ORIGINAL ARTICLE

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

Toshio Kitawaki[1], Norimitsu Kadowaki[1], Keiko Fukunaga[1], Yasunari Kasai[2], Taira Maekawa[2], Katsuyuki Ohmori[3], Tatsuya Itoh[4], Akira Shimizu[4], Kiyotaka Kuzushima[6], Tadakazu Kondo[1], Takayuki Ishikawa[1], Takashi Uchiyama[1]

[1]Department of Hematology and Oncology, [2]Center for Cell and Molecular Therapy, Department of Transfusion Medicine and Cell Therapy, [3]Department of Clinical Laboratory Medicine, [4]Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan; [5]Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Japan

Correspondence and offprint requests

Norimitsu Kadowaki, M.D., Ph.D.

Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University

54 Shogoin Kawara-cho, Sakyo-ku, Kyoto 606-8507, Japan

E-mail: kadowaki@kuhp.kyoto-u.ac.jp

Tel: +81-75-751-3155

Fax: +81-75-751-4963

Table of Contents category

Clinical investigation

Word count

4034

Abstract

Objectives. Prognosis of elderly patients with acute myeloid leukemia (AML) remains dismal. To explore the potential of immunotherapy for improving the clinical outcome of 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 4 patients 5 times at 2-week intervals. To facilitate DC migration to lymph nodes, injection sites were pretreated with killed *Streptococcus pyogenes* OK-432 1 day before. DCs were co-injected with OK-432 to induce maturation and IL-12 production *in vivo*.

Results. Anti-leukemic responses were observed by an interferon-γ enzyme-linked immunospot assay or a tetramer assay in 2 of the 4 patients. In an HLA-A*2402-positive patient, induction of $CD8^+$ T cell responses to WT1- and hTERT-derived peptides were observed, indicating cross-priming *in vivo*. The 2 patients with anti-leukemic immunity showed longer periods of disease stabilization than the other 2

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.

Keywords

acute myeloid leukemia; dendritic cells; immunotherapy; apoptotic cells; OK-432

Introduction

The 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 outcome 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 anti-leukemic 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 (LCDCs) showed that the generation of LCDCs was feasible only in 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 LCDCs 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.

There are several parameters to enhance the immunogenicity of MoDC vaccines. (i) 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]. (ii) Among DC maturation-inducing factors, microbial components that trigger the production of IL-12 are beneficial to induce effective adaptive immunity [16]. (iii) 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 may generate optimal DCs that retain IL-12 production. (iv) Inflammation in the skin before DC injection facilitates the DC migration to draining lymph nodes, leading to a stronger immune response [18-19]. (v) 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 anti-leukemic immunity in 2 of 4 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 non-adherent 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% dimethylsulfoxide 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 that were killed by 120 Gy γ-irradiation and 48-hour serum-free culture in RPMI1640 (Wako Pure Chemical Industries, Osaka, Japan). To examine the cross-presenting capacity of DCs, HLA-A*2402-positive, immature 3d-DCs were pulsed with HLA-A*2402-negative, Epstein-Barr virus-transformed lymphoblastoid cell lines (LCLs) that were killed as described above.

DCs were matured with OK-432 (0.1 KE/mL) and prostaglandin E_2 (1 μg/mL) (MP Biomedicals, Solon, OH) for 6 hours, and co-cultured with autologous T cells at a DC: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 by the 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 x 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 less than 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-h 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) from 10 liters of 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). The endotoxin level in the KLH preparation examined by the supplier was <0.1 I.U./mg. After 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 x 10^7 DCs were intradermally injected at four sites in bilateral arms and thighs.

Twenty-four hours before the 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 5 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 (DTH) tests and 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 CMV (amino acids 328-336; QYDPVAALF) [29]. All peptides were purchased from Multiple Peptide Systems (San Diego, CA). 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 DTH test

Four x 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 a diameter than the antigen-unpulsed control were 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 x 10^4 cells/well. As responder cells, fresh and *in vitro* stimulated MNCs from PB and BM were plated with fresh MNCs at 1-2 x 10^5 cells/well and *in vitro* stimulated MNCs at 1-2 x 10^4 cells/well. After overnight incubation, spots were developed using 3-Amino-9-ethylcarbazole (Sigma Chemical, St. Louis, MO) 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 [29]. Fresh and *in vitro* stimulated MNCs were stained with a tetramer and FITC-conjugated anti-CD8 mAb (BD Biosciences) and analyzed by flow cytometery (FACSCalibur™; BD Biosciences) [29].

Results

In vitro assays to optimize the generation of DCs

To optimize the 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 (3d-DCs) with

6-day differentiated DCs (6d-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 naive $CD4^+$ T cells (Supplementary Fig. S1), indicating that 3d-DCs have comparable functions with 6d-DCs. Next, we examined the capacity of 3d-DCs to cross-present apoptotic cell-associated antigens. At the DC: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 LCLs 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 (LPS) leads to DC exhaustion [17], as indicated by loss of the 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. Up-regulation 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 durations of exposure to OK-432 at the start of culture. As short as 2-hour exposure up-regulated 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 LPS [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 naive $CD4^+$ 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 24h-culture with or without OK-432, and tended to produce a lower amount of IL-12p70 upon OK-432 stimulation as compared with non-cryopreserved DCs (Supplementary Fig. S2A, C), similar levels of CD83 and CD86 expression was induced by OK-432 in both DCs (Supplementary Fig. S2B). Thus, although cryopreservation of immature DCs impaired their functions to some extent, cryopreserved DCs largely retained the viability and expressions 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, 4 patients were eligible for the DC vaccination (Table 1). In these patients, more than 5×10^7 DCs for 5 vaccinations could be generated from a single apheresis. Autologous apoptotic leukemic cells were added to DCs as antigens at leukemic cell:DC ratios of 1:3.3 to 1:6.5, depending

on the numbers of collected leukemic cells (Supplementary Table 1). Status of PB and BM at the time of apheresis are shown in Supplementary Table 1. Representative data of surface molecule expressions on DCs are shown in Supplementary Fig. S3.

