the two patients in the APA group who were diagnosed with bilateral aldosterone hypersecretion by AVS without ACTH stimulation responded well to the ACTH stimulation test. They underwent laparoscopic adrenalectomy and were histologically proven to have had an adrenal adenoma. After adrenalectomy, their hypertension and hypokalemia were cured. In two patients in the IHA group diagnosed with unilateral aldosterone hypersecretion by AVS without ACTH stimulation, no adrenal tumor was detected on CT scanning, although histological diagnosis could not be made. They were treated medically with aldosterone receptor blockers. Our results do not agree with a previous study by Seccia *et al.* (37), which showed that ACTH stimulation during AVS is confounding for diagnosis of an APA. However, Seccia *et al.* (37) used a selectivity index cutoff (>1.1) that was shown to be unreliable by others (36, 38, 39), justifying caution for the validity of their findings.

Earlier studies found that PAC levels in IHA patients are usually higher than those in essential hypertension patients. In this study, as shown in Table 1 and Fig. 3, the baseline PAC did not differ between the IHA group and the non-PA group. All patients in this study met the screening criteria of ARR greater than 20 at least once in an outpatient setting. Thus, most of those in the non-PA group had low renin hypertension, and they were substantially different from common essential hypertension patients. Moreover, the accurate diagnostic criteria for IHA have yet to be defined. Some reports consider it the part of PA not diagnosed as APA, whereas others regard it as bilateral aldosterone secretion. Pathologically, IHA is defined as hyperplasia of zona glomerulosa that hypersecrete aldosterone. However, most patients with IHA do not undergo surgery, and pathology-based diagnosis of IHA is rare. Therefore, the differential diagnosis between IHA and non-PA hypertension is usually based on the confirmatory diagnostic tests for PA. However, these tests do not always clearly discriminate between patients with PA and those without it. Rossi *et al.* (40) found that the AUC for diagnosis of PA by the saline infusion and captopril challenge tests were relatively low (0.811 and 0.785, respectively). Therefore, the non-PA group in the present study may have included IHA patients, and vice versa. However, the aim of this study was to detect patients highly suspicious of an APA that called for a definitive diagnostic test of PA subtype. The difference is important because an APA can be cured by surgery, and both essential hypertension and IHA benefit from medical treatments.

Our study is limited by its retrospective nature. A prospective, large-scale study is required to confirm the diagnostic accuracy of the rapid ACTH stimulation test under dexamethasone suppression in the diagnosis of an APA.

In conclusion, this study shows that the ACTH stimulation test is a supportive procedure in the diagnosis of an APA among patients suspected of PA. Although this test was not definitive enough for the final diagnosis of an APA, it can be performed in an outpatient setting and does not require special technique or devices. We rather suggest using this test to select candidates for a definitive subtype diagnostic test among those with suspected or confirmed PA (Fig. 4).

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Cross-priming of CD8$^+$ T cells in vivo by dendritic cells pulsed with autologous apoptotic leukemic cells in immunotherapy for elderly patients with acute myeloid leukemia

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Objective. **The prognosis for elderly patients with acute myeloid leukemia (AML) remains dismal. To explore the potential of immunotherapy for improving clinical outcomes for these patients, we performed a phase I clinical trial of dendritic cell (DC)–based immunotherapy for elderly patients with AML.**

Materials and Methods. **Autologus monocytes were obtained after reducing tumor burden by chemotherapy. Immature DCs induced with granulocyte-macrophage colony-stimulating factor and interleukin-4 were pulsed with autologous apoptotic leukemic cells as antigens. DCs were administered intradermally to four patients five times at 2-week intervals. To facilitate DC migration to lymph nodes, injection sites were pretreated with killed *Streptococcus pyogenes* OK-432 one day before. DCs were coinjected with OK-432 to induce maturation and interleukin-12 production in vivo.**

Results. **Antileukemic responses were observed by an interferon-γ enzyme-linked immunospot assay or a tetramer assay in two of four patients. In a human leukocyte antigen–A*2402-positive patient, induction of CD8$^+$ T-cell responses to WT1- and human telomerase reverse transcriptase–derived peptides were observed, indicating cross-priming in vivo. The two patients with antileukemic immunity showed longer periods of disease stabilization than the other two patients.**

Conclusions. **This study demonstrates the immunogenicity of autologous DCs that cross-present leukemia-associated antigens from autologous apoptotic leukemic cells in vivo in elderly patients with AML.**

Management of elderly patients with acute myeloid leukemia (AML) remains a challenge because of a high rate of therapy-related mortality and chemotherapy resistance [1]. Antigen-specific immunotherapy, which is less toxic and kills leukemic cells through different mechanisms than chemotherapy, has the potential capacity to improve the clinical outcomes of these patients. Recent identification of several leukemia-associated antigens prompted us to develop immunotherapy for elderly patients with AML [2].

Active immunization by peptide vaccines can induce antileukemic immunity and clinical responses in AML [3–6]. Clinical trials of dendritic cell (DC)–based immunotherapy for AML have also been reported [7–12]. However, the trial using leukemic cell–derived DCs showed that the generation of leukemic cell–derived DCs was feasible in only a limited number of patients, and even in vaccinated patients the treatment could not induce clinical responses [9]. This may be due to lower immunostimulatory activity of leukemic cell–derived DCs than monocyte-derived DCs (MoDCs) [13]. Recently, the efficient generation of MoDCs from patients with AML has been demonstrated in vitro [14], providing a rationale for the use of MoDCs in immunotherapy for AML.

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There are several parameters to enhance the immunogenicity of MoDC vaccines. Whereas monocytes are cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 conventionally for 5 to 7 days to induce DCs, a shorter period of culture is sufficient to induce equivalently potent DCs [15]. Among DC maturation–inducing factors, microbial components that trigger the production of IL-12 are beneficial to induce effective adaptive immunity [16]. An extended period of stimulation with microbial components results in DC exhaustion in which DCs lose the capacity to produce IL-12 [17]. Thus, a short-term stimulation can generate optimal DCs that retain IL-12 production. Inflammation in the skin before DC injection facilitates DC migration to draining lymph nodes, leading to a stronger immune response [18,19]. Using apoptotic whole tumor cells as antigens may be instrumental in inducing multivalent immune responses [20].

We performed in vitro assays to optimize these parameters. Based on the results of these assays, we conducted a phase I clinical trial of immunotherapy for elderly patients with AML at the second or later remission setting, using DCs loaded with autologous apoptotic leukemic cells. The treatment was well-tolerated and safe and induced antileukemic immunity in two of four patients, which was associated with transient disease stabilization. Importantly, in one patient, cross-priming of leukemia antigen–specific $CD8^+$ T cells in vivo was explicitly demonstrated. This study indicates the safety and immunogenicity of immunotherapy using MoDCs that cross-present leukemic cell antigens in elderly patients with AML.

Materials and methods

Generation, maturation, and cryopreservation of DCs for in vitro assays

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers by density gradient centrifugation using Lympholyte H (Cedarlane, Ontario, Canada). Monocytes were purified using anti–CD14-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), or enriched by plastic adherence by incubating PBMCs at 37°C for 2 hours and removing nonadherent cells by pipetting. Monocytes were cultured with 800 IU/mL GM-CSF (Primmune, Kobe, Japan) and 500 IU/mL IL-4 (Primmune) in CellGro DC medium (CellGenix Technologie Transfer, Freiburg, Germany) for 3 days (3d-DCs) or 6 days (6d-DCs). In some experiments, 3d-DCs were frozen in CP-1 freezing medium (Kyokuto Pharmaceutical Industrial, Tokyo, Japan). CP-1 contains 12% hydroxymethyl starch and 10% dimethyl sulfoxide in normal saline and was mixed with 8% human serum albumin before use. DCs were matured with 0.1 KE/mL OK-432 (Picibanil; Chugai Pharmaceuticals, Tokyo, Japan), a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of *Streptococcus pyogenes* (group A) [21].

In vitro analysis of DC functions

Flow cytometric analysis, measurement of IL-12p70 production, T-cell–stimulatory capacity of DCs for allogeneic naive $CD4^+$ T cells, and the cytokine profile of $CD4^+$ T cells primed with DCs were analyzed as described previously [15,22].

Uptake of apoptotic cells by DCs and the cross-presenting capacity of DCs

Efficiency of uptake of apoptotic cells by DCs was assessed as described previously [23] using myeloid leukemia cell lines K562, OUN-1 [24] (Dr. Yasukawa, Ehime University, Japan), and a T-cell leukemia cell line MT2, which were killed by 120 Gy γ-irradiation and 48-hour serum-free culture in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan). To examine the cross-presenting capacity of DCs, human leukocyte antigen (HLA)-A*2402–positive, immature 3d-DCs were pulsed with HLA-A*2402–negative, Epstein-Barr virus–transformed lymphoblastoid cell lines, which were killed as described here. DCs were matured with OK-432 (0.1 KE/mL) and prostaglandin E_2 (1 μg/mL) (MP Biomedicals, Solon, OH, USA) for 6 hours, and cocultured with autologous T cells at a DC-to-T cell ratio of 1:10. IL-2 (50 IU/mL; Teceleukin; Shionogi & Co., Ltd., Osaka, Japan) was added on the next day. For a positive control, DCs pulsed with HLA-A*2402–restricted EBNA3B peptide (TYSAGIVQI; KURABO Industries, Osaka, Japan) were used. Expansion of EBNA3A- and EBNA3B-specific $CD8^+$ T cells were evaluated by HLA tetramer staining [25].

