	DAK+DC
14	54/F	Unknown	Brain, lung	–	Chemotherapy + radiation	DAK
15	38/F	Ovary	Pt	+	Chemotherapy	DAK+DC
16	76/M	Lung	Pl	+	Chemotherapy	DAK
17	31/M	Colon	Liver, LN	+	Chemotherapy	DAK
18	52/F	Ovary	Pt, LN	+	Chemotherapy	DAK+DC
19	53/F	Breast	Bone	+	Chemotherapy	DAK+DC
20	66/F	Pancreas	Pt	+	Chemotherapy	DAK+DC
21	63/M	Liver	Lung	+	Chemotherapy	DAK+DC
22	65/F	Stomach	Pt	+	Chemotherapy	DAK

Pt: Peritoneum; Pl: pleural membrane; LN: lymph nodes; DC: DC vaccine; DAK: DC-activated lymphocytes.

clinicians are now planning depletion of Tregs to improve the therapeutic effect of tumor immunotherapies such as monocyte-derived dendritic cell (DC)-based vaccine therapy and activated lymphocyte infusion therapy (17, 18). In fact, several reports have shown that depletion of Tregs resulted in increased immune responses toward tumors in animal tumor models (19-22). However, reports analyzing the relationships between the quantity of Tregs and the effects of DC-based immunotherapy in clinical settings are extremely rare (23).

The main purpose of the present study was to examine whether measurement of Tregs in peripheral blood mononuclear cells from cancer patients could represent a marker for predicting the therapeutic effect of immunotherapy.

Patients and Methods

Patients. Twenty-two inoperable cancer patients with multiple metastases were examined according to a protocol approved by the Kyushu University Ethics Committee, Japan. The inclusion criteria were: histologically confirmed cancer, not amenable to cure by any standard therapy; performance status of 0, 1 or 2 on the ECOG scale; a minimum estimated life expectancy of 3 months; adequate hematological, hepatic and renal function; age of >18 years and the presence of obtainable tumor cells. The clinical details of the patients are summarized in Table I.

Study design. Each patient received an intravenous injection of 1-10×10^8 tumor-pulsed DC-activated lymphocytes (DAK) and/or a subcutaneous injection of 2-30×10^6 mature DCs loaded with necrotic autologous tumor cells (DC vaccine) into the left supraclavicular area every 2 or 4 weeks. In principle, this immunotherapy was continued for as long as possible in the outpatient clinic.

Preparation of DAK and DC vaccine. Autologous tumor-pulsed DCs (DC vaccine) were prepared as described previously (24). Briefly, immature DCs were prepared from PBMCs using recombinant human granulocyte/monocyte colony-stimulating factor (GM-CSF; 200 ng/ml; Novartis Pharma, Basel, Switzerland) and recombinant human IL-4 (500 U/ml; Ono, Tokyo, Japan) for 7 days. Tumor cells obtained from the tumor masses or malignant effusions were lysed by five freeze-thaw cycles (necrotic tumor cells). The immature DCs were incubated with the necrotic tumor cells overnight and then cultured for 2 days in medium containing tumor necrosis factor α (TNF-α; 1000 U/ml; R&D Systems, Minneapolis, MN, USA) and prostaglandin E2 (PGE2; 1 μg/ml; Sigma, St. Louis, MO, USA).

For preparation of the DAKs, non-adherent cells among each patient's PBMCs were cultured with tumor-pulsed DCs for 1 week in Hy-medium containing 175 JRU/ml of human recombinant IL-2 (Nipro, Tokyo, Japan).

