Freeze-Thawing Procedures Have No Influence on the Phenotypic and Functional Development of Dendritic Cells Generated from Peripheral Blood CD14+ Monocytes

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Abstract: Little is known about the potential influence of cryopreservation on the biologic activities of dendritic cells (DCs). In this study, we examined the effects of freeze-thawing on the phenotypic and functional development of human DCs obtained from granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood CD14+ cells. CD14+ cells were cultured, immediately or after freeze-thawing, with granulocyte-macrophage CSF and interleukin-4 for 9 days, and then with added tumor necrosis factor-α for another 3 days. For both fresh and freeze-thawed monocytes, immature DCs harvested on day 6 and mature DCs harvested on day 9 of culture were examined under the same conditions. Cells were compared with regard to their 1) capacities for antigen endocytosis and chemotactic migration (immature DCs), and 2) allogeneic mixed lymphocyte reaction and antigen-specific cytotoxic T lymphocyte responses (mature DCs). Freeze-thawing did not affect the viability or subsequent maturation of DCs at any stage of development. Furthermore, essentially no difference was observed in phenotype or function between cells generated from fresh or cryopreserved/thawed cells. Although this study design was limited with the use of fetal bovine serum, the observation still suggests that freeze-thawing does not affect viability, phenotype, subsequent maturation, or functions of DCs at any stage of maturation.

Key Words: dendritic cells, cryopreservation

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Dendritic cells (DCs) are potent antigen-presenting cells that have been characterized as a natural adjuvant[1–3] and have been examined in several studies for their possible clinical application in immunotherapy against cancer.[4–7] Immature DCs can capture and process exogenous antigens,[8] while in response to maturation stimuli, they start to express enhanced costimulatory molecules and migrate into secondary lymphoid organs to select and stimulate antigen-specific T cells.[2,9] However, only a few studies have examined the influence of cryopreservation/thawing on the subsequent expansion of culture-generated DCs, the expression of surface molecules, and the functional capacities of immature and mature DCs.

In the present study, isolated CD14+ cells, rather than plastic adherent cells that have been used in most other studies, were cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) for 9 days and then with added tumor necrosis factor-α (TNF-α) for 3 additional days according to the method described in other studies.[10] To investigate the influence of preceding cryopreservation and thawing on the subsequent development of DCs, we examined the phenotypic and functional development of DCs, including endocytic, chemotactic migratory, and antigen-presenting capacities at different maturation steps, ie, monocytes, immature DCs, and mature DCs. Our results suggest that freeze-thawing does not affect the induction of functional mature DCs.

MATERIALS AND METHODS

Culture Medium and Cytokines

The following culture media were used to generate DC: RPMI 1640 (GIBCO-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Catalog no. 30071, lot no.AHK9081, KitHycone, Logan, UT) and 1000 units/mL penicillin, 100 μg/mL streptomycin (GIBCO-BRL). Recombinant human GM-CSF was provided by Kirin Brewery-Company (Tokyo, Japan). Recombinant human IL-4, TNF-α, and IL-2 were purchased from R & D Systems (Minneapolis, MN).

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Procedure for Obtaining Monocyte-Derived DCs from Mobilized Peripheral Blood CD14+ Cells

Peripheral blood mononuclear cells (PBMCs) from healthy donors were collected by leukapheresis after mobilization with granulocyte-CSF (G-CSF) as previously described.[11] Small aliquots of cells (about 2 × 10^8 cells) remaining in the tubing system were used for this experiment after informed consent was obtained. The experimental program was approved by the IRB of National Cancer Center Hospital in Tokyo. Cells were washed twice with phosphate-buffered saline and $CD14^+$ cells were then positively selected with MicroBeads (Miltenyi Biotec, Bergish Glandbach, Germany) according to the manufacturer's instructions. Bead-bound cells were analyzed immediately after selection by flow cytometry and found to be composed of >90% $CD14^+$ cells. Isolated $CD14^+$ cells (approximately 1 × 10^6 cells/mL) were cultured in 25-cm^2 flasks (Corning Inc., Corning, NY) in culture medium supplemented with GM-CSF (50 ng/mL) and IL-4 (50 ng/mL) for 6 days to allow them to differentiate into immature DCs. Thereafter, immature DCs were further cultured with GM-CSF (50 ng/mL), IL-4 (50 ng/mL), and TNF-α (50 ng/mL) for 3 days to differentiate into mature DCs. Half of culture medium was replaced with the same volume of the fresh medium containing the cytokines every 3 days.

Freeze-Thawing Procedures

Freezing was performed as follows: the cells were resuspended in a freezing medium containing 10% DMSO and 90% fetal bovine serum at a cell concentration of 5 × 10^6 cells/mL per tube (CryoTubes™, catalog no. 375353, Nunc A/S, Denmark) and slowly frozen to −80°C overnight using a Cryo 1°C NALGEN Freezing Container (Nalge Nunc International, Rochester, NY) filled up with isopropyl alcohol. The frozen cells were then transferred to a liquid nitrogen tank for storage for 4 weeks. Thawing was performed as follows: the frozen cells in a freezing tube were thawed in a 37°C water bath and then washed with culture medium containing 10% acid citrate dextrose solution (Sigma-Aldrich, Steinheim, Germany) by centrifugation at 250*g* force for 10 minutes at room temperature. The thawed cells were counted using trypan blue dye and cultured overnight at 37°C in 5% CO_2 with an appropriate combination of cytokines for subsequent assay. To evaluate the effect of freezing/thawing procedure, the recovery rate in each step was calculated as follows:

Alive cell number per tube after thawing /Alive cell number per tube before freezing × 100 (%)

Definition of Cell Samples

Each stage in the maturation of DCs and points of cryopreservation/thawing are illustrated in Figure 1. Cryopreservation was performed at each maturation stage to obtain the following cell types for experiments: 1) 2 types of monocytes [fresh (fr/mono) and thawed monocytes (th/mono)], 2) 3 types of immature DCs harvested on day 6 of culture [freshly cultured immature DCs (fr/iDC), thawed immature DCs (th/iDC), and immature DCs generated from the culture of th/mono (iDCs from th/mono)], and 3) 4 types of mature DCs [freshly cultured mature DCs (fr/mDC), thawed mature DCs (th/mDC), mature DCs generated from th/iDCs (mDCs from th/iDC), and mature DCs generated from th/mono (mDCs from th/mono)]. At each maturation stage, the number of viable cells, cell surface markers, and function of cells were examined. The number of viable cells was counted with trypan blue dye under the microscope and the results represent the average of 4 calculations.

Flow Cytometric Analysis

Cell surface markers were examined at each maturation stage using the following monoclonal antibodies: fluorescent isothiocyanate (FITC)-conjugated anti-CD14 and HLA-DR antibodies, and phycoerythrin (PE)-conjugated anti-CD1a, CD80, and CD86 antibodies, which were purchased from Becton Dickinson (San Jose, CA), and PE-labeled anti-CD83 antibody, which was purchased from Coulter-Immunotech (Miami, FL). Flow cytometry was performed using a FACSCalibur (Becton Dickinson). Expression rates of the surface markers were evaluated with the fluorochrome-conjugated isotype IgG intensity as a control. The results represent the percentage of positive cells calculated from 10^4 total events.

Endocytosis Assay with FITC-Dextran

FITC-Dextran (MW 40000; Sigma, St Louis, MO) was added to the cell suspensions at a final concentration of 1 mg/mL, and the cells were cultured for 45 minutes at either 37°C or 4°C as a control. After incubation, cells were washed twice with cold phosphate-buffered saline and analyzed by flow cytometry.

Transmigration Assay

To evaluate the chemotactic effects of DCs on stimulation, a previously described, the double-chamber system was used with a minor modification.[12,13] Briefly, polycarbonate membranes with 8-μm pore size filters (Chemotaxicell™; Kurabo, Osaka, Japan) were placed on 24-well culture plates to separate the upper and lower chambers. IL-16 (TECHNE, Minneapolis, MN) solution was diluted to concentrations from 0.0001 to 10 nmol/L with 500 μL of culture medium and placed in the lower chambers. Immature and mature DCs were then added to the upper chambers at 1 × 10^5 cells /100 μL. After incubation for 4 hours at 37°C, the cells migrated to the lower chambers and those larger than 12 μm were counted using a Coulter counter.

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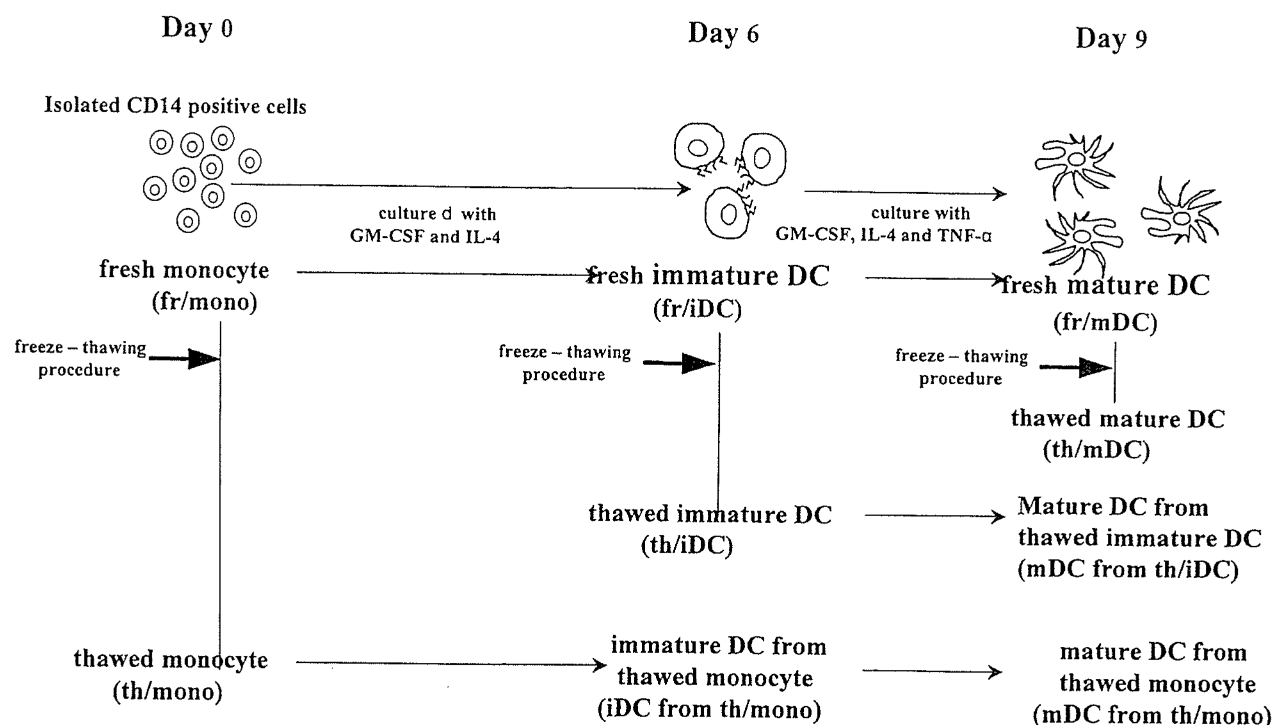

