A Phase I Study of α-Galactosylceramide (KRN7000)–Pulsed Dendritic Cells in Patients with Advanced and Recurrent Non–Small Cell Lung Cancer

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ABSTRACT

Purpose: **Human Vα24 natural killer T (NKT) cells bearing an invariant Vα24JαQ antigen receptor, the counterpart of murine Vα14 NKT cells, are activated by a specific ligand, α-galactosylceramide (αGalCer, KRN7000), in a CD1d-dependent manner. I.v. administration of αGalCer-pulsed dendritic cells (DC) induces significant activation and expansion of Vα14 NKT cells in the lung and resulting potent antitumor activities in mouse tumor metastatic models. We did a phase I dose escalation study with αGalCer-pulsed DCs in lung cancer patients.**

Experimental Design: **Patients with advanced non–small cell lung cancer or recurrent lung cancer received i.v. injections of αGalCer-pulsed DCs (level 1: 5 × 10^7/m^2; level 2: 2.5 × 10^8/m^2; and level 3: 1 × 10^9/m^2) to test the safety, feasibility, and clinical response. Immunomonitoring was also done in all completed cases.**

Results: **Eleven patients were enrolled in this study. No severe adverse events were observed during this study in any patient. After the first and second injection of αGalCer-pulsed DCs, dramatic increase in peripheral blood Vα24 NKT cells was observed in one case and significant responses were seen in two cases receiving the level 3 dose. No patient was found to meet the criteria for partial or complete responses, whereas two cases in the level 3 group remained unchanged for more than a year with good quality of life.**

Conclusions: **In this clinical trial, αGalCer-pulsed DC administration was well tolerated and could be safely done even in patients with advanced disease.**

INTRODUCTION

A unique lymphocyte subpopulation, natural killer T (NKT) cells, are characterized by the coexpression of an invariant antigen receptor and natural killer (NK) receptors (1, 2). Human NKT cells express the invariant Vα24JαQ paired with the Vβ11 antigen receptor, whereas murine NKT cells express the invariant Vα14Jα281 receptor paired with Vβ8.2, Vβ7, or Vβ2. NKT cells are activated by a specific glycolipid antigen, α-galactosylceramide (αGalCer), in a CD1d-dependent manner (3–6). CD1d is a HLA class Ib antigen-presenting molecule, and is well conserved through mammalian evolution with a lack of allelic polymorphism (7). After activation, human Vα24 NKT cells and murine Vα14 NKT cells show strong antitumor activity against various malignant tumors *in vitro* and *in vivo* (8–12) and produce high levels of cytokines, such as IFN-γ and interleukin (IL)-4 rapidly, thereby activating other effector cells; they also play regulatory roles in a wide range of immune responses (1, 13–17).

αGalCer-pulsed dendritic cells (DC) activated Vα14 NKT cells and eradicated established metastatic tumor foci in models of mouse liver metastasis, suggesting that the administration of αGalCer-pulsed DCs may exert greater antitumor activity than the direct administration of αGalCer (18). The i.v. injection of αGalCer-pulsed DCs induces the activation and expansion of endogenous Vα14 NKT cells in the lung parenchyma (19, 20). These observations in murine models suggest that similar antitumor effects in the human lung would be expected when αGalCer-pulsed DCs are administered.

Human Vα24 NKT cells play crucial roles in various immune responses, including antitumor activity (21, 22). Decreased numbers of Vα24 NKT cells in human peripheral blood mononuclear cells (PBMC) have been shown in several diseases, including lung cancer (23–27). However, the precise mechanisms underlying the reduction in the number of NKT cells in peripheral blood are not yet understood. In a recent phase I study of αGalCer administration in humans, direct i.v. injections of αGalCer induced a significant elevation of cytokines in the serum (28).

Based on these findings, we did a phase I study of αGalCer-pulsed DC treatment in patients with recurrent or advanced non–small cell lung cancer. The major goal of this study was to confirm the safety profile of αGalCer-pulsed DC

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immunotherapy. No severe adverse events were observed. We detected a dramatic expansion of peripheral blood Vα24 NKT cells in one patient receiving $1 \times 10^9/m^2$ αGalCer-pulsed DCs.