All the patients completed the 5 vaccinations safely (Table 1). In all the patients, grade 1-2 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 the 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 DTH tests and/or IFN-γ ELISPOT assays in 3 patients except Patient #4 (Table 1 and data not shown). Two patients (Patients #2 and #3) showed induction of immune responses to leukemia-associated antigens. In Patient #2, who was HLA-A*2402-negative, IFN-γ ELISPOT assays using autologous leukemic cell-pulsed DCs revealed the induction of anti-leukemic immunity in PBMCs and BMMCs without *in vitro* stimulation after the 4th vaccination (Figs. 3A). The anti-leukemic immune response was still detected 1 month

after the 5th 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 anti-leukemic immunity at subsequent time points in this patient because the patient developed leukocytopenia probably owing to progression of MDS.

In Patient #3, who was HLA-A*2402-positive, HLA-A*2402-restricted peptides from WT1 and hTERT were used in immunological monitoring. CMVpp65$_{328\text{-}336}$ peptide was used as a positive control in ELISPOT assays (Fig. 4B). No responses to the leukemia-associated antigens were observed until the 4th vaccination. However, 2 months after the 5th vaccination, positive responses to the modified WT1$_{235\text{-}243}$ and the hTERT$_{461\text{-}469}$ 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$_{235\text{-}243}$ peptide/HLA-A*2402 tetramer also bound to the natural WT1$_{235\text{-}243}$ 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 5th vaccination. No responses were detected in PBMCs or BMMCs without *in vitro* stimulation (data not shown). Thus, the vaccinations induced HLA class I-restricted, anti-leukemic immunity, indicating that the DCs cross-presented leukemia-associated

antigens *in vivo*. In addition, in Patient #2, leukemic cell-reactive T cells were detected in BM (Fig. 3), the main tumor site in leukemia.

Clinical outcome

The 2 patients with anti-leukemic immunity had longer periods of disease stabilization than the other 2 patients without anti-leukemic immunity (Fig. 5A). Notably, in Patient #3, the percentages of leukemic cells in BM dropped from 11% to 5.2% during the second month after the 5th vaccination, when a positive anti-leukemic immunity was observed (Fig. 5B). Thus, these observations suggest that induction of anti-leukemic 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. Induction of anti-leukemic immunity was observed in 2 of 4

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 the patients prevented us from drawing any definitive conclusion regarding clinical efficacy from the present trial. However, longer periods of disease stabilization observed in the 2 patients with anti-leukemic immunity compared to the other 2 patients without anti-leukemic immunity implied that induction of anti-leukemic immunity might have impacted on the clinical course of these patients.

There are several features in the method of DC vaccination in this trial: (i) short-term 3-day culture to generate DCs in an attempt to reduce labor, cost, and time, (ii) use of whole leukemic cells as antigens to induce multivalent immune responses, (iii) use of the microbial adjuvant OK-432 as a maturation-inducing factor to generate Th1-inducing DCs, (iv) *in vivo* maturation of DCs to avoid DC exhaustion by extended stimulation *in vitro* with OK-432, and (v) 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 [30-33]. Furthermore, MoDCs has been shown to cross-present apoptotic

leukemic cells to $CD8^+$ T cells *in vitro* [34]. 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 naive $CD4^+$ 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 (TLR) 4 [35-38]. We showed that, like LPS [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 TLR ligands are better inducers of Th1-type and cytotoxic T cell responses [17, 39-40]. Moreover, a clinical trial suggests superiority of briefly matured DCs in pediatric patients with cancer [41]. 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 [42-43]. 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 [42-43], 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 anti-leukemic immunity, which rapidly declined after the cessation of vaccination. Maintenance of anti-leukemic immunity might lead to improvement of clinical efficacy, and might be fulfilled by increasing the number of vaccination, 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 anti-leukemic $CD8^+$ T cell response, peptide vaccination may be added following 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 of elderly patients with AML, which has been difficult to be achieved by other therapeutic approaches.

Support and Financial Disclosure Declaration

This study was supported by Coordination, Support and Training Program for Translational Research from Ministry of Education, Culture, Sports, Science, and Technology of Japan, and The Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor, and Welfare, Japan.

The authors declare no competing financial interests.

Acknowledgements

We thank Satoshi Teramukai, Harue Tada, and Masanori Fukushima (Department of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital) for patient

enrollment, Maki Utsumi for her excellent technical assistance, and physicians for referral of patients.

This study was supported by Coordination, Support and Training Program for Translational Research from Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1. Erba HP. Prognostic Factors in Elderly Patients with AML and the Implications for Treatment. Hematology. 2007;2007:420-428.

2. Smits ELJM, Berneman ZN, Van Tendeloo VFI. Immunotherapy of Acute Myeloid Leukemia: Current Approaches. Oncologist. 2009;14:240-252.

3. Schmitt M, Schmitt A, Rojewski MT, et al. RHAMM-R3 peptide vaccination in patients with acute myeloid leukemia, myelodysplastic syndrome, and multiple myeloma elicits immunologic and clinical responses. Blood. 2008;111:1357-1365.

4. Rezvani K, Yong ASM, Mielke S, et al. Leukemia-associated antigen-specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. Blood. 2008;111:236-242.

5. Oka Y, Tsuboi A, Taguchi T, et al. Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. Proc Natl Acad Sci U S A. 2004;101:13885-13890.

6. Keilholz U, Letsch A, Busse A, et al. A clinical and immunologic phase 2 trial of Wilms tumor gene product 1 (WT1) peptide vaccination in patients with AML and MDS. Blood. 2009;113:6541-6548.

7. Fujii S, Shimizu K, Fujimoto K, et al. Treatment of post-transplanted, relapsed patients with hematological malignancies by infusion of HLA-matched, allogeneic-dendritic cells (DCs) pulsed with irradiated tumor cells and primed T cells. Leuk Lymphoma. 2001;42:357-369.

8. Li L, Giannopoulos K, Reinhardt P, et al. Immunotherapy for patients with acute myeloid leukemia using autologous dendritic cells generated from leukemic blasts. Int J Oncol. 2006;28:855-861.

9. Roddie H, Klammer M, Thomas C, et al. Phase I/II study of vaccination with dendritic-like leukaemia cells for the immunotherapy of acute myeloid leukaemia. Br J Haematol. 2006;133:152-157.

10. Kitawaki T, Kadowaki N, Kondo T, et al. Potential of dendritic cell immunotherapy for

relapse after allogeneic hematopoietic stem cell transplantation, shown by WT1 peptide- and keyhole limpet hemocyanin-pulsed, donor-derived dendritic cell vaccine for acute myeloid leukemia. Am J Hematol. 2008;83:315-317.