Clinical trial protocol

The protocol was approved by the Ethics Committee, Graduate School and Faculty of Medicine, Kyoto University. Each patient gave written informed consent in accordance with the Declaration of Helsinki. The primary and secondary objectives were the assessment of safety and immunological and clinical responses, respectively.

Autologous leukemic cells were harvested before induction chemotherapy. Patients were required to be between 16 and 79 years of age and have a diagnosis of AML according to World Health Organization criteria [26,27]. Patients were excluded if they had another concurrent malignancy, an active autoimmune disease, positivity for blood-borne infectious agents, or a history of penicillin allergy (because OK-432 contains penicillin). Patients were enrolled if 5×10^7 or more leukemic cells were harvested. Thereafter, patients were treated with chemotherapy. More than 4 weeks after the last chemotherapy, patients proceeded to the DC vaccination if leukemic cells in bone marrow (BM) were <20%. In addition, to assess the clinical efficacy of DC vaccination, the presence of an evaluable lesion in BM, which was defined as 0.1% or more of leukemic cells by flow cytometry, was required. Furthermore, patients should have an Eastern Cooperative Oncology Group performance status of 0 to 2 and adequate vital organ functions. Patients were excluded if they had eligibility for hematopoietic stem cell transplantation or an uncontrollable infection. Concomitant chemotherapy and radiotherapy were prohibited.

DC vaccine generation

DC vaccines were generated from autologous monocytes under current Good Manufacturing Practice conditions. Autologous leukemic cells to be used as antigens were obtained as mononuclear cells (MNCs) by density gradient centrifugation over Ficoll-Hypaque (GE Healthcare, Buckinghamshire, UK) from BM and/or peripheral blood (PB) samples. MNCs were frozen in CP-1 freezing medium and stored at −150°C. Before added to DCs, MNCs were killed by 120 Gy

γ-irradiation and 48 hours serum starvation. Killing of MNCs was confirmed by the percentage of Annexin V–positive cells being 90% or more by flow cytometry and reduced uptake of [^{3}H]-thymidine to the baseline level.

Apheresis products, which were obtained with COBE Spectra (Caridian BCT, Lakewood, CO, USA) from 10 L blood, were processed by elutriation using Elutra (Caridian BCT) to enrich monocytes. At the time of apheresis, no leukemic cells were observed in the PB of the patients, as assessed by a routine clinical laboratory test. Monocytes were cultured with 800 U/mL GM-CSF and 500 U/mL IL-4 in CellGro DC medium in gas-permeable plastic bags (VueLife 118; CellGenix Technologie Transfer) at 37°C, 5% CO_2 to generate immature DCs. After 48 hours, DCs were pulsed with autologous apoptotic leukemic cells and 2 μg/mL keyhole-limpet hemocyanin (KLH; Biosyn Corporation, Carlsbad, CA, USA). The endotoxin level in the KLH preparation examined by the supplier was <0.1 IU/mg. After an additional 24 hours, DCs were frozen as immature DCs in CP-1 freezing medium and stored at −150°C.

Administration of the DC vaccine

A total of 1×10^7 DCs were intradermally injected at four sites in bilateral arms and thighs. Twenty-four hours before DC administration, the injection sites were pretreated by 0.2 KE/site OK-432. At the time of DC administration, DCs were thawed and mixed with 1 KE OK-432. Then, the mixture of DCs and OK-432 was injected. The DC administration was repeated at 2-week intervals for five administrations.

Monitoring of immunological and clinical responses

Antigen-specific immune responses were assessed at indicated time points. Immune responses to KLH and autologous leukemic cells were tested by skin delayed-type hypersensitivity tests and interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays. In addition, in a HLA-A*2402–positive patient, immune responses to HLA-A*2402–restricted peptides derived from leukemia-associated antigens were examined by IFN-γ ELISPOT assay and HLA tetramer staining. The peptides used in the assays were the natural $WT1_{235-243}$ peptide (CMTWNQMNL) [24], the modified $WT1_{235-243}$ peptide (CYTWNQMNL) [28], the human telomerase reverse transcriptase $(hTERT)_{461-469}$ peptide (VYGFVRACL) [29], and the lower matrix 65-kd phosphoprotein (pp65) of cytomegalovirus (CMV) (amino acids 328–336; QYDPVAALF) [30]. All peptides were purchased from Multiple Peptide Systems (San Diego, CA, USA). Both PBMCs and BM mononuclear cells (BMMCs) were subjected to assays before and after 1-week in vitro stimulation with antigen- or peptide-pulsed DCs in the presence of 15 U/mL IL-2 (Teceleukin). To evaluate clinical responses, percentages of leukemic cells in BM were monitored by morphology and flow cytometry at indicated time points.

Skin delayed-type hypersensitivity test

The 4×10^5 antigen-pulsed DCs were intradermally injected in the forearm. Sizes of induration and erythema were measured 48 hours later. Erythema that was 1.5-fold or larger in diameter than the antigen-unpulsed control was considered positive.

IFN-γ ELISPOT assay

IFN-γ ELISPOT assays (Mabtech, Nacka Strand, Sweden) were performed using antigen-pulsed DCs and peptide-pulsed C1R-A*2402 (Dr. Masafumi Takiguchi, Kumamoto University, Kumamoto, Japan). Stimulator cells were plated at 2×10^4 cells/well. As responder cells, fresh and in vitro–stimulated MNCs from PB and BM were plated with fresh MNCs at 1 to 2×10^5 cells/well and in vitro–stimulated MNCs at 1 to 2×10^4 cells/well. After overnight incubation, spots were developed using 3-amino-9-ethylcarbazole (Sigma Chemical, St Louis, MO, USA) and counted by KS ELISPOT compact (Carl Zeiss MicroImaging, Tokyo, Japan). Numbers of specific spot-forming cells were calculated by subtracting the number of spots with unpulsed DCs from the number of spots with antigen-pulsed DCs.

HLA tetramer staining

Natural $WT1_{235-243}$ peptide/HLA-A*2402 tetramer was purchased from Medical & Biological Laboratories (Nagoya, Japan). Modified $WT1_{235-243}$ peptide/HLA-A*2402 tetramer and a peptide derived from the HIV envelope (env) protein/HLA-A*2402 tetramer were produced as described previously [30]. Fresh and in vitro–stimulated MNCs were stained with a tetramer and fluorescein isothiocyanate–conjugated anti-CD8 monoclonal antibody (BD Biosciences) and analyzed by flow cytometery (FACSCalibur; BD Biosciences) [30].

Results

In vitro assays to optimize generation of DCs

To optimize generation of DCs, we performed in vitro functional assays. We first compared DCs differentiated from monocytes in the presence of GM-CSF and IL-4 for 3 days with 6-day differentiated DCs conventionally used in clinical trials. After 24-hour exposure to OK-432, both 3d-DCs and 6d-DCs showed similar levels of surface molecule expressions, IL-12p70 production, and T-cell stimulatory capacity for allogeneic naïve $CD4^+$ T cells (Supplementary Figure E1; online only, available at www.exphem.org), indicating that 3d-DCs have functions comparable with 6d-DCs. Next, we examined the capacity of 3d-DCs to cross-present apoptotic cell–associated antigens. At the DC-to-apoptotic cell ratio of 1:1, 11% to 33% of immature 3d-DCs incorporated apoptotic leukemia cell lines (Fig. 1A). Moreover, HLA-A*2402–positive DCs pulsed with killed lymphoblastoid cell lines from an HLA-A*2402–negative donor induced expansion of $CD8^+$ T cells specific for the HLA-A*2402–restricted epitopes of EBNA3A and EBNA3B (Fig. 1B), indicating the capacity of DCs to cross-present apoptotic cell–derived antigens.

An extended period of exposure of DCs to lipopolysaccharide leads to DC exhaustion [17], as indicated by loss of IL-12–producing capacity by DCs. To examine whether OK-432 induces DC exhaustion, we analyzed the maturation kinetics of OK-432–stimulated 3d-DCs. Upregulation of the surface molecules (Fig. 2A) and IL-12p70 production (Fig. 2B) became evident 4 and 8 hours after OK-432 stimulation, respectively. Maximal levels of surface molecule expressions and IL-12p70 production were observed at 48 hours. Next, we examined how many hours of exposure to OK-432 is sufficient to elicit a maturation signal to DCs, using 3d-DCs that were cultured for a total of 48 hours with different

