A Phase I Study of *In vitro* Expanded Natural Killer T Cells in Patients with Advanced and Recurrent Non–Small Cell Lung Cancer

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Abstract

Purpose: Human Vα24 natural killer T (Vα24 NKT) cells bearing an invariant Vα24JαQ antigen receptor are activated by a glicolipid ligand α-galactosylceramide (αGalCer; KRN7000) in a CD1d-dependent manner. The human Vα24 NKT cells activated with αGalCer and interleukin-2 have been shown to produce large amounts of cytokines, such as IFN-γ, and also exerting a potent killing activity against various tumor cell lines. We did a phase I study with autologous activated Vα24 NKT cell therapy.

Experimental Design: Patients with advanced or recurrent non–small cell lung cancer received i.v. injections of activated Vα24 NKT cells (level 1: $1 \times 10^7/m^2$ and level 2: $5 \times 10^7/m^2$) to test the safety, feasibility, and clinical response of this therapeutic strategy. Immunomonitoring was also done in all cases.

Results: Six patients were enrolled in this study. No severe adverse events were observed during this study in any patients. After the first and second injection of activated Vα24 NKT cells, an increased number of peripheral blood Vα24 NKT cells was observed in two of three cases receiving a level 2 dose of activated Vα24 NKT cells. The number of IFN-γ-producing cells in peripheral blood mononuclear cells increased after the administration of activated Vα24 NKT cells in all three cases receiving the level 2 dose. No patient was found to meet the criteria for either a partial or a complete response.

Conclusions: The clinical trial with activated Vα24 NKT cell administration was well tolerated and carried out safely with minor adverse events even in patients with advanced diseases.

A unique lymphocyte subpopulation, consisting of invariant natural killer T (iNKT) cells, is characterized by the coexpression of an invariant antigen receptor and natural killer (NK) receptors (1–4). Human iNKT cells express the invariant Vα24JαQ paired with the Vβ11 antigen receptor and are activated by a specific glycolipid antigen α-galactosylceramide (αGalCer) in a CD1d-dependent manner. CD1d is an HLA class Ib antigen-presenting molecule, which is well conserved through mammalian evolution with a lack of allelic polymorphism (5, 6). After activation, human Vα24 NKT cells show a strong antitumor activity against various malignant tumors both *in vitro* and *in vivo* (2, 7, 8) and produce high levels of cytokines, such as IFN-γ and interleukin-4 (IL-4), thereby activating other antitumor effector cells (9–12). Decreased numbers of Vα24 NKT cells in human peripheral blood mononuclear cells (PBMC) have been shown in patients with malignant diseases (13–15). At the same time, functional alterations of Vα24 NKT cells have also been reported after *in vitro* stimulation with αGalCer in patients with some malignant diseases (13, 16–18). For the patients possessing severely decreased or functionally deficient Vα24 NKT cells, the expansion and activation of these cells *in vitro* and the subsequent adoptive transfer may be therapeutically beneficial. The *in vitro* expansion of Vα24 NKT cells has been reported to be successful in the presence of αGalCer and IL-2 in both healthy volunteers and cancer-bearing patients (19–21).

Based on theses findings, we carried out a phase I study using *in vitro* expanded Vα24 NKT cells in patients with recurrent or advanced non–small cell lung cancer. The goal of this study was to confirm the safety profile of activated Vα24 NKT cell immunotherapy, and no severe adverse events were observed.

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We detected an increased number of Vα24 NKT cells and also an increased number of IFN-γ-producing cells in the PBMCs of the patients receiving $5 \times 10^7/m^2$ *in vitro* expanded Vα24 NKT cells.

Patients and Methods

Patient eligibility criteria. Patients between 20 and 80 years of age, with a histologic or cytologic diagnosis of non–small cell lung cancer for which no standard treatment was available, were eligible for the study. Further inclusion criteria were a performance status of 0, 1, or 2; an expected survival of ≥6 months; normal or near normal renal, hepatic, and hematopoietic function; and no chemotherapy or radiotherapy received for at least 4 weeks before enrollment. In enrolled patients, $V\alpha24^+V\beta11^+$ NKT cells were detected at a level of >100 cells in 1 mL peripheral blood by flow cytometry. The exclusion criteria were a positive response to HIV, hepatitis C virus, or human T-cell lymphotrophic virus antibodies; positive for hepatitis B antigen; the presence of active inflammatory disease or active autoimmune disease; a history of hepatitis; pregnancy or lactation; concurrent corticosteroid therapy; and evidence for another active malignant neoplasm. The histologic type, tumor-node-metastasis classification, and the antitumor effect of treatment were classified according to the general rules for clinical and pathologic recording of lung cancer as described by the Japan Lung Cancer Society.

Clinical protocol and study design. The study was carried out in the Department of Thoracic Surgery, Chiba University Hospital, Japan, according to the standards of Good Clinical Practice for Trials on Medicinal Products in Japan. The protocol was approved by the Institutional Ethics Committee (no. 1972). In addition, this trial underwent *ad hoc* reviews by the Chiba University Quality Assurance Committee on Cell Therapy.

The study design is illustrated in Fig. 1. Written informed consent was obtained from all of the patients before undergoing a screening evaluation to determine eligibility. Clinical and laboratory assessments were conducted once a week, consisting of a complete physical examination and standard laboratory values. Any adverse events and changes in laboratory values were graded according to the National Cancer Institute Common Toxicity Criteria version 2.0. All patients underwent an assessment of the tumor status at baseline and 4 weeks after the second NKT cell administration (7 weeks after study entry). Disease progression was defined as >25% increase in target lesions and/or the appearance of new lesions.

