Improvement of Impaired Immunological Status of Patients with Various Types of Advanced Cancers by Autologous Immune Cell Therapy

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Abstract. *We evaluated the immunological status of patients with various solid tumors by flow cytometry of immune cell populations and their frequencies in peripheral blood samples. The change in immunological status was also analyzed in patients given autologous immune cell therapy, such as αβT cell, γδT cell, NK cell or DC vaccine therapy. The frequency of regulatory T-cells (Tregs) was shown to be high in patients with cancers of the lung (squamous carcinoma cells), head and neck, esophagus and uterus, although there were no significant differences in effector cell population or Th1/2 ratio between various types of cancers except for a few. The cellular immunological status was impaired in most patients with advanced solid tumors before immune cell therapy and the impaired T-cell immune status was restored by infusion of effector cells, such as αβT cells or γδT cells, although the number of NK cells in the peripheral blood did not always increase after autologous NK cell therapy. The concurrent αβT cell therapy and DC vaccine therapy could successfully increase the number of CD8+ T-cells in the peripheral blood of patients with various types of cancers. Two or three injections of αβT cells could potentially reduce Tregs frequency prior to DC vaccine, as well as the concurrent αβT cell and DC vaccine therapy. However, an increase in the Tregs frequency was observed in some patients who received NK cell therapy. These findings suggest that it is necessary to include or combine certain types of immune cell therapy when the Tregs frequency of cancer patients is high before or after autologous immune cell therapy.*

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Immune cell therapy is one of the regenerative medical treatments in which autologous or allogeneic cells are cultured and processed *ex vivo* and utilized for the treatment of various diseases (1). Autologous immune cell therapy is potent for various malignancies, such as the clinical use of sipuleucel-T for prostate cancer (2). Sipuleucel-T was approved for personalized immune cell therapy for metastatic hormone-refractory prostate cancer by the U.S. Food and Drug Administration in 2010. For patients with various malignancies, several methods of immune cell therapy have become available, such as adoptive T-cell immunotherapy using *ex vivo*-expanded cytotoxic T lymphocytes (3-5). Furthermore, dendritic cell (DC)-based vaccines have also been employed in clinical trials or practice of cancer immunotherapy (6, 7).

The Seta Clinic Group is one of the pioneers that introduced immune cell therapy against cancer in Japan and provides several types of autologous immune cell therapy, such as αβT cell, γδT cell, natural killer (NK) cell or DC vaccine therapy (8). In a previous study, it was shown that αβT cell therapy increased the number of T-cell subsets but not that of B or NK cells. Additionally, the number and percentage of regulatory T-cells (Tregs) decreased significantly in the patients who received αβT cell therapy (9). We also reported a survival advantage in advanced lung cancer patients who received adoptive T cell immunotherapy using αβT cells (10). For the adjuvant therapy of lung cancer patients, adoptive activated T lymphocyte immunotherapy has been found to have the potency to yield favorable effects in combination with other conventional therapies, such as chemotherapy or radiotherapy (11). The repeated infusions of activated $CD8^+$ T-cells could contribute to the increase in cytotoxic activity in a patient's immune system and the infused autologous T lymphocytes, including central memory $CD8^+$ T-cells, may be restored in the body with the increased number of $CD8^+$ T-cells and improve the inverted CD4/CD8 T-cell ratio in the peripheral blood (9). These findings

suggest that, for αβT cell therapy, $CD8^+$ T-lymphocytes actively proliferate and become the predominant cell type (over 60% on average) during the culture period, thus showing their potency to promote killer activities and decrease the number of Tregs.

Recently, the adoptive infusion of innate immune cells, such as NK cells or γδT cells, has also been investigated in clinical trials on patients with hematological malignancies and solid tumors. We previously reported that zoledronate-activated γδT cell therapy could increase the number and frequency of $CD3^+V\gamma9^+\gamma\delta$T cells in the peripheral blood of patients with various solid tumors (12). For advanced non-small lung cancer refractory to the standard therapy, an increased number of NKT cells was observed in 6 of 17 patients after several injections of α-galactosylceramide (GalCer)-pulsed interleukin-2 (IL-2)/granulocyte-macrophage colony-stimulating factor (GM-CSF)-cultured peripheral blood mononuclear cells (PBMCs) (13).

In the Seta Clinic Group, we decide on the type of personalized immune cell therapy on the basis of not only tumor characterization but also the immunological status of the cancer patient. Therefore, we examine peripheral blood samples obtained from patients with various types of cancer by flow cytometry (FCM) before immune cell therapy. In this study, we initially analyzed in detail the immunological status of advanced cancer patients by FCM and compared immune cell populations and their frequencies between patients with various advanced solid tumors. Additionally, we also studied the changes in the immunological status of the cancer patients receiving autologous immune cell therapy, such as αβT cell, γδT cell, NK cell or DC vaccine therapy.