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; expected survival of 6 months or more; and normal or near-normal renal, hepatic, and hematopoietic function. Additionally, enrolled patients had received no chemotherapy or radiotherapy for at least 6 weeks before enrollment. NKT cells were detected at a level of >10 cells in 1 mL peripheral blood by flow cytometry. Exclusion criteria were a positive response to HIV, hepatitis C virus, or human T-cell lymphotrophic virus antibodies; positive for hepatitis B surface 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. 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 described by Japan Lung Cancer Society.

Clinical Protocol and Study Design. The study was done at 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. 333). Additionally, this trial underwent *ad hoc* reviews by the Chiba University Quality Assurance Committee on Cell Therapy.

The study design is shown in Fig. 1. Written informed consent was obtained from all of the patients before undergoing a screening evaluation to determine eligibility. Extensive clinical and laboratory assessments were conducted weekly and consisted of a complete physical examination and standard laboratory values. Adverse events and changes in laboratory values were graded according to National Cancer Institute Common Toxicity Criteria version 2.0. All patients underwent an assessment of tumor status at baseline and 4 weeks after the fourth αGalCer-pulsed DC administration (12 weeks after study entry). Disease progression was defined as >25% increase in target lesions and/or the appearance of new lesions.

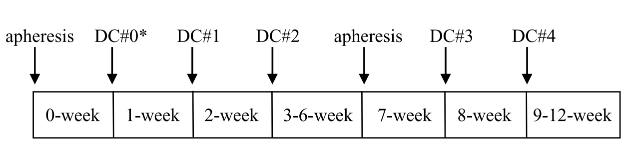


Fig. 1 Experimental design of αGalCer-pulsed APC administration. *, injection of αGalCer nonpulsed APCs. The patients received αGalCer-pulsed APC containing DCs (DC#1, DC#2, DC#3, and DC#4). The timings of apheresis and DC administration are shown.

Preparation of Antigen-Presenting Cells Containing Dendritic Cells from Peripheral Blood. All procedures were done according to Good Manufacturing Practice standards. Eligible patients underwent peripheral blood leukapheresis (COBE Spectra, Gambro BCT, Inc., Lakewood, CO), and PBMCs were collected and further separated by density gradient centrifugation (OptiPrep, Nycomed Amersham, Oslo, Norway). PBMCs were washed thrice and resuspended in AIM-V (Invitrogen Corp., Carlsbad, CA) with 800 units/mL of human granulocyte macrophage colony-stimulating factor (GeneTech Co., Ltd., China) and 100 Japanese reference units per milliliter of recombinant human IL-2 (Imunace, Shionogi, Osaka, Japan). The cultured cells were pulsed with 100 ng/mL of specific ligand, αGalCer (KRN7000; Kirin Brewery, Gunma, Japan) on the day before administration. After 7 to 14 days of cultivation, the cells were harvested and washed thrice and resuspended in 100 mL 2.5% albumin in saline. Patients received the cultured cells i.v. The criteria for αGalCer-pulsed DC administration included a negative bacterial culture 48 hours before DC injection, cell viability >70%, and an endotoxin test 48 hours before DCs injection with a result <0.7 Ehrlich units/mL. To evaluate the effect of DCs themselves, we first prescribed an αGalCer-nonpulsed DC injection (DC#0; Fig. 1).

APCs containing DCs were administered in a dose-escalation design at a dose level per cohort of 5×10^7, 2.5×10^8, and 1×10^9 cells/m^2/injection (Fig. 1).

Dendritic Cell Phenotype Evaluation. The phenotypes of monocytes and DCs were determined with an EPICS XL-MCL flow cytometer (Beckman Coulter, Marseilles, France). The monoclonal antibodies (mAb) used were FITC-labeled anti-HLA-DR, CD83, CD14; phycoerythrin-labeled anti-CD11c, CD80, CD56; and Cychrome-labeled anti-CD86, CD40, CD3 (Becton Dickinson, San Diego, CA). Isotype-matched control mAbs were used as negative controls.

Immunologic Monitoring. PBMC samples were obtained at least twice before DC administration and weekly until 4 weeks after the final treatment.

Flow Cytometric Analysis of Peripheral Blood Vα24+Vβ11+ Natural Killer T Cells. Frequencies of $V\alpha24^+V\beta11^+$NKT cells in PBMCs were assessed by flow cytometry. Mononuclear cells were three-color stained with FITC-conjugated anti–T-cell receptor (TCR) Vα24 mAb (C15; Immunotech, Marseilles, France), phycoerythrin-conjugated anti-TCR Vβ11 mAb (C21, Immunotech), and Cychrome-conjugated anti-CD3ε mAb (UCTH1; PharMingen). The stained cells were subjected to flow cytometry and the percentages of $V\alpha24^+V\beta11^+CD3^+$ cells among mononuclear cells were calculated. Then, the NKT cell numbers (counts/mL) were estimated from the PBMC cell counts.