Flow cytometry analysis. Both $CD4^+CD25^{high}$ and $CD4^+FOXP3^+$ T-cells are generally considered to be Tregs. In order to identify the Tregs more precisely, correlation analyses between $CD4^+CD25^{high}$ and $CD4^+FOXP3^+$ T-cells were performed using PBMCs obtained from 10 healthy volunteers and the 22 advanced cancer patients. The population of $CD4^+CD25^{high}$ T-cells was determined by flow cytometric analysis by a three-color staining method as described previously (11, 25). The PBMCs were stained with anti-CD25, anti-CD4 and anti-CD8 antibodies (BD

Biosciences, Tokyo, Japan). Intracellular staining of FOXP3 was conducted using a PE-conjugated anti-human FOXP3 Staining Set (clone PCH101; e-Bioscience, San Diego, CA, USA) according to the manufacturer's instructions. Two- or three-color flow cytometry was performed using a FACSCalibur™ (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). The $CD25^{high}$ cells or $FOXP3^{+}$ cells after gating of $CD4^{+}$ lymphocytes were evaluated as Tregs. The percentage of Tregs among the total $CD4^{+}$ T-cells was represented as % Tregs.

Statistical analysis. Fisher's exact probability test was used for the statistical analyses. The data were analyzed with a SAS statistical software package (Abacus Concepts, Berkeley, CA, USA). Values of $p<0.05$ were considered to indicate statistical significance. The estimated probability of survival was calculated using the Kaplan-Meier method. The Mantel Cox log-rank test was used to compare curves between the high and low Tregs.

Results

Clinical details of cancer patients. All 22 patients were evaluated as having progressive disease at the time of entry into the immunotherapy (Table I). Overall, 19 patients had received prior second-line chemotherapy, 1 patient had received prior radiotherapy, 2 patients had received prior combination therapy with chemotherapy and radiotherapy, and 18 patients had received prior surgery. All 22 patients received DC-based immunotherapy for as long as possible in the outpatient clinic. Briefly, 9 patients received combination therapy with an intravenous injection of DAK and DC vaccine, while 13 patients received DAK alone. No severe adverse reactions except for low-grade fever occurred during the observation period. As we evaluated before initiation of the therapy (inclusion criteria), all the patients survived for more than 3 months after the therapy (Table II). Subsequently, 16 patients died within 23 months of the therapy, and 6 patients currently survive, more than 28 months after the therapy.

% Tregs in cancer patients and healthy volunteers. Representative flow cytometric data from a stage IV gastric cancer patient are shown (Figure 1A and 1B). Overall, 5% and 6% of the total $CD4^{+}$ T-cells were $CD25^{low}$ and $CD25^{high}$ T-cells, respectively (Figure 1A). Most of the $CD4^{+}CD25^{high}$ T-cells were also $FOXP3^{+}$ and most of the $CD25^{-/low}$ cells were $FOXP3^{-}$ (Figure 1B). When the populations of $CD4^{+}CD25^{high}$ and $CD4^{+}FOXP3^{+}$ T-cells were expressed as percentages of the total $CD4^{+}$ T-cells, $CD4^{+}CD25^{high}$ and $CD4^{+}FOXP3^{+}$ T-cells showed a significant positive correlation ($p<0.001$; Figure 1C). Therefore, the $CD4^{+}CD25^{high}$ T-cells were defined as Tregs in the present study. Representative flow cytometric data from a normal volunteer and a lung cancer patient are shown in Figure 2A. The number of Tregs was significantly higher in the cancer patient than in the normal volunteer.

Table II. *Relationship between % Tregs and survival time after the immunotherapy.*

Case no.	Age/ gender	Survival time (Months)	Status	% Tregs	Immunotherapy
Short-survival group (12 patients)					
8	58/M	4	Death	2.75	DAK
9	60/M	4	Death	6.66	DAK
4	63/F	5	Death	3.73	DAK + DC
11	49/F	6	Death	9.16	DAK
3	64/F	6	Death	8.66	DAK
10	77/M	6	Death	5.71	DAK
2	56/M	7	Death	11.98	DAK
5	70/M	7	Death	8.40	DAK + DC
6	70/M	7	Death	2.96	DAK + DC
1	59/F	9	Death	1.35	DAK
7	65/F	9	Death	4.91	DAK
12	79/M	11	Death	3.14	DAK
Medium-survival group (4 patients)					
13	73/M	12	Death	5.85	DAK + DC
14	54/F	12	Death	3.01	DAK
15	38/F	16	Death	4.89	DAK + DC
16	76/M	23	Death	2.58	DAK
Long-survival group (6 patients)					
17	31/M	28	Alive	3.44	DAK
18	52/F	32	Alive	2.05	DAK + DC
19	53/F	34	Alive	4.27	DAK + DC
20	66/F	34	Alive	4.73	DAK + DC
21	63/M	36	Alive	2.93	DAK + DC
22	65/F	38	Alive	2.83	DAK

DC: DC vaccine DAK: DC-activated lymphocytes.