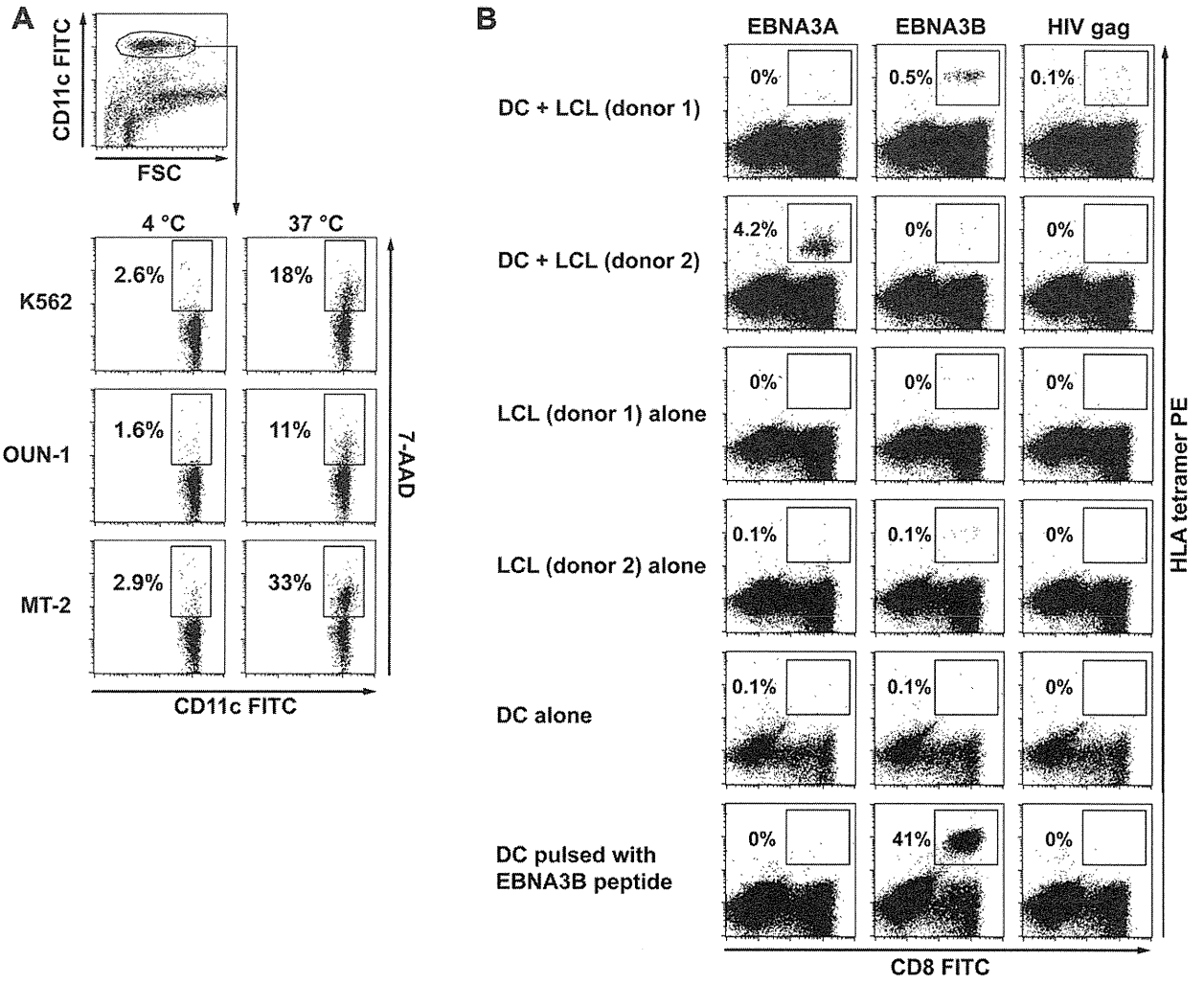

Figure 1. 3d-DCs incorporate apoptotic cells and cross-present cell-associated antigens. (**A**) Uptake of apoptotic cells by 3d-DCs. Apoptotic K562, OUN-1, and MT2 were labeled with 7-aminoactinomycin D (7-AAD) (20 μg/mL), and cocultured with immature 3d-DCs at a DC-to-apoptotic cell ratio of 1:1. After 4 hours of incubation at 4°C or 37°C, cells were stained with fluorescein isothiocyanate–conjugated anti-CD11c monoclonal antibody and analyzed by flow cytometry. Cells positive for both CD11c and 7-AAD were considered to be DCs that had phagocytosed apoptotic cells. (**B**) The cross-presenting capacity of DCs. Immature 3d-DCs from a HLA-A*2402–positive donor were pulsed with apoptotic HLA-A*2402–negative donor-derived lymphoblastoid cell lines (LCLs), matured with OK-432 and prostaglandin E_2, and cocultured with autologous T cells. For a positive control, DCs pulsed with the EBNA3B peptide were used as a stimulator. After 7 days, expansions of EBNA3A- and EBNA3B-specific $CD8^+$ T cells were evaluated by HLA tetramer staining. Dead cells are excluded by staining with propidium iodide. Numbers shown indicate percentages of tetramer-positive cells among $CD8^+$ cells. Representative data from two experiments are shown.

durations of exposure to OK-432 at the start of culture. As short as 2-hour exposure upregulated CD83 and CD86 (Fig. 2C) and induced IL-12p70 production (Fig. 2D) during the subsequent 46-hour culture without OK-432. Although at the time of 8-hour exposure, the induction of CD83, CD86 (Fig. 2A), and IL-12p70 (Fig. 2B) was low, 8-hour exposure was sufficient to induce maximal levels of CD83 and CD86 expression (Fig. 2C) and IL-12p70 production (Fig. 2D). Notably, although initial 24-hour exposure to OK-432 induced the maximal levels of CD83 and CD86 expression (Fig. 2C), DCs did not produce a detectable level of IL-12p70 during the last 24-hour culture (Fig. 2D). These data indicate that, like lipopolysaccharide [17], OK-432–induced IL-12p70 production was limited within the first 24 hours and most active between 8 and 24 hours after OK-432 stimulation. The functional significance of ongoing IL-12p70 production by DCs in priming naïve $CD4^+$ T cells was supported by the data that 3d-DCs matured with OK-432 for 6 hours showed a superior capacity to induce IFN-γ–producing T cells to those matured for 24 hours (Fig. 2E). Thus, extended stimulation with OK-432 induces DC exhaustion. To avoid it, we decided to administer immature DCs together with OK-432 to patients and to induce DC maturation in vivo.

It is convenient to prepare a large number of DCs from a single batch of apheresis and freeze them in aliquots. We assessed the effect of cryopreservation on DCs. Whereas cryopreserved immature 3d-DCs showed somewhat higher percentages of dead cells after 24-hour culture with or

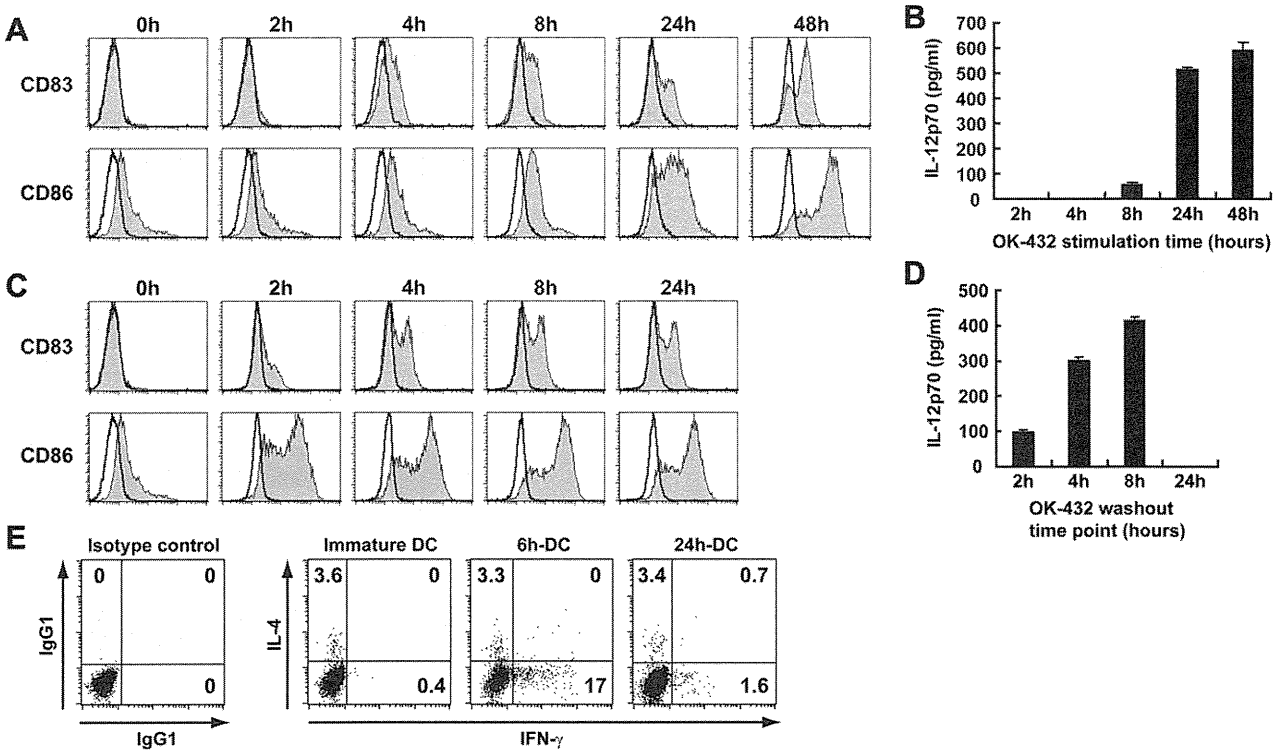

Figure 2. Short-term stimulation with OK-432 is optimal to generate Th1-inducing mature DCs. (**A**, **B**) Immature 3d-DCs were cultured in the presence of OK-432 (0.1 KE/mL) for indicated time periods, then harvested and analyzed. (**C**, **D**) Immature 3d-DCs were cultured in the presence of OK-432 for indicated time periods, washed, replated, and further cultured for a total of 48 hours. Cells and supernatants harvested at 48 hours were analyzed. (**A**, **C**) Expression of CD83 and CD86 was analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms indicate staining with isotype controls. (**B**, **D**) IL-12p70 production in culture supernatants of DCs (5×10^5 cells/mL) were measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. (**E**) Naïve $CD4^+$ T cell differentiation induced by DCs. Immature 3d-DCs were matured with OK-432 (0.1 KE/mL) for 6 or 24 hours and cocultured with allogeneic naïve $CD4^+$ T cells for 7 days. Cytokine profiles of T cells were analyzed by intracellular cytokine staining. Numbers indicate percentages of cells in each quadrant. Representative data from four experiments are shown.

without OK-432, and tended to produce a lower amount of IL-12p70 upon OK-432 stimulation as compared with non-cryopreserved DCs (Supplementary Figure E2A, C; online only, available at www.exphem.org), similar levels of CD83 and CD86 expression were induced by OK-432 in both DCs (Supplementary Figure E2B; online only, available at www.exphem.org). Thus, although cryopreservation of immature DCs impaired their function to some extent, cryopreserved DCs largely retained the viability and expression of immunostimulatory molecules. Considering the practical convenience to prepare a stock of DCs at one time, we decided to freeze DCs as immature DCs. Taken together, these data demonstrate that DCs generated in the present study are capable of inducing $CD8^+$ T-cell responses to apoptotic cell–derived antigens, and that immature DCs can be cryopreserved without critical loss of functions.