FIGURE 1. Definition of cell maturation stage and scheme of the experimental framework. CD14$^+$ cells were isolated from G-CSF-mobilized PBMCs using MACS, resuspended at 1×10^6 cells/mL, and cultured in the presence of GM-CSF (50 ng/mL) and IL-4 (50 ng/mL) for 6 days to generate immature DCs. TNF-α (50 ng/mL) was then added to the culture to induce mature DCs after an additional 3 days. Freshly isolated and cultured cells were resuspended in freezing medium at 5×10^6 cells/mL, cryopreserved for 4 weeks in liquid nitrogen, and then rapidly thawed for experimental use. Using this experimental culture system, 3 types of immature DCs and 4 types of mature DCs were generated.

Allogeneic Mixed Lymphocyte Reaction (allo-MLR)

Allo-MLR was performed according to a protocol previously described.[14] Briefly, irradiated (30 Gy) mature DCs were washed 3 times with RPMI 1640 medium, including 10% fetal bovine serum and plated into 96-well round-bottomed microplates at various cell concentrations. Allogeneic CD3$^+$ T lymphocytes as a responder cell were isolated from PBMCs by human T cell enrichment columns (R&D, Minneapolis, MN). Responder cells were added at 5×10^4 cells/well in a final total volume of 200 μL, and plates were incubated for 5 days. The cocultured cells were pulsed with 1 μCi [^{3}H]methylthymidine (5 Ci/mmol; Amersham Life Science, Buckinghamshire, UK) per well for 16 hours and then harvested and analyzed by a liquid scintillation counter.

Induction of Cytotoxic T Lymphocytes (CTL)

Mature DCs (1×10^5) prepared from HLA-A24$^+$ donors were loaded with 10 μM Epstein-Barr virus (EBV)-derived peptide (TYGPVFMCL: purity >95%) (Genetics, Huntsville, AL),[15] which is capable of binding to HLA-A2402 for 2 hours. In 24-well plates, autologous purified CD8$^+$ T lymphocytes, which were isolated from PBMCs by magnetic cell sorting using CD8 Microbeads (Miltenyi Biotec) as a source of effector cells, were cocultured at a ratio of 2:1 with mature DCs in 2 mL of CTL medium (RPMI 1640) and AIM-V serum-free medium (1:1), supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 1% penicillin-streptomycin, 1% nonessential amino acid (GIBCO-Invitrogen, Aukland, New Zealand), and 100 IU/mL IL-2 (Shionogi Pharmaceutical, Osaka, Japan) for 10 days.

Half of the medium was changed every 3 days. BEC-2 (HLA-A2402) and Bamb-2 (HLA-A1/A26) generated by EBV-transformed B-lymphoblastoid cell lines from EBV$^+$ healthy donor (kindly provided by Dr. K. Itoh, Kurume University, Kurume, Japan)[16] as target cells were also loaded at a concentration of 1×10^6 cells/mL with 10 μM of EBV peptide for 2 hours. The effector cells were cocultured with target cells in a total volume of 200 μL at effector to target (E:T) ratios of 2:1, 5:1, and 10:1, respectively, in 96-well round-bottom microplates. After overnight incubation, the concentration of interferon (IFN)-γ in the supernatant was