Detection of IFN-γ Messenger RNA in Sorted Natural Killer T Cell by Reverse Transcription-PCR. The levels of IFN-γ production in peripheral blood NKT cells were assessed by semiquantitative PCR. Two hundred thousand PBMCs separated from fresh peripheral blood were suspended in 200 μL of a complete culture medium in 96-well round-bottom plates and stimulated with 10 μg/mL of immobilized anti-CD3ε mAb (OKT3, American Type Culture Collection, Manassas, VA) for 14 hours as previously described (20, 27).

Then, the cells were harvested and stained with FITC-conjugated anti-TCR Vα24 mAb and phycoerythrin-conjugated anti-TCR Vβ11 mAb. Dead cells were gated out by propidium iodide staining, and TCR Vα24 Vβ11 double-positive cells were collected by a FACSVantage cell sorter (Becton Dickinson) in 96-well round-bottom plates containing 20 μL lysis buffer [40 mmol/L Tris-HCl (pH 8.5), 60 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L DTT, 0.5% NP40, 0.05 units/μL RNasin (Promega, Madison, WI)] at a density of 100 cells/well.

Sorted $V\alpha24^+V\beta11^+$ NKT cells (100 cells/well) were treated for 10 minutes at room temperature in lysis buffer. Whole cell lysates were reverse-transcribed in 8 μL 5× buffer [100 mmol/L Tris-HCl (pH 8.5), 150 mmol/L KCl, 7.5 mmol/L $MgCl_2$, 25 mmol/L DTT, 0.5 mg/mL bovine serum albumin], 0.5 μL of oligo $pd(T)_{12\text{-}18}$ (500 μg/mL; Amersham Pharmacia Biotech, Piscataway, NJ), 2 μL deoxynucleotide triphosphates (2 mmol/L), 0.2 μL Superscript II (40 units, Life Technologies), and diethylpyrocarbonate double-distilled water in a total volume of 40 μL. The reaction was done at 42°C for 50 minutes and then inactivated at 70°C for 15 minutes. One microliter of the reaction mixture was used for PCR analysis done in 4 μL buffer (100 mmol/L KCl, 2.5 mmol/L MgCl, 0.05% gelatin), 0.4 μL deoxynucleotide triphosphates (10 mmol/L), 1 μL each of primers for IFN-γ (5′-GAGCCAAATTGTCTCCTTTTACTT, 3′-GTAGGCAGGACAACCATTACTGGG, 10 mmol/L), or Cα (5′-GCAAACGCCTTCAACAACAGC, 3′-CCACTTTCAG-GAGGAGGATTCG, 10 mmol/L), 1 unit of Taq polymerase (5 units/μL, Takara Shuzo, Co., Ltd., Shiga, Japan), and double-distilled water in a total volume of 20 μL. PCR was done at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for 30 cycles for IFN-γ and Cα on a Takara PCR Thermal Cycler SP (Takara Shuzo). To obtain a standard curve, 2.7×10^3 copy numbers of PCR products amplified with IFN-γ and Cα primer pairs were serially diluted and subjected to PCR. Each PCR product was hybridized with a ^{32}P-labeled IFN-γ or Cα probe and band intensities were quantified by an automated densitometer (Fujix BAS2500, Fujifilm I&I, Co. Ltd., Tokyo, Japan). The copy numbers of IFN-γ and Cα in each sample were estimated from standard curves. Relative IFN-γ mRNA = copy numbers of IFN-γ / copy numbers of Cα.

RESULTS

Patient Characteristics. In accordance with the protocol, a total of 11 patients were enrolled in the study from April 2001 to December 2002. Two patients in the level 1 group dropped out because their primary disease became worse during treatment and another treatment was prescribed. Patient characteristics are summarized in Table 1. Two patients were stage IV primary lung cancer and nine were recurrent lung cancer after surgical treatment. The study included nine patients with adenocarcinoma, one patient with squamous cell carcinoma, and one patient with large cell carcinoma. Ten patients received previous treatments, including two who underwent surgical resection, four cases who received radiation therapy, and six cases who received chemotherapy >6 weeks before enrollment in the study.