Table I. *Characteristics of 816 patients with various malignancies whose immunological status was analyzed by flow cytometry.*

Characteristics	No. of patients (%)
Primary site or cancer type	
Head and neck	34 (4.2)
Lung	138 (16.9)
Colorectum	124 (15.2)
Stomach	78 (9.6)
Esophagus	35 (4.3)
Small intestine	9 (1.1)
Pancreas	95 (11.6)
Biliary tract	33 (4.0)
Liver	30 (3.7)
Breast	54 (6.6)
Ovary	65 (8.0)
Uterus	50 (6.1)
Prostate	15 (1.8)
Bladder	17 (2.1)
Kidney	12 (1.5)
Malignant melanoma	11 (1.3)
Peritoneum	8 (1.0)
Brain tumor	5 (0.6)
Bone & soft tissue sarcoma	3 (0.4)
No. of treatments	
0	101 (12.4)
1-5	259 (31.7)
6≤	456 (55.9)
Regimen	
αβT	646 (79.2)
γδT	59 (7.2)
NK	23 (2.8)
DC	306 (37.5)

Materials and Methods

Patients. From May 2012 to July 2014, the immunological status of 873 patients with unresectable advanced or recurrent cancers was evaluated by FCM before autologous immune cell therapy. Most patients had been given chemotherapy, such as cytotoxic drugs, molecular target drugs or hormones. In this study, the immunological status of 816 cancer patients, excluding those with hematological malignancies or rare cancers, was analyzed in detail: (number of cases, primary site or type of cancer) 34, head and neck; 138, lung; 124, colorectum; 78, stomach; 35, esophagus; 9, small bowel; 95, pancreas; 33, bile duct; 30, liver; 54, breast; 65, ovary; 50, uterus; 15, prostate; 17, bladder; 12, kidney; 11, malignant melanoma; 8, peritoneum; 5, brain; and 3, bone and soft tissue malignancies (Table I).

Immune cell therapy. For effector cell therapy, we prepared αβT or γδT cells cultured *ex vivo* with interleukin 2 (IL2) and an immobilized antibody to CD3 or IL2 and bisphosphonate, respectively (10, 12). For NK cell therapy, we prepared activated NK cells cultured *ex vivo* with IL2 and other cytokines. For DC vaccine therapy, we collected PBMCs from the patients by leukapheresis and the adherent cell fraction was used for the DC culture using a medium supplemented with IL4 and GM-CSF. The DCs pulsed with tumor-specific peptides or the autologous tumor lysate were injected subcutaneously into cancer patients (14, 15). The combination of immune cell therapy with chemotherapy or radiotherapy was not prohibited, although immune cell therapy was carried out on a different day to avoid cytotoxic damage of the αβT cells, γδT cells, NK cells or DCs when the patients underwent conventional standard therapy. Immune cell therapy was discontinued when cancer progressed, as determined by diagnostic imaging or on the basis of clinical symptoms, during the course of the immune cell therapy consisting of six injections.

Immunological status assessment. For the immunological status of the cancer patients, we examined immune cell populations and their frequencies in the peripheral blood samples obtained from the patients before immune cell therapy by FCM as previously reported (9). Phenotypes of PBMCs were studied by whole-blood staining with OptiLyse C lysis solution. Absolute cell number was determined using Flow-Count™ fluorosphere internal standard beads. The OptiLyse C, Flow-Count™ beads and monoclonal antibodies (mAbs) against CD3, CD4, CD8, CD45, CD56, TCR pan αβ, TCR pan γδ, and TCR Vγ9 were purchased from Beckman Coulter (Brea, CA, USA). Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) was used with gradient centrifugation to isolate PBMCs. We utilized the isolated PBMCs for Foxp3 staining and

Table II. *Immune cell populations and frequencies in patients with various advanced solid tumors.*