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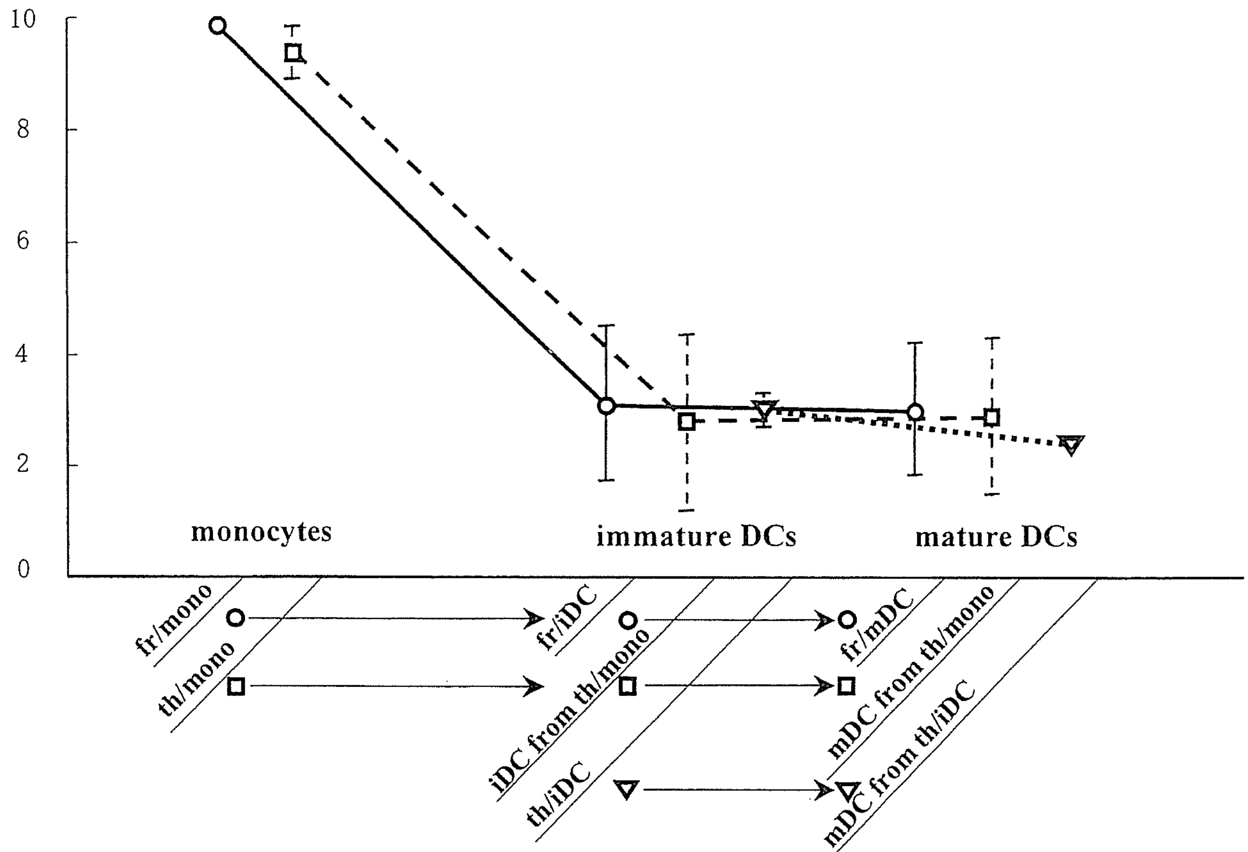

FIGURE 2. Comparison of the number of viable cells at each stage of maturation. Freshly isolated $CD14^+$ cells and cryopreserved $CD14^+$ cells were cultured with GM-CSF and IL-4 for 6 days and then cultured with GM-CSF, IL-4, and TNF-α for an additional 3 days. The initial number of each type of cell was 1×10^7. While cell viability was maintained at >90% through the culture period, the number of viable cells of fr/mDCs, mDCs from th/mono, and mDCs from th/iDCs decreased to 3.0×10^6, 2.9×10^6, and 2.4×10^6, respectively. This decrease was most prominent in the progression from monocytes to immature DCs, and there was no difference in the number of viable cells between fresh and cryopreserved cells. Data represent the mean ± SD of 6 independent experiments in triplicate.

measured using ELISA according to the manufacturer's instructions (IMMUNOTECH, Marseille, France).

Cytotoxicity Assay

Cytotoxicity assays were set up using the LDH-Cytotoxic Test Kit (Wako, Osaka, Japan) in 96-well round-bottomed plates in 200 μL of phosphate-buffered saline supplemented with 0.1% bovine serum albumin according to the manufacturer's protocol. Effector cells were cocultured with target cells (BEC-2 and Bamb-2) at E:T ratios of 2:1, 5:1, and 10:1, respectively, in 96-well round-bottomed microplates. The plates were incubated overnight, 100 μL of supernatant was transferred to 96-well flat-bottomed microtiter plates, 100 μL of chromogenic substrate for LDH was added, and the cells were incubated for 45 minutes in the dark for color development. Subsequently, 100 μL of stop solution was added and the absorbance at 560 nm was determined using an ELISA plate reader. The percentage of target-cell lysis was calculated as follows:

[(experimental LDH release) − (target spontaneous LDH release)] × 100/[(target total LDH release) − (target spontaneous LDH release)]