Table 1 Characteristics of enrolled patients

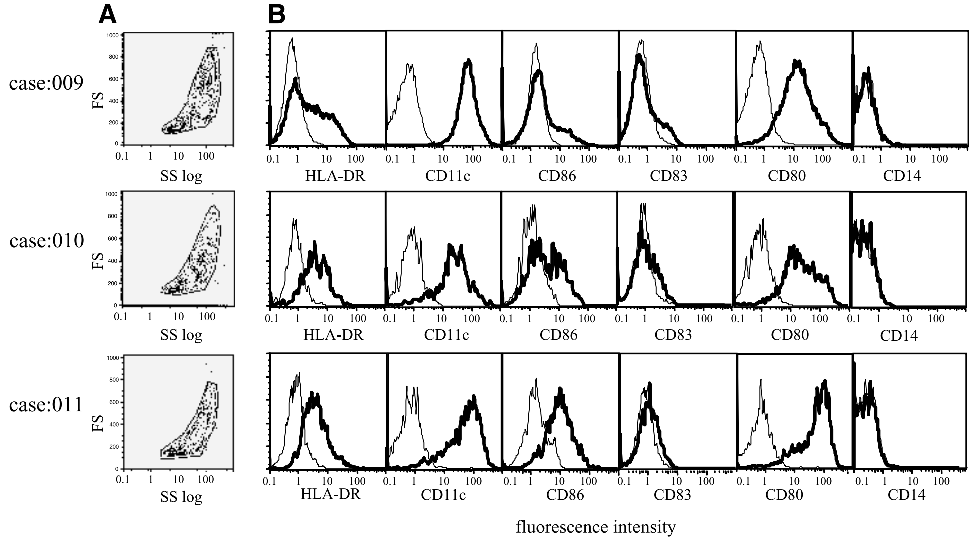
Total number entered	11
Completions/dropouts	9/2
Median age (range)	69.0 (63-78)
Gender, male/female	8/3
PS, 0/1/2	9/2/0
Histology, Ad*/Sq**/large***	9/1/1
Clinical staging, IV/recurrence	2/9
Prior treatment: surgey/radiation/chemotherapy/none	2/4/6/1

NOTE. Values represent numbers of patients.
Abbreviations: PS, performance status; Ad, adenocarcinoma; Sq, squamous cell carcinoma.

Phenotypes of Antigen-Presenting Cells Containing Dendritic Cells. The phenotypes of APCs containing DCs prepared for each administration were analyzed by flow cytometry for each administration. DC-rich population (i.e., large, granular lymphocytes) were electrically gated by forward and side scatter parameters ($FS^{high}SS^{high}$). Representative profiles for three patients in the level 3 group are shown in Fig. 2*B*. In all preparations, the $FS^{high}SS^{high}$ cells showed an immature monocyte-derived DC phenotype expressing HLA-DR, CD11c, CD80, and CD86. The expression of CD83 was marginal. All preparations were found to be negative for CD14. In addition, the percentages of $CD3^+$ cells, $CD3^-CD56^+$ cells, and $CD3^-CD56^-$ cells in the administered cells (four times per patient in level 3) are shown with SDs (Fig. 2*C*). The administered cells contained substantial numbers of $CD3^+$ cells in addition to $CD3^-$ cells.

Adverse Events. No major (above grade 2) toxicity or severe side effects were observed in any patient (Table 2). One patient in the level 1 group (case 001) experienced transient flush and headache after DC injection. One patient in the level 3 group (case 010) experienced general fatigue (grade 2) during the first course and headache 1 day after the second DC administration. This patient showed a striking expansion in the number of peripheral blood NKT cells (see Fig. 3, case 010). Cystitis (grade 2) observed in this patient will not be related to the treatment and cured by antibiotics prescription. In addition, an elevation in the serum potassium level (grade 1 and 2) was observed in two patients in the level 1 group and one patient in the level 2 group, an elevation in the serum creatinine level (grade 1) was observed in one patient in the level 1 group, and an elevation in the total-bilirubin level (grade 1) was observed in one patient in the level 2 group.