Preparation of activated Vα24NKT cells from peripheral blood. All procedures were done according to the Good Manufacturing Practice standards. Eligible patients underwent peripheral blood leukapheresis (Spectra, COBE). PBMCs were collected and then further separated by density gradient centrifugation (OptiPrep, Axis-Shield, Oslo, Norway). After washing three times, the cells were resuspended in AIM-V (Invitrogen Corp., Carlsbad, CA) with 100 JRU/mL of recombinant human IL-2 (Imunace, Shionogi, Japan) and 100 ng/mL of αGalCer (KRN7000; Kirin Brewery Co., Gunma, Japan). Restimulation with αGalCer-pulsed autologous PBMCs was done on days 7 and 14. After 14 or 21 days of cultivation, the cells were harvested, washed thrice, and then resuspended in 100 mL of 2.5% albumin in saline. The patients received the cultured cells i.v. The criteria for activated Vα24 NKT cell administration included a negative bacterial culture 48 hours before Vα24NKT cell injection, cell viability >70%, and an endotoxin test 48 hours before cell injection with a result <0.7 EU/mL. The activated Vα24 NKT cells were given in a dose escalation design at a dose level per cohort of 1×10^7 and 5×10^7 cells/m^2 per injection. The cell dose represents the $V\alpha24^+V\beta11^+$ NKT cell number but not the bulk population of the cells.

Activated Vα24NKT cell phenotype evaluation. The phenotypes of peripheral blood lymphocytes and activated Vα24NKT cells were determined by a flow cytometry analysis. The monoclonal antibodies (mAb) used were FITC-labeled anti-TCR Vα24 mAb (C15; Immunotech, Marseilles, France), anti-CD8 mAb (HIT8a; BD Biosciences PharMingen, San Diego, CA), phycoerythrin-labeled anti-TCR Vβ11 mAb (C21; Immunotech), anti-CD56 mAb (B159; BD Biosciences PharMingen), and cychrome-labeled anti-CD3ε mAb (UCTH1; BD Biosciences PharMingen), anti-CD4mAb (RPA-T4; BD Biosciences PharMingen). Phycoerythrin-labeled αGalCer-loaded CD1d tetramer was prepared in our laboratory using a baculovirus expression system kindly provided by Dr. M. Kronenberg (La Jolla Institute, La Jolla, CA; ref. 22). Isotype-matched control mAbs were used as negative controls. To determine the percentages of Vα24NKT cells in the *in vitro* αGalCer/IL-2–cultured PBMCs, we presented the values of $V\alpha24^+V\beta11^+CD3^+$ cells because the percentages of αGalCer/CD1d tetramer-positive cells and $V\alpha24^+V\beta11^+CD3^+$ cells are basically identical (Supplementary Fig. S1).

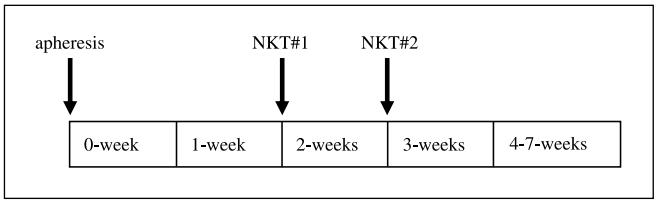

Fig. 1. Experimental design of activated Vα24 NKT cell administration. The patients received *in vitro* expanded activated Vα24 NKT cells (*NKT#1* and *NKT#2*). Timing for both apheresis and activated Vα24 NKT cell administration.

^{51}Cr release cytotoxic assay. Target Daudi cells (B lymphoma) and PC-13 cells (lung large cell carcinoma; 5×10^6) were labeled with 100 μCi sodium chromate (Amersham LIFE SCIENCE, Little Chalfont, Buckinghamshire, England) for 1 hour. Activated NKT cells were seeded into 96-well round-bottomed plates at the indicated effector/target ratios on ^{51}Cr-labeled Daudi cells or PC-13 cells (1×10^4). Radioactivity released from lysed target cells was counted on a γ-counter after 4 hours of incubation at 37°C in 5% CO_2 incubator. The percentage of ^{51}Cr release was calculated by the following formula: % specific lysis = (sample cpm − spontaneous cpm) × 100 / (maximum cpm − spontaneous cpm) as described (19). Spontaneous cpm was calculated from the supernatant of target cells alone, and the maximum release was obtained by adding 1 N HCl to the target cells. The data are expressed as the mean value of triplicate cultures with SDs.

Cytokine measurement. The amount of cytokines in the culture supernatant was measured by ELISA. Cultured Vα24 NKT cells were plated at 2×10^5 per well with 1×10^6 irradiated PBMCs that had been previously pulsed for 2 to 3 hours with 100 ng/mL αGalCer. The supernatants were collected after 24 to 48 hours of activation and stored −80°C. IFN-γ and IL-4 were measured using ELISA kits (BD Biosciences, San Jose, CA) according to the manufacturer's instructions.

Flow cytometric analysis of the peripheral blood NKT cells and NK cells. PBMC samples were obtained from the patients twice before Vα24 NKT cell administration and weekly until 4 weeks after the final injection. The frequencies of Vα24 NKT cells ($V\alpha24^+V\beta11^+CD3^+$), NK cells ($CD3^-CD56^+$), T cells ($CD3^+$ cell), and other cells, including B cells ($CD3^-CD56^-$ cells) in PBMCs, were assessed by flow cytometry using automated full blood counts. The frequency of monocyte was calculated from the cytogram findings. The absolute numbers of these cells were also calculated.