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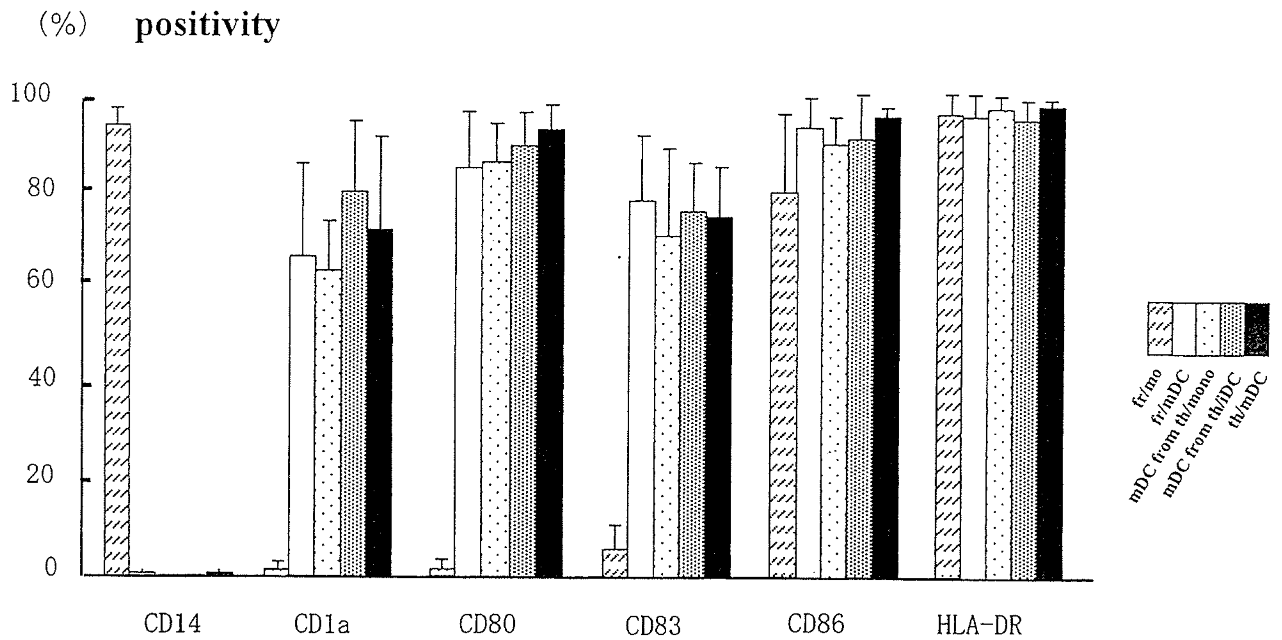

FIGURE 3. Surface markers on cells cultured for 9 days. Characteristics of mature DCs, ie, down-regulation of CD14 and up-regulation of CD1a, CD83, and HLA-DR, were observed on cells cultured for 9 days. Each marker for mature DCs was expressed similarly on cells generated from cryopreserved CD14+ cells, iDCs, and mDCs. Data represent the mean ± SD of 5 independent experiments in triplicate.

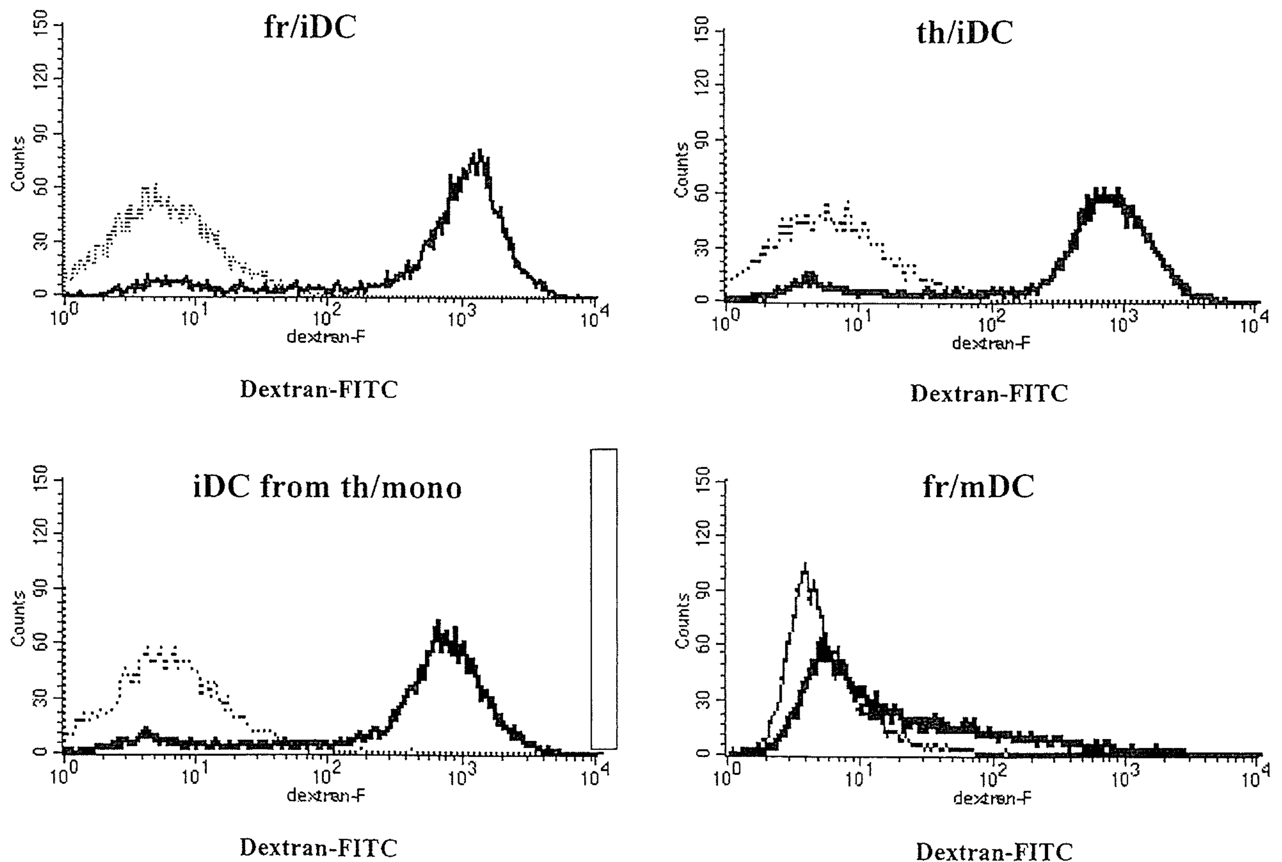

FIGURE 4. Endocytic capacity for dextran in each type of immature DCs. Dextran was added at a final concentration of 1 mg/mL to each type of immature DCs, and the cells were then incubated for 45 minutes at either 37°C (thick lines) or 4°C (thin lines) as controls. The values shown in the flow cytometry profiles are the mean fluorescence intensity indexes. These results show that cryopreserved immature DCs retained their endocytic capacity for antigens.