Immunologic Monitoring. Immunologic assays were done for the nine patients who completed the study. The frequency of peripheral blood NKT cells in all patients was measured by fluorescence-activated cell sorting analysis. As shown in Fig. 3*B*, one patient (case 010) in the level 3 group showed dramatic increase in the circulating NKT cell number after the first and second αGalCer-pulsed DC administration. The absolute numbers of Vα24 NKT cells decreased transiently to a nadir around 1 to 2 days after the αGalCer-pulsed DC injection, and subsequently increased >20-fold 3 days after second αGalCer-pulsed DC injection. The increased levels were sustained for at least 1 week. This sharp fluctuation, however, could not be detected after the third and fourth αGalCer-pulsed DC injection. The frequency of NKT cells of



C

	CD3+	CD3-CD56+	CD3-CD56-
009	52.2±8.5	23.8±12.9	15.4±10.3
010	83.2±3.4	12.7±3.4	4.1±2.5
011	70.2±13.3	17.3±11.8	11.5±12.1

Fig. 2 Flow cytometric analysis of αGalCer-pulsed APC (GM/IL-2 DC). *A*, forward scatter/side scatter (*FS*/*SS*) profiles with gates of the administered cells; dashed line, gate for *B*; solid line, gate for *C*. *B*, expression levels of HLA-DR, CD11c, CD86, CD83, CD80, and CD14 were assessed by flow cytometric analysis at the time of administration. Solid lines, background staining with an isotype control. Bold lines, staining profiles of the indicated molecules. Data are for three patients in the level 3 group. *C*, summary of the contents of cells administered. Each patient received αGalCer-pulsed APCs (GM/IL-2 DC) four times. The mean percentages of the indicated fractions in the administered cells are shown with SDs.

this patient (case 010) before entering the study was ~0.03% of all PBMCs. The number of peripheral blood NKT cells from the other two patients in the level 3 group (cases 009 and 011) increased only after the first αGalCer-pulsed DC administration (Fig. 3*A* and *C*). In the remaining six cases in the level 1 and level 2 groups, no clear relationship was found between the number of circulating NKT cells and αGalCer-pulsed DC administration.

In addition, we monitored IFN-γ production from Vα24 NKT cells in PBMCs. IFN-γ production in Vα24 NKT cells increased following the administration of αGalCer-pulsed DCs in the one case in which the number of circulating NKT cells changed strikingly (Fig. 4, case 010). After the third and fourth administration of αGalCer-pulsed DCs, no obvious elevation in the level of IFN-γ production was detected.

Clinical Outcome. Nine cases could be evaluated at the end of the clinical trial period. From chest X-ray and computed tomography findings, there were no cases of complete response or partial response, five cases of no change, and four cases of disease progression. Three patients receiving dose level 3 were followed up for 23 to 26 weeks after the clinical trial period and all were classified as no change.

DISCUSSION

The primary aim of this study was to assess the feasibility and toxicity of adoptive immunotherapy by using αGalCer-pulsed DCs in patients with advanced or recurrent non–small cell lung cancer. Our results indicate that αGalCer-pulsed DC therapy has no major side effects and is well tolerated even in patients with advanced stages of lung cancer.

Table 2 Patient profiles and adverse events observed

Level events (grade)	Case	Age/gender	Diagnosis	Cancer lesion	Pretreatment	Adverse
1	001	65/F	Rec	Lung	OP	Hot flash(I), headache(I), hyperkalemia(II), cretinine(I)
	002	63/M	Rec	Lung	None	Hyperkalemia(II)
	003	68/M	Rec	Lung	CT	None
2	006	65/M	Rec	Lung, brain, bone	CT, RT	Hyperbilirubinemia
	007	78/M	Rec	Lung	RT	Hyperkalemia(I)
	008	67/M	Rec	Lung	RT	None
3	009	61/M	Primary	Lung, bone, adrenal gland	RT	None
	010	66/F	Rec	Lung, pleura	OP, CT	Headache(I), cytitis(II), general fatigue(II)
	011	74/F	Primary	Lung, pleura, bone	CT	None

Abbreviations: M, male; F, female; rec, recurrence; primary, primary lung cancer; ST, surgical treatment; CT, chemotherapy; RT, radiation therapy.