11. Lee J-J, Kook H, Park M-S, et al. Immunotherapy using autologous monocyte-derived dendritic cells pulsed with leukemic cell lysates for acute myeloid leukemia relapse after autologous peripheral blood stem cell transplantation. J Clin Apher. 2004;19:66-70.

12. Van Tendeloo VF, Van de Velde A, Van Driessche A, et al. Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. Proc Natl Acad Sci U S A. 2010;107:13824-13829.

13. Draube A, Beyer M, Wolf J. Activation of autologous leukemia-specific T cells in acute myeloid leukemia: monocyte-derived dendritic cells cocultured with leukemic blasts compared with leukemia-derived dendritic cells. Eur J Haematol. 2008;81:281-288.

14. Royer P-J, Bougras G, Ebstein F, et al. Efficient monocyte-derived dendritic cell generation in patients with acute myeloid leukemia after chemotherapy treatment: Application to active immunotherapy. Exp Hematol. 2008;36:329-339.

15. Dauer M, Obermaier B, Herten J, et al. Mature Dendritic Cells Derived from Human

Monocytes Within 48 Hours: A Novel Strategy for Dendritic Cell Differentiation from Blood Precursors. J Immunol. 2003;170:4069-4076.

16. Sporri R, Reis e Sousa C. Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function. Nat Immunol. 2005;6:163-170.

17. Langenkamp A, Messi M, Lanzavecchia A, Sallusto F. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat Immunol. 2000;1:311-316.

18. MartIn-Fontecha A, Sebastiani S, Hopken UE, et al. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. J Exp Med. 2003;198:615-621.

19. Nair S, McLaughlin C, Weizer A, et al. Injection of Immature Dendritic Cells into Adjuvant-Treated Skin Obviates the Need for Ex Vivo Maturation J Immunol. 2003;171:6275-6282.

20. Berard F, Blanco P, Davoust J, et al. Cross-priming of naive CD8 T cells against melanoma antigens using dendritic cells loaded with killed allogeneic melanoma cells. J Exp Med. 2000;192:1535-1544.

21. Fujimoto T, Duda RB, Szilvasi A, Chen X, Mai M, O'Donnell MA. Streptococcal preparation OK-432 is a potent inducer of IL-12 and a T helper cell 1 dominant state. J Immunol.

1997;158:5619-5626.

22. Kitawaki T, Kadowaki N, Sugimoto N, et al. IgE-activated mast cells in combination with pro-inflammatory factors induce Th2-promoting dendritic cells. Int Immunol. 2006;18:1789-1799.

23. Nouri-Shirazi M, Banchereau J, Bell D, et al. Dendritic Cells Capture Killed Tumor Cells and Present Their Antigens to Elicit Tumor-Specific Immune Responses. J Immunol. 2000;165:3797-3803.

24. Ohminami H, Yasukawa M, Fujita S. HLA class I-restricted lysis of leukemia cells by a CD8+ cytotoxic T-lymphocyte clone specific for WT1 peptide. Blood. 2000;95:286-293.

25. Kuzushima K, Hayashi N, Kudoh A, et al. Tetramer-assisted identification and characterization of epitopes recognized by HLA A*2402-restricted Epstein-Barr virus-specific CD8+ T cells. Blood. 2003;101:1460-1468.

26. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood. 2002;100:2292-2302.

27. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114:937-951.

28. Tsuboi A, Oka Y, Udaka K, et al. Enhanced induction of human WT1-specific cytotoxic T lymphocytes with a 9-mer WT1 peptide modified at HLA-A*2402-binding residues. Cancer Immunol Immunother. 2002;51:614-620.

29. Kuzushima K, Hayashi N, Kimura H, Tsurumi T. Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8+ T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. Blood. 2001;98:1872-1881.

30. Kokhaei P, Choudhury A, Mahdian R, et al. Apoptotic tumor cells are superior to tumor cell lysate, and tumor cell RNA in induction of autologous T cell response in B-CLL. Leukemia. 2004;18:1810.

31. Ferlazzo G, Semino C, Spaggiari GM, Meta M, Mingari MC, Melioli G. Dendritic cells efficiently cross-prime HLA class I-restricted cytolytic T lymphocytes when pulsed with both apoptotic and necrotic cells but not with soluble cell-derived lysates. Int Immunol. 2000;12:1741-1747.

32. Hoffmann TK, Meidenbauer N, Dworacki G, Kanaya H, Whiteside TL. Generation of Tumor-specific T Lymphocytes by Cross-Priming with Human Dendritic Cells Ingesting Apoptotic Tumor Cells. Cancer Res. 2000;60:3542-3549.

33. Galea-Lauri J, Wells JW, Darling D, Harrison P, Farzaneh F. Strategies for antigen choice

and priming of dendritic cells influence the polarization and efficacy of antitumor T-cell responses in dendritic cell-based cancer vaccination. Cancer Immunol Immunother. 2004;53:963-977.

34. Spisek R, Chevallier P, Morineau N, et al. Induction of leukemia-specific cytotoxic response by cross-presentation of late-apoptotic leukemic blasts by autologous dendritic cells of nonleukemic origin. Cancer Res. 2002;62:2861-2868.

35. Itoh T, Ueda Y, Okugawa K, et al. Streptococcal preparation OK432 promotes functional maturation of human monocyte-derived dendritic cells. Cancer Immunol Immunother. 2003;52:207-214.

36. Kuroki H, Morisaki T, Matsumoto K, et al. Streptococcal preparation OK-432: a new maturation factor of monocyte-derived dendritic cells for clinical use. Cancer Immunol Immunother. 2003;52:561-568.

37. Okamoto M, Furuichi S, Nishioka Y, et al. Expression of Toll-Like Receptor 4 on Dendritic Cells Is Significant for Anticancer Effect of Dendritic Cell-Based Immunotherapy in Combination with an Active Component of OK-432, a Streptococcal Preparation. Cancer Res. 2004;64:5461-5470.

38. Nakahara S, Tsunoda T, Baba T, Asabe S, Tahara H. Dendritic cells stimulated with a bacterial product, OK-432, efficiently induce cytotoxic T lymphocytes specific to tumor rejection

peptide. Cancer Res. 2003;63:4112-4118.

39. Dohnal AM, Graffi S, Witt V, et al. Comparative evaluation of techniques for the manufacturing of dendritic cell-based cancer vaccines. J Cell Mol Med. 2009;13:125-135.