Patients, feasibility, and safety

Thirteen patients were recruited to the study for the leukemic-cell harvest at the onset of AML. After chemotherapy, four patients were eligible for DC vaccination (Table 1). In these patients, $>5 \times 10^7$ DCs for five vaccinations could be generated from a single apheresis. Autologous apoptotic leukemic cells were added to DCs as antigens at leukemic cell-to-DC ratios of 1:3.3 to 1:6.5, depending on the numbers of collected leukemic cells (Supplementary Table E1; online only, available at www.exphem.org). Status of PB and BM at the time of apheresis are shown in Supplementary Table E1 (online only, available at www.exphem.org). Representative data of surface molecule expressions on DCs are shown in Supplementary Figure E3 (online only, available at www.exphem.org).

All of the patients completed the five vaccinations safely (Table 1). In all the patients, grade 1 to 2 fever and grade 2 skin reactions at the injection sites were observed. The fever was resolved within 2 days after vaccination and most likely related to administration of OK-432. The skin reactions at the injection sites were transient and characterized by erythema, pruritus, and tenderness. No significant toxicities to vital organs or signs of autoimmunity were observed.

Induction of antigen-specific immune responses to KLH and leukemic cells

Induction of an immune response to KLH was detected by skin delayed-type hypersensitivity tests and/or IFN-γ ELISPOT assays in three patients, with the exception of patient no. 4

Table 1. Patient characteristics and results of the DC vaccination

Patient no.	Age/Sex	Diagnosis	DC vaccination was started		LC in BM at the first vaccination[a] (%)	Adverse effects[b]	Immune response		Clinical response	Died at (days after the last vaccination)
			After the last CT (d)	After diagnosis (d)			KLH	LC		
1	76/F	AML-MRC	82	93	1.8	Fever (1) Injection site reaction (2)	Yes	No	PD Died of sepsis with leukemia	186
2	75/M	AML-MRC	40	155	0.6	Fever (1) Injection site reaction (2)	Yes	Yes	Transient disease stabilization Died of leukemia	391
3	70/M	AML-MRC	44	344	2.9	Fever (2) Injection site reaction (2)	Yes	Yes	Transient disease stabilization Died of sepsis with leukemia	192
4	66/M	AML M2	67	144	0.2	Fever (1) Injection site reaction (2)	No	No	PD Died of leukemia	66

AML-MRC = acute myeloid leukemia with myelodysplasia-related changes; CT = chemotherapy; F = female; LC = leukemic cells; M = male; PD = progressive disease.

[a]Percentages of leukemic cells in bone marrow were determined by flow cytometry.

[b]Numbers in parentheses indicate grade of toxicity according to the National Cancer Institute-Common Terminology Criteria for Adverse Events version 3.0.

(Table 1 and data not shown). Two patients (patient nos. 2 and 3) showed induction of immune responses to leukemia-associated antigens. In patient no. 2, who was HLA-A*2402–negative, IFN-γ ELISPOT assays using autologous leukemic cell–pulsed DCs revealed the induction of antileukemic immunity in PBMCs and BMMCs without in vitro stimulation after the fourth vaccination (Fig. 3A). The antileukemic immune response was still detected 1 month after the fifth vaccination in in vitro–stimulated PBMCs and BMMCs (Fig. 3B), but was no longer detected without in vitro stimulation (Fig. 3A). We could not test antileukemic immunity at subsequent time points in this patient because the patient developed leukocytopenia, probably owing to progression of myelodysplastic syndrome.

In patient no. 3, who was HLA-A*2402–positive, HLA-A*2402–restricted peptides from WT1 and hTERT were used in immunological monitoring. $CMVpp65_{328-336}$ peptide was used as a positive control in ELISPOT assays (Fig. 4B). No responses to the leukemia-associated antigens were observed until the fourth vaccination. However, 2 months after the fifth vaccination, positive responses to the modified $WT1_{235-243}$ and the $hTERT_{461-469}$ peptides were detected in in vitro–stimulated PBMCs by HLA tetramer staining (Fig. 4A) and an IFN-γ ELISPOT assay (Fig. 4B), respectively. The PBMCs binding to the modified $WT1_{235-243}$ peptide/HLA-A*2402 tetramer also bound to the natural $WT1_{235-243}$ peptide/HLA-A*2402 tetramer (Fig. 4A), indicating that these cells were capable of recognizing the natural WT1 peptide presented on leukemic cells. These responses were short-lived and almost completely disappeared 3 months after the fifth vaccination. No responses were detected in PBMCs or BMMCs without in vitro stimulation (data not shown). Thus, the vaccinations induced HLA class I–restricted, antileukemic immunity, indicating that the DCs cross-presented leukemia-associated antigens in vivo. In addition, in patient no. 2, leukemic cell-reactive T cells were detected in BM (Fig. 3), the main tumor site in leukemia.

Clinical outcomes

The two patients with antileukemic immunity had longer periods of disease stabilization than the other two patients without antileukemic immunity (Fig. 5A). Notably, in patient no. 3, the percentages of leukemic cells in BM dropped from 11% to 5.2% during the second month after the fifth vaccination, when a positive antileukemic immunity was observed (Fig. 5B). Thus, these observations suggest that induction of antileukemic immunity was associated with extended the periods of disease stabilization in these patients.

Discussion

Novel therapies with less toxicity are necessary for intractable AML in elderly patients. In this study, we conducted a phase I clinical trial of immunotherapy for such patients using DCs pulsed with autologous apoptotic leukemic cells.

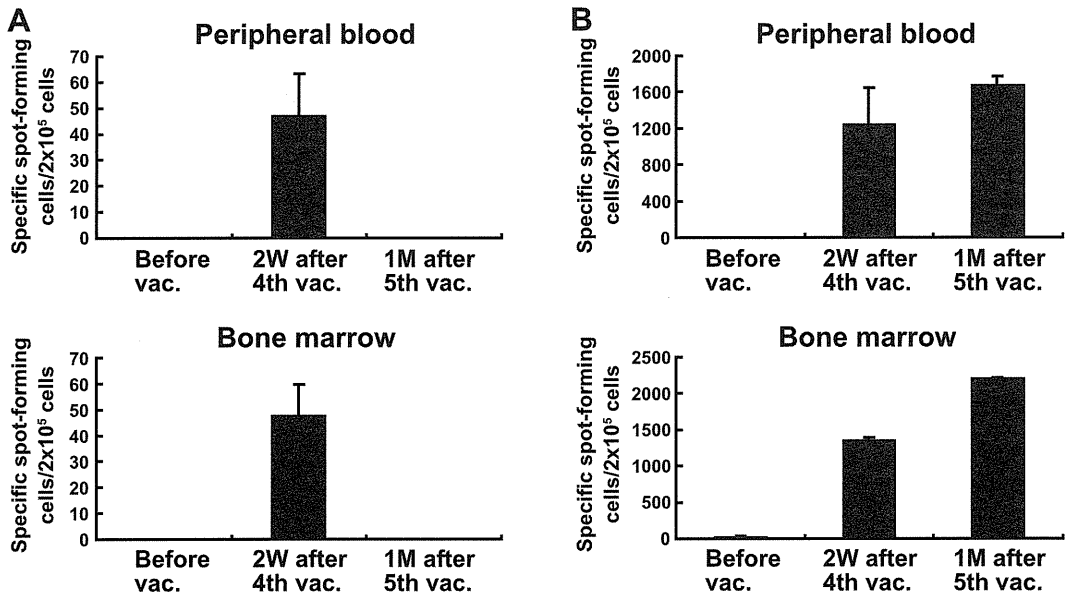

Figure 3. IFN-γ ELISPOT assay in patient no. 2. MNCs from PB and BM were obtained at indicated time points and subjected to IFN-γ ELISPOT assays directly after isolation (**A**) or after 1 week of stimulation with antigen-pulsed DCs (**B**). In IFN-γ ELISPOT assays, 2×10^5 MNCs (**A**) and 1×10^4 MNCs (**B**) were incubated with 1×10^4 leukemic cell–pulsed or unpulsed DCs. Numbers of specific spot-forming cells per 2×10^5 MNCs, calculated by subtracting numbers of spots with unpulsed DCs from numbers of spots with leukemic cell–pulsed DCs. Error bars indicate the standard deviation of duplicate measurements.