Single-cell enzyme-linked immunospot assay. The PBMCs were washed thrice with PBS and then were stored in liquid nitrogen until use. For detecting IFN-γ-secreting cells, 96-well filtration plates (Millipore, Bedford, MA) were coated with mouse anti-human IFN-γ (10 μg/mL; Mabtech, Nacka Strand, Sweden). PBMCs (5×10^5 per well) were incubated for 16 hours with or without αGalCer (100 ng/mL) in 10%FCS containing RPMI. Phorbol 12-myristate 13-acetate (10 μg/mL) plus ionomycin (10 nmol/L) was used as a positive control. After culture, the plates were washed and incubated with biotinylated

anti-IFN-γ (1 μg/mL; Mabtech). Spot-forming cells were quantified by a microscopy. In our enzyme-linked immunospot assay protocol, the majority of the IFN-γ-producing cells detected after αGalCer stimulation for 16 hours was $CD56^+$ NK and NKT cells (Supplementary Table S1).

Results

Patient characteristics. In accordance with the protocol, a total of six patients were enrolled in the study from July 2003 to March 2004. The patient characteristics are summarized in Table 1. All patients showed recurrent lung cancer after surgical treatment. The study included four patients with adenocarcinoma and two patients with squamous cell carcinoma. All patients received previous treatments, including two cases who had undergone an incomplete surgical resection of the recurrent legions, one who had received radiation therapy, and four who had received chemotherapy >4 weeks before enrollment into this study.

Phenotypes of activated Vα24NKT cells cultured with αGalCer and IL-2. The surface phenotypes of activated Vα24 NKT cells were analyzed by flow cytometry for each administration. Representative profiles (Vα24/Vβ11 and CD3/CD56) of a patient in the level 2 (case 005) are shown in Fig. 2A. Freshly isolated PBMCs contained very small percentages of $V\alpha24^+V\beta11^+$ NKT cells (0.06%; Fig. 2A, *top left*). After cultivation in the presence of αGalCer and IL-2, this population efficiently expanded (25%, 2 weeks and 21.5%, 3 weeks; Fig. 2A, *top center* and *right*). The CD3-negative population decreased after cultivation (Fig. 2A, *bottom*). The percentages of NKT cells, $CD3^+$ cells, and $CD3^-CD56^+$ cells in the cultured cells of six patients (before, 2 weeks, and 3 weeks after cultivation) are summarized in Table 2. The CD4/CD8 expression of αGalCer/CD1d tetramer-positive cells was also investigated. $CD8^+$ and $CD4^-CD8^-$ phenotype were dominant in one patient (case 005), and $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ phenotypes were all observed in other two patients in the levels 2 (Fig. 2B). It is interesting to note that we detected a substantial number of $CD8^+V\alpha24^+V\beta11^+$ NKT cells, which correlated with the findings of a previous report (23). Next, functional evaluations of the levels of cytotoxic activities and cytokine production were done. Activated Vα24 NKT cells simulated with αGalCer and IL-2 showed efficient cytotoxic activities against human tumor cell lines, including Daudi cells and PC-13 cells (Fig. 2C). In addition, they also produced a large amount of IFN-γ and a small amount of IL-4 in response to a rechallenge with αGalCer-pulsed PBMCs (Fig. 2D). The number of $V\alpha24^+V\beta11^+$ NKT cells in the PBMC cultures for 1 to 3 weeks increased efficiently with some variations (Fig. 2E). The expansion rate of Vα24 NKT cells in six donors averaged 1,290-fold (range: 50- to 3,460-fold) for the 14-day culture period and 2,380-fold (range: 280- to 5,250-fold) for the 21-day culture period, and these results are consistent with those shown in our previous report (19).

Adverse events. No major (grade >2) toxicity or severe side effects were observed in any patients (Table 1). One patient in the level 1 group (case 001) experienced a transient flush and headache soon after the Vα24 NKT cell injection, but no additional treatment was required. One patient in the level 1 group (case 003) experienced transient arrhythmia a few hours after the Vα24 NKT cell injection, and the symptom disappeared in several minutes without any medication. One patient in the level 2 group (case 004) experienced a mild facial paralysis (numbness) on day 42 (21 days after the final injection). A zygomatic bone metastasis was found after precise medical check-up. The numbness was improved by a radiation therapy on the metastatic legion in the zygomatic bone. One patient in the level 2 group (case 006) experienced an increased fever (grade 1) a few hours after the Vα24 NKT cell administration, which abated 6 hours after the use of loxoprofen sodium. Regarding the laboratory data, an elevation in the serum glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase (day 42), and total bilirubin level (day 22) was observed in one patient in the level 1 group (case 001); a lactate dehydrogenase elevation (day 28) was observed in one patient in the level 1 group (case 003); and an elevation in the γ-GTP level (day 22) was observed in one patient in the level 2 group (case 006). The Common Terminology Criteria for Adverse Events grade of these abnormal data was categorized as grade 1, and no cases needed any additional treatment.