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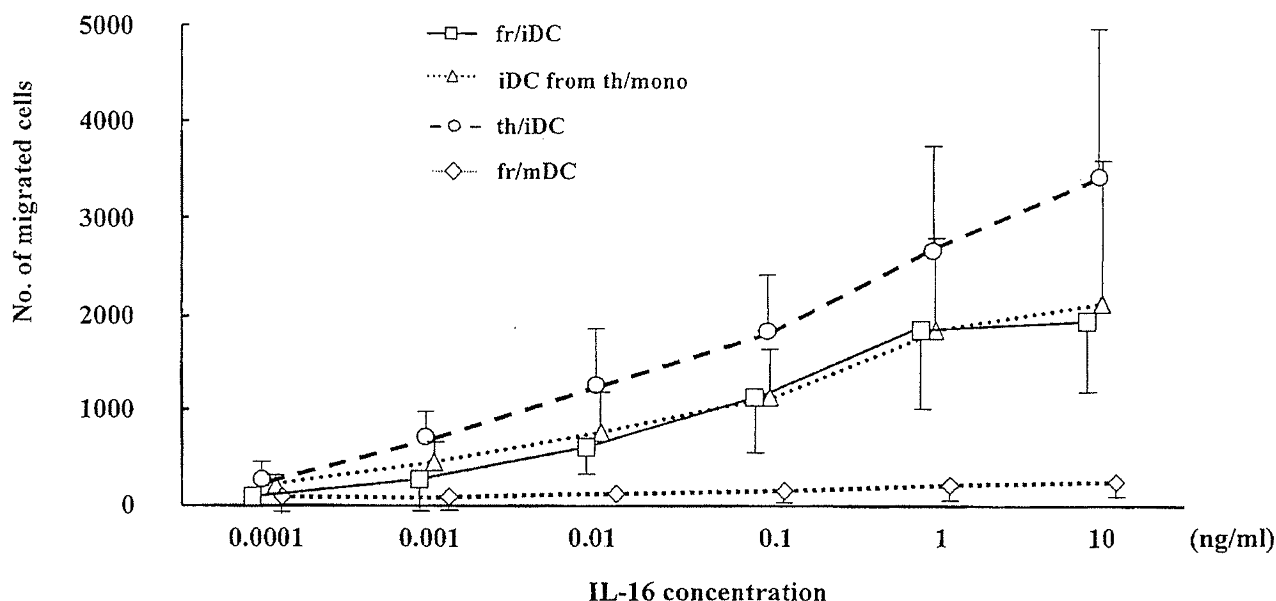

FIGURE 5. Chemotactic migratory capacity of each type of immature DCs. Each type of immature DCs showed similar dose-dependent migration activity upon stimulation with IL-16, while fresh mature DCs did not. Data are mean values of triplicate runs and represent the mean ± SD of 5 independent experiments in triplicate.

Statistical Analysis

Values are presented as the mean ± SD. The Mann-Whitney *U* test was used to compare values between subgroups with StatView version 4.0, and P values < 0.05 were considered statistically significant.

RESULTS

Surface Marker Analysis of Freshly Harvested or Cryopreserved Cells

When freshly isolated CD14$^+$ cells were cultured with GM-CSF and IL-4 for 6 days, the number of viable cells decreased from 1×10^7 to $3.1 \pm 1.2 \times 10^6$. No further change in the cell number was seen during the subsequent 3-day culture period with GM-CSF, IL-4, and TNF-α (Fig. 2). There were no statistically significant differences in the number of viable cells between fresh and cryopreserved cells at each maturation stage.

Cell Surface Markers of Cells Derived from Fresh and Cryopreserved CD14$^+$ Cells, iDCs, and mDC

As shown in Figure 3, mature DCs derived from fresh CD14$^+$ cells exhibited a phenotype typical of DCs, ie, down-regulation of CD14 to <1% and up-regulation of HLA-DR >95%. The expressions of CD83, a maturation marker of DCs, on cells cultured from fresh CD14$^+$ cells at 6 and 9 days of culture were, respectively, 7.9 ± 9.0% and 79.3 ± 13.1%. These results suggest that most cells at 6 days were immature DCs, while those at 9 days were mature DCs. The expression levels of costimulatory molecules on mature DCs, CD80 and CD86, were 88.8 ± 11.8% and 94.2 ± 8.7%, respectively. Regarding the expression of other surface markers, including CD14, CD1a, CD80, CD83, CD86, and HLA-DR, there were no essential differences between DCs that were generated from fresh CD14$^+$ cells and cryopreserved CD14$^+$ cells, for either iDCs or mDCs. The recovery rate of monocyte, immature DCs, and mature DCs in the freezing/thawing procedure were 95.1%, 91.2%, and 68.6%, respectively. These results indicate that cryopreserved CD14$^+$ cells and immature DCs are still capable of differentiating into mature DCs in the same manner as fresh cells.