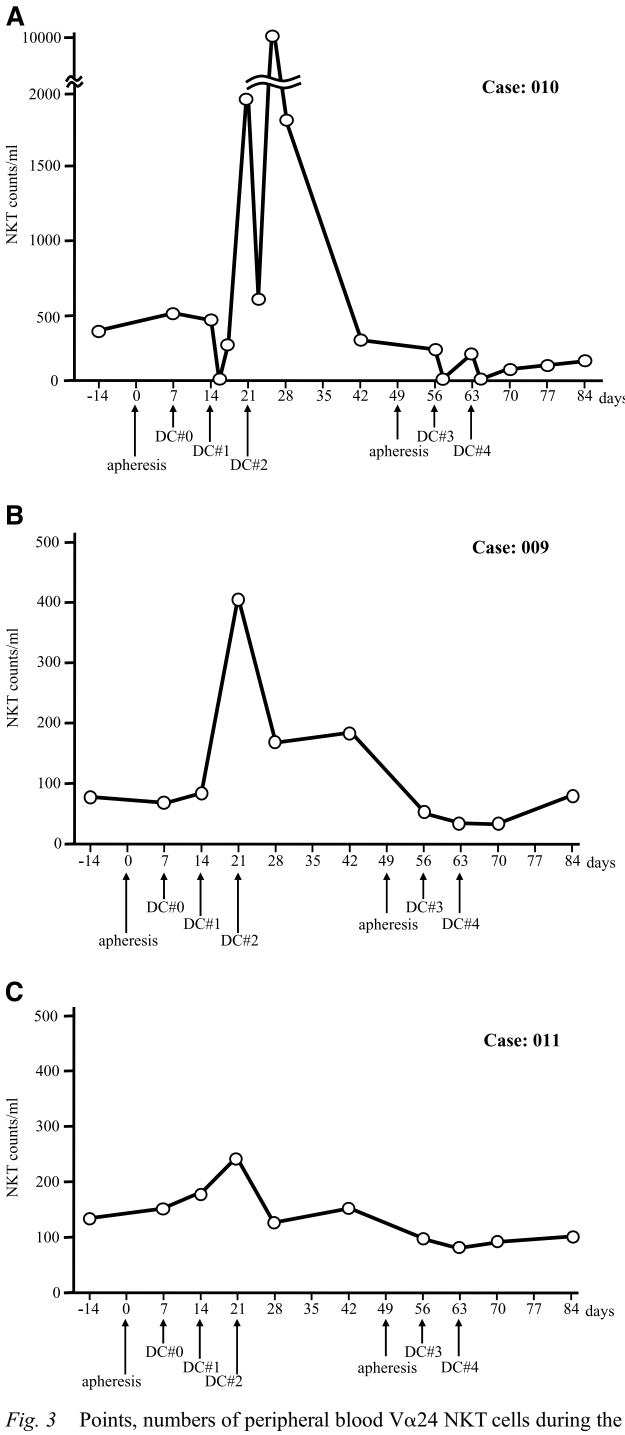


Fig. 3 Points, numbers of peripheral blood Vα24 NKT cells during the course of treatment in three patients in the level 3 group. The numbers and percentages of peripheral blood Vα24 NKT cells (Vα24$^+$Vβ11$^+$cells) during the course of treatment in three patients in the level 3 group were assessed by flow cytometric analysis. The frequency of Vα24 NKT cells in the total PBMCs of each patients is as follows; case 009, 0.01%; case 010, 0.03%; and case 011, 0.012%.

There were no clinical symptoms suggesting the development of an autoimmune disease during the observation period. Furthermore, the therapy was safe and done on outpatients. Although activated liver NKT cells in mice cause severe hepatitis (29, 30), slight liver dysfunction was detected in only one patient in the level 2 group, and this patient recovered without additional treatment. This could be due to the low frequency of αGalCer-reactive NKT cells in humans compared with mice (31).

We detected hyperbilirubinemia (T-bilirubin, 2.3 mg/dL, reference range 0.2-1.2 mg/dL) in patient 6 on day 35 (2 weeks after the second α-GalCer-pulsed DC administration). This patient showed the following: asparate aminotransferase (GOT,30 units/L, reference range 13-33 units/L), alanine aminotransferase (GPT, 28 units/L, reference range 8-42 units/L), and lactate dehydrogenase (LDH, 237 units/L, reference range 119-229 units/L). The patient had an episode of fluctuating increased T-bilirubin levels (1.5-2.0 mg/mL) without treatment. Thus, the elevated T-bilirubin in patient 6 on day 35 seems not to be related to our cell therapy. As for the relationship with hemolysis, patient 6 on day 35 showed potassium and LDH level at the upper limits (potassium, 4.9 mEq/L, reference range 3.5-5.0 mEq/L; LDH, 237 units/L, reference range 119-229 units/L), whereas the LDH level of this patient before entry was 272 units/L LDH. Thus, we cannot exclude the possibility of hemolysis in patient 6 on day 35; however, it is more likely that this is a feature unique to this patient. In any event, no treatment for the elevated T-bilirubin was required.

