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Galectin-9 ameliorates acute GVH disease through the induction of T-cell apoptosis

Kazuki Sakai[*1,2], *Eri Kawata*[*1], *Eishi Ashihara*[1], *Yoko Nakagawa*[1], *Akira Yamauchi*[3], *Hisayuki Yao*[1], *Rina Nagao*[1], *Ruriko Tanaka*[1], *Asumi Yokota*[1], *Miki Takeuchi*[1], *Hideyo Hirai*[1], *Shinya Kimura*[4], *Mitsuomi Hirashima*[5], *Norio Yoshimura*[2] *and Taira Maekawa*[1]

[1] Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, Kyoto, Japan
[2] Department of Transplantation and Regenerative Surgery, Kyoto Prefectural University of Medicine, Japan
[3] Department of Cell Regulation, Faculty of Medicine, Kagawa University, Japan
[4] Division of Hematology, Respiratory Medicine, and Oncology, Department of Internal Medicine, Faculty of Medicine, Saga University, Japan
[5] Department of Immunology and Immunopathology, Faculty of Medicine, Kagawa University, Japan

Galectins comprise a family of animal lectins that differ in their affinity for β-galactosides. Galectin-9 (Gal-9) is a tandem-repeat-type galectin that was recently shown to function as a ligand for T-cell immunoglobin domain and mucin domain-3 (Tim-3) expressed on terminally differentiated $CD4^+$ Th1 cells. Gal-9 modulates immune reactions, including the induction of apoptosis in Th1 cells. In this study, we investigated the effects of Gal-9 in murine models of acute GVH disease (aGVHD). First, we demonstrated that recombinant human Gal-9 inhibit MLR in a dose-dependent manner, involving both Ca^{2+} influx and apoptosis in T cells. Next, we revealed that recombinant human Gal-9 significantly inhibit the progression of aGVHD in murine BM transplantation models. In conclusion, Gal-9 ameliorates aGVHD, possibly by inducing T-cell apoptosis, suggesting that gal-9 may be an attractive candidate for the treatment of aGVHD.

Key words: Apoptosis · Galectin-9 · GVH disease

Supporting Information available online

Introduction

Galectins comprise a family of animal lectins that preferentially bind to β-galactosides through a carbohydrate recognition domain. The 15 members of the galectin family are divided into three groups based on structural similarities [1, 2]. Among the members of the galectin family, Galectin-9 (Gal-9) belongs to the group of bivalent tandem repeat galectins and has two homologous carbohydrate recognition domains connected by a linker peptide [3]. Gal-9 was initially characterized as an eosinophil-specific chemoattractant [4] and, subsequently, Gal-9 was found to possess a variety of biological functions, including roles in cell differentiation, aggregation, adhesion, the induction of cell death, and the inhibition of cancer metastasis [5–8].

Gal-9 is a ligand for T-cell immunoglobin domain and mucin domain-3 (Tim-3) expressed on terminally differentiated $CD4^+$

Correspondence: Dr. Eishi Ashihara
e-mail: ash0325@kuhp.kyoto-u.ac.jp, ash@koto.kpu-m.ac.jp

*These authors contributed equally to this work.

Th1 cells. Gal-9 induces the loss of IFN-γ-producing Th1 cells, thereby negatively regulating Th1 autoimmunity in EAE [9]. The suppression of immune reactions by Gal-9 has also been shown in other Th1-mediated disease models [10, 11]. In the current study, we investigated the immunosuppressive effects of Gal-9 using a murine BM transplantation (BMT) model. We demonstrated that recombinant human (rh) Gal-9 suppresses the induction of acute GVH disease (aGVHD) by inducing T-cell apoptosis.

Results

Gal-9 inhibits MLR through the induction of T-cell apoptosis

We first examined whether Gal-9 inhibited MLR. Splenic mononuclear cells were isolated from C57BL/6 and B6D2F1 female mice, and then 5×10^6 C57BL/6 splenocytes (responders) were cocultured with 1×10^5 irradiated B6D2F1 splenocytes (stimulators) in the presence of various concentrations of Gal-9. Gal-9 suppressed the MLR in a dose-dependent manner (Fig. 1A). To investigate whether Gal-9 induced T-cell apoptosis, enriched T cells isolated from the spleens of C57BL/6 mice were cultured in anti-CD3 Ab-coated plates in the presence of serial dilutions of rhGal-9 for 72 h. Similar to a previous report [5], Gal-9 induced apoptosis in a dose-dependent manner (Fig. 1A). We also investigated the effect of Gal-9 on the proliferation of T cells stimulated with anti-CD3 and anti-CD28 Ab. Gal-9 inhibited T-cell proliferation by more than 90% at a dose of 0.03 μM (Fig. 1B). Since the galectin family members bind to sugar chains containing β-galactoside such as lactose [6], we investigated whether lactose inhibited the effects of Gal-9. The addition of 30 mM lactose almost completely abrogated Gal-9-induced cell proliferation (Fig. 1C). To investigate whether Ca^{2+} influx could be involved in the effects of Gal-9 binding to β-galactoside, we performed fluo-4/AM assays of Ca^{2+} influx in response to 1.0 μM Gal-9 treatment. An influx of Ca^{2+} was observed in splenic T cells 10–20 ms after Gal-9 treatment, and lactose reduced the influx of Ca^{2+} induced by Gal-9 (Fig. 1D). Taken together, these results suggest that Gal-9