Table 1. Profiles of each patient and any adverse events observed

Level	Case	Age/gender	Diagnosis	Cancer lesion	PS	Pre-Tx	Adverse events
I	001	66/F	Recurrence after complete surgical resection, adenocarcinoma	Lung, pleura	0	ST, CT	Hot flash, headache, GOT, GPT, total bilirubin elevation
I	002	78/M	Recurrence after complete surgical resection, squamous cell carcinoma	Lung, SCLN; mediastinal LN	1	RT	None
I	003	78/M	Recurrence after complete surgical resection, squamous cell carcinoma	Lung	1	CT	Arrhythmia, LDH elevation
II	004	66/M	Recurrence after complete surgical resection, adenocarcinoma	Lung	0	CT	Facial paralysis
II	005	55/M	Recurrence after complete surgical resection, adenocarcinoma	Lung, pleura	0	CT	Headache, general fatigue
II	006	61/F	Recurrence after complete surgical resection	Lung, brain	0	ST	Fever, γ-GTP elevation

Abbreviations: SCLN, supraclavicular lymph node; LN, lymph node; PS; performance status; pre-Tx, treatment after recurrence and before clinical trial; ST, surgical treatment of the recurrent lesions; RT, radiation therapy; CT, chemotherapy; GOT, glutamic-oxalacetic transaminase; GPT, glutamic-pyruvic transaminase; LDH, lactate dehydrogenase.