We observed one episode of cystitis in patient 10 between the administration of DC#3 and DC#4. Three days after the administration of DC#3, the patient experienced frequent urination, painful urination, and hematuria, and antibiotics were prescribed. No bacterial assessment of the urine was done because the symptoms were very mild and disappeared within a day. The transferred cells (DC#3) were tested for bacterial contamination and none was noted. As we did not observe cystitis after the administration of DC#1, DC#2, or DC#4, it is unlikely that the cystitis was caused by the therapy. In addition, because no

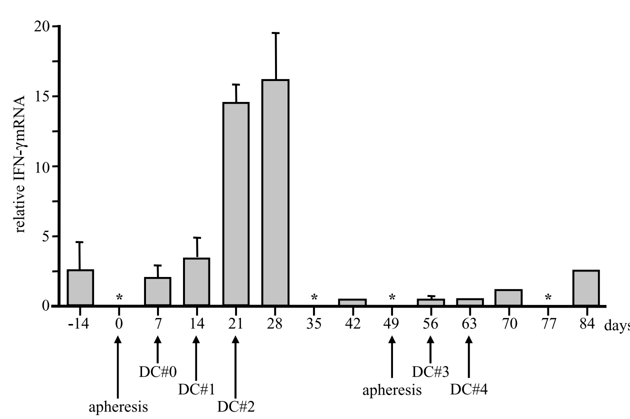


Fig. 4 Increased expression of IFN-γ in Vα24 NKT cells after administration of αGalCer-pulsed DCs. The ability to produce IFN-γ in peripheral blood Vα24 NKT cells during the course of treatment in case 010 was determined by quantitative reverse transcription-PCR. Whole PBMC was activated with immobilized anti-CD3ε overnight. Vα24$^+$Vβ11$^+$ NKT cells were sorted and quantitative reverse transcription-PCR for IFN-γ and Cα was done. Columns, relative IFN-γ mRNA (copy numbers of IFN-γ mRNA normalized to the copy numbers of TCR Cα mRNA in 100 Vα24$^+$Vβ11$^+$ NKT cells) for triplicate cultures; bars, SD. *, not detected.

increase in the level of peripheral blood NKT cells was observed after the administration of DC#3 or DC#4, it is also unlikely that the cystitis was the result of changes in NKT cells.

As for elevations in potassium, we observed two episodes in level 1 and one episode in level 2 treatment. We observed no episode of potassium elevation in level 3 treatment in which the highest doses of cells were administered. Nine patients received four cell administrations. Thus, although we cannot exclude the possibility of a temporal association, we do not believe there is any link between the administration of cells and potassium elevation. As for elevation in serum creatinine, we observed one episode in level 1; however, for the same reason as above, there seems to be no relationship between treatment and creatinine elevation. It is possible that the elevation in potassium was due to hemolysis, whereas no simultaneous elevation in T-bilirubin or LDH was detected. Thus, it is more likely that the patient experienced a transient reduction in kidney function. In fact, the levels of potassium were at the upper limit of the reference range (4.8 mEq/L) before entry into the study. However, no abnormalities in kidney function were detected in any of the patients with elevated potassium levels in the creatinine clearance test.

In contrast to other DC-based clinical trials, in which DCs were induced in medium containing granulocyte macrophage colony-stimulating factor and IL-4 (GM/IL-4 DC; refs. 32–36), the DCs used in our phase I study were prepared by stimulation with granulocyte macrophage colony-stimulating factor and IL-2 (GM/IL-2 DC). Our preliminary results showed that GM/IL-2 DCs stimulate NKT cells to proliferate very efficiently at levels similar to stimulated by GM/IL-4 DCs.[4] In our GM/IL-2 DCs, CD1d molecules are expressed on various cell subsets, including DCs, macrophages, T cells, and B cells. The presentation of αGalCer is independent from transporters associated with antigen processing and clearly different from that of peptide presentation by class I MHC molecules (1, 37). Thus, several cell types other than DCs may also be involved in αGalCer presentation and the resulting expansion of NKT cells.