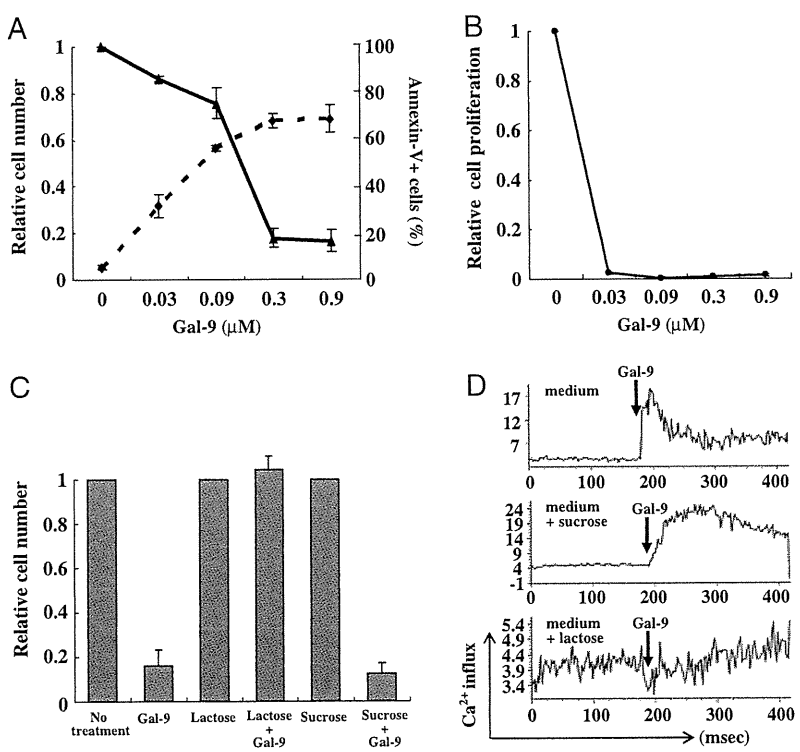

Figure 1. Gal-9 inhibits MLR through the induction of T-cell-apoptosis by Ca^{2+} influx in response to β-galactoside binding. (A) Responder C57BL/6 and irradiated (30 Gy) stimulator B6D2F1 splenocytes were cocultured with rhGal-9 for 10 days. MLR was evaluated by the modified MTT assay (relative cell number, solid line). Enriched T cells obtained from the spleens of C57BL/6 mice were cultured with plate-bound anti-CD3 Ab and rhGal-9 for 72 h. Apoptosis was evaluated by Annexin-V staining (dotted line). (B) Enriched T cells were cultured with plate-bound anti-CD3 Ab, soluble anti-CD28 Ab (5 μg/mL), recombinant mouse IL-2 (20 ng/mL), and rhGal-9. Cell proliferation was assessed by a modified MTT assay after 72 h incubation. (C) A modified MTT assay using splenic T cells after 72 h incubation in the presence of lactose or sucrose (30 mM) and/or rhGal-9 was performed. The data shows relative cell number compared with that without stimulation of lactose, sucrose, or rhGal-9. (D) Splenic T cells in RPMI1640 medium containing 10% FCS with or without sucrose or lactose were loaded with fluo-4/AM (5 μM) and intracellular Ca^{2+} influx after the administration of Gal-9 (1.0 μM; arrow) was measured by FACS Canto II. Data are (A–C) mean ± SD of three experiments (in (B) the SD are smaller than the symbol size and are not visible) and (D) are representative of three independent experiments.

induces T-cell apoptosis, presumably through the modulation of Ca^{2+} influx induced by Gal-9 binding to β-galactoside, resulting in the suppression of MLR.

Gal-9 treatment ameliorates aGVHD

Allogeneic T-cell-depleted BM (TCD-BM) cells and splenocytes from C57BL/6 mice were transplanted into irradiated B6D2F1 mice, and aGVHD was allowed to develop, at which point rhGal-9 was administered i.p. at a dose of 3 or 30 μg/mouse for 14 consecutive days. aGVHD was evaluated by clinical score [12]. The administration of Gal-9 at a dose of 30 μg/mouse significantly ameliorated aGVHD as compared with vehicle-treated mice or mice treated at the dose of 3 μg/mouse (Fig. 2A). However, Gal-9 did not prolong the survival of Gal-9-treated mice as compared with the untreated mice (data not shown). We evaluated pathological scores for

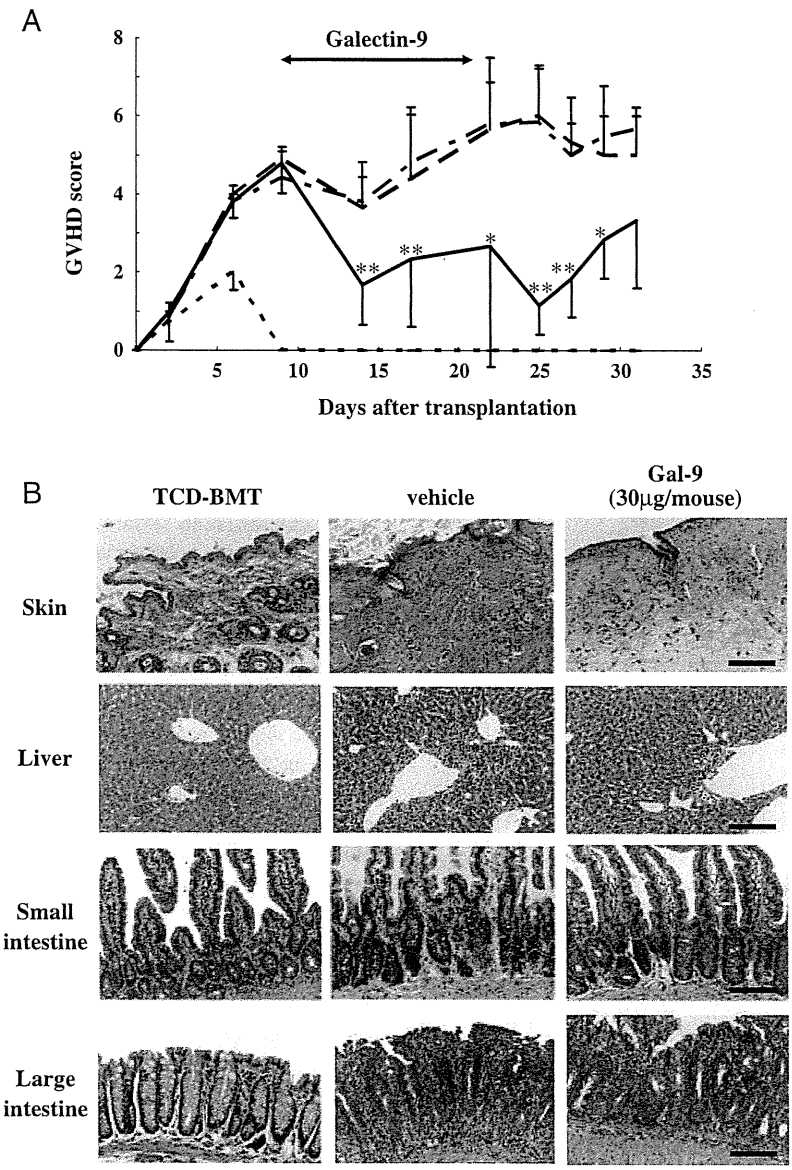

Figure 2. rhGal-9 ameliorates aGVHD in BMT model using mononuclear splenocytes. (A) B5D2F1 mice ($n = 10$ *per* group) were transplanted either with C57BL/6 TCD-MB cells alone and left untreated (dotted line) or with TCD-BM cells and C57Bl/6 mononuclear splenocytes, and once aGVHD had developed, were treated for 14 days with rhGal-9 (3 μg/mouse, dashed line; 30 μg/mouse, solid line) or vehicle alone (dashed, dotted line). Clinical scores were evaluated once or twice a wk. *$p<0.05$; **$p<0.01$ *versus* vehicle control mice (Student's t-test). Data are representative of two independent experiments. (B) Histopathological analysis of tissue from recipient mice in the experiments described in (A) stained with hematoxylin and eosin. Tissues were harvested on the day following the administration of rhGal-9 for 14 days. Original magnification, × 200. Each scale bar indicates 100 μm.