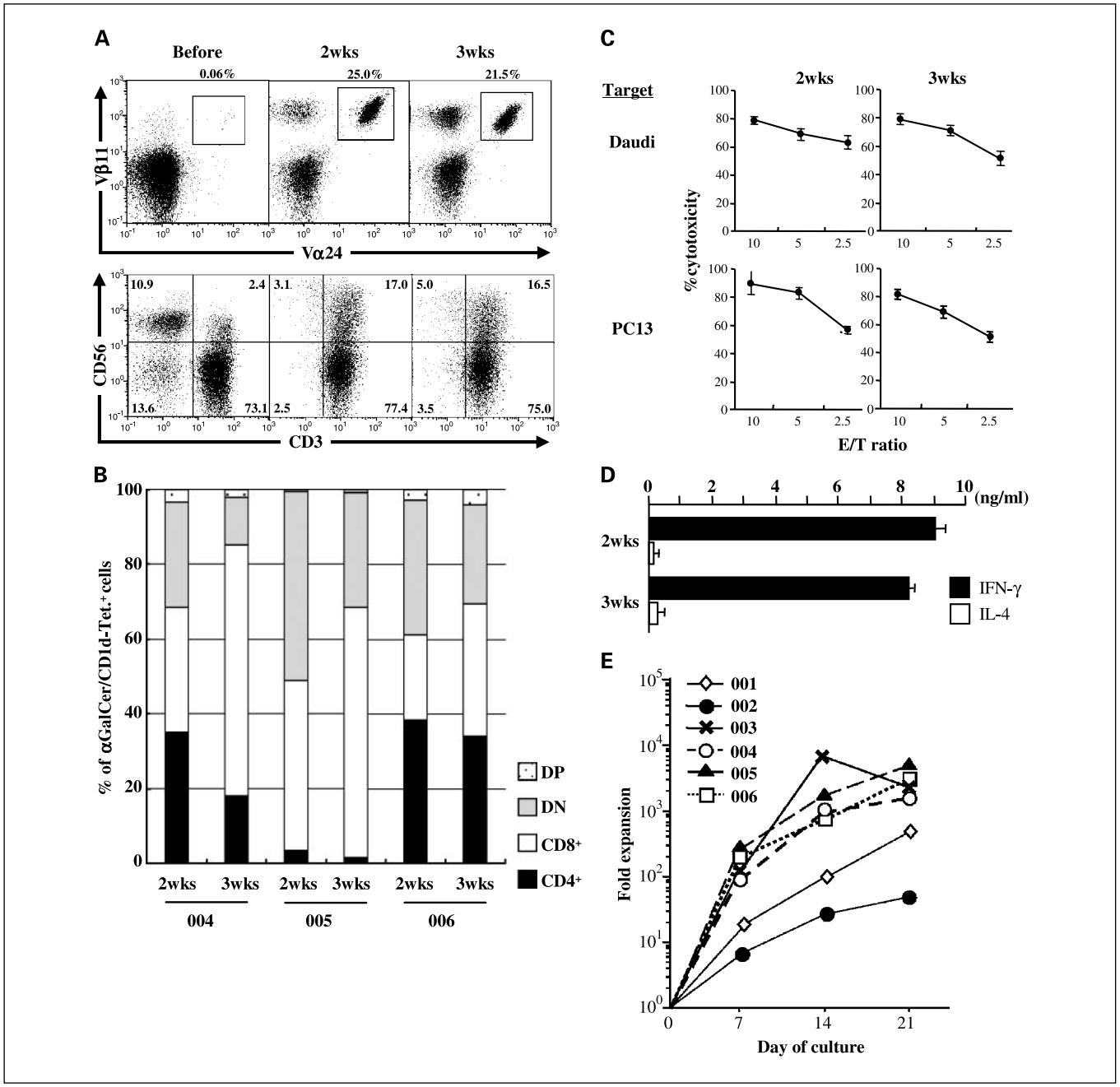

Fig. 2. Phenotypic and functional evaluation of activated Vα24 NKT cells. PBMCs from a patient were cultured with αGalCer and IL-2 for 2 or 3 weeks, and whole cultured cells were used for analysis. *A,* flow cytometric analysis of precultured (*Before*) or cultured cells (*2 wks* or *3 wks*). Top, percentages of $V\alpha24^+V\beta11^+$ NKT cells. Bottom, NK cells ($CD56^+CD3^-$), NKT cells ($CD56^+CD3^+$), and T cells ($CD3^+CD56^-$). Representative results of a patient (case 005) in the level 2 group. *B,* CD4/CD8 expression on αGalCer/CD1d tetramer-positive cells. Cultured cells were stained with phycoerythrin-labeled αGalCer/CD1d tetramer followed by FITC-labeled anti-CD8 mAb and cychrome-labeled anti-CD4 mAb. CD4/CD8 expression was determined by a flow cytometry analysis at the end of the 2- or 3-week culture period. *C,* cytotoxic activity of *in vitro* expanded Vα24 NKT cells. Whole cultured cells for 2 or 3 weeks were incubated with two target cell lines (1×10^4 per well) for 4 hours at various effector/target ratios (*E/T ratio*) in triplicate. Percentages of specific ^{51}Cr release with SD. *D,* cytokine production from activated Vα24 NKT cells. After 2- or 3-week cultivation with αGalCer and IL-2, whole cells were collected and seeded onto a 96-well plate (2×10^5 per well) in triplicate. Restimulation was done with αGalCer-pulsed, irradiated, autologous PBMCs for 24 hours, and the amounts of cytokines (IFN-γ and IL-4) in the culture supernatant were determined by ELISA. *E,* expansion of Vα24 NKT cells after cultivation with αGalCer and IL-2. The PBMCs were cultured with αGalCer (100 ng/mL) and IL-2 (100 JRU/mL). On days 7, 14, and 21, all cultured cells were harvested, and the total cell number and the percentage of $V\alpha24^+V\beta11^+$ NKT cells were evaluated by a flow cytometry analysis. The fold increase in $V\alpha24^+V\beta11^+$ NKT cells was determined as follows: [(total number of live cells recovered on days 7, 14, and 21) × (%$V\alpha24^+V\beta11^+CD3^+$ cells)] / [(total number of live cells before culture) × (%$V\alpha24^+V\beta11^+CD3^+$ cells before culture)]. Number (*001-006*) represent each enrolled patient.

Immunologic monitoring. Immunologic assays were done for the six patients who completed the study. The frequency of peripheral blood $V\alpha24^+V\beta11^+$ NKT cells in all patients was measured by a flow cytometry analysis (Fig. 3A-F). A level 1 patient (case 003) showed increased circulating Vα24 NKT cells on days 35 to 49 (Fig. 3C). Two patients in the level 2 group showed increases in the Vα24 NKT cells after the administration on day 28 (Fig. 3D) or on days 21 and 28

Table 2. Profiles of αGalCer/IL-2–cultured PBMCs

Level	Case	-NKT cell (%)			$CD3^+$ cell (%)			$CD3^-CD56^+$ cell (%)		
		Before	2 wk	3 wk	Before	2 wk	3 wk	Before	2 wk	3 wk
I	001	0.02	3.5	3.2	75.5	76.0	65.4	10.9	21.8	32.8
I	002	0.01	0.1	0.3	44.1	34.9	51.8	ND	ND	ND
I	003	0.01	10.3	2.5	76.4	93.3	ND	10.4	6.6	ND
II	004	0.04	3.3	1.3	51.4	96.0	95.7	28.0	3.6	3.8
II	005	0.06	25.0	21.5	75.5	94.4	91.5	10.9	3.1	5.0
II	006	0.05	2.5	2.0	78.9	64.2	59.2	10.8	34.0	38.3

NOTE: NKT cells, $V\alpha24^+V\beta11^+$ cells.
Abbreviation: ND, not done.

(Fig. 3E) compared with the initial NKT cell numbers detected (before or on day 14: before 1st injection). The absolute numbers of Vα24 NKT cells decreased transiently to a nadir around 1 day after the Vα24 NKT cell injection in these three cases. The number of NK cells slightly increased in one patient on day 28 (Fig. 3D), but no obvious increase was detected in the other four patients. In addition, we monitored the absolute numbers of peripheral blood $CD3^+$ cells, $CD3^-CD56^-$ cells, and monocytes in patients 005 and 006 (Fig. 3G and H). We observed a slight increase in the number of peripheral blood $CD3^+$ cells, but no obvious changes in the number of $CD3^-CD56^-$ cells or monocytes.

Concurrently, we assessed the ability to produce IFN-γ after restimulation with αGalCer. The patient PBMCs were stimulated with αGalCer for 16 hours, and the IFN-γ-producing cells in the culture were assessed by an enzyme-linked immunospot assay. The number of cells with IFN-γ production increased 1 and 2 weeks after the second injection in a patient in level 1 (case 001, Fig. 4A) and a level 2 patient (case 004, Fig. 4C), immediately after the first injection in a patient in level 2 (case 005, Fig. 4D), and 2 weeks after the second injection in case 006 (Fig. 4E). Regarding the production of IL-4, we did not observe a detectable number of αGalCer-dependent IL-4-producing cells in any cases in our assay system (data not shown).

Clinical outcome. All six cases were evaluated at the end of the clinical trial period. From the chest X-ray and computed tomography findings, there were no cases of complete response or partial response, four cases of stable disease (cases 001, 003, 005, and 006), and two cases of progressive disease (cases 002 and 004). Two patients receiving the level 2 dose were followed up for 9 and 12 months after the clinical trial period, and both of them were classified as stable disease.