The % Tregs of the total $CD4^{+}$ T-cells among the PBMCs from the 22 cancer patients before DC-based immunotherapy was significantly higher than that among the PBMCs from the healthy volunteers (Figure 2B).

Relationship between survival prognosis and % Tregs among PBMCs before DC-based immunotherapy. The 22 patients were divided into three groups according to their survival time after the therapy (Table II). Overall, 12 patients died within 11 months after the therapy (short-survival patients) and 6 patients survived for more than 24 months after the therapy (long-survival patients). The remaining 4 patients survived for more than 11 months, but less than 24 months after the therapy (medium-survival patients). Nine out of the 12 short-survival patients, 2 out of the 4 medium-survival patients and 2 out of the 6 long-survival patients received the DAK therapy alone. The other patients received the DAK+DC vaccine therapy.

When the % Tregs values before the therapy were compared among the three survival groups, the % Tregs was significantly lower for the long-survival patients than for the

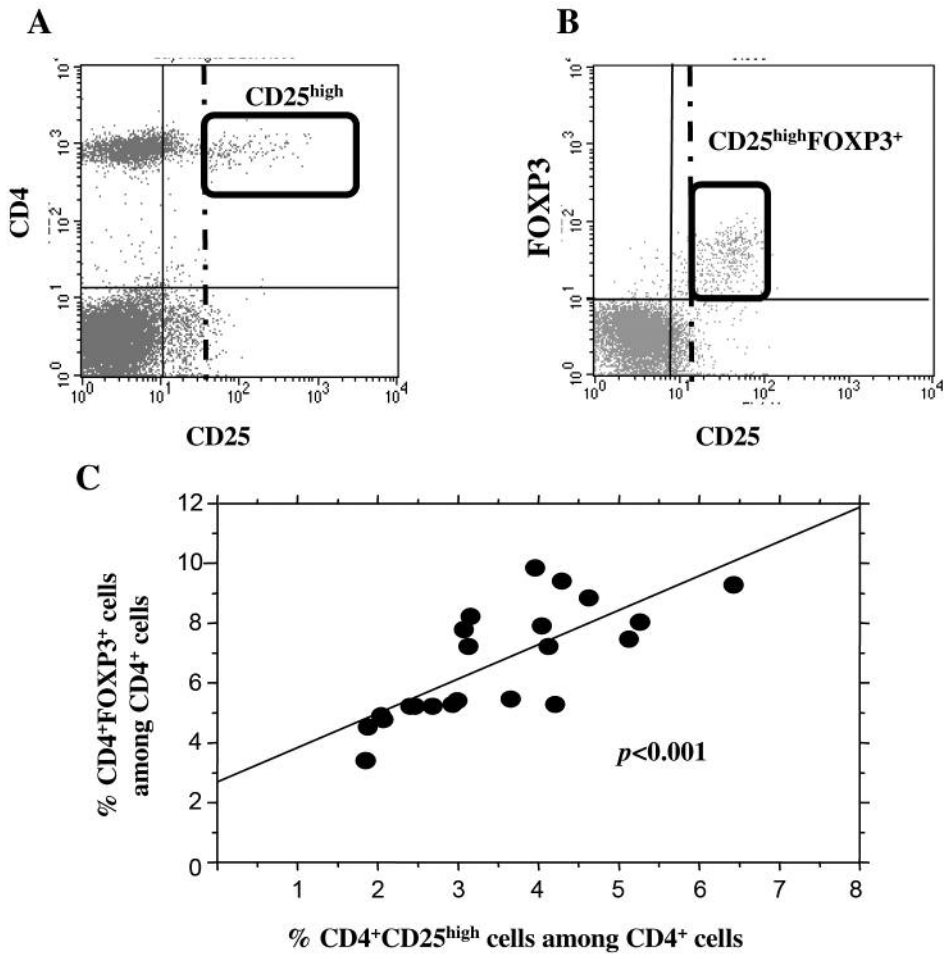
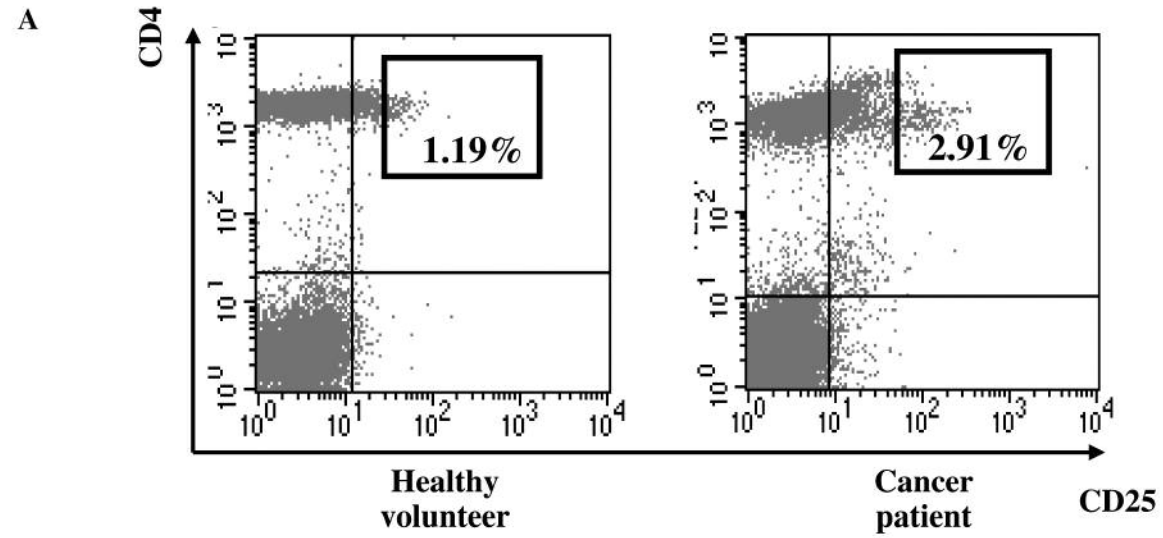