Endocytic Capacity of Fresh or Cryopreserved DC

Three samples representing immature DCs, ie, fr/iDCs, th/iDCs, and iDCs from th/mono, showed well-preserved endocytic capacities for FITC-dextran, compared with freshly isolated mature DCs as a negative control (Fig. 4).

Chemotactic Migratory Capacity of Freshly Isolated Immature DCs and Cryopreserved Immature DC

IL-16 is a soluble ligand for CD4 and is known to induce chemotaxis of immature DCs but not mature DCs.[13] In our

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experiments, 3 different types of immature DCs, ie, fr/iDCs, th/iDCs, and iDCs from th/mono, showed dose-dependent migration in response to IL-16. However, fresh mature DCs did not migrate even at higher concentrations of IL-16 (Fig. 5).

Allogeneic T Lymphocyte Stimulatory Capacity of Different Mature DCs Samples

We investigated whether freezing-thawing affected the proliferative response of allogeneic CD3$^+$ cells, as measured by the incorporation of [^{3}H]TdR, and found that freezing-thawing did not affect allo-MLR responses (Fig. 6).

Specific CTL Induction Capacity of Mature DC

The production of IFN-γ in coculture with mature DCs samples and BEC-2 (HLA-A24$^+$, as a target cell) was dependent on the E:T ratio, which ranged from 2 to 10 (Fig. 7A), while no productive activity was observed with Bamb-2 (HLA-A24$^-$) as a negative control target. Furthermore, the results of measurement of LDH to analyze specific lysis of target cells were parallel to those of the IFN-γ release assay (Fig. 7B). Taken together, these results indicate that several aspects of DCs function were not disturbed by cryopreservation/thawing at any stage of maturation.

DISCUSSION

In this study, different populations of DCs lineage, ie, CD14$^+$ cells isolated from PBMC, immature, and mature DCs, were frozen and thawed at different points of maturation. Thereafter, cells were cultured with GM-CSF, IL-4, and TNF-α. As a result, cryopreservation for 4 weeks at any stage of maturation did not affect subsequent viability, cell phenotype, including CD80, CD83, and CD86, or function, including allogeneic MLR activity. The same trends regarding the effect of cryopreservation on cell surface marker[17–19] and MLR activity[20] in DCs have been recently reported. Furthermore, Sato et al.[21] reported that there was no difference in the phenotype or allogeneic MLR reaction before and after freeze/thaw of DCs generated from cord blood CD34$^+$ cells.

Although one of the most important characteristics of DCs is the HLA-restricted capability to generate cytotoxic T lymphocyte (CTL), no previous studies with the exception of the Feuerstein et al. report[22] have investigated CTL-induction capability after cryopreservation of DCs. In this study, we tested this aspect of DC function using EBV peptide bound to HLA-A24$^+$ and showed that the CTL-induction ability of DCs at any stage of maturation was preserved after cryopreservation/thawing. Migration activity is another im-

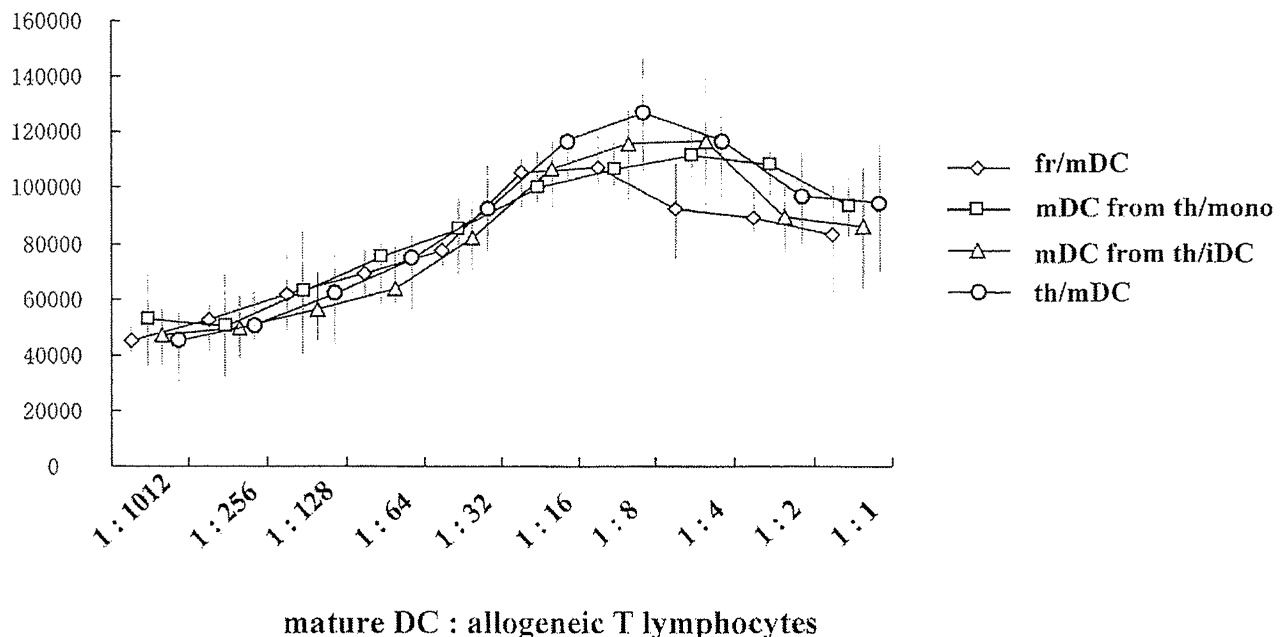

FIGURE 6. Allogeneic T lymphocyte stimulatory capacity of mature DCs. Allogeneic T lymphocytes, as a responder, were plated at 5 × 10^4 per well with increasing numbers of each type of irradiated (30 Gy) mature DCs as a stimulator. After 5 days of culture, the proliferative response was shown to depend on the DC concentration. Results are presented as the mean ± SD of 6 individual experiments in triplicate.