Discussion

The primary aim of this study was to assess the feasibility and toxicity of adoptive immunotherapy using activated Vα24 NKT cells in patients with advanced or recurrent non–small cell lung cancer. Because this is the first clinical trial for the administration of Vα24 NKT cells activated *in vitro* with αGalCer and IL-2, we did the trial very carefully. Our results indicate that activated Vα24 NKT cell therapy has no major side effects and is well tolerated with minor adverse events, even in patients with advanced stages of lung cancer. There were no clinical symptoms suggesting the development of an autoimmune disease during the observation period. Furthermore, the therapy was safe with minor adverse events, and it can be easily done on an outpatient basis. Although activated iNKT cells in the mouse liver induced severe hepatitis (24, 25), only slight liver dysfunction was detected in two patients in the level 1 group and one patient in the level 2 group. These patients recovered without any additional treatments. This could be due to the limited number of activated Vα24 NKT cells that migrated into the liver of the patients receiving the Vα24 NKT cell administration.

We chose PBMCs as a source of Vα24 NKT cells for administration. An effective method to obtain a large number of purified, functional Vα24 NKT cells from human peripheral blood has been reported (21). In this report, autologous whole PBMCs were used as antigen-presenting cells instead of the additional preparation of monocyte-derived dendritic cells for stimulation. Our culture system was similar to that described in this report. The expansion of Vα24 NKT cells was sufficient (Fig. 2E); thus, this simple procedure seemed to be the most appropriate for the clinical use. The fold increase in the Vα24 NKT cell number varied among the patients (Fig. 2E), and it seems to be partially depended on the initial frequency of Vα24 NKT cells (see Table 2). We previously reported that the number of Vα24 NKT cells in the peripheral blood significantly decreased in patients with lung cancer (14). We, therefore, set relatively tight entry criteria (>100 mL) regarding the number of peripheral blood Vα24 NKT cells. The number of $V\alpha24^+V\beta11^+CD3^+$ NKT cells and αGalCer/CD1d tetramer-positive cells were almost identical in the cultures with αGalCer and IL-2 (Supplementary Fig. S1); therefore, the expansion of Vα24-negative αGalCer/CD1d tetramer-reactive T cells reported by Gadola et al. seemed to be negligible in our culture system (26).

Deficiencies in IFN-γ production or the proliferative potential of Vα24 NKT cells have recently been reported in some patients with advanced malignancies (13, 15, 17). In prostate cancer patients, *ex vivo* expansion of αGalCer-activated Vα24 NKT cells as well as their IFN-γ production was decreased (13). The number of peripheral blood Vα24 NKT cells was decreased in the cancer patients regardless of the tumor type or tumor load, but the ability to produce IFN-γ at a per cell basis did not decrease (15). Vα24 NKT cells from different types of solid cancer patients failed to proliferate even with αGalCer stimulation, thus producing reduced levels of cytokines compared with those from healthy individuals (17). In lung cancer patients, a reduced proliferative response of Vα24 NKT cells to αGalCer was detected, and the reduction was partially

recovered by granulocyte-colony stimulating factor (27). These studies indicate that Vα24 NKT cells in cancer patients have some numerical and functional defects. In contrast, the number and the function of Vα24 NKT cells has been reported not to be suppressed in glioma patients (28). We previously reported the ability to produce IFN-γ in peripheral blood Vα24 NKT cells to be preserved in non–small cell lung cancer patients (14). The reason for the discrepancy between the NKT cell function in the lung cancer patients in our report (14) and that in the report by Konishi et al. (27) is still unclear at this time. However, the levels of expansion and the ability to produce IFN-γ in our prepared activated Vα24 NKT cells were sufficient for the use of clinical trials (Table 2; Fig. 2). Although we still do not know whether there is functional alteration in the Vα24 NKT cells in the lung, we detected an accumulation of Vα24 NKT cells in the cancerous lesion in the lung, thus suggesting the occurrence of antitumor reactions against tumor cells (14).

In patients with either decreased or functionally altered Vα24 NKT cells, the expansion and activation of these cells *in vitro* and subsequent adoptive transfer should be therapeutically beneficial, if these deficiencies can be rectified through *in vitro* cultivation. From the results shown in this report, *in vitro* expanded Vα24 NKT cells seemed to be functional regarding IFN-γ production (Fig. 2). More importantly, we detected an increased number of IFN-γ-producing cells in the peripheral blood after the injection of activated Vα24 NKT cells. The timing of the increase detected was from a day to 2 weeks (Fig. 4). Because the number of injected activated Vα24 NKT