Table 1. Histological scores of aGVHD after Gal-9 treatment[a)]

	TCD-BMT	Allo-BMT vehicle	Allo-BMT Gal-9 3 μg	Allo-BMT Gal-9 30 μg
Small intestine	3.5±1.0‡	12.3±3.5	7.8±2.8*	8.2±3.0*
Large intestine	2.2±1.5‡	19.4±2.2	16.7±3.9*	14.8±3.2*
Liver	0.75±0.46‡	9.6±2.0	4.8±1.6†	5.5±2.3†
Total score	6.4±1.6‡	41.3±3.6	29.3±6.3†	28.6±4.6†

[a)] Scores (0–4) for each parameter were assessed on coded slides as described in the *Materials and methods*. Small intestine: villous blunting, crypt regeneration, loss of enterocyte brush border, luminal sloughing of cellular debris, crypt cell apoptosis, outright crypt destruction, and lamina propria lymphocyte infiltrate. Large intestine: crypt regeneration, crypt cell apoptosis, surface colonocyte, colonocyte vacuolization, surface colonocyte attenuation, outright crypt destruction, and lamina propria lymphocyte infiltrate. Liver: portal tract expansion by an inflammatory cell infiltrate, lymphocytic infiltrate of bile ducts, bile duct epithelial cell apoptosis, bile duct epithelial cell sloughing, vascular endothelialitis, parenchymal apoptosis, parenchymal microabscesses, parenchymal mitotic figures, hepatocellular cholestasis, and hepatocellular steatosis. Total GVHD score represents the mean ± SD of the sum of scores for small intestine, large intestine, and liver for each animals in indicated group ($n = 6$–9). *$p<0.05$ *versus* Allo-BMT treated with vehicle; †$p<0.01$ *versus* Allo-BMT treated with vehicle; ‡$p<0.005$ *versus* Allo-BMT treated with vehicle.

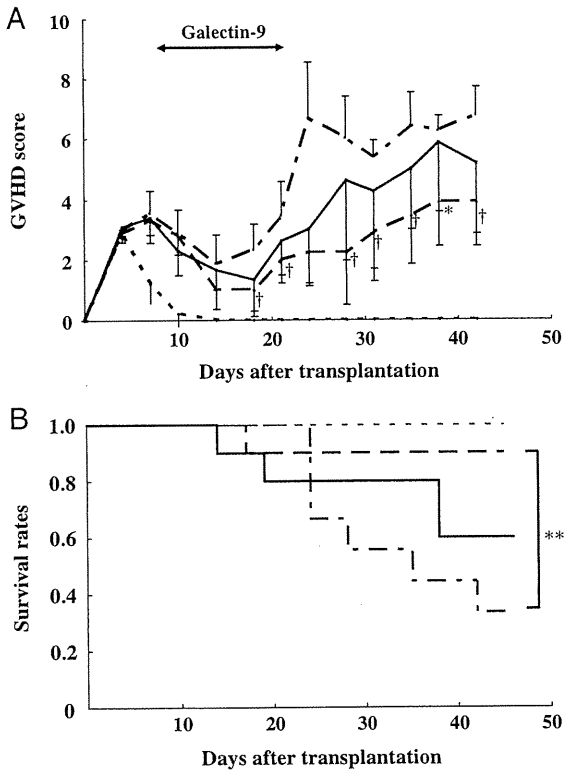

Figure 3. rhGal-9 ameliorates aGVHD in a BMT model using splenic $CD4^+$ T cells. (A) Mice were treated and evaluated as described in Fig. 2A but substituting splenic $CD4^+$ T cells for mononuclear splenocytes. *$p<0.01$; †$p<0.005$ *versus* vehicle control mice (Student's t-test). Data are representative of two independent experiments. (B) Survival curves of the $CD4^+$ T-cell-transplanted mice described in (A). **$p<0.05$ *versus* vehicle control mice (Log-rank test).

the small and large intestines and livers of transplanted mice using a semi-quantitative scoring system described previously [13]. Treatment with Gal-9 at a dose of 3 or 30 μg/mouse significantly decreased damage to these organs in recipient mice (Fig. 2B, Table 1).

We next investigated whether rhGal-9 ameliorated aGVHD induced by the infusion of splenic $CD4^+$ T cells. T-cell-depleted BM (TCD-BM) cells and $CD4^+$ T cells from C57BL/6 mice were transplanted into irradiated B6D2F1 mice. In this system, administration of rhGal-9 significantly ameliorated aGVHD (Fig. 3A). Moreover, administration of Gal-9 at a dose of 3 μg/mouse also prolonged survival as compared with untreated mice ($p<0.05$, Fig. 3B).

We next investigated the effects of rhGal-9 treatment on $CD4^+$ T-cell subsets. Peripheral blood leukocytes from recipient mice were collected for flow cytometric analysis on the day after a 14-day administration of Gal-9. To analyze the effect of Gal-9 on donor lymphocytes, splenic mononuclear cells from GFP Tg mice were used for the induction of aGVHD. For flow cytometry, the gating parameter was first set to isolate the lymphocyte population of peripheral blood leukocytes, and then set for GFP^+ cells. The frequency of $CD4^+$/Tim-3^+ cells was slightly decreased, whereas, the frequency of $CD4^+$/$CD25^+$ and $CD25^+$/Foxp3^+ Treg was slightly increased, by the Gal-9 treatment (Fig. 4A, Supporting Information Fig. 1); however, the changes were not statistically significant. We also investigated the effects of Gal-9 on cytokine production. Peripheral blood from recipient mice treated with vehicle or rhGal-9 (30 μg/mouse) was collected on the day after a 14-day administration of Gal-9. The production of TNF-α and IL-17 was suppressed by Gal-9 treatment ($p = 0.09$ and $p = 0.03$, respectively). Although the production of IFN-γ was decreased, there was no significant difference between the vehicle and Gal-9 treatment groups. IL-2 and IL-10 were unaffected by Gal-9 (Fig. 4B).

Discussion

aGVHD is a serious complication of allogeneic BMT, initiated by damage to the gastrointestinal tract, especially the intestinal mucosa. In the gastrointestinal tract, the bacterial product lipopolysaccharide translocates from the intestinal lumen to the blood circulation and stimulates the secretion of IL-1α and TNF-α from host Mϕ, resulting in the activation of host APC and the