Primary site or cancer type	CD3 (cells/μl)	CD4 (cells/μl)	CD8 (cells/μl)	Vγ9γδT (cells/μl)	NK (cells/μl)	Th1/2 ratio	Treg (%)
Head & neck	467 (344-830)	253 (163-458)	177 (145-288)	12 (5-19)	175 (131-301)	3.2 (2.1-4.4)	7.9 (6.0-11.4)***
Lung	770 (536-1049)	438 (284-635)	268 (166-389)	11 (5-27)	222 (147-312)#	2.9 (1.8-4.9)	7.1 (5.4-9.3)
Colorectum	917 (646-1224)	567 (403-729)	251 (180-435)	18 (7-39)	221 (147-320)#	3.0 (1.9-5.0)	6.1 (4.6-7.7)***
Stomach	836 (652-1058)	516 (391-686)	239 (151-344)	14 (6-45)	222 (165-317)#	3.9 (2.1-5.2)	5.8 (4.7-7.5)**/***
Esophagus	536 (386-1003)	335 (198-470)	202 (114-354)	8 (3-19)	176 (125-272)	3.7 (2.0-6.9)	7.3 (5.1-10.8)
Small intestine	612 (498-986)	414 (296-475)	225 (123-505)	10 (6-21)	264 (136-341)	3.4 (2.2-4.9)	5.7 (4.7-7.0)
Pancreas	708 (484-991)	446 (307-668)	205 (120-292)*	12 (5-23)	162 (107-260)	3.3 (2.1-5.4)	6.9 (5.1-9.4)
Biliary tract	740 (511-986)	482 (297-603)	211 (134-314)	10 (6-32)	186 (122-292)	2.4 (1.6-3.7)	7.2 (5.5-9.0)
Liver	822 (585-1138)	445 (333-821)	261 (187-407)	11 (5-23)	168 (122-264)	3.7 (2.5-5.0)	6.9 (5.4-9.2)
Breast	878 (688-1087)	466 (366-657)	279 (200-390)	16 (6-31)	196 (121-284)	3.4 (2.3-5.9)	7.1 (5.7-9.1)
Ovary	934 (673-1236)	533 (384-738)	303 (213-437)*	20 (8-50)	147 (105-225)#	3.3 (1.7-5.1)	5.6 (4.6-7.1)**/***
Uterus	752 (442-1059)	386 (213-637)	262 (154-330)	16 (6-49)	178 (113-236)	3.3 (1.9-5.1)	7.5 (5.8-10.3)**
Prostate	681 (275-1050)	396 (165-595)	269 (118-343)	10 (5-27)	250 (147-301)	2.9 (2.3-5.1)	7.0 (5.3-8.0)
Bladder	578 (443-1242)	359 (248-556)	227 (118-419)	14 (6-24)	228 (181-383)	3.2 (1.9-4.5)	7.1 (5-8.5)
Kidney	918 (501-1104)	424 (326-619)	252 (150-399)	11 (3-33)	182 (119-275)	1.8 (1.5-3.4)	7.1 (4.1-8.8)
Malignant melanoma	787 (647-980)	407 (346-642)	283 (238-429)	12 (8-18)	166 (109-265)	3.4 (1.5-5.1)	6.1 (5.2-6.9)
Peritoneum	910 (547-1283)	596 (380-925)	281 (168-391)	6 (3-69)	174 (134-201)	2.4 (1.9-4.4)	6.0 (3.1-9.3)
Brain tumor	527 (337-1227)	240 (166-535)	250 (154-585)	19 (9-83)	232 (161-259)	1.8 (1.4-5.2)	8.5 (6.3-13.1)
Bone & soft tissue sarcoma	778 (307-1490)	466 (199-655)	282 (90-723)	8 (6-156)	220 (203-295)	2.9 (1.3-4.2)	7.8 (7.7-8.0)

Data represents median (first and third quartiles). #The number of NK cells for ovarian cancer was significantly lower than that for lung, colorectal or gastric cancer. *The number of $CD8^+$ T-cells for ovarian cancer was significantly higher than that for pancreatic cancer. **The Treg frequency in uterine cancer was significantly higher than that in ovarian or gastric cancer. ***The Treg frequency in head and neck cancer was significantly higher than that in ovarian, colorectal or gastric cancer.

cytokine production assay. For Foxp3 staining, the PBMCs were fixed and permeabilized using a fixation/permeabilization kit (BioLegend, San Diego, CA, USA) and Foxp3 was stained with anti-Foxp3 mAb (clone 259D, BioLegend). For intracellular cytokine production assay, the PBMCs were suspended in conditioned medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Grand Island, NY, USA) containing phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO, USA), ionomycin (Sigma-Aldrich), and brefeldin A (Sigma-Aldrich). The cells were incubated at 37˚C in a humidified atmosphere with 5% CO_2 for 4 h for the IFN-γ/IL-4 assay. After the activated cells were fixed and permeabilized, intracellular cytokines were stained with an anti-IFN-γ (Beckman Coulter) or -IL-4 (Beckman Coulter) antibody. The blood samples obtained from the cancer patients were quickly brought to the clinical laboratory of SRL, Inc. (Tokyo, Japan), a company which offers clinical laboratory testing, and examined by FCM.

Clinical trial of NK cell therapy. The clinical trial, which was approved by the research ethics committee of Seta Clinic (approval number: SCG12067) and registered at the University Hospital Medical Information Network Clinical Trial Registry (UMIN-CTR) in Japan (UMINID: 000008046), was performed to assess the feasibility of culturing NK cells for autologous NK cell therapy as the primary endpoint and to monitor the safety of the therapy and immunological status as the secondary endpoints. The inclusion criteria in this study were as follows: (i) the patients should have histopathologically confirmed malignant tumors, (ii) their performance status score should be in the range of 0-2, (iii) there should be no serious abnormalities in bone marrow, liver or renal functions and (iv) written informed consent should have been obtained. This clinical trial involved 19 patients with advanced malignancies and PBMCs were collected every two weeks. The *ex vivo*-expanded NK cells were given to each patient six times at two-week intervals. Concerning the secondary endpoints, adverse events were monitored on the basis of Common Terminology Criteria of Adverse Events (CTCAE), ver. 4.0 (16), while the immunological status of the patients who received NK cell therapy was also monitored by FCM of peripheral blood.

Statistical analyses. Statistical analysis was conducted using the JMP 11.1.1 software (SAS Institute, Cary, NC, USA). A chi-square or Fisher's exact test was performed to evaluate the significance of difference between results. Additionally, the Kruskal-Wallis and Steel-Dwass tests were also carried out. A p-value of <0.05 was considered statistically significant.