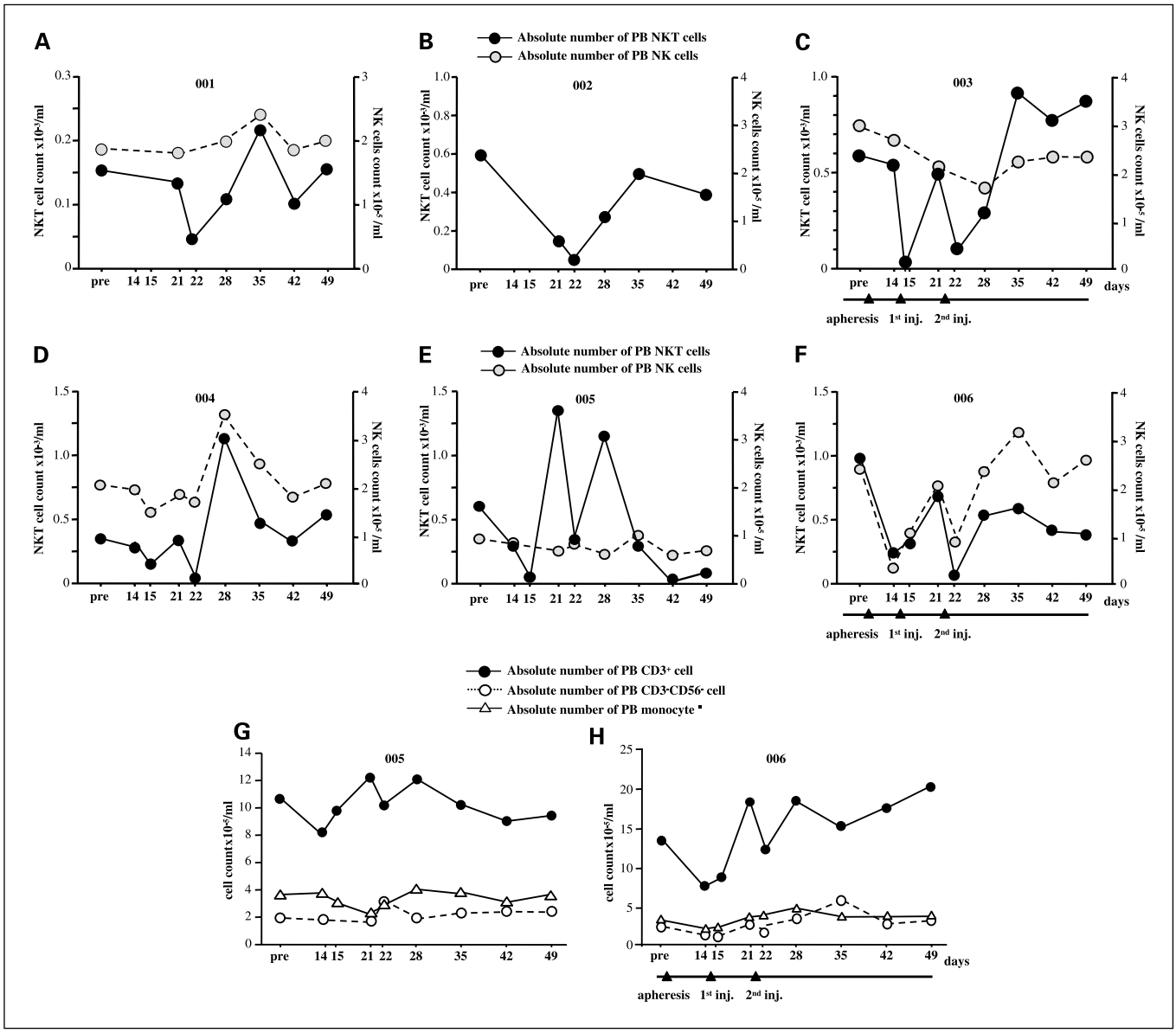

Fig. 3. Immunologic monitoring of PBMCs of patients with Vα24 NKT cell administration. Absolute number of peripheral blood NKT cells ($V\alpha24^+V\beta11^+$ cells) and NK cells ($CD56^+CD3^-$ cells; *A-F*) and that of T cells ($CD3^+$ cell), other cells including B cell ($CD3^-CD56^-$ cells), and monocytes (*G* and *H*) during the course of treatment in each patient. With the use of the results of a flow cytometry analysis and automated full blood counts (Chiba University Hospital), the absolute number of NKT cells, NK cells, T cells, and other cells was calculated. The absolute number of monocytes was calculated from the cytogram. 1st inj., first *in vitro* expanded NKT cell injection; 2nd inj., second *in vitro* expanded NKT cell injection.