40. Felzmann T, Huttner KG, Breuer SK, et al. Semi-mature IL-12 secreting dendritic cells present exogenous antigen to trigger cytolytic immune responses. Cancer Immunol Immunother. 2005;54:769-780.

41. Dohnal A, Witt V, Hügel H, Holter W, Gadner H, Felzmann T. Phase I study of tumor Ag-loaded IL-12 secreting semi-mature DC for the treatment of pediatric cancer. Cytotherapy. 2007;9:755-770.

42. de Vries IJM, Krooshoop DJEB, Scharenborg NM, et al. Effective Migration of Antigen-pulsed Dendritic Cells to Lymph Nodes in Melanoma Patients Is Determined by Their Maturation State. Cancer Res. 2003;63:12-17.

43. Morse MA, Coleman RE, Akabani G, Niehaus N, Coleman D, Lyerly HK. Migration of human dendritic cells after injection in patients with metastatic malignancies. Cancer Res. 1999;59:56-58.

Figure legends

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-AAD (20 μg/mL), and co-cultured with immature 3d-DCs at a DC:apoptotic cell ratio of 1:1. After 4 hours of incubation at 4°C or 37°C, cells were stained with FITC-conjugated anti-CD11c mAb 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 LCLs, matured with OK-432 and prostaglandin E_2, and co-cultured 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.

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, re-plated 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 x 10^5 cells/mL) were measured by ELISA. Error bars indicate the standard deviation of duplicate measurements. (E) Naive $CD4^+$ T cell differentiation induced by DCs. Immature 3d-DCs were matured with OK-432 (0.1 KE/mL) for 6 or 24 hours and co-cultured with allogeneic naive $CD4^+$ 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.

Figure 3 IFN-γ ELISPOT assay in Patient #2. MNCs from peripheral blood (PB) and bone marrow (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 x 10^5 MNCs (A) and 1 x 10^4 MNCs (B) were incubated with 1 x 10^4 leukemic cell-pulsed or unpulsed

DCs. Numbers of specific spot-forming cells per 2 x 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 #3. (A) HLA tetramer staining. MNCs from peripheral blood were obtained at indicated time points, stimulated for 1 week with DCs pulsed with the modified $WT1_{235-243}$ peptide, stained with PE-labeled peptide/HLA-A*2402 tetramers and FITC-labeled anti-CD8 mAb, 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 x 10^4 MNCs were incubated with 2 x 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 non-specific spots. Thus, the data before vaccination are not shown. Numbers of specific spot-forming cells per 2 x 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 bone marrow as determined by flow cytometry in 4 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 #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.