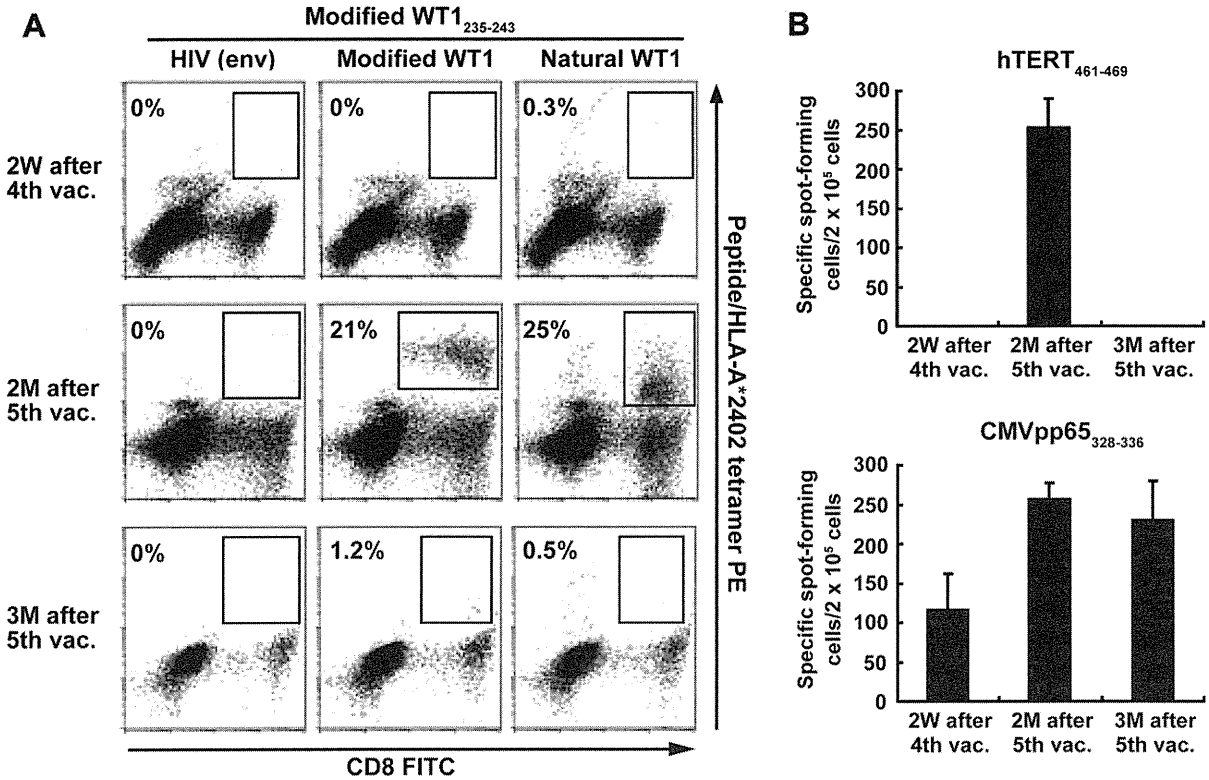

Figure 4. Immune responses in patient no. 3. (**A**) HLA tetramer staining. MNCs from PB were obtained at indicated time points, stimulated for 1 week with DCs pulsed with the modified $WT1_{235-243}$ peptide, stained with phycoerythrin-labeled peptide/HLA-A*2402 tetramers and fluorescein isothiocyanate–labeled anti-CD8 monoclonal antibody, and analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Numbers indicate percentages of tetramer-positive cells among $CD8^+$ cells. (**B**) IFN-γ ELISPOT assay. MNCs were stimulated for 1 week with DCs pulsed with the $hTERT_{461-469}$ or $CMVpp65_{328-336}$ peptide, and subjected to IFN-γ ELISPOT assays. In the assays, 2×10^4 MNCs were incubated with 2×10^4 C1R-A*2402 pulsed with or without the $hTERT_{461-469}$ or $CMVpp65_{328-336}$ peptide. Before vaccination, the assay was performed using DCs as a stimulator, which induced many nonspecific spots. Thus, the data before vaccination are not shown. Numbers of specific spot-forming cells per 2×10^5 MNCs, calculated by subtracting numbers of spots with unpulsed C1R-A*2402 from numbers of spots with antigen-pulsed C1R-A*2402, were depicted. Error bars indicate the standard deviation of duplicate measurements.

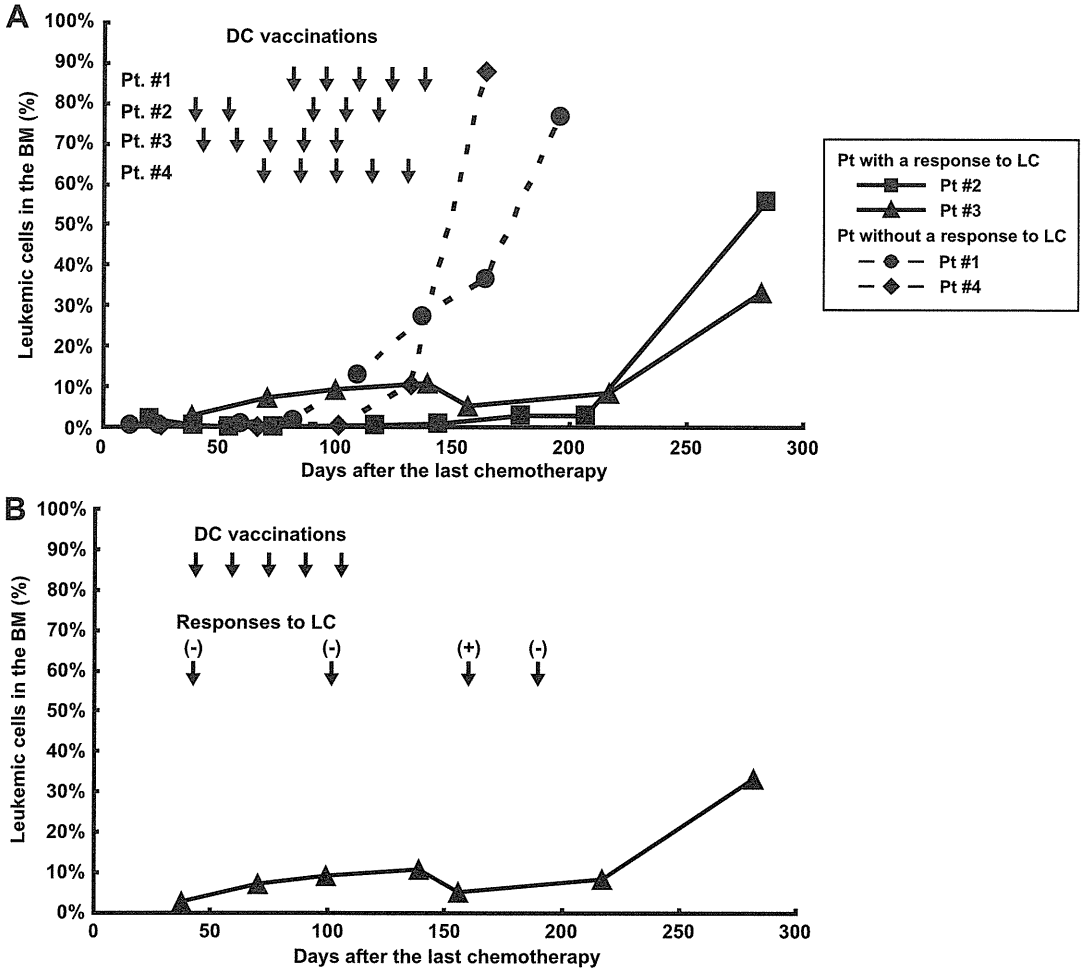

Figure 5. Clinical courses during the DC vaccination. (**A**) Percentages of leukemic cells in BM as determined by flow cytometry in four vaccinated patients are shown. Solid lines indicate patients with immune responses to leukemic cells (LCs) (patients 2 [■] and 3 [▲]). Dashed lines indicate patients without immune responses to LCs (patients 1 [●] and 4 [◆]). Arrows indicate time points when DC vaccines were administered to each patient. (**B**) The clinical course of patient no. 3. Arrows indicate time points when immunological monitoring was performed. Plus (+) or minus (−) signs indicates that immune responses to leukemic cells were detected or not detected at that time point, respectively.

Induction of antileukemic immunity was observed in two of four vaccinated patients. This is the first study that demonstrates cross-priming of $CD8^+$ T cells by DCs pulsed with apoptotic leukemic cells in vivo in humans, thus providing a proof of principle of this approach. The limited number of patients prevented us from drawing any definitive conclusion regarding clinical efficacy from the present trial. However, longer periods of disease stabilization observed in the two patients with antileukemic immunity compared to the other two patients without antileukemic immunity implied that induction of antileukemic immunity might have impacted on the clinical course of these patients.

There are several features in the method of DC vaccination in this trial: short-term 3-day culture to generate DCs in an attempt to reduce labor, cost, and time; use of whole leukemic cells as antigens to induce multivalent immune responses; use of the microbial adjuvant OK-432 as a maturation-inducing factor to generate Th1-inducing DCs; in vivo maturation of DCs to avoid DC exhaustion by extended stimulation in vitro with OK-432; and prior induction of inflammation at the injection sites to facilitate DC migration to draining lymph nodes.

We used autologous apoptotic leukemic cells as antigens because several studies have shown that apoptotic cells are more efficiently cross-presented by DCs to $CD8^+$ T cells than soluble antigens such as tumor lysate [31–34]. Furthermore, MoDCs has been shown to cross-present apoptotic leukemic cells to $CD8^+$ T cells in vitro [35]. Apoptotic cells as antigens also have advantages over peptides, in that the DCs have the ability to process multiple antigens from the apoptotic cells and present those antigens on their own HLA molecules. In this study, we clearly showed that MoDCs cross-presented leukemia-associated antigens WT1 and hTERT from apoptotic leukemic cells. Furthermore, T cells reactive to leukemic cells were detected in BM.