Results

Patients and treatments. For the two-year period, the immunological status of 816 patients with different types of cancers was analyzed by FCM of peripheral blood before treatment. The number of treatment sessions and the regimen of the immune cell therapy for the 816 patients are shown in Table I. Four hundred fifty-six (55.9%) out of the 816 patients completed six or more immune cell therapy injections. However, 259 patients (31.7%) received less than

Table III. *Tregs frequency in 105 patients with non-small cell lung cancer.*

Tregs frequency	Squamous cell carcinoma	Adenocarcinoma
<10%	50% (10/20)	85.9% (73/85)
10%≤	50% (10/20)	14.1% (12/85)

six treatment sessions due to disease progression, as determined by imaging diagnosis or clinical progression of the malignancies; the six treatment sessions were given every 2 weeks for 3 months as one course. As per their request, 47 patients (5.8%) did not eventually receive the immune cell therapy even after assessment of their immunological status. Furthermore, 54 patients (6.6%) were unable to receive the immune cell therapy because their condition worsened during the cell preparation period. For the immune cell therapy regimens, αβT cell, γδT cell and NK cell therapies were given to 646, 59 and 23 patients, respectively (79.2, 7.2 and 2.8%). Three hundred and six patients received DC vaccine therapy (37.5%). Three hundred twenty-one patients were given more than two types of immune cell therapy and 225 patients received αβT cell therapy prior to or concurrently with DC vaccine therapy (70.1%).

Immunological status of patients with various types of cancer. Table II shows the immune cell populations and their frequencies for the patients with various types of cancer before receiving autologous immune cell therapies. The number of $CD8^+$ T-cells was significantly lower in patients with pancreatic cancer than in those with ovarian cancer. However, for NK cells, the cell number was notably lower in the ovarian cancer patients than in those with lung, colorectal or gastric cancers. For CD3, CD4 and Vγ9γδ T cells, or Th1/2 ratio, there was no significant difference in the T-cell population between patients with various types of cancers. It was found that the Tregs frequency among $CD4^+$ T-cells was significantly higher in the patients with head and neck cancer than in those with ovarian, colorectal or gastric cancer. The Tregs frequency was also significantly higher in patients with uterine cancer than in those with ovarian or gastric cancer. Figure 1 shows the Tregs frequency in the various types of cancer and the percentage of patients with more than 10% Tregs frequency. The patients with head and neck, lung, esophageal, uterine or brain tumors showed higher Tregs frequencies (32.4, 22.5, 25.7, 26.0 or 40.0%, respectively). In addition, for non-small cell lung cancer, the Tregs frequency was significantly higher in the patients with squamous cell carcinoma than in those with adenocarcinoma (Table III).

Table IV. *Characteristics of 19 cancer patients injected with autologous NK cells.*

No	Age	Gender	Primary site	Distant metastasis	Combined therapy
1	86	M	Bladder	-	-
2	55	M	Breast	Bone, lung	-
3	67	F	Colorectum	Liver, lymph node	C,M
4	65	F	Breast	Liver	H
5	39	F	Head and neck	-	C
6	58	F	Uterus	Bladder, lung, lymph node	-
7	61	F	Breast	vertebra, bone, lymph node	H
8	58	M	Colorectum	Liver, lung	-
9	61	M	Liver	Lung	-
10	71	M	Prostate	Bone	H
11	78	F	Uterus	Lung, vagina	C
12	54	F	Colorectum	Liver	R,C,M
13	74	M	Prostate	Lung, bone	H
14	46	F	Uterus	Liver, muscle	-
15	62	M	Lung	Bone, lymph node	R,C,M
16	60	F	Breast	Lung, lymph node	C,M
17	76	M	Liver	Lymph node	M
18	46	F	Ovary	Lymph node, peritoneum	-
19	71	F	Colorectum	Lymph node	C

R, radiotherapy; C, chemotherapy; M, molecular-targeted drug; H, hormone therapy.

Change in immunological status by immune cell therapy. We evaluated the change in immunological status, in terms of the numbers of $CD8^+$T, Vγ9γδT and NK cells, as well as Tregs frequency, of the patients given various autologous immune cell therapies. We were able to analyze the change in the immunological status of 98 out of the 456 patients who completed the six or more injections as part of the immune cell therapy. For the patients given αβT cells only, the numbers of $CD8^+$T, Vγ9γδT and NK cells increased in 76.6% (36/47), 40.4% (19/47), and 44.7% (21/47) of the patients, respectively, including five patients who showed a simultaneous increase in the number of all three effector cells (Figure 2). For αβT cell therapy, a decrease in Tregs frequency was observed in 24 patients (51.1%), although the Tregs frequency increased up to more than 10% in three patients. Out of the five patients given γδT cell therapy, although all patients received αβT cell therapy previously, four showed Vγ9γδT cell up-regulation (80.0%). For one patient whose number of Vγ9γδT cells in peripheral blood did not increase and whose Tregs frequency increased up to 20.0%, it was difficult to expand the γδT cells *ex vivo* and we failed to provide a sufficient number of γδT cells to the patient. Changes in the immunological status of five patients who received NK cell therapy varied in terms of the numbers of $CD8^+$T, Vγ9γδT and NK cells or Tregs frequency. Concurrent DC vaccine and αβT cell therapies, DC vaccine therapy with two or three prior αβT cell infusions and DC