Fig. 1

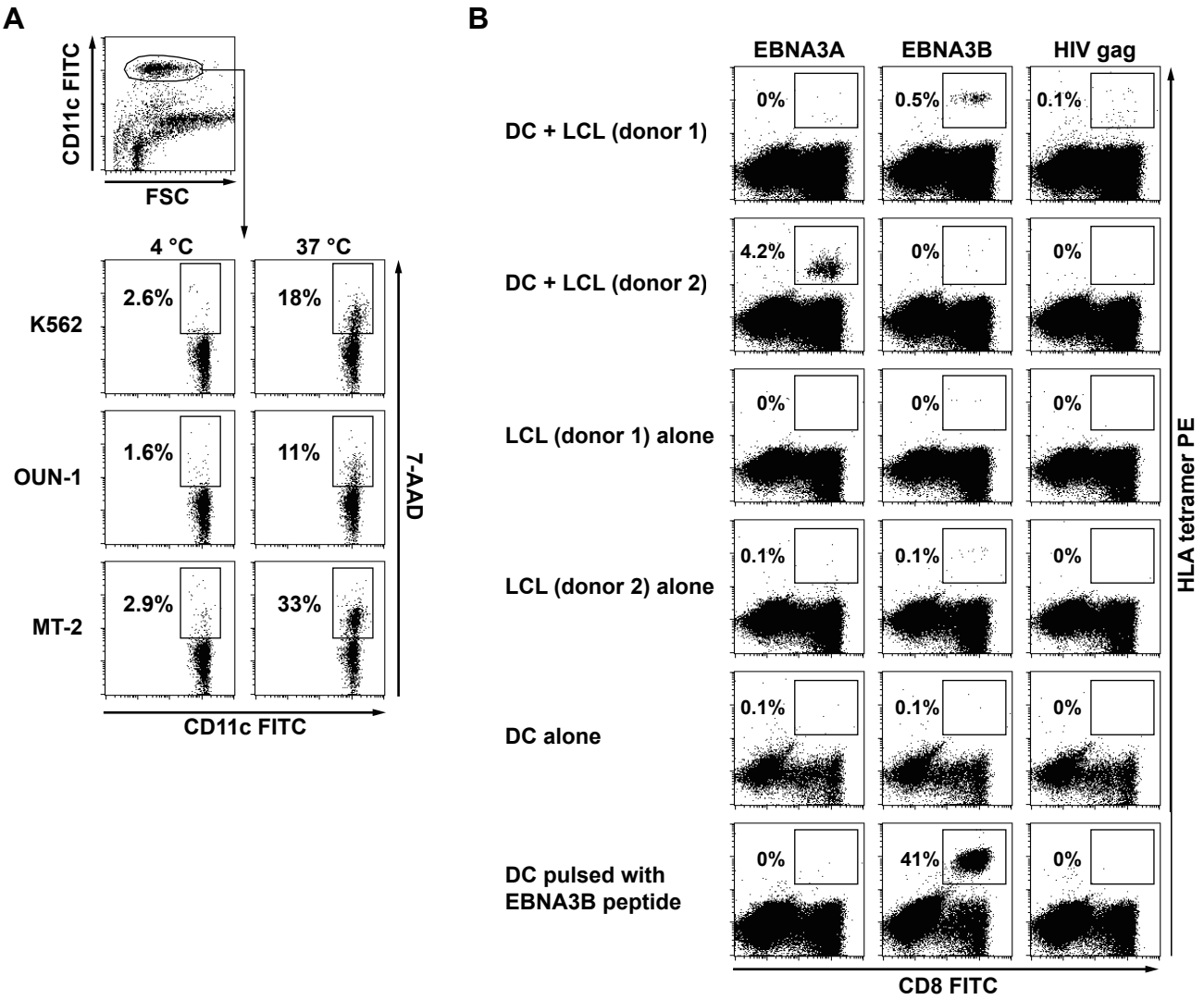
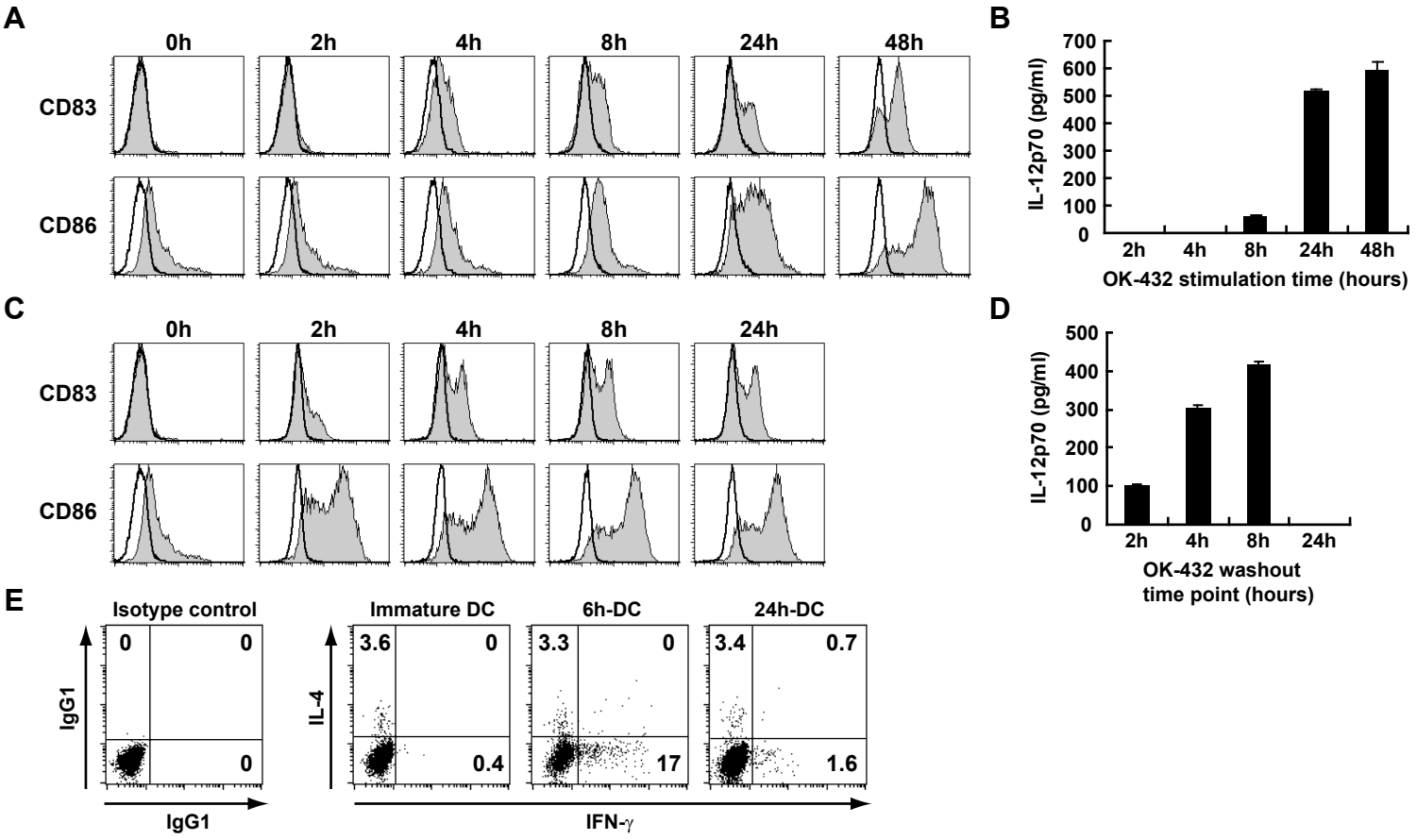

A
CD11c FITC
FSC
4 °C
37 °C
K562
2.6%
18%
OUN-1
1.6%
11%
MT-2
2.9%
33%
7-AAD
CD11c FITC
B
EBNA3A
EBNA3B
HIV gag
DC + LCL (donor 1)
0%
0.5%
0.1%
DC + LCL (donor 2)
4.2%
0%
0%
LCL (donor 1) alone
0%
0%
0%
LCL (donor 2) alone
0.1%
0.1%
0%
DC alone
0.1%
0.1%
0%
DC pulsed with EBNA3B peptide
0%
41%
0%
HLA tetramer PE
CD8 FITC

Fig. 2


A
0h
2h
4h
8h
24h
48h
CD83
CD86
B
IL-12p70 (pg/ml)
700
600
500
400
300
200
100
0
2h
4h
8h
24h
48h
OK-432 stimulation time (hours)
C
0h
2h
4h
8h
24h
CD83
CD86
D
IL-12p70 (pg/ml)
500
400
300
200
100
0
2h
4h
8h
24h
OK-432 washout
time point (hours)

E
Isotype control
Immature DC
6h-DC
24h-DC
IgG1
IL-4
0
0
0
0
3.6
0
0.4
3.3
0
17
3.4
0.7
1.6
IgG1
IFN-γ