The levels of circulating NKT cells are decreased in primary lung cancer patients, but the ability to produce IFN-γ is normal compared with healthy controls (20). Moreover, our previous reports showed that i.v.-injected αGalCer-pulsed mouse DCs migrate to the lung and activate endogenous Vα14 NKT cells *in situ* (19). The monitoring of Vα14 NKT cell number in the lung of mice receiving αGalCer-pulsed DCs showed a significant increase for at least 7 days (19). Furthermore, the number of Vα24 NKT cells in human lung seem to be equivalent to the number of Vα14 NKT cells in mouse lung and considerably increased numbers of Vα24 NKT cells have been found to infiltrate tumor legions (20). Therefore, we chose i.v. injection as the route of administration of αGalCer-pulsed DCs, as αGalCer-pulsed DCs activate lung NKT cells very efficiently. As for monitoring the injected DCs in circulation, there is a report suggesting that only a limited fraction of the i.v. administered DCs can be detected in the peripheral blood (38); thus, we did not include monitoring of the circulating DCs.

Primary lung cancer is hard to cure although the primary tumor lesion is small enough to be diagnosed at early stage. Approximately half or more of patients with lung carcinomas who underwent complete resection had clinically undetectable local or distant micrometastases (39–41). These patients probably had microscopic lesions that could not be removed by surgery. This emphasizes the importance of preoperative or postoperative immunotherapy to suppress the growth of micrometastasis. For this purpose, among lymphocytes possessing antitumor activity, cells for tumor surveillance, such as NK and NKT cells, should be most appropriate. Furthermore, because intrapulmonary recurrence is one of the common patterns of recurrence during postoperative follow-up periods, the control of intrapulmonary metastasis seems particularly important for improving prognosis. Immunotherapy would seem to be most beneficial when used as a postsurgical adjuvant therapy because the residual tumor would probably be at its minimum after complete resection. From this point of view, non–small cell lung cancer patients undergoing radical surgery may be the optimal candidates for immunotherapy aimed at NKT cell activation in the lung.

To detect changes in the function of Vα24 NKT cells after treatment, we used a semiquantitative reverse transcription-PCR assay with cell sorting. We prepared 100 sorted NKT cells and examined their copy numbers of IFN-γ mRNA normalized to the copy numbers of TCR Cα mRNA (Fig. 4). This was the only way to evaluate the function of Vα24 NKT cells in PBMCs. Although this analysis cannot determine whether the increase in IFN-γ mRNA reflects an increase in cell number or an increase on a per cell basis, the change reflects an increase on a population basis. The analysis of peripheral blood lymphocytes may not reflect the status of lymphocytes in the lung where tumor eradication might be occurring, but it is the only reliable method for the repeated detection of immune reactions in humans presently available. As shown in Fig. 4, the production of IFN-γ was up-regulated after the first two injections of αGalCer-pulsed DCs, although we detected only one case (case 010) with a high frequency of Vα24 NKT cells in the PBMC. This is the first observation that the function of Vα24 NKT cells is modulated after the administration of αGalCer-pulsed DCs in humans.

There are only a few reports of clinical studies using αGalCer against malignant diseases. Giaccone et al. (28) reported a rapid disappearance of NKT cells from the PBMC and slight increased serum level of IFN-γ, IL-12, granulocyte macrophage colony-stimulating factor, and tumor necrosis factor-α after the first i.v. injection of soluble αGalCer. Similar to our results, the changes were observed only in patients with relatively high NKT cell numbers before treatment. However, we did not detect IL-4 or IFN-γ in the peripheral blood of all patients. Nieda et al. (42) reported a decrease in the number of lymphocytes not only in peripheral blood NKT cell subsets but also in NK cell, T-cell, and B-cell subsets, after the administration of αGalCer pulsed GM/IL-4 DCs. In mouse models, the mechanism for the rapid disappearance of splenic NKT cells after αGalCer administration seems to be the down-regulation of surface TCR and NK1.1 markers (43–45). We also observed a rapid disappearance of Vα24 NKT cells from the peripheral blood in case

[4] S. Motohashi and T. Nakayama, unpublished observation.

010 (Fig. 3*B*). However, the most remarkable observation was a dramatic expansion of circulating NKT cells a few day after the transient disappearance. This dramatic expansion was not observed in previous reports (28, 42). We are currently performing a more comprehensive study with increased numbers of patients.

In summary, our results indicate that αGalCer-pulsed DC administration is well tolerated and that this therapy can be done safely even in patients with advanced disease. With greater numbers of treatments, we may have more conclusive findings about immune responses and antitumor responses. Furthermore, a combination of this αGalCer-pulsed DC therapy with potentially additive or synergistic therapeutic maneuvers may provide more prominent antitumor responses.

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