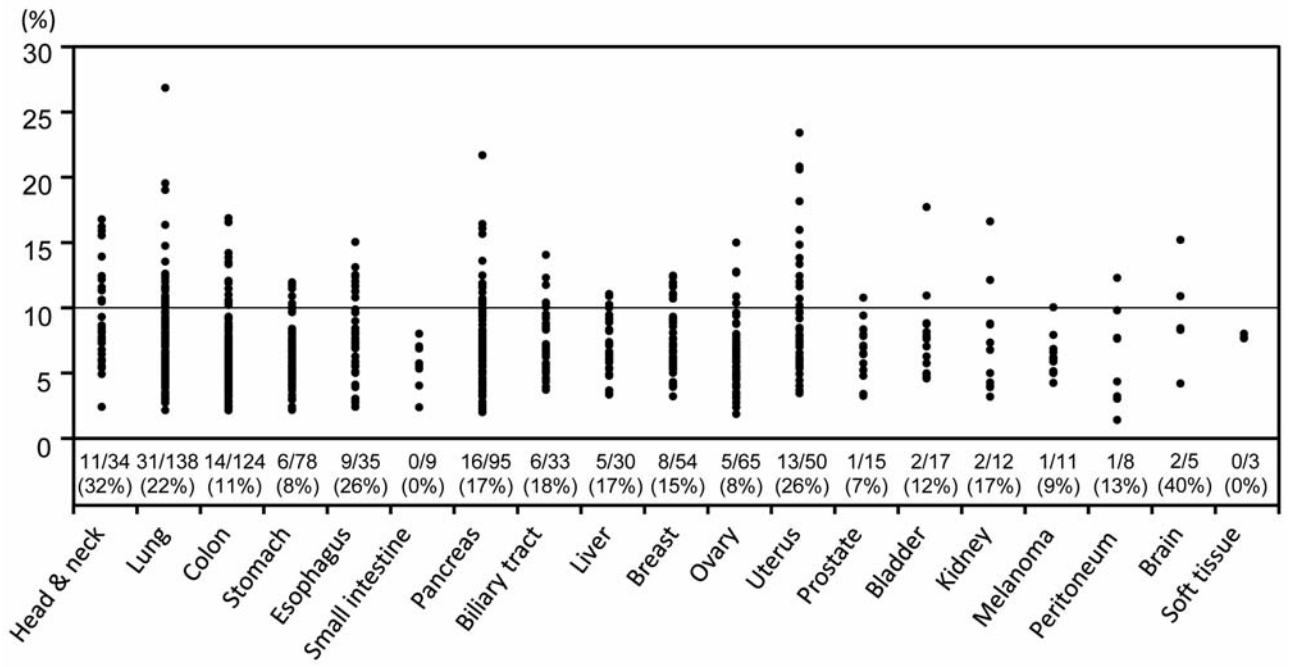

Figure 1. *Tregs frequency in various types of cancer and percentage of patients with more than 10% Tregs frequency. Higher Tregs frequencies were found in the patients with head and neck, lung, esophageal, uterine or brain tumors, although the Tregs frequency was less than 10% in more than 90% of patients with stomach, small intestine, ovarian or prostate cancers, melanoma and soft tissue sarcomas.*

vaccine therapy only were performed in 18, 22 and 1 out of 41 patients with various solid tumors, respectively (Figure 3). For the patients given DC vaccines, the numbers of $CD8^+$ T-cells, Vγ9γδT cells and NK cells increased in 56.1% (23/41), 48.8% (20/41) and 34.1% (14/41) of the patients, respectively. A decrease in Tregs frequency was observed in 34.1% of the patients injected with DC vaccines. A significant increase in the number of αβT cells was found in the patients concurrently given DC vaccine and αβT cell therapies compared with those given DC vaccine therapy with two or three αβT cell injections given previously.

Clinical trial of NK cell therapy for patients with advanced solid tumors. Nineteen patients with advanced or recurrent cancer were enrolled in this study. The age range was from 39-86 years old (median age=61 years); the numbers of males and females were 8 and 11, respectively. Seventeen of the 19 patients had progressive or recurrent cancer and two patients were administered autologous NK cells as adjuvant therapy after surgery. Patients' characteristics are shown in Table IV. Various malignancies were registered for this clinical trial, including lung, liver and colorectal cancers, as well as sarcomas. We successfully expanded a sufficient amount of cells *ex vivo* for NK cell therapy in 13 of the 19 patients (68.4%) at the initial injection of NK cells. Safety as a secondary end-point was evaluated using CTCAE, ver. 4.0. A grade 1 itching was observed in only one patient with hepatocellular carcinoma, which disappeared after the administration of anti-histamines. No hematological toxicity related to the NK cell therapy was observed. For 10 patients who completed the six or more injections as part of the NK cell therapy, changes in the numbers of CD8+T, Vγ9γδT and NK cells, as well as Tregs frequency, were observed as shown in Figure 4. An increase in the number of NK cells in peripheral blood was observed in 2 of the 10 patients, although there was no significant difference in the number of injected NK cells among the 10 patients. The Tregs frequency increased in four patients given NK cell therapy; however, in one patient, the Tregs frequency decreased from 13.0% to 10.2%.