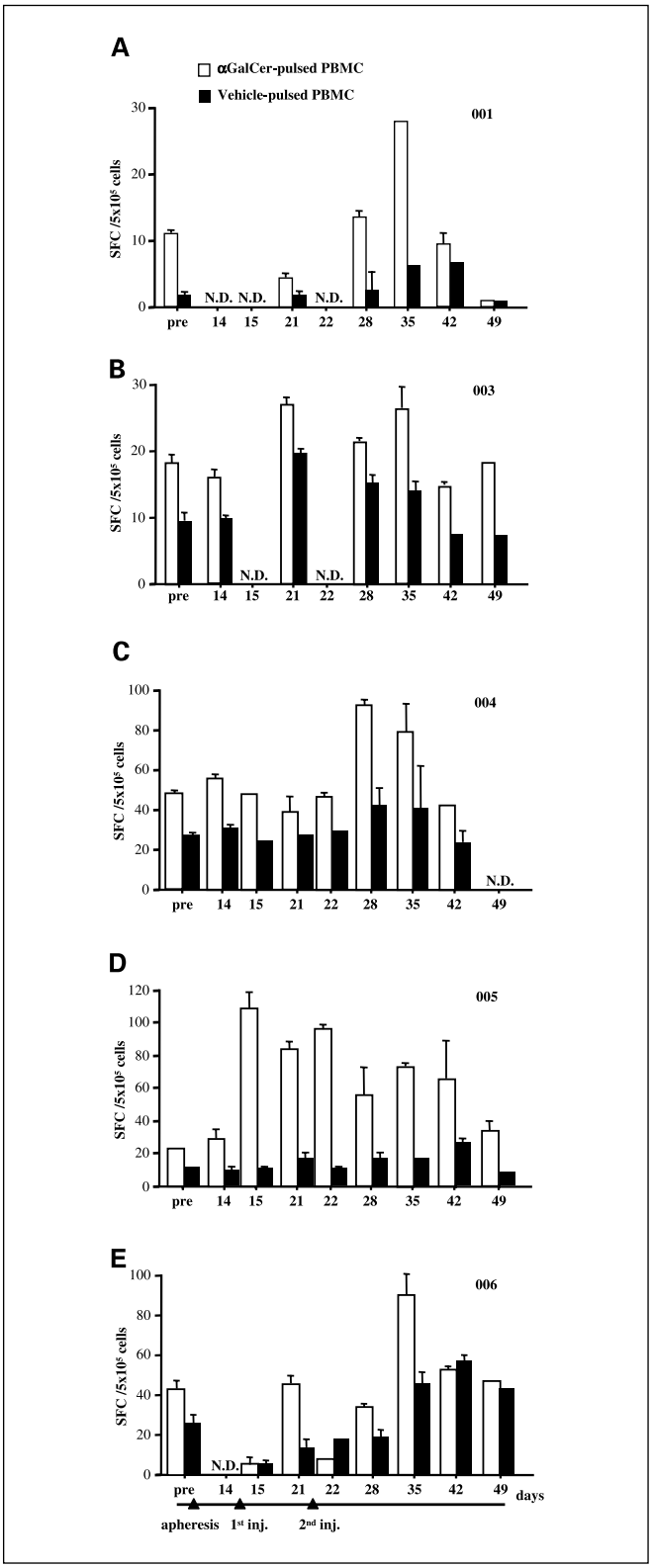

Fig. 4. Monitoring of IFN-γ-producing cells in PBMCs of patients with Vα24 NKT cell administration. Detection of αGalCer-reactive IFN-γ-producing cells by enzyme-linked immunospot assay. Cryopreserved PBMCs were thawed and cultured overnight with either αGalCer or vehicle. The presence of IFN-γ-producing cells was quantified by an enzyme-linked immunospot assay. Spot number of IFN-γ with SD for triplicate cultures. N.D., not done; 1st inj., first *in vitro* expanded NKT cell injection; 2nd inj., second *in vitro* expanded NKT cell injection; SFC, spot-forming cells.

cells was within the order of 1×10^8, it is very likely that some important change occurred in patients after the administration of activated Vα24 NKT cells, such as a robust expansion of endogenous Vα24 NKT cells and the activation of NK cells. In fact, the specific activation of iNKT cells has been reported to lead to a rapid induction of extensive NK cell proliferation and cytotoxicity, partially depending on the IFN-γ production by iNKT cells (9, 10, 29, 30). Regarding the transient decrease in the number of Vα24 NKT cells observed after NKT cell administration (Fig. 3C, D, and E), such a decrease was also observed in our previous clinical trials with αGalCer-pulsed dendritic cells (31). We do not know the reason at this time, but a possibility for the down-modulation of Vα24 NKT cell receptors from the cell surface has been suggested (32–34).

Immunotherapy methods with αGalCer or αGalCer-pulsed dendritic cells in patients with malignant diseases have been recently described (31, 35–37). In these trials, it is expected that the given αGalCer or αGalCer-pulsed dendritic cells induces the activation of Vα24 NKT cells *in situ*. We detected an increase in the number of Vα24 NKT cells in the peripheral blood of patients receiving αGalCer-pulsed dendritic cells (31). The most characteristic difference in the present study from these studies is that the activation process of Vα24 NKT cells with αGalCer presented on dendritic cells is done *in vitro*. We detected notable immune responses *in vivo* after the administration of activated Vα24 NKT cells (Figs. 3 and 4). Although we need to more precisely assess the nature of immune responses induced by the administration of activated Vα24 NKT cells with an increased number of patients, the results shown in this report provide an alternative procedure of cancer immunotherapy aimed at the activation of Vα24 NKT cells and the subsequent activation of NK cells.

No clear antitumor effect was observed in the present clinical trials. However, these trials are still small scale; thus, we need to await the findings of relatively large-scale studies to evaluate the clinical efficacy of this immunotherapy with activated Vα24 NKT cells. A study with a relatively longer monitoring period (at least a few years) may help to answer the question whether this immunotherapy can induce a long stable disease status in advanced cancer patients. In addition, a combined immunotherapy with the administration of αGalCer or αGalCer-pulsed dendritic cells is expected to result in a more effective clinical effect.

Primary lung cancer is hard to cure, although the primary tumor lesions tend to be small enough to be diagnosed at an early stage. Approximately half or more of patients with lung carcinomas who undergo complete resection had clinically undetectable local or distant micrometastases (38–40). These findings point to the importance of preoperative or postoperative immunotherapy to suppress the growth of micrometastasis. For this purpose, among the lymphocytes possessing antitumor activity, cells for tumor surveillance, such as NK and NKT cells, are considered to be the most appropriate. From this point of view, non–small cell lung cancer patients undergoing radical surgery may thus be the optimal candidates for immunotherapy aimed at Vα24 NKT cell activation.

In summary, immunotherapy of activated Vα24 NKT cell administration is well tolerated, and it can be carried out safely with minor adverse events even in patients with advanced disease. *In vivo* immunologic responses, including the elevation of IFN-γ-producing cells in the peripheral blood, were detected

after the administration of activated Vα24 NKT cells. With a greater number of treatments, we should eventually obtain more conclusive evidence regarding evaluation of the antitumor effect of this therapy. Furthermore, a combination of this therapy with a potentially additive or synergistic therapeutic strategy may also result in a more prominent antitumor effect.

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