Fig. 3

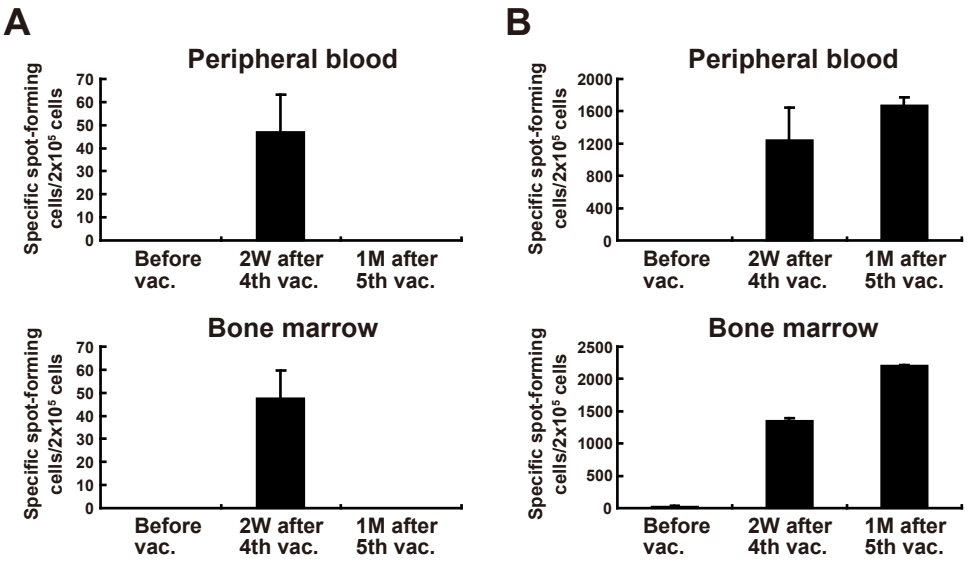
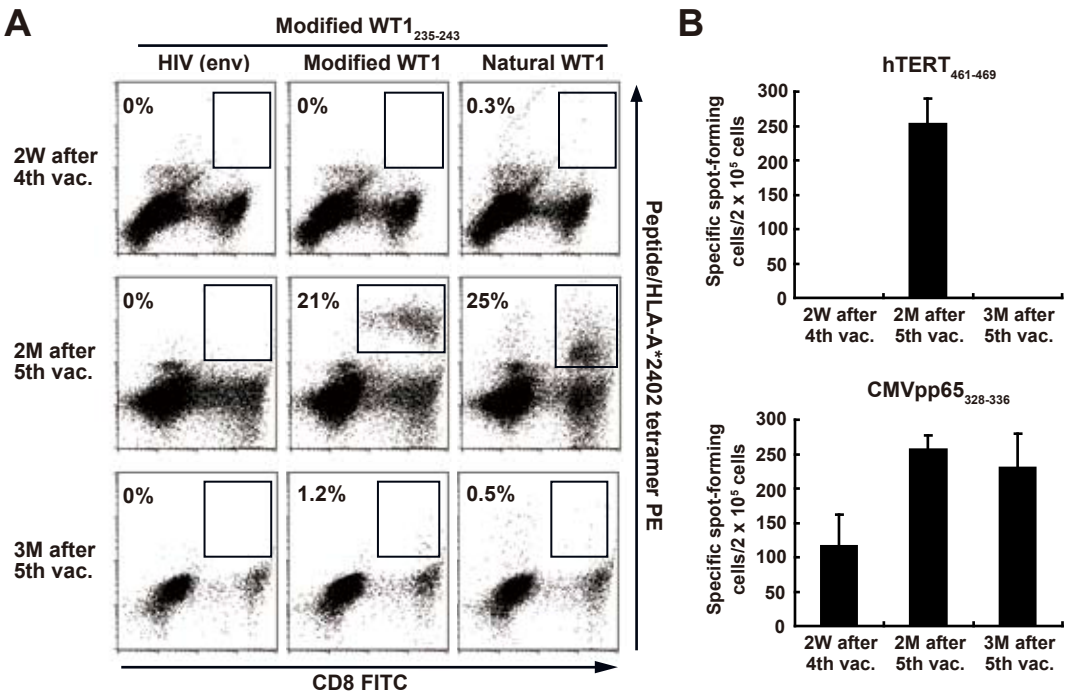

A
Peripheral blood
Specific spot-forming cells/2x10^5 cells
70
60
50
40
30
20
10
0
Before vac.
2W after 4th vac.
1M after 5th vac.
Bone marrow
Specific spot-forming cells/2x10^5 cells
70
60
50
40
30
20
10
0
Before vac.
2W after 4th vac.
1M after 5th vac.
B
Peripheral blood
Specific spot-forming cells/2x10^5 cells
2000
1600
1200
800
400
0
Before vac.
2W after 4th vac.
1M after 5th vac.
Bone marrow
Specific spot-forming cells/2x10^5 cells
2500
2000
1500
1000
500
0
Before vac.
2W after 4th vac.
1M after 5th vac.

Fig. 4


A
Modified WT1235-243
HIV (env)
Modified WT1
Natural WT1
2W after 4th vac.
2M after 5th vac.
3M after 5th vac.
0%
0%
0.3%
0%
21%
25%
0%
1.2%
0.5%
Peptide/HLA-A*2402 tetramer PE
CD8 FITC

B
hTERT461-469
Specific spot-forming cells/2 x 10^5 cells
300
250
200
150
100
50
0
2W after 4th vac.
2M after 5th vac.
3M after 5th vac.
CMVpp65328-336
Specific spot-forming cells/2 x 10^5 cells
300
250
200
150
100
50
0
2W after 4th vac.
2M after 5th vac.
3M after 5th vac.