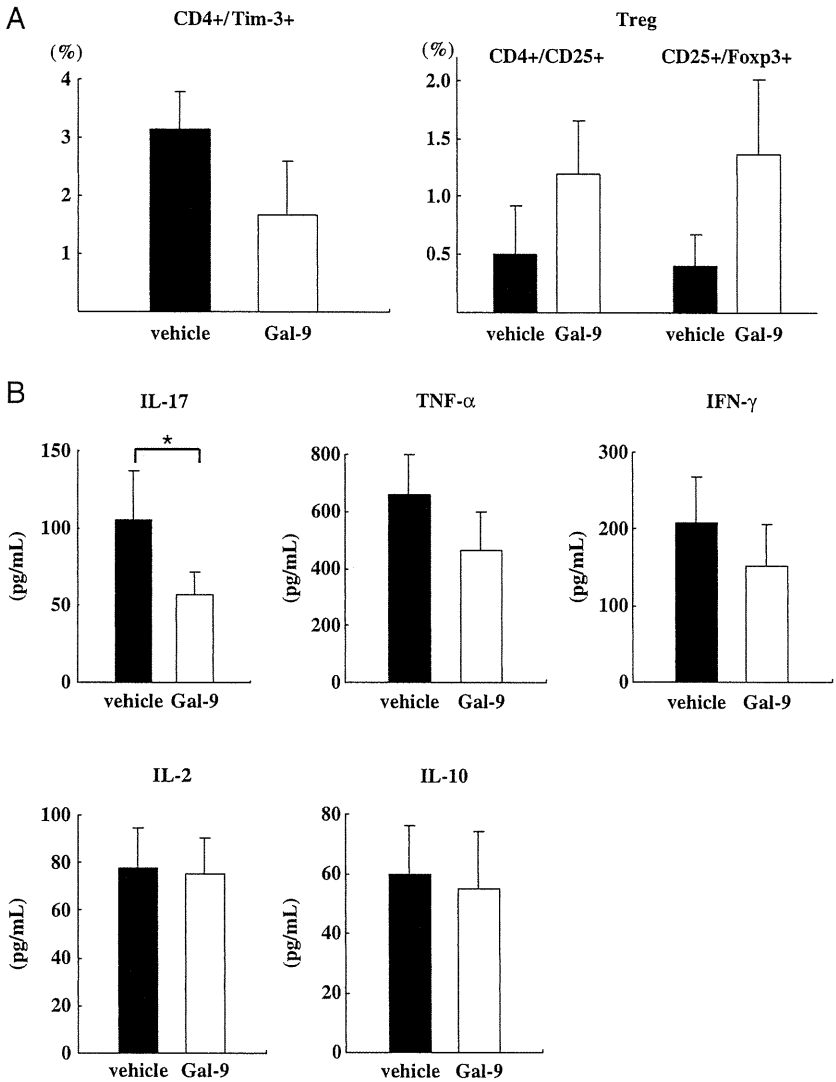

Figure 4. Effects of Gal-9 treatment on $CD4^+$ T-cell subsets and cytokine production. (A) TCD-BM cells obtained from C57Bl/6 mice and splenic mononuclear cells obtained from GFP Tg mice were transplanted into B6D2F1 mice. After induction of aGVHD, vehicle control or 30 μg of rhGal-9 was administered for 14 days. Peripheral blood cells were harvested on the day following the 14 day's administration and T-cell subsets were analyzed by FACS. Data are mean+SD ($n = 3$ *per* group) of two independent experiments giving consistent results. (B) Peripheral blood was collected from the recipient mice on the day following the administration of rhGal-9 or vehicle control for 14 days and the serum was separated to assess cytokine levels ($n = 4$–5 *per* group). Cytokine assays were performed using a Bio-Plex™ Pro Mouse Cytokine Assay. Data are mean+SD of three independent experiments. *$p<0.05$ *versus* vehicle control mice (Student's t-test).

differentiation of donor T cells to Th1 cells. Alloreactive Th1 cells release IFN-γ and IL-2, which promote the differentiation of cytotoxic T cells and inflammatory cytokine storms. The consequence of these immune responses is the development of aGVHD in the target organs [14].

In the current study, Gal-9 was found to suppress MLR in a dose-dependent manner, and it was revealed by FACS analysis that Gal-9 treatment increased the number of Annexin-V^+ T cells *in vitro*. Moreover, Gal-9 inhibited the proliferation of $CD3^+$ T cells stimulated with anti-CD3 and anti-CD28 Abs at a dose of only 0.03 μM. Differences in inhibitory doses of Gal-9 between MLR (Fig. 1A) and the T-cell proliferation stimulated with anti-CD3 and anti-CD28 Ab (Fig. 1B) could be due to the activation of DC by Gal-9 in the MLR [15, 16]. We have previously demonstrated that apoptosis is induced in both activated $CD4^+$ and $CD8^+$ T cells, although particularly in the $CD4^+$ T cells through the Ca^{2+}–calpain–caspase-1 pathway [5]. We confirmed here that Gal-9 induces an influx of Ca^{2+} into splenic T cells and that the influx was inhibited by co-treatment with lactose as previously reported [5]. Our findings indicate that Gal-9 induces apoptosis in splenic T cells through Ca^{2+} influx induced by β-galactoside binding, resulting in the suppression of MLR.

We investigated the immunosuppressive effects of Gal-9 in a murine model of aGVHD. rhGal-9 was administered when mice

exhibited symptoms of aGVHD. To clarify the effect of Gal-9 on aGVHD, we administered Gal-9 starting on the day when the influence of radiation was no longer apparent and aGVHD became pronounced. rhGal-9 amerliorated aGVHD clinical scores, and histological analysis revealed that Gal-9 significantly suppressed damage in target organs. However, survival rates in the Gal9-treated mice were not significantly affected as compared with untreated mice (data not shown). One reason for this lack of effect on survival may be the short duration of rhGal-9 treatment. The form of Gal-9 used in the current study is an rh form of the protein and, when rhGal-9 was administered to mice for 2 wk, neutralizing Ab against rhGal-9 were produced and the activity of Gal-9 was diminished (unpublished observations from Dr. Niki in GalPharma). In our preliminary experiments, aGVHD was not controlled any further upon longer treatment (data not shown); thus, we terminated rhGal-9 treatment after 2 wk. Nevertheless, the clinical scores and histological damages induced by aGVHD were improved, even with this short Gal-9 treatment.

The effects of rhGal-9 on aGVHD were also investigated in a $CD4^+$ T-cell-transplanted model. rhGal-9 at a dose of 3 μg/mouse ameliorated aGVHD and prolonged survival as compared with untreated mice. Together with the results of the mononuclear splenocyte-transplanted model, these results indicated that Gal-9 is an effective agent for the treatment of aGVHD. Interestingly, Gal-9 at the higher dose (30 μg/mouse) did not ameliorate aGVHD in the $CD4^+$ T-cell-transplanted model. The reason for this is not clear; however, $CD8^+$ T cells were not transfused into recipient mice in this murine model and aGVHD might not be as aggressive in this model as in the whole splenocyte-transplanted model. We speculate that Gal-9 at the lower dose suppresses aGVHD progression through ;Tim-3, which is expressed on $CD4^+$ T cells. The higher dose of Gal-9, on the other hand, might also activate APC cells [15, 16]. Thus, the inhibitory effects on aGVHD due to the induction of apoptosis may be negated by the APC activation at a higher dose. However, further investigation is necessary to clarify the mechanism by which Gal-9 acts in the context of activated immune functions.

Using splenic mononuclear cells from GFP Tg mice for the induction of aGVHD, we analyzed the effects of Gal-9 treatment on subsets of donor-derived $CD4^+$ T cells. Gal-9 treatment decreased numbers of $CD4^+$/Tim-3^+ T cells and given that Tim-3 is a receptor for Gal-9 [9], these results suggest that Gal-9 may directly induce apoptosis in $CD4^+$/Tim-3^+ T cells. $CD4^+$/$CD25^+$ or $CD25^+$/$Foxp3^+$ Treg numbers in the circulation were, on the other hand, increased by Gal-9 treatment. Treg are involved in the pathogenesis of autoimmune diseases, as well as in immunotolerance in GVHD [17–19]. We have previously shown that there is a significant decrease in $CD4^+$/$Foxp3^+$ splenic cells in Gal-9-deficient mice as compared with WT mice, and that Gal-9 induces the differentiation of $CD4^+$/$CD62L^+$ naive T cells into $Foxp3^+$/$CD4^+$/$CD25^+$ Treg cells [10]. More interestingly in our current study, although $CD4^+$/Tim-3^+ T-cell numbers were decreased by Gal-9 treatment, Treg numbers were increased. These results suggest that Treg precursors do not express Tim-3 and that Gal-9 induces the proliferation of Treg cells through receptors other than Tim-3. Additional studies are warranted to clarify the precise mechanisms of induction of Treg cell expansion by Gal-9.