Figure 1. *$CD4^+CD25^{high}$ T-cells and $CD4^+FOXP3^+$ T-cells existing in PBMCs. A, PBMCs were stained with anti-CD25 and anti-CD4 antibodies and analyzed by 2-color flow cytometry. Representative data showing $CD25^{high}$ and $CD25^{low}$ populations in $CD4^+$T-cells. B, PBMCs were stained with anti-CD25, anti-CD4 and FOXP3 antibodies and analyzed by 3-color flow cytometry. Representative data shows the expression of CD25 and FOXP3 within the $CD4^+$T-cells. C, Correlation between the percentage of $CD25^{high}$ T-cells and $FOXP3^+$ T-cells to total $CD4^+$T-cells analyzed by Spearman's correlation using PBMCs from 12 cancer patients and 9 healthy volunteers.*

short-survival patients (p=0.026; Figure 3A). At 3 months after the therapy, the % Tregs was measured again in 7 short-survival patients, 2 medium-survival patients and 6 long-survival patients (Figure 3B). The % Tregs values tended to decrease after the therapy in all three groups. However, the differences did not reach statistical significance (Figure 3B). When the % Tregs values before and after the therapy were compared in the individual patients, 5, 1 and 1 of the 7 short-survival patients showed decreased, no change and increased values after the therapy (Figure 4, left panel), respectively. The 2 medium-survival patients showed decreased and increased values after the therapy, respectively (Figure 4, middle panel). Among the 6 long-survival patients, 2, 1 and 3 patients showed decreased values, no change and increased values after the therapy (Figure 4, right panel), respectively.