Fig. 5

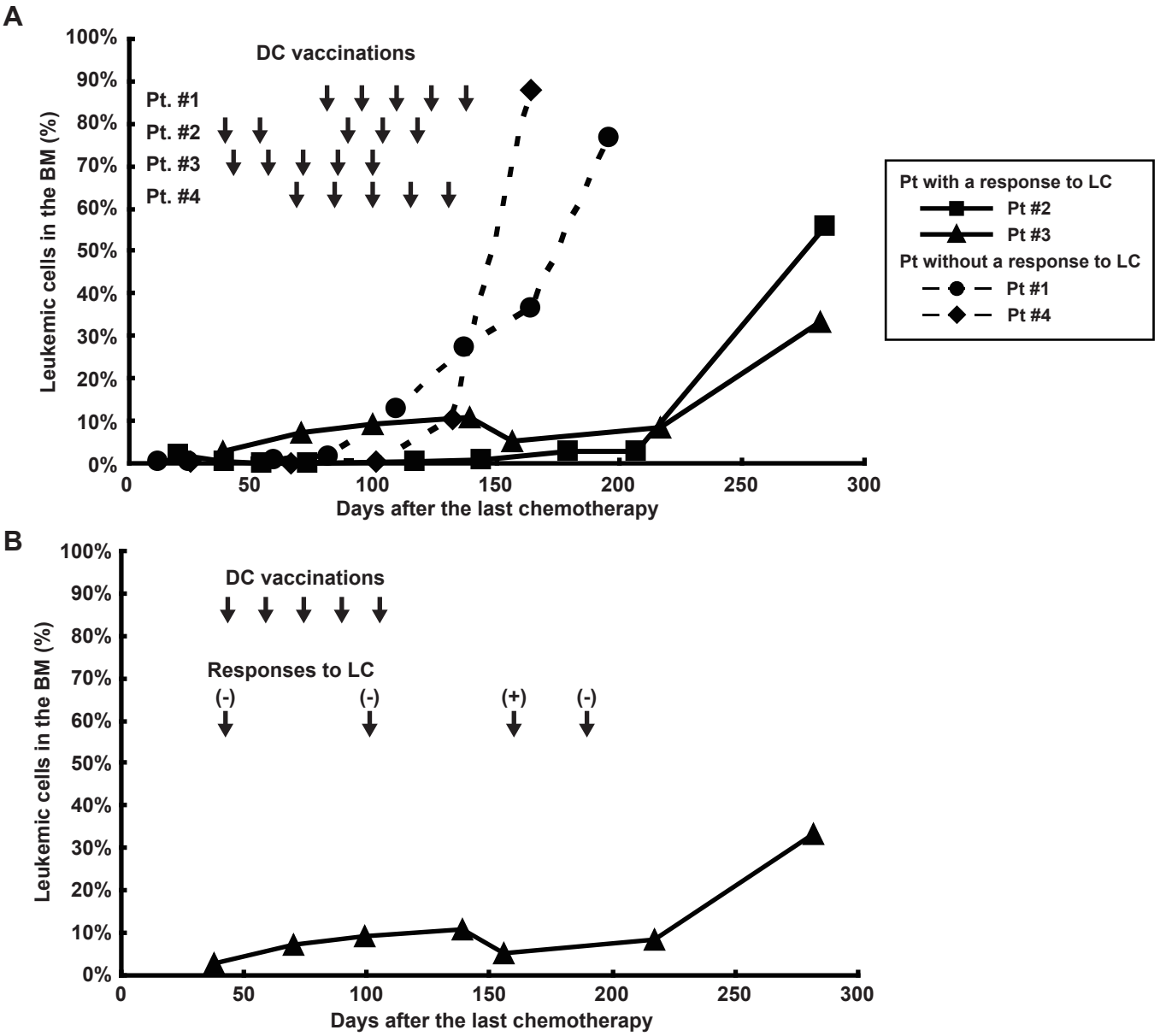

A
DC vaccinations
Pt. #1
Pt. #2
Pt. #3
Pt. #4
Leukemic cells in the BM (%)
Days after the last chemotherapy
Pt with a response to LC
Pt #2
Pt #3
Pt without a response to LC
Pt #1
Pt #4
B
DC vaccinations
Responses to LC
(-)
(-)
(+)
(-)
Leukemic cells in the BM (%)
Days after the last chemotherapy

Table 1 Patient characteristics and results of the DC vaccination

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

Vac: vaccination, CT: chemotherapy, LC: leukemic cells, BM: bone marrow, AML-MRC: Acute myeloid leukemia with myelodysplasia-related changes, PD: Progressive disease

*Percentages of leukemic cells in bone marrow were determined by flow cytometry. **Numbers in parenthesis indicate grade of toxicity according to the National Cancer Institute-Common Terminology Criteria for Adverse Events version 3.0.

Supplementary figure legends

Supplementary Figure 1 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 (ELISA). 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 co-cultured with DCs at indicated DC:T cell ratios. On day 4, 1 μCi 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 PHA as a positive control. Representative data from three experiments are shown.

Supplementary Figure 2 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 V-positive 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 x 10^5 cells/mL) induced by 24-hour stimulation with OK-432 was measured by ELISA. Error bars indicate the standard deviation of duplicate measurements. Representative data from four experiments are shown.

Supplementary Figure 3 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 7-AAD. Numbers indicate percentages of cells in each quadrant. Representative data from Patient #1 are shown.