Analysis of cytokine levels revealed that Gal-9 suppressed the production of IL-17, as well as TNF-α and IFN-γ, in recipient mice. Th17 cells, which are IL-17-producing $CD4^+$ T cells [20], play a crucial role in orchestrating the immune reactions that participate in autoimmune diseases [21, 22]. Recent reports indicate that IL-17-producing Th17 cells augment aGVHD [23–25]. Given that Tim-3 is expressed in Th17 cells [10, 26, 27], the current findings suggest that Gal-9 induces apoptosis in Th17 and Th1 cells, resulting in decreased production of IL-17- and Th1-related cytokines such as TNF-α and IFN-γ. Furthermore, it has been reported that Th17 and Treg cells are reciprocally induced [28]. We speculate that Gal-9 treatment suppresses Th17 differentiation, redirecting the process to Treg differentiation. During aGVHD, the IFN-γ levels in Gal-9-treated mice were decreased, although not significantly, whereas the level of IL-17 was significantly suppressed. Since $CD4^+$ T cells can reciprocally differentiate into Th1 and Th17 cells [23], we speculate that the $CD4^+$ T cells that survived might have differentiated into IFN-γ-producing Th1 cells, resulting in only a slight suppression of IFN-γ.

In conclusion, Gal-9 was found to ameliorate aGVHD in a murine model of BMT through the induction of T-cell apoptosis. The results of the current study suggest that Gal-9 may be useful for the treatment of aGVHD and, in light of previously reported data from other allogeneic transplantation models [10, 11, 29], our results suggest that Gal-9 is a promising biological agent for the suppression of immune reactions.

Materials and methods

Mice

Six- to ten-wk-old C57BL/6 mice ($H\text{-}2^b$) and B6D2F1 female mice ($H\text{-}2^{b/d}$) were purchased from Oriental Bio Service (Kyoto, Japan) and SLC Japan (Osaka, Japan), respectively. GFP Tg mice [30, 31] were kindly provided by Dr. Miyazaki (Osaka University, Japan). Mice were maintained on a 12:12 hour light–dark cycle in a specific pathogen-free animal facility at the Kyoto University. Approval for the animal protocols was obtained from the Committee on Animal Research of the Kyoto University Faculty of Medicine.

Isolation of T cells

T-cell populations of interest were obtained from mice using a magnetic cell sorting separation system (Miltenyi, Gladbach, Germany), according to the manufacturer's instructions. A mouse Pan T Isolation Kit (Miltenyi) was used for the isolation of $CD3^+$ T cells. For the isolation of $CD90.2^+$ and $DC4^+$ T cells, an

anti-CD90.2 Ab and an anti-CD4 Ab (Miltenyi) were used, respectively. Isolated cells were stained with an anti-CD3ε Ab (BD Bioscience, Tokyo, Japan) or an anti-CD4 Ab, and the purity of the isolated or depleted cell populations were analyzed by FACS Canto II using Diva software (BD Bioscience).

MLR

Splenocytes were isolated from spleens of C57BL/6 and B6D2F1 mice after cervical dislocation. Spleens were crushed through a sterile mesh and then were filtered through a 70-μm Cell Strainer (BD Bioscience). Single-cell suspensions were prepared in RPMI1640 (Gibco, Tokyo, Japan) containing 10% heat-inactivated FCS (Invitrogen, Tokyo, Japan), 2 mM L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco), and then mononuclear splenocytes were obtained by Ficoll-Hypaque density centrifugation (Lympholyte-M; Cedarlane, Ontario, Canada). Responder C57BL/6 splenocytes (5×10^6 cells/100 μL *per* well) and irradiated (30 Gy) stimulator B6D2F1 splenocytes (1×10^5 cells/100 μL *per* well) were cocultured in a flat-bottom 96-well plate (BD Bioscience) in the presence of serial dilutions of rhGal-9 for 10 days. MLR was evaluated by a modified MTT assay [32, 33] using a Cell-counting kit-8 (Dojindo Laboratory, Kumamoto, Japan). Data represent the means of four samples for each concentration of Gal-9.

The effect of Gal-9 on T-cell proliferation, stimulated by anti-CD3 and anti-CD28 Ab was evaluated in cultures of enriched splenic T cells from C57BL/6 mice. The purity of the isolated $CD3^+$ T-cell population was more than 95%. T cells were cultured (2.5×10^5/well) in 96-well anti-CD3 Ab-coated plates (BD Bioscience) with soluble anti-CD28 Ab (5 μg/mL; BD Bioscience) and recombinant mouse IL-2 (20 ng/mL; Wako, Osaka, Japan) with or without serial dilutions of rhGal-9. After 72 h, cell proliferation was assessed using a modified MTT assay. Data represent the means of four samples for each concentration of Gal-9.

Analysis of apoptosis

Enriched $CD90.2^+$ T cells were cultured in anti-CD3 Ab-coated plates in the presence or absence of Gal-9 for 72 h. The purity of the enriched cell population was ≥95%, and CD3 expression was confirmed. Apoptosis was determined using an Annexin V-FITC Apoptosis Detection Kit I (BD Bioscience), according to the manufacturer's instructions. Cells were analyzed by FACS Canto II using Diva software. To assess the requirement for β-galactoside binding in Gal-9-induced apoptosis, we performed a modified MTT assay using splenic T cells in the presence of 30 mM lactose or sucrose (Wako).

Ca^{2+} influx

Splenic T cells in RPMI1640 medium containing 10% FCS were loaded with fluo-4/AM (5 μM) (Invitrogen) at 37°C for 30 min. Cells were washed and resuspended in medium, and then intracellular Ca^{2+} influx induced in response to rhGal-9 (1.0 μM) was measured using FACS Canto II. Data were analyzed using FlowJo software (Tree Star, Ashland, OR) We also evaluated the requirement of β-galactoside binding in Ca^{2+} influx by culturing cells in the presence of lactose or sucrose (30 mM).