To determine whether the % Tregs before therapy was a prognostic factor for patient survival, Kaplan-Meier estimates were calculated and a log-rank test was performed. The % Tregs value before the therapy was dichotomized at 4.98% on the basis of the mean value of the 22 patients (4.98±2.69%) or approximately 2-fold the mean value of the healthy volunteers. The difference in survival time between the high % Tregs (>4.98%; n=7) and low % Tregs (<4.98%; n=15) groups was significant (p=0.003%; Figure 5A). To investigate whether the different immunotherapy regimens (DAK alone or DAK+DC vaccine) affected the prognosis, the % Tregs for each therapy was divided as above. For the DAK alone therapy, the Kaplan-Meier survival curves of the high % Tregs (n=5) and low % Tregs (n=8) groups



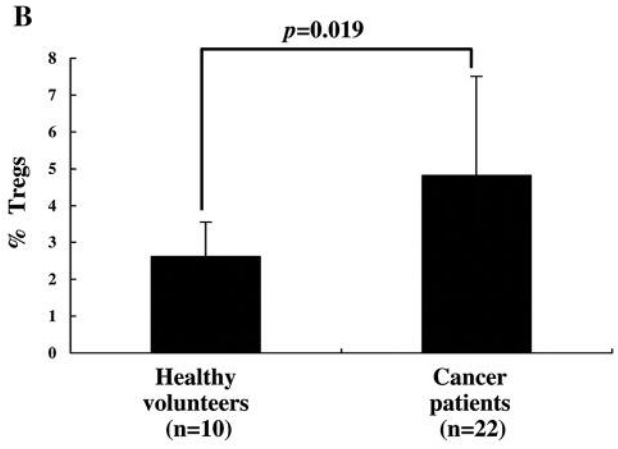


Figure 2. *Measurement of Tregs in PBMCs by 2-color flow cytometry analysis. A, Left panel: representative flow cytometric data from a healthy volunteer. Right panel: representative flow cytometric data from a patient with lung cancer. B, Tregs in PBMCs from 10 healthy volunteers and 22 cancer patients listed in Table I as determined by 2-color flow cytometry. % Tregs are represented as the percentage of Tregs among the total CD4+ T-cells.*

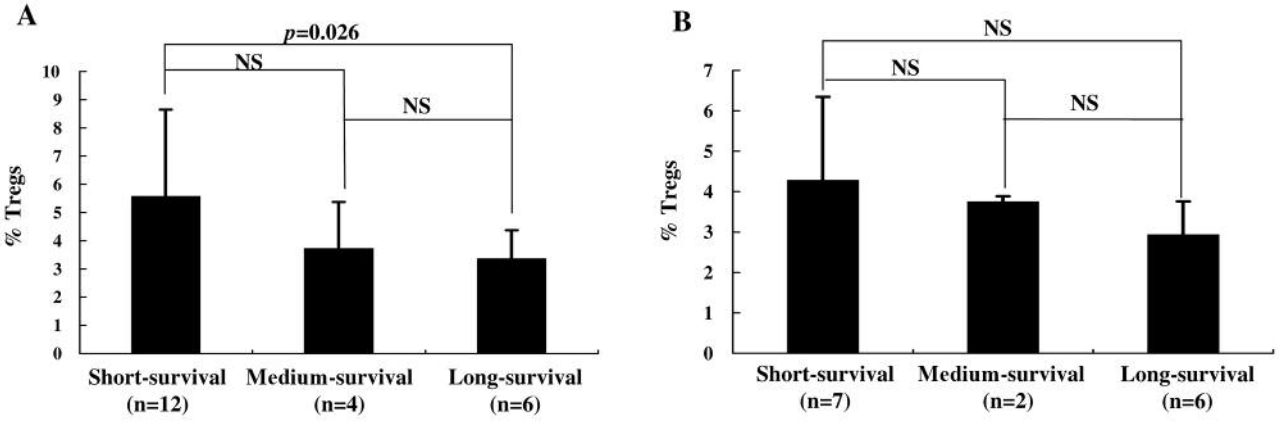


Figure 3. *Relationship between % Tregs and survival time. A, Relationship between % Tregs before the immunotherapy and survival time. Patients were divided into three groups according to survival time after the immunotherapy. % Tregs are represented as a percentage of total CD4+T-cells. B, Relationship between % Tregs after the immunotherapy and survival time. % Tregs were re-examined at 3 months after the immunotherapy.*