A murine study has shown that DC maturation not by inflammatory cytokines but by pathogen-derived components is crucial for DCs to acquire the capacity to differentiate naïve $CD4^+$ T cells into effector T cells [16]. We used OK-432, a preparation of killed *Streptococcus pyogenes* [21], which strongly triggers DC maturation through Toll-like receptor 4 [36–39]. We showed that, like lipopolysaccharide [17], longer stimulation with OK-432 induces DC exhaustion, resulting in the reduced capacity of DCs to induce Th1 responses. Several preclinical studies have shown that DCs briefly exposed to Toll-like receptor ligands are better inducers of Th1-type and cytotoxic T-cell responses [17,40,41]. Moreover, a clinical trial suggests superiority of briefly matured DCs in pediatric patients with cancer [42]. In this trial, we administered immature DCs together with OK-432 to avoid DC exhaustion before administration. The induction of IFN-γ detected by the ELISPOT assay implied IL-12 production by DCs in vivo.

Only a small proportion of intradermally administered DCs reach draining lymph nodes [43,44]. In a mouse model, pretreatment of administration sites with inflammatory cytokines enhance DC migration to regional lymph nodes [18]. Based on this finding, we pretreated administration sites with a low dose of OK-432. Because of unavailability of a cell-processing facility for cells labeled with indium-111 oxyquinoline [43,44], we could not evaluate the efficiency of DC migration to lymph nodes. Whether this administration procedure is superior to others should be evaluated in future studies.

In this study, multiple vaccinations were required to elicit antileukemic immunity, which rapidly declined after cessation of vaccination. Maintenance of antileukemic immunity might lead to improvement of clinical efficacy, and might be fulfilled by increasing the number of vaccinations, which was, however, impossible in this study because of the limited availability of autologous leukemic cells. Thus, if a peptide is available for the induced antileukemic $CD8^+$ T-cell response, peptide vaccination may be added after DC vaccination. Furthermore, blockade of immunosuppressive mechanisms may be combined.

In conclusion, we demonstrated the feasibility, safety, and immunogenicity of DC-based immunotherapy for elderly patients with AML. Cross-priming of $CD8^+$ T cells by DCs pulsed with autologous apoptotic leukemic cells was provoked in vivo. The results were promising, yet further intensification of vaccine potency is clearly required. This novel therapeutic approach may lead to improvement of clinical outcomes in elderly patients with AML, which has been difficult to achieve with other therapeutic approaches.

Acknowledgments

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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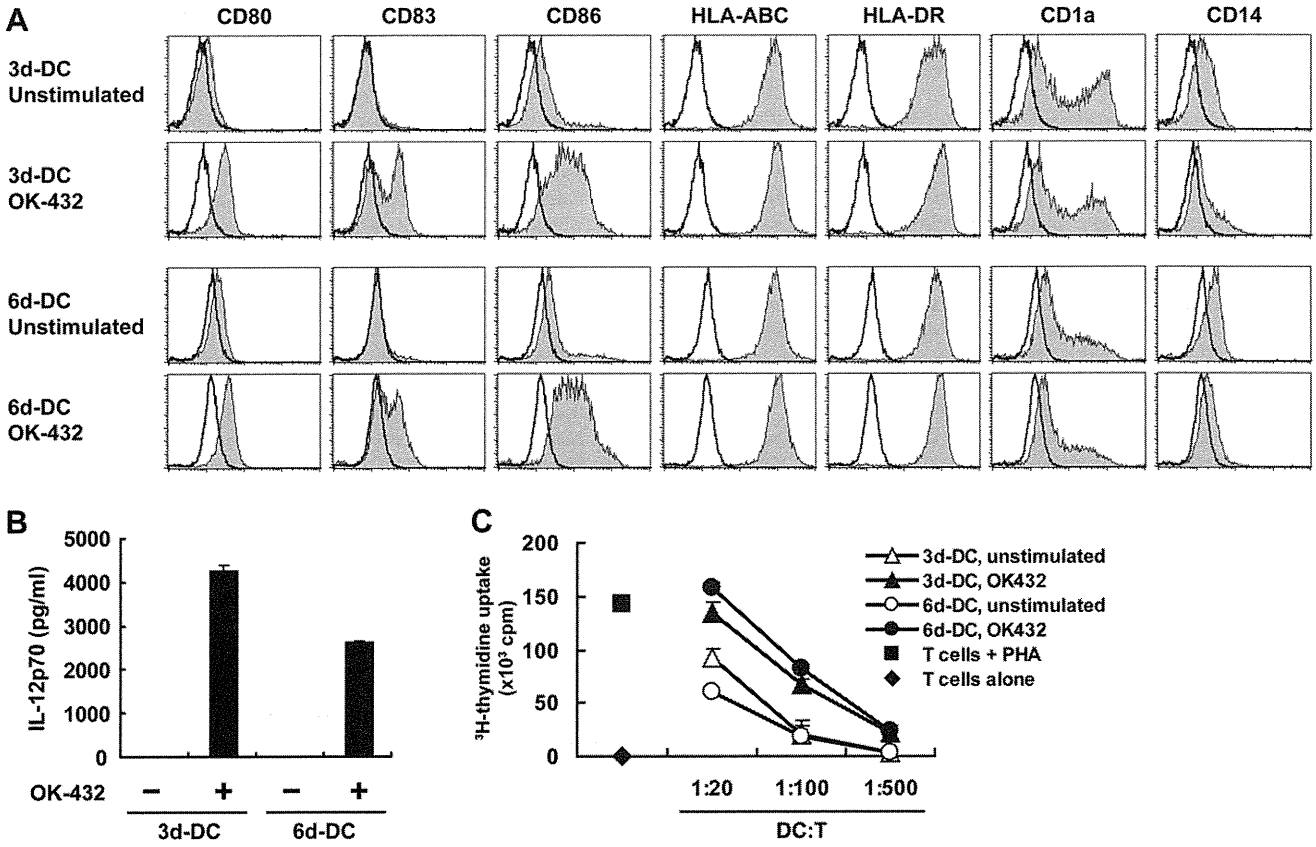
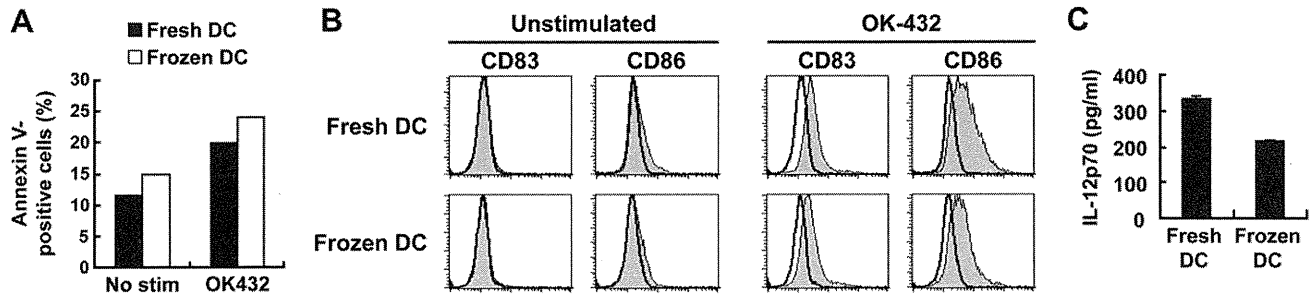
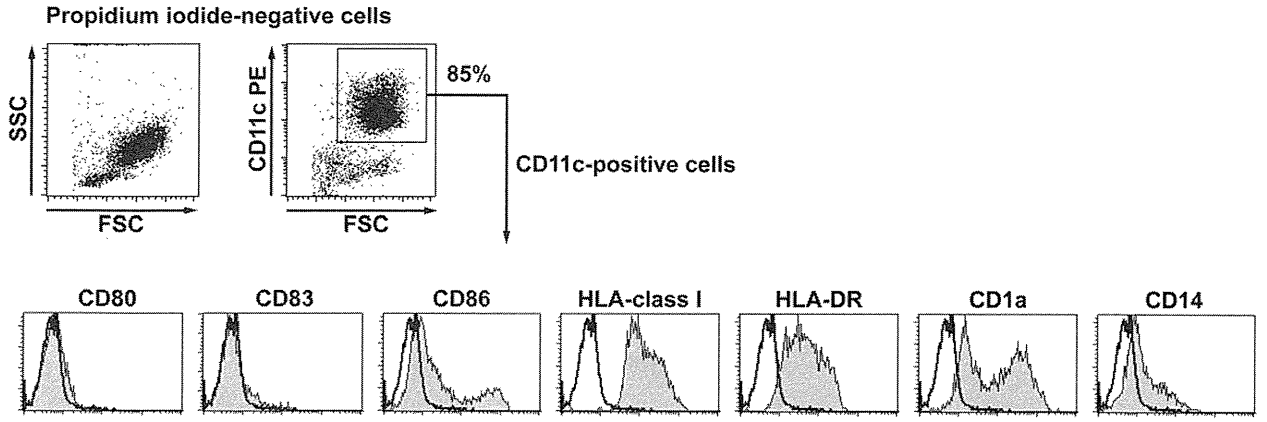


Supplementary Figure E1. 3d-DCs and 6d-DCs have comparable T-cell stimulatory capacity. (**A**) Expressions of surface molecules on DCs. Unstimulated or OK-432-stimulated DCs were analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms indicate staining with isotype controls. (**B**) IL-12p70 production by DCs (5×10^5 cells/mL) stimulated with OK-432 (0.1 KE/mL) for 24 hours was measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. (**C**) Proliferation of naive $CD4^+$ T cells stimulated with DCs. Allogeneic naive $CD4^+$ T cells were cocultured with DCs at indicated DC to T-cell ratios. On day 4, 1 Ci of [^{3}H]-thymidine was added. After 16 hours of further incubation, thymidine uptake was counted. Naive $CD4^+$ T cells were stimulated with 10 μg/mL phytohemagglutinin as a positive control. Representative data from three experiments are shown.