BMT

WT C57BL/6 mice were sacrificed by cervical dislocation, and femurs and tibiae were removed. BM cells were collected by flushing bones with RPIM1640 containing 10% FCS using a 21-gauge needle, and then the cells were filtered and washed twice by centrifugation at 1500 rpm for 5 min. After the BM cells were suspended in buffer (1 × PBS containing 5 mM EDTA and 0.5% BSA), T-cell-depletion was performed using an anti-CD90.2 Ab. Residual $CD90.2^+$ cells were routinely less than 0.5% of purified BM cells. TCD-BM cells were resuspended in 1 × PBS prior to injection. Mononuclear splenocytes from WT C57BL/6 mice were used as the source of allogeneic T cells. Recipient B6D2F1 mice received a myeloablative dose (9 Gy) of total body irradiation from an X-ray irradiator. Six to eight hours later, each recipient mouse was injected i.v. with 4×10^6 TCD-BM cells alone or with 5×10^6 mononuclear splenocytes. Where indicated, the ability of rhGal-9 to ameliorate aGVHD induced by splenic $CD4^+$ T cells was evaluated. The purity of the enriched $CD4^+$ T-cell population was more than 95%. Recipient B6D2F1 mice received a myeloablative dose (9 Gy) of total body irradiation, and then each recipient mouse was injected i.v. with 4×10^6 TCD-BM cells with or without 2×10^6 $CD4^+$ splenocytes.

aGVHD and Gal-9 treatment

aGVHD clinical scores were evaluated once or twice a wk [12]. After aGVHD developed, recipient mice were treated with rhGal-9 (3 or 30 μg/mouse) or vehicle by i.p. injection for 14 consecutive days. Mice that exhibited aGVHD score of greater than 7 were sacrificed and added to the Kaplan–Meier statistics the same day, as previously reported [34]. Histopathological evaluation of aGVHD was performed as follows. Tissues (liver, skin, and small and large intestines) from the recipient mice were placed in 10% formalin, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin. Slides were coded without reference to prior treatment and examined in a blinded fashion by one individual (E.A.). A semi-quantitative scoring system was used as previously described [13]. Seven parameters were scored for the small intestine (villous blunting, crypt regeneration, loss of enterocyte brush border, luminal sloughing of cellular debris, crypt cell apoptosis, outright crypt destruction, and lamina propria lymphocyte infiltrate) and large intestine (crypt regeneration, crypt cell apoptosis, surface colonocyte, colonocyte vacuolization, surface colonocyte attenuation, outright crypt

destruction, and lamina propria lymphocyte infiltrate); and ten parameters were scored for the liver (portal tract expansion by an inflammatory cell infiltrate, lymphocytic infiltrate of bile ducts, bile duct epithelial cell apoptosis, bile duct epithelial cell sloughing, vascular endothelialitis, parenchymal apoptosis, parenchymal microabscesses, parenchymal mitotic figures, hepatocellular cholestasis, and hepatocellular steatosis). The scoring system was as follows: 0 = normal; 0.5 = focal and rare; 1 = focal and mild; 2 = diffuse and mild; 3 = diffuse and moderate; and 4 = diffuse and severe. Scores were added to provide a total score for each organ. The maximum score for the small intestine and large intestine was 28, and for the liver, 40.

Phenotypic analysis of $CD4^+$ T-cell subsets

Peripheral blood leukocytes from recipient mice were collected for flow cytometric analysis of $CD4^+$ T-cell subsets on the day following the administration of Gal-9 for 14 days. To analyze donor lymphocytes, the splenic mononuclear cells obtained from GFP Tg mice were used for the induction of aGVHD. The gating parameters were first set to isolate the lymphocyte population of peripheral blood leukocytes, and then set to isolate GFP^+ cells. T-cell subsets were analyzed using the following Ab: anti-mouse CD4-PerCP-Cy5.5 (eBioscience, San Diego, CA), anti-mouse Tim-3-PE (eBioscience), anti-CD25-PE, and anti-Foxp3-allophycocyanin (eBioscience). Cells were analyzed by FACS Canto II using the Diva software (BD Bioscience).

Cytokine assays

Peripheral blood was collected from mice treated with vehicle or 30 μg of rhGal-9 to assess cytokine production on the day following the administration of Gal-9 for 14 days. Serum cytokines were measured using a Bio-Plex™ Pro Mouse Cytokine Assay (BioRad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Statistical analysis

Differences in aGVHD scores and serum cytokine levels between groups were determined using the Student's *t*-test. Differences in aGVHD histological scores were determined using the Mann–Whitney's *U*-test. Differences in survival among groups of mice were evaluated with a log-rank test of Kaplan–Meier survival curves. A p value of <0.05 was considered statistically significant.

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Abbreviations: **aGVHD:** acute GVH disease · **BMT:** BM transplantation · **Gal-9:** galectin-9 · **rh:** recombinant human · **TCD:** T-cell-depleted · **Tim-3:** T-cell immunoglobin domain and mucin domain-3

Full correspondence: Dr. Eishi Ashihara, Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
Fax: +81-75-751-4283
e-mail: ash0325@kuhp.kyoto-u.ac.jp

Current address: Dr. Eishi Ashihara, Department of Molecular Cell Physiology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kamigyo-ku, Kyoto 602-8566, Japan
Fax: +81-75-251-0295
e-mail: ash@koto.kpu-m.ac.jp

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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

Toshio Kitawaki[a], Norimitsu Kadowaki[a], Keiko Fukunaga[a], Yasunari Kasai[b], Taira Maekawa[b], Katsuyuki Ohmori[c], Tatsuya Itoh[d], Akira Shimizu[d], Kiyotaka Kuzushima[e], Tadakazu Kondo[a], Takayuki Ishikawa[a], and Takashi Uchiyama[a]

[a]*Department of Hematology and Oncology;* [b]*Center for Cell and Molecular Therapy, Department of Transfusion Medicine and Cell Therapy;* [c]*Department of Clinical Laboratory Medicine;* [d]*Department of Experimental Therapeutics, Kyoto University Hospital, Kyoto, Japan;* [e]*Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Japan*

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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.

Offprint requests to: 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

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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