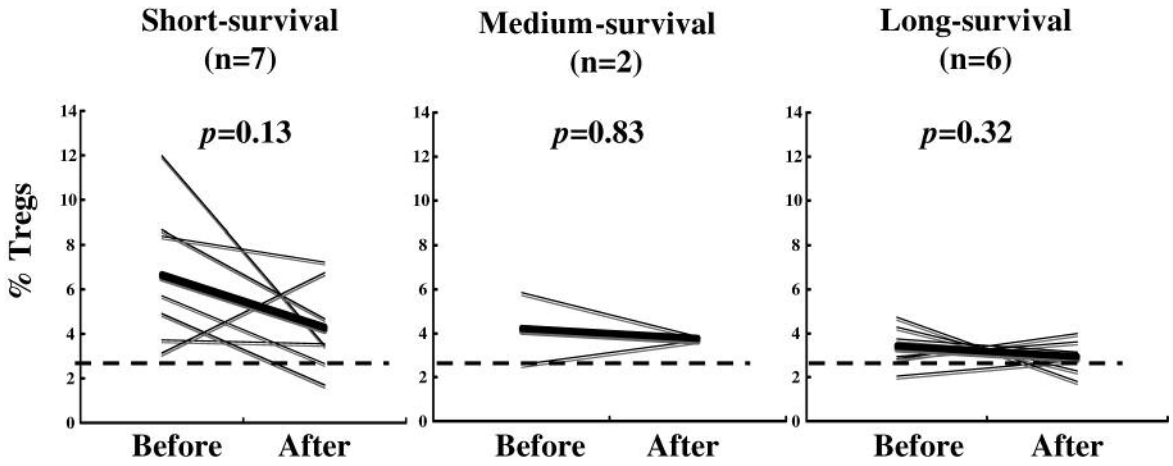
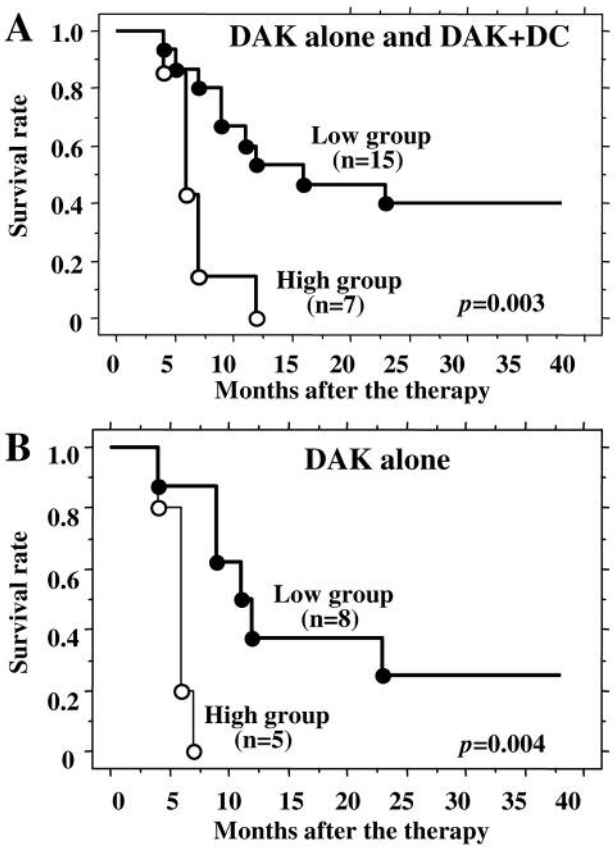