Discussion

Various immune cell therapies have been employed, such as adoptive T-cell immunotherapy or DC-based vaccines in clinical trials or practices of cancer immunotherapy. We previously reported on the safety and feasibility of the tumor-lysate-loaded DC vaccine by electroporation in phase I clinical trials and the clinical utilization of αβT cell, γδT cell and other DC vaccine therapies for the treatment of human solid tumors (8, 15). For the DC-based cancer vaccine, there exist some reports that describe the lack of a clinical response

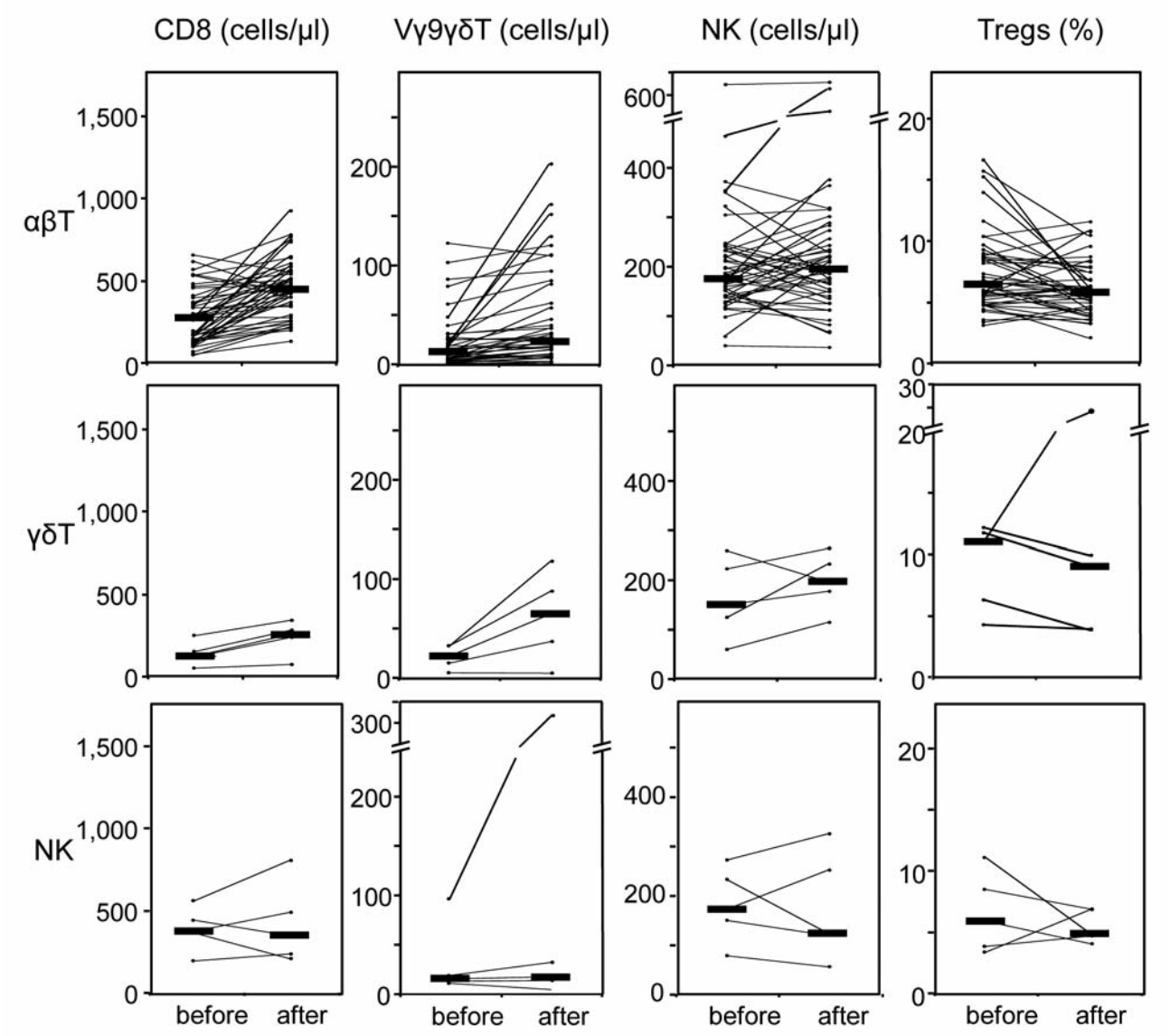

Figure 2. *Change in immunological status following autologous effector cell therapy of cancer patients. Immunological status in terms of the numbers of CD8+T, Vγ9γδT and NK cells, as well as Tregs frequency, was analyzed in patients before and after αβT, γδT or NK cell therapy.*