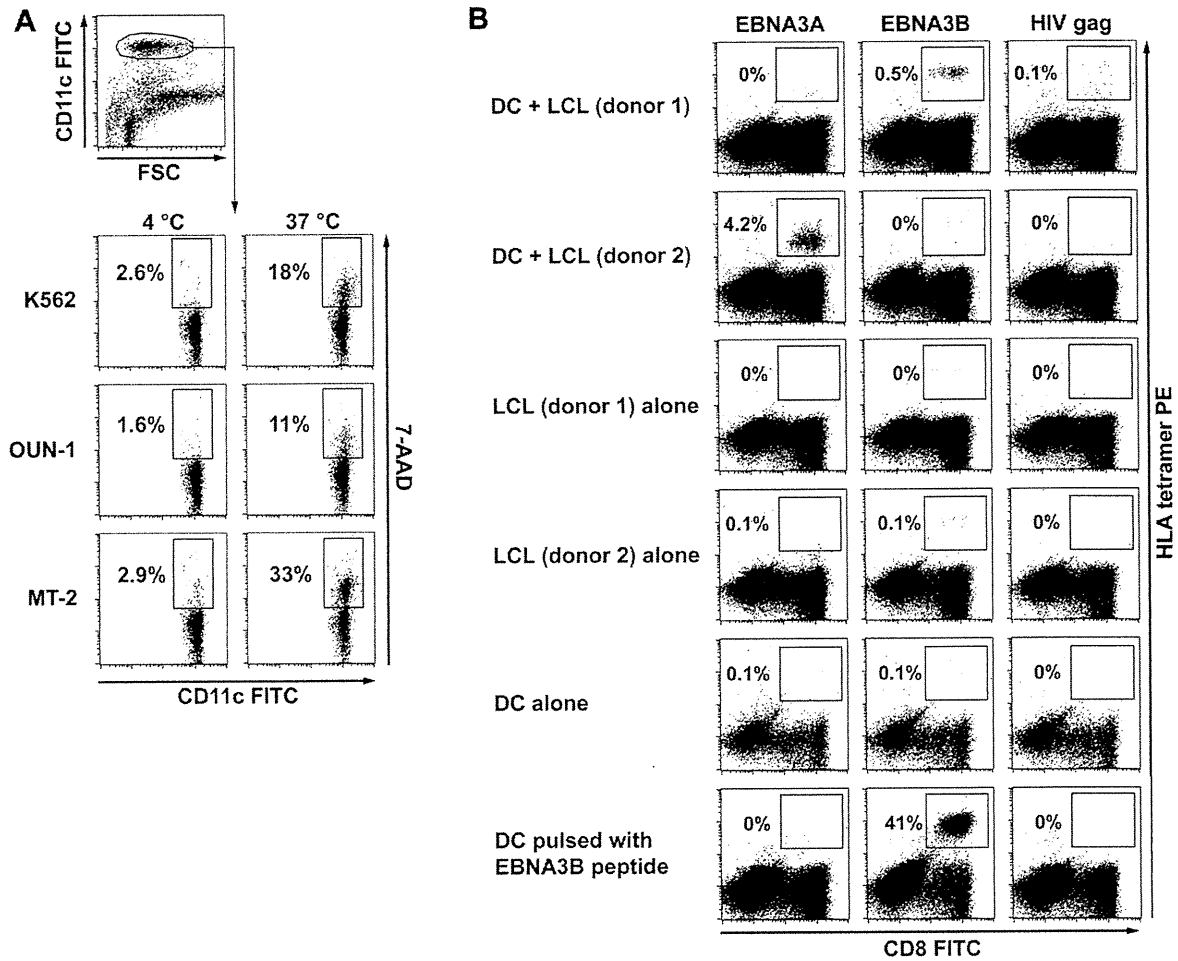

Figure 1. 3d-DCs incorporate apoptotic cells and cross-present cell-associated antigens. (**A**) Uptake of apoptotic cells by 3d-DCs. Apoptotic K562, OUN-1, and MT2 were labeled with 7-aminoactinomycin D (7-AAD) (20 μg/mL), and cocultured with immature 3d-DCs at a DC-to-apoptotic cell ratio of 1:1. After 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

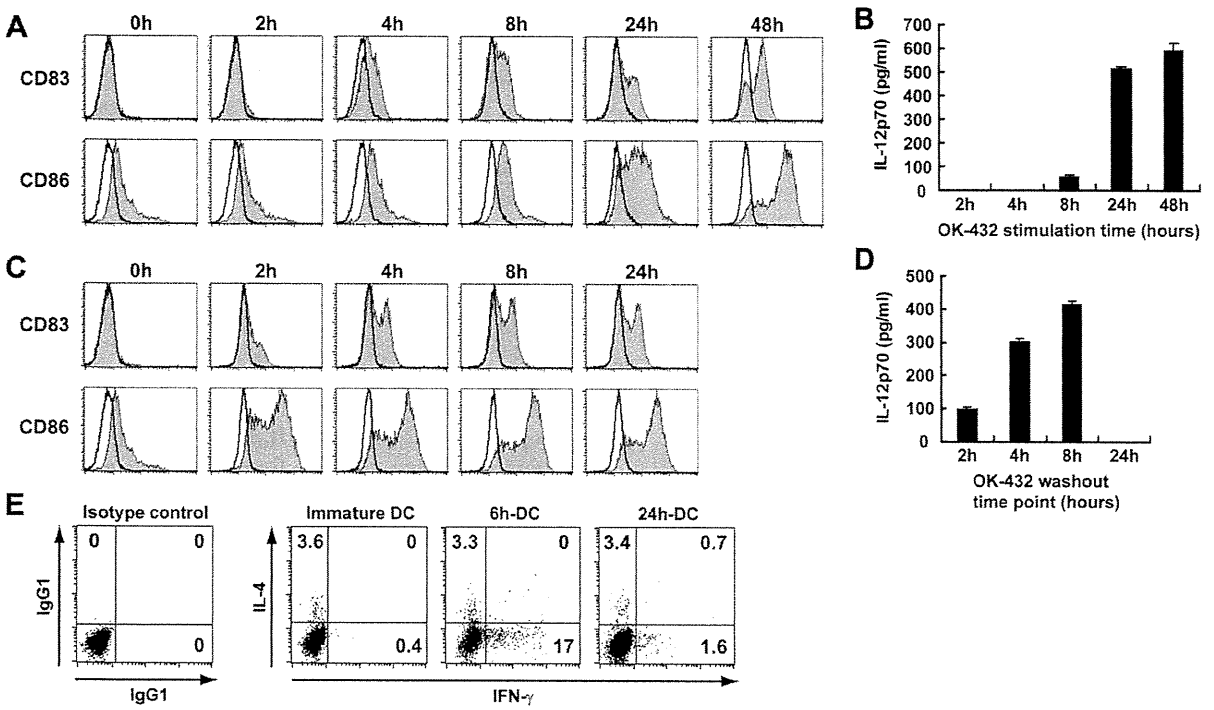

Figure 2. Short-term stimulation with OK-432 is optimal to generate Th1-inducing mature DCs. (**A**, **B**) Immature 3d-DCs were cultured in the presence of OK-432 (0.1 KE/mL) for indicated time periods, then harvested and analyzed. (**C**, **D**) Immature 3d-DCs were cultured in the presence of OK-432 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.

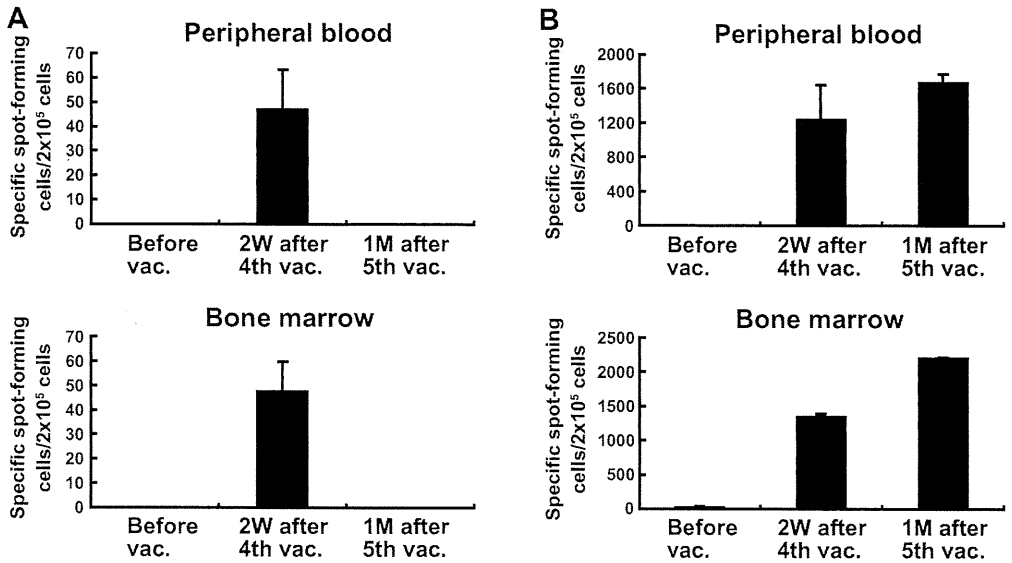

Figure 3. IFN-γ ELISPOT assay in patient no. 2. MNCs from PB and BM were obtained at indicated time points and subjected to IFN-γ ELISPOT assays directly after isolation (**A**) or after 1 week of stimulation with antigen-pulsed DCs (**B**). In IFN-γ ELISPOT assays, 2×10^5 MNCs (**A**) and 1×10^4 MNCs (**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.