Supplementary Figure E2. Effects of cryopreservation on immature 3d-DCs. (**A**) Viability of fresh and frozen 3d-DCs after 24 hours of incubation with or without OK-432 (0.1 KE/mL) were evaluated by staining with Annexin-V. Percentages of Annexin-Vpositive cells are indicated. (**B**) Expression of surface molecules on fresh and frozen DCs after 24 hours of incubation with or without OK-432. (**C**) IL-12p70 production by fresh and frozen DCs (5×10^5 cells/mL) induced by 24-hour stimulation with OK-432 was measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. Representative data from four experiments are shown.



Supplementary Figure E3. Expression of surface molecules on DCs for vaccination. Cryopreserved DCs from patients were thawed, stained, and analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Numbers indicate percentages of cells in each quadrant. Representative data from patient no. 1 are shown.

Supplementary Table E1. DC vaccine generation

	At the time of apheresis				
Patient no.	Days after the last CT	PB WBC (/L)	PB Mo (%)	BM LC[a] (%)	Antigen dose (LC:DC)
1	74	4700	7	0.9	1:5
2	31	3000	9	2.0	1:6.5
3	43	3900	15	0[b]	1:6
4	46	4800	16	0.3	1:3.3

CT = chemotherapy; LC = leukemic cells; Mo = monocytes.
[a]Percentages of leukemic cells in bone marrow were determined by flow cytometry.
[b]Patient 3 was in complete remission at the time of apheresis. The patient subsequently relapsed and became eligible for DC vaccination.

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A New Indicator of a Favorable Prognosis in Locally Advanced Renal Cell Carcinomas: γδ T-Cells in Peripheral Blood

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Abstract. *Background: Although human γδ T-cells that express Vγ2Vδ2–bearing T-cell receptor (Vγ2Vδ2T-cells) have recently received considerable attention in the development of novel cancer immunotherapies, consensus has not yet been reached regarding the physiological relevance of this T-cell subset in the context of cancer immunosurveillance. Clinical trials of adoptive immunotherapy using autologous Vγ2Vδ2T-cells have been applied to patients with advanced renal cell carcinoma (RCC) and some clinical benefits have been reported. In the present study, we investigated the correlation between the proportion of γδ T-cells in peripheral before surgery in patients with locally advanced RCC and those clinical outcomes. Materials and Methods: Of 41 patients who underwent surgery for RCC, 13 patients had stage III disease without metastasis. These stage III patients were stratified into two groups based on the peripheral γδ T-cell proportion being greater or less than 8.7% before surgery and were followed-up for up to 137 months (median 129 months). Results: Remarkably, an obvious difference was found in the overall survival and cause-specific survival rate between the two groups. In 6 patients with a higher proportion of γδ T-cells, one patient had lung metastasis but there were no cancer deaths. In contrast, 5 out of 7 patients with a lower proportion of γδ T-cells died during the study and 4 out of 7 patients died due to RCC. Conclusion: An increase in the proportion of peripheral γδ T-cells is a favorable prognostic factor for patients with locally advanced RCC.*

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Key Words: Renal cell carcinoma, gamma-delta T-cell, prognosis factor.

Human Vγ2Vδ2T-cells recognize nonpeptide antigens and exhibit cytotoxity against various tumor cell lines and may play an important role in future immunosurveillance systems. Vγ2Vδ2T-Cells are major subsets of peripheral blood γδ T-cells. Vγ2Vδ2T-cells are also activated by synthesized pyrophosphomonoester derivatives and nitrogen-containing bisphosphonates, such as zoledronic acid, with interleukin-2 (IL-2) *in vitro*. Based on these findings, Vγ2Vδ2T-cells have recently attracted considerable attention in the development of novel cancer immunotherapies, and several different approaches have been designed and employed in clinical trials (1-3).

We previously reported that a patient with lung metastasis after radical nephrectomy for renal cell carcinoma (RCC) had six cycles of adoptive immunotherapy using autologous *in vitro*-activated Vγ2Vδ2T-cells followed by low-dose IL-2 and zoledronic acid intravenous infusion. Complete response was achieved, which has been maintained for 3 years without any additional treatment (4). We also reported that Vγ2Vδ2T-cells exert effects on a variety of tumor cells (5) and there is an increase in the number of Vγ2Vδ2-T cells in some patients with RCC (6). However, consensus has not been reached regarding the physiological relevance of Vγ2Vδ2T-cells in the context of immunosurveillance for cancer. We therefore investigated the prognostic significance of the proportion of γδ T-cells in peripheral blood in patients with locally advanced RCC.

Materials and Methods

Patients. From December 1997 to August 1998, 41 patients underwent surgery for RCC at the Department of Urology, Tokyo Women's Medical University Hospital. We have already reported that 10 out of the 41 RCC patients showed increased proportions of peripheral blood γδ T-cells in T-cells (6). These patients were followed up at the Tokyo Women's Medical University Hospital and our associated hospitals for up to 137 months after the surgery. Of 41 patients, 29 were men and 12 were women, who ranged in age from 33 to 78 (median age, 59) years at the time of surgery. Also included were 32 healthy individuals, ranging in age from 19 to 73 (median age, 40) years, who had never suffered from malignancies or bacterial infections, such as tuberculosis, typhoid, and tularemia, as we have already reported (6). Computed tomography (CT) was conducted twice yearly after the surgery for up to 5 years and then once a year for at least 10 years.

Isolation of peripheral blood mononuclear cells (PBMC) and flowc ytometric analysis. PBMC were isolated from the heparinized peripheral blood of both RCC patients and healthy subjects by Ficoll-Conray density-gradient centrifugation performed at 1500 rpm for 30 minutes, as described previously (6). The following monoclonal antibodies (mAbs) were used to identify fresh PBMC during immunofluorescence analysis: Fluorescein isothiocyanate (FITC)-conjugated-anti-Vδ2 chain mAb (Immu389, Beckman Coulter, California, USA; or 15D, Serotech Ltd, Kidlington, Oxford, UK), FITC-conjugated-anti-Vγ2 chain mAb (Immu360; Beckman Coulter), FITC-conjugated-anti-pan-γ/δT-cell receptor mAb (Immu515; Beckman Coulter), Phycoerythrin (PE)-conjugated-anti-CD3 mAb (SK7; Becton Dickinson Immunocytometory Systems, San Jose, CA, USA). The stained T-cells were examined by two-color flow cytometric analysis using an EPICS CS or XL flow cytometer (Beckman Coulter), as described previously (6). During all staining procedures, the cells were kept on ice.

Statistical analysis. Statistical analyses were conducted to test the differences between two items using the log-rank test and Kaplan-Meier estimator. We used the Stat View 5.0J software package (Abacus Concepts, Inc, CA, USA).

Results

The proportion of γδ T-cells among $CD3^+$ cells derived from healthy individuals was 4.3±2.2% (data not shown). Forty-one RCC patients were divided into groups of RCC development stage according to the UICC 2002 classification (Table I). The flow cytometric results of PBMC from patient no. 6 are shown in Figure 1 as representative data. Predominant expansion of Vγ2Vδ2T-cells in γδ T-cells was also seen in those from the other patients with expansion of γδ T-cells in PBMC. Four patients (18.2%) in stage I, 1 patient (50.0%) in stage II and 5 patients (38.5%) in stage III showed an increased proportion of peripheral blood γδ T-cells in their $CD3^+$ cells. The numbers of patients in each stage were different from those numbers that we previously reported because of the different UICC classification (6). We focused on the patients with stage III disease, because of their high risk of RCC recurrence (7). Thirteen patients with stage III clear cell RCC were classified into two groups, patients 1-6 and 7-13, according to their having a γδ T -ell

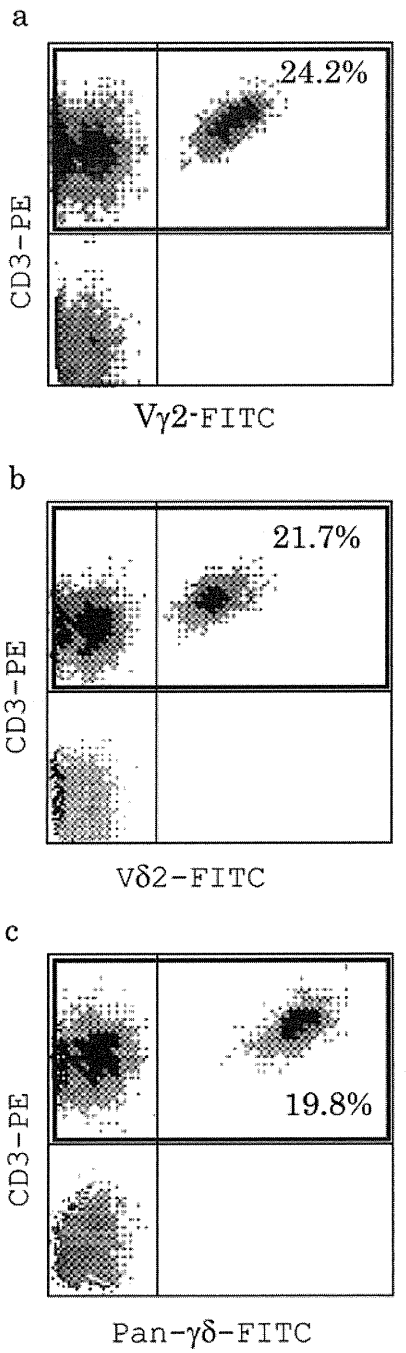

Figure 1. *Predominant expansion of TCR Vγ2/Vδ2-bearing T-cells in RCC patients. PBMC obtained from patient 6 were stained with several combinations of PE-conjugated-anti-CD3 and, anti-TCR Vγ2 (a), anti-TCR Vδ2 (b) and FITC-conjugated anti-pan-TCR γδ (c) mAbs. Figures indicate the percentage of stained cells in $CD3^+$ PBMC.*

Table I. *Distribution of patients with elevated γ/δ T-cells in the different stages of RCC development.*

	Stage of RCC development[a]			
	I	II	III	IV
Number of patients with expanded γ/δ T-cell populations[b]	4/22 18.2%	1/2 50.0%	5/13 38.5%	0/4 0%

[a]According to UICC 2002 classification. [b]The proportion of γ/δ T-cells exceeded 8.7% (4.3±4.7%, average of healthy controls±2SD) of PBMC.