Fig. S1

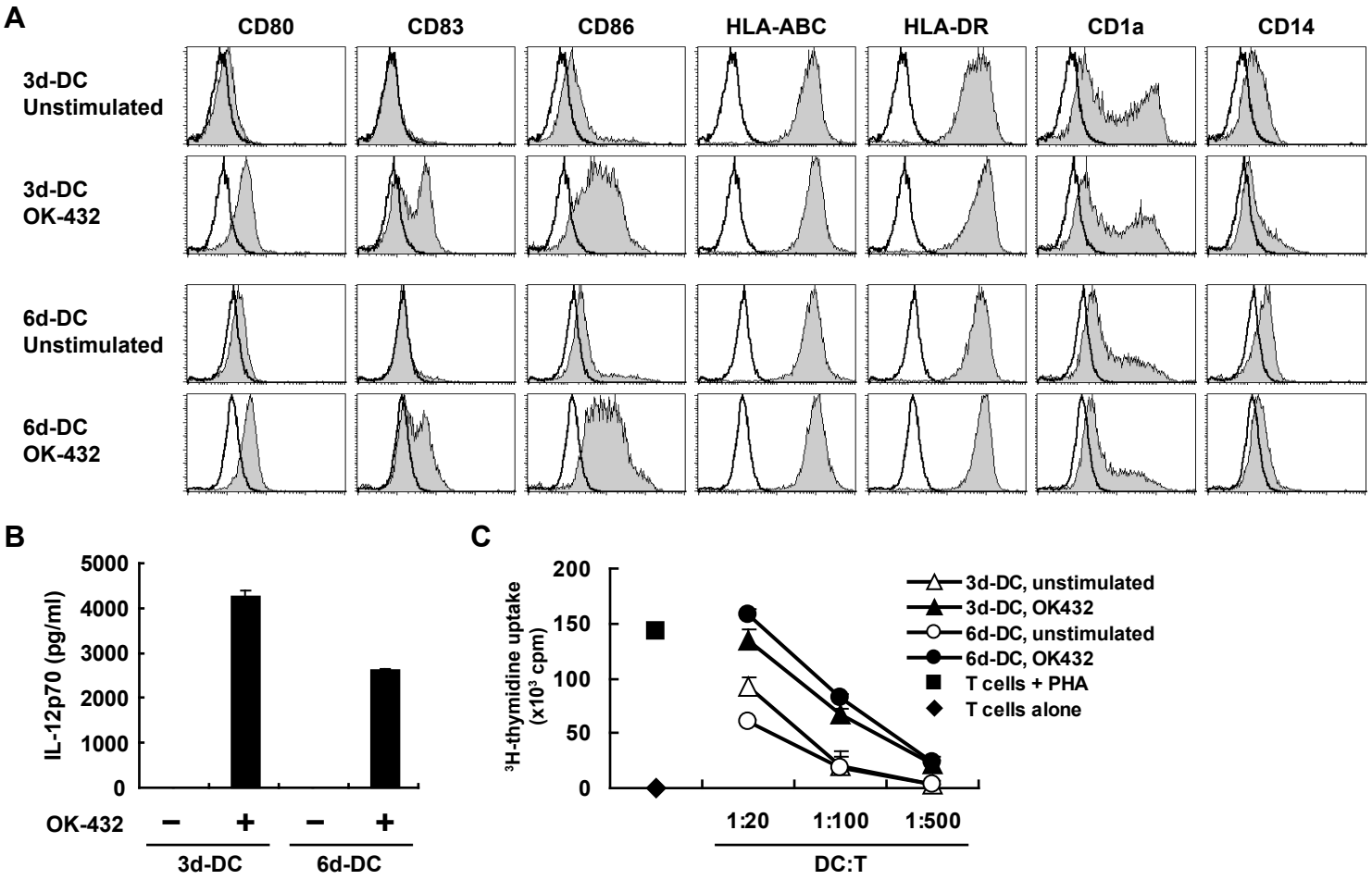

A
CD80
CD83
CD86
HLA-ABC
HLA-DR
CD1a
CD14
3d-DC Unstimulated
3d-DC OK-432
6d-DC Unstimulated
6d-DC OK-432
B
IL-12p70 (pg/ml)
5000
4000
3000
2000
1000
0
OK-432
−
+
−
+
3d-DC
6d-DC
C
³H-thymidine uptake (x10³ cpm)
200
150
100
50
0
3d-DC, unstimulated
3d-DC, OK432
6d-DC, unstimulated
6d-DC, OK432
T cells + PHA
T cells alone
1:20
1:100
1:500
DC:T

Fig .S2

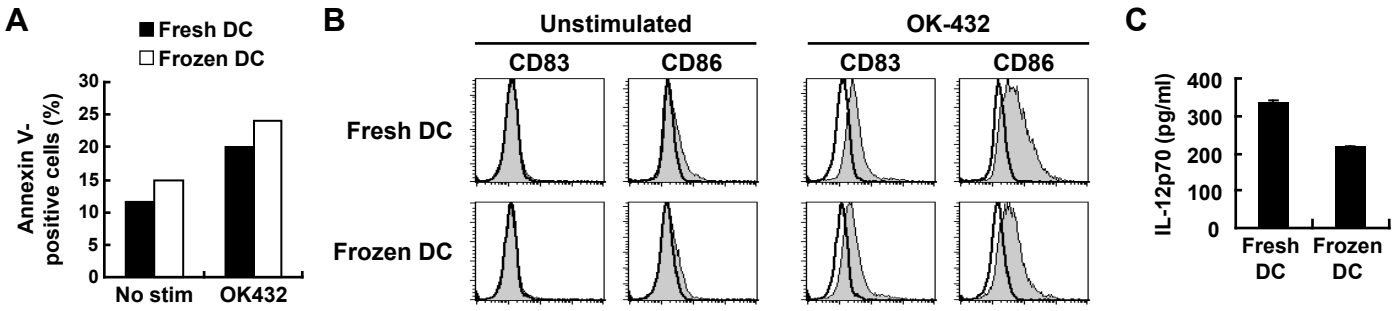
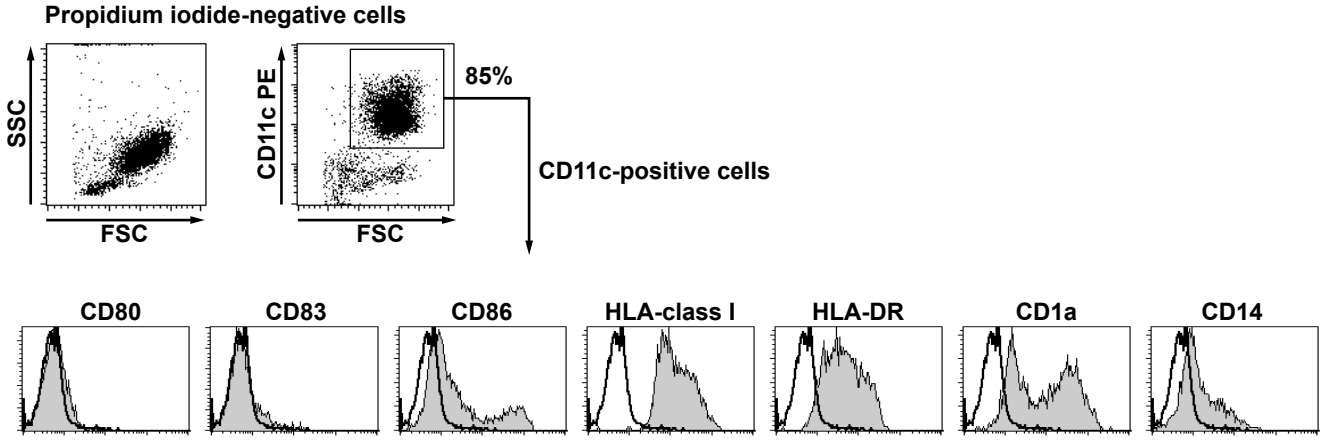

A
Fresh DC
Frozen DC
Annexin V-positive cells (%)
30
25
20
15
10
5
0
No stim
OK432
B
Unstimulated
OK-432
CD83
CD86
CD83
CD86
Fresh DC
Frozen DC
C
IL-12p70 (pg/ml)
400
300
200
100
0
Fresh DC
Frozen DC

Fig. S3


Propidium iodide-negative cells
SSC
FSC
CD11c PE
FSC
85%
CD11c-positive cells
CD80
CD83
CD86
HLA-class I
HLA-DR
CD1a
CD14

Supplementary Table 1 DC vaccine generation

	At the time of apheresis				Antigen dose
Patient	Days after the last CT	PB WBC	PB Mo	BM LC*	LC:DC
#1	74	4700/µl	7%	0.9%	1:5
#2	31	3000/µl	9%	2.0%	1:6.5
#3	43	3900/µl	15%	0%**	1:6
#4	46	4800/µl	16%	0.3%	1:3.3

CT: chemotherapy, Mo: monocytes, LC: leukemic cells

*Percentages of leukemic cells in bone marrow were determined by flow cytometry. **Patient #3 was in complete remission at the time of apheresis. The patient subsequently relapsed and became eligible for DC vaccination.