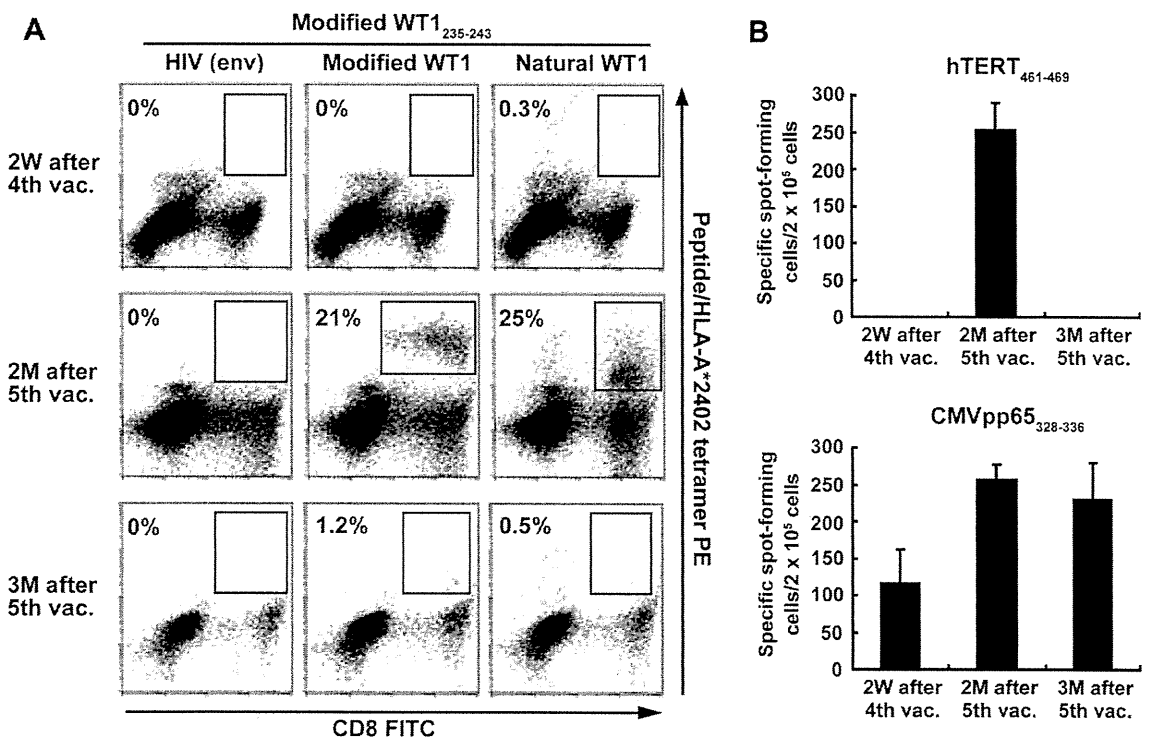

Figure 4. Immune responses in patient no. 3. (**A**) HLA tetramer staining. MNCs from PB were obtained at indicated time points, stimulated for 1 week with DCs pulsed with the modified $WT1_{235-243}$ peptide, stained with phycoerythrin-labeled peptide/HLA-A*2402 tetramers and fluorescein isothiocyanate–labeled 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.

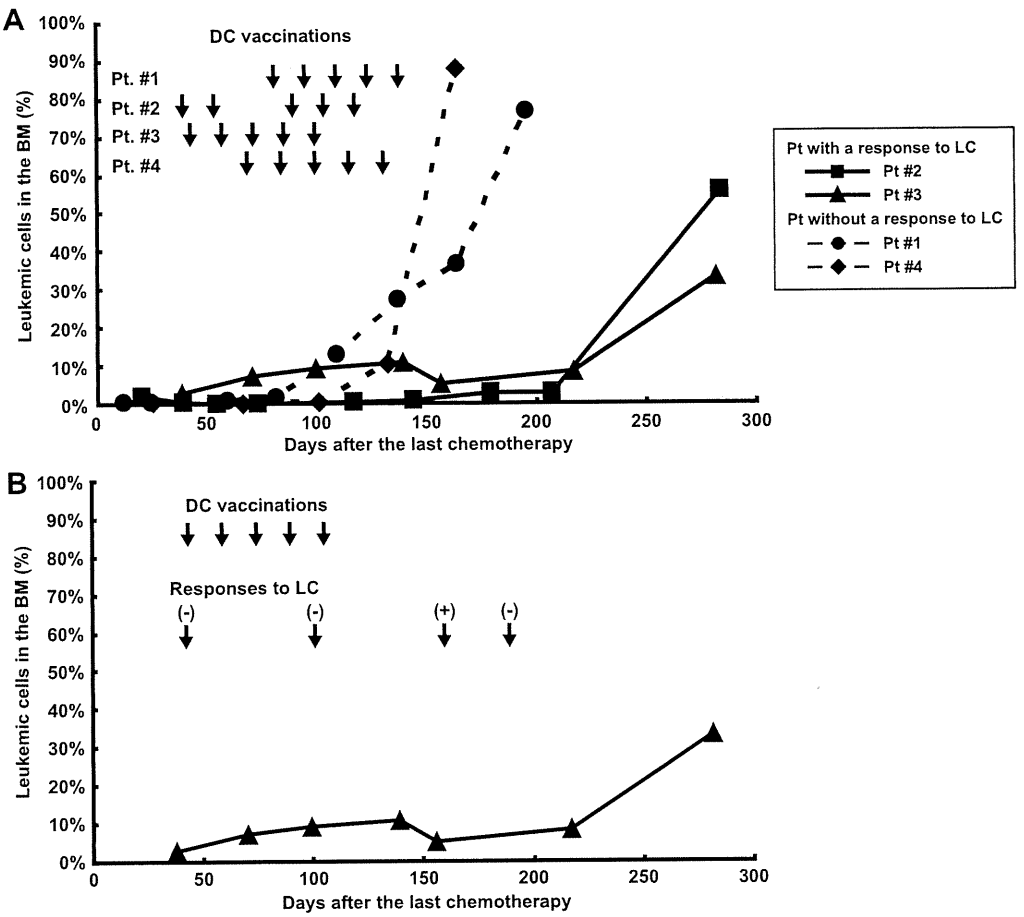

Figure 5. Clinical courses during the DC vaccination. (**A**) Percentages of leukemic cells in BM as determined by flow cytometry in four vaccinated patients are shown. Solid lines indicate patients with immune responses to leukemic cells (LCs) (patients 2 [■] and 3 [▲]). Dashed lines indicate patients 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.

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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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