Figure 4. *Change of % Tregs before and after the immunothyerapy. % Tregs before and after the immunotherapy was compared in individuals (thin lines) of three survival groups. Thick line: average of % Tregs before and after the immunotherapy. Dotted line: average of % Tregs from 10 healthy volunteers.*



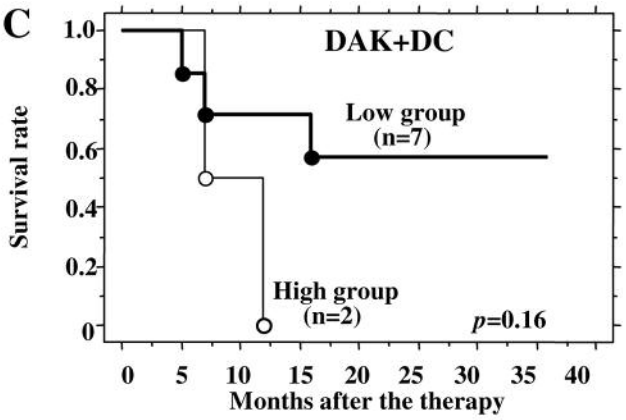


Figure 5. *Relationship between % Tregs before the immunotherapy and survival prognosis. Patients were divided into two groups: high % Tregs group and low % Tregs group. % Tregs was dichotomized at 4.98% on the basis of the mean value of the 22 patients or approximately 2-fold the mean value of the 10 healthy volunteers.*

differed significantly (p=0.004; Figure 5B). On the other hand, for the DAK+DC vaccine therapy, the Kaplan-Meier survival curves of the high % Tregs (n=2) and low % Tregs (n=7) groups showed no significant difference (p=0.16: Figure 5C).

Discussion

The present study revealed for the first time that the % Tregs in the PBMCs before therapy may be an effect prediction parameter of immunotherapy for advanced cancer patients.

It was not easy to obtain a suitable clinical outcome for our cancer patients by immunotherapy alone, since most of our patients had very advanced-stage tumors that were evaluated as being resistant to standard therapies. In fact, in our previous phase I/II study for patients with disseminated carcinomas, Kaplan-Meier survival analysis indicated that <50% of patients would survive for more than 1 year after combination therapy with DCs and CD3-activated lymphocytes (26). This implies the necessity of selecting patients in whom immunotherapy can bring about therapeutic benefits, such as prolongation of the survival period.

In the present study, the $CD4^+$ T-cells showing high expression of CD25, *i.e.* $CD4^+CD25^{high}$ T-cells were evaluated as Tregs, since most of the $CD4^+CD25^{high}$ T-cells also expressed FOXP3 (Figure 1C). When the relationship between the % Tregs value before the therapy and the survival period was analyzed, the % Tregs of long-survival patients was significantly lower than that of short-survival patients (Figure 3A). Importantly, the % Tregs of long-survival patients was very similar to that of the healthy volunteers (Figures 2B and 3A). In addition, some patients showed a significant decrease in the % Tregs after the therapy, even among the short-survival patients showing a high % Tregs before the therapy (Figure 4, left panel). These findings suggested that the % Tregs before the therapy may be useful as an effect prediction marker in immunotherapies for advanced cancer patients. As expected, when the patients showing >4.98% Tregs were evaluated as high Tregs patients on the basis of approximately 2-fold the mean % Tregs of healthy volunteers, the survival time was significantly shorter than that of the low Tregs patients (Figure 5A). In particular, the data indicate that the % Tregs may be an effect prediction marker in DAK alone therapy (Figure 5B). On the other hand, a similar result was not demonstrated in the patients treated with DAK+DC vaccine (Figure 5C). Thus no definite answer concerning this point was established. Only a few of the short-survival patients (3 out of the 12 patients) compared with the long-survival patients (4 out of the 6 patients) received the DC vaccine (Table II), which may have affected the results. However, it is still unclear whether % Tregs is an effective prediction marker for the DC vaccine. Nevertheless, for the first time, a possible clinical application was shown for Tregs in immunotherapy. In addition, it is noteworthy that the % Tregs changed after these immunotherapies.

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