despite good immunological responses, which is due to the diminished immune competence of patients with large tumor burdens. Recently, improving the global immune dysfunction has been shown to be important, especially immune suppression by Tregs or myeloid-derived suppressor cells (MDSCs). Antibodies against cytotoxic T lymphocytes (CTL)-associated antigen-4 (CTLA4) or programmed death-1 (PD1), which are immune checkpoint inhibitors, are clinically used for melanoma; they can reverse the immune suppression and activate T-cells (17, 18). Thus, it is important to reveal the immunological status, especially immune suppression, in the tumor microenvironment in patients with various types of cancer before immune cell therapy. For colorectal cancers, it was found that the Tregs frequency increased significantly in both peripheral blood and cancer tissue, and $Foxp3^{high}$ and CD45RA- Tregs in cancer tissue correlated with tumor metastases by dividing Tregs into subgroups based on Foxp3 and CD45RA (19). Therefore, it is possible to carry-out immune monitoring of the tumor microenvironment during immune cell therapy, as well as to evaluate the immunological competence of patients before and after treatment by detailed sub-group analysis of Tregs frequency. In this study, we showed that there were no notable differences in the numbers of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells, Vγ9γδT cells and NK cells,

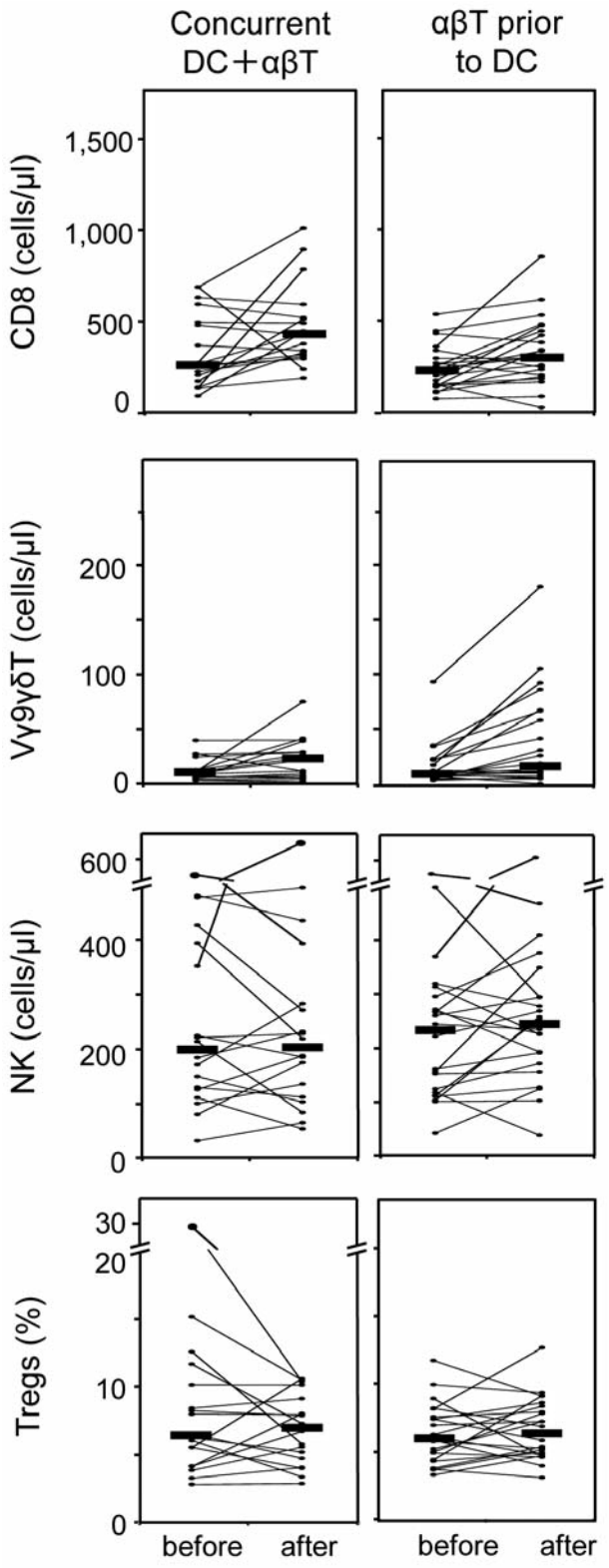

Figure 3. *Change in immunological status following DC vaccine therapy of cancer patients. Immunological status was compared between patients concurrently given DC vaccine and αβT cell therapy and those given DC vaccine with two or three αβT cell injections given previously.*

or Th1/2 ratio in the peripheral blood of patients with various types of cancer, with the exception of ovarian, head and neck or pancreatic cancer. Tregs levels in the peripheral blood or in tumor-infiltrating lymphocytes of patients with some solid tumors were reported to be higher than those of healthy volunteers (20, 21). Our data also indicated that the Tregs frequency in patients with head and neck, lung, esophagus, uterus or brain tumors was significantly higher than that in patients with other solid tumors. Furthermore, the Tregs frequency was significantly higher in patients with lung squamous cell carcinoma than in those with adenocarcinoma, as well as in patients with head and neck or esophageal tumors in whom squamous cell carcinoma is predominant. Black *et al*. reported that Tregs were present in all lung tissues examined but with significant enrichment in adenocarcinoma compared to squamous cell carcinoma (22). However, our present data suggest that Tregs could be induced more frequently in the peripheral blood of patients with squamous cell carcinoma than in those with other types of cancer.