Table II. *Characteristics of the study group. Patient demographics, clinical and pathological characteristics and outcome are summarized.*

Patient no.	Age at surgery (years)	Gender	Lymphocyte numbers (/μl)	γδ T-cells (%)	T Stage	Tumor size (cm)	Grade	Survival (months)*	Recurrence		Outcome
									Site	Time after surgery (months)	
1	76	F	2970	12.4	T3a	7.5	2	137			Survival
2	33	F	2804	9.6	T3a	15.0	2	130			Survival
3	62	M	1348	34.5	T3a	7.0	3	131			Survival
4	62	M	2042	30.2	T3b	10.0	2	126			Survival
5	59	F	1673	10.5	T3b	4.5	1	135			Survival
6	47	F	1916	19.8	T3a	9.0	3	129	Lung	45	Survival
7	70	F	1953	3.3	T3b	10.0	2	135	Lung	125	Death due to RCC
8	65	M	2508	1.2	T3a	7.0	2	67	Lung	66	Death due to RCC
9	67	M	2282	2.1	T3a	4.5	1	105			Death due to AMI
10	62	M	1865	0.4	T3b	6.0	2	10	Lung	8	Death due to RCC
11	54	M	1710	1.7	T3a	2.7	2	128			Survival
12	53	M	1770	5.6	T3a	9.0	3	52	Lung/bone	24	Death due to RCC
13	55	M	2297	6.8	T3a	8.0	2	129			Survival

*Patients 1-6: 131.3±4.0 months, patients 7-13: 89.4±47.6 months, $p<0.05$. Since there were no cancer deaths in the study period in the former group, statistical analysis has not yet been completed. The difference between the two groups was, however, significant. AMI: Acute myocardial infarction.

proportion higher or lower than 8.7% (the mean±2×standard deviation (SD)for healthy individuals is 4.3%±2×2.2%). There was no inherent dichotomy in the clinical and pathological characteristics between these two groups (Table II). Surprisingly, obvious differences were observed in the overall and the cause-specific survival rates (Figure 2a and b), but no significant difference was found in the disease-free survival rate by log-rank tests (Figure 3). In patients 1-6, there were no deaths due to cancer during the study and only one patient experienced lung metastasis, 45 months after the surgery. In contrast, among patients 7-13, four patients died due to RCC at 10, 52, 67 and 135 months after the surgery and another due to acute myocardial infarction 105 months after the surgery. In addition, four patients from this group developed metastasis (Table II). Interestingly, the proportion of peripheral blood γδ T-cells was only 0.5% at 18 months after lung metastasis developed in patient no. 7, compared with 2.9% at 6 years after lung metastasis developed in patient no.1, who is still alive (data not shown). In the patients with stage I and II RCC, whose proportions of peripheral blood γδ T-cells were elevated to more than 8.7% of $CD3^+$ cells, there have been no deaths due to RCC (data not shown)

Discussion

While evidence has been accumulating that Vγ2Vδ2-bearing γδ T-cells exert cytotoxic activity against a wide spectrum of tumor cells *in vitro* as well as *in vivo*, the physiological role of this T-cell subset remains enigmatic (8). We previously suggested that γδ T-cells may provide innate immunity against RCC, based on the findings that the proportion of γδ T-cells increased with an increase in the disease stage and that the percentage of these cells returned to normal levels after nephrectomy (6). In this study, we clearly demonstrated that increased levels of γδ T-cells were indeed correlated with the overall survival rate in patients with locally advanced RCC. Table II shows there was no significant difference in the actual numbers of lymphocytes between these two groups. The increased proportion of γδ T-cells was due to an increase

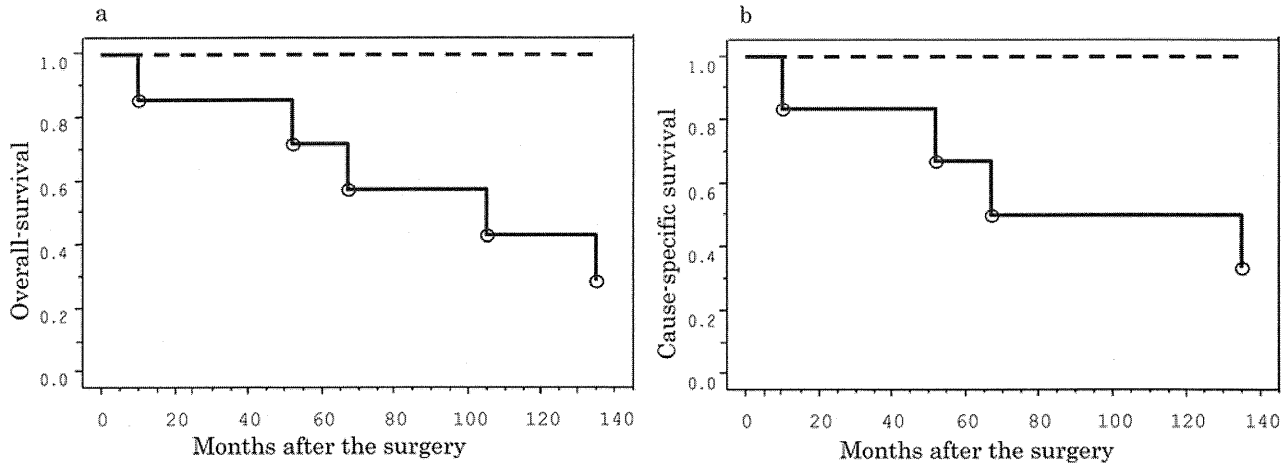

Figure 2. *The overall (a) and the cause-specific (b) survival rates were analyzed by Kaplan-Meier survival analysis. The dotted line indicates these rates for patients with an increased proportion of peripheral blood γδ T-cells before the surgery and the solid line indicates these rates in the patients without an increased proportion of γδ T-cells.*

in the number of γδ T-cells. This result strongly supports the hypothesis that γδ T-cells should play a cardinal role in cancer immunosurveillance and indicates that an increase in the proportion of peripheral blood γδ T-cells is a favorable prognostic factor for patients with locally advanced RCC. There was no significant difference in the disease-free survival rate between these two groups. One explanation might be that only a small number of patients were followed up and we should draw up a project to obtain proof of the concept that an increased proportion of γδ T-cells in peripheral blood before radical nephrectomy may prevent recurrence of RCC in a large number of patients. Five out of 41 RCC patients with stage I or II localized RCC showed increased levels of γδ T-cells and it is difficult to explain the roles of γδ T-cells in these case because none of these patients developed recurrence of RCC. In patient 6, the proportion of γδ T-cells decreased from 19.8% to 4.4% at four months after the surgery and to 2.9% at the time of RCC recurrence. Peripheral blood γδ T-cells did not increase at the time of recurrence. This indicates that recurrent RCC may escape from the immunosurveillance system of γδ T-cells or that γδ T-cells might not be able to respond to RCC because of the immunosuppressive mechanisms of the regulatory cells. Finke et al. reported that immunosuppressive myeloid dendritic cells and regulatory cells increased in peripheral blood of patients with advanced RCC (9, 10). In patient 7, the proportion of γδ T-cells decreased from 3.3% to 0.5% at the time of recurrent RCC. It is difficult to explain whether the metastatic RCC developed because of the decreased proportion of γδ T-cells or because metastatic RCC had immunosuppressive mechanisms which reduced the proportion of γδ T-cells. Nevertheless, whatever mechanisms are involved in decreasing the proportion of γδ T-cells after

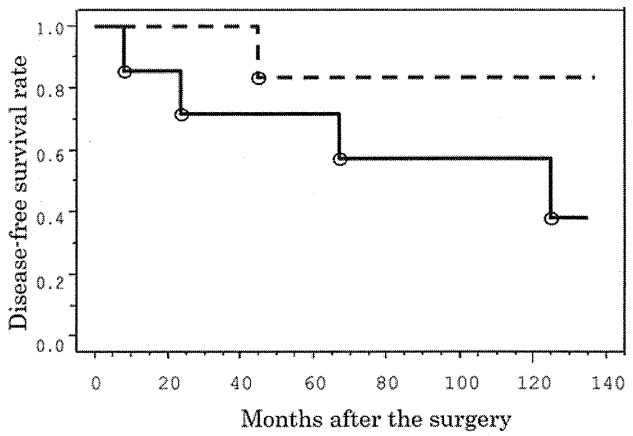

Figure 3. *The disease-free rates were analyzed by Kaplan-Meier survival analysis and log-rank test. The dotted line indicates these rates for the patients with an increased proportion of peripheral blood γδ T-cells before the surgery and the solid line indicates these rates in the patients without an increased proportion of γδ T-cells. There was no significant difference in the disease-free rate between these two groups.*

the development of recurrent RCC, the resulting reduction clearly influences the prognosis. This finding provides an impetus to undertake further investigation for cancer immunotherapy using γδ T-cells.

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