In the present study, as well as in our previous studies, after αβT cell or γδT cell therapy, the cellular immunological status was impaired in most patients with advanced solid tumors and the impaired T-cell immune status was restored by effector cell infusions. These data show that FCM enables the assessment of a cancer patient's immune status following immunotherapy, such as αβT-cell or γδT-cell therapy. Previous reports showed that DC-based immunotherapy not only restored antigen-specific immunity but also decreased the Tregs frequency in the peripheral blood of patients with melanoma or glioma (23, 24). In this study, most of the patients who were given DC vaccine therapy previously or concurrently received αβT-cell therapy because the number of CD8+ T cells was low and/or the Tregs frequency was high before treatment. For the patients who concurrently received αβT cell and DC vaccine therapy, a significant increase in the number of CD8+ T cells was observed compared with patients who received several αβT cell therapies prior to DC vaccine administration, while there were no significant differences in the numbers of NK cells and Vγ9γδ T-cells or Tregs frequency. When the cancer patients receive DC vaccine, it is necessary to repeat the αβT cell therapy to increase or maintain the number of CD8+T cells. However, to reduce the high Tregs frequency, two or three αβT cell injections may be sufficient.

In the clinical trial of adoptive immunotherapy using autologous NK cells, NK cell therapy was partially effective in patients with recurrent malignant gliomas, although all the patients exhibited transient fever (25). In the present clinical trial, grade 1 itching was the solitary adverse event and adoptive immunotherapy of autologous NK cells was safe in combination with conventional therapy for patients with various types of cancer. Presently, we can successfully expand NK cells *ex vivo* in more than 90% of patients by improved methods, although the success rate was 68.4% in this study

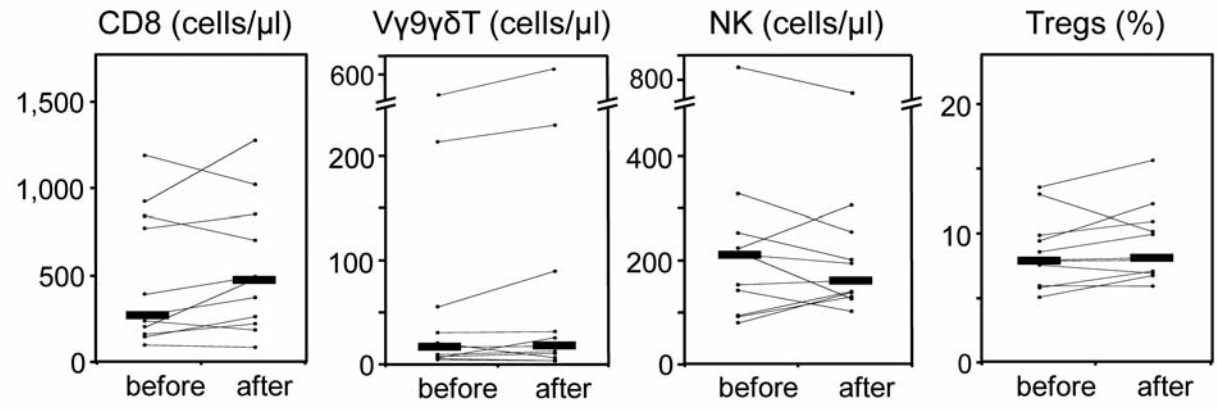

Figure 4. *Change in immunological status of cancer patients in the clinical trial of autologous NK cell therapy. Immunological status in terms of the numbers of CD8+T, Vγ9γδT and NK cells, as well as Tregs frequency, was analyzed in 10 patients with six or more injections of ex-vivo-activated autologous NK cells.*

(data not shown). The number of peripheral NK cells increased in only 2 of 10 patients in the clinical trial of NK cell therapy, although the number of effector cells increased in the blood for most of the patients injected with αβT cells or γδT cells. Some investigators reported that NK cells moved to the spleen, lymph nodes, lung, liver and gastrointestinal tissue and adoptively transferred NK cells could move to the tumor site and persist *in vivo*, which correlated with the antitumor effect observed (26, 27). Geller *et al*. reported that the adoptive transfer of haploidentical NK cells after lymphodepleting chemotherapy may be limited by reconstituting recipient Tregs for patients with recurrent ovarian and breast cancer (28). Thus, for NK cell therapy, it is important to reduce the Tregs frequency and, apparently, αβT cell therapy could be one way of reducing the high Tregs frequency observed in the four patients administered with *ex vivo*-expanded autologous NK cells in the clinical trial.

In conclusion, the Tregs frequency in peripheral blood was particularly high in patients with head and neck, esophageal, uterine and lung cancers. Autologous immune cell therapy, such as αβT cell, γδT cell, NK cell or DC vaccine therapy, could have the potential to increase the number of effector cells and reduce Tregs frequency, but it is necessary to include or combine other immune cell therapies when the Tregs frequency of cancer patients becomes high after autologous immune cell therapy.

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