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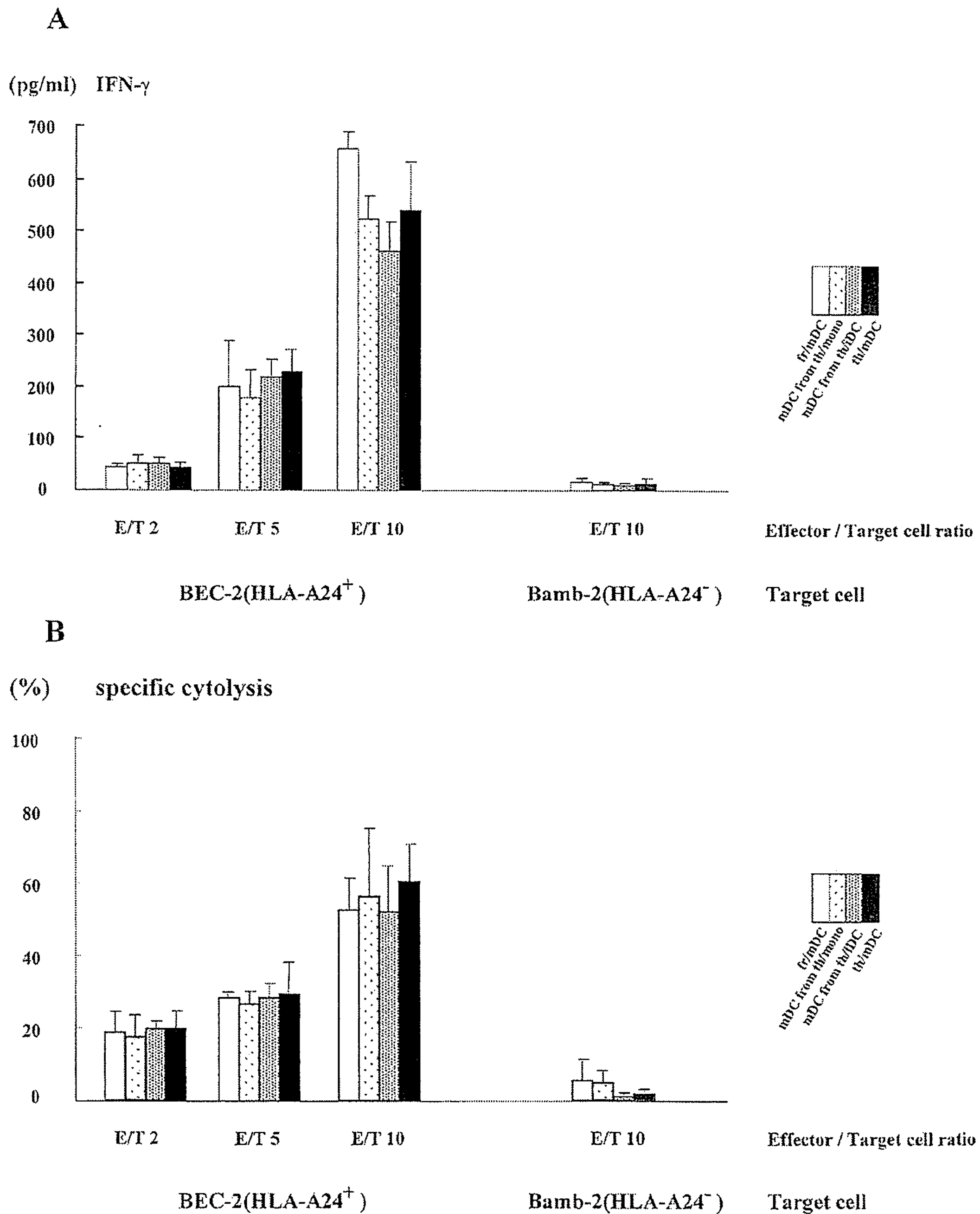

FIGURE 7. Specific CTL induction capacity of each type of mature DCs. Mature DCs prepared from HLA-A24$^+$ donors were pulsed with HLA-A24-restricted EBV peptides for 2 hours. Pulsed mature DCs were cultured with autologous CD8$^+$ cells as an effector cell with 100 IU/ mL IL-2 for 10 days. Stimulated effector cells were harvested and cocultured with BEC-2 cells (HLA-A24$^+$), which served as target cells, at effector-to-target (E:T) ratios of 2:1, 5:1, and 10:1, respectively, in triplicate. After overnight incubation, CTL specificity was measured by the IFN-γ concentration in the supernatant (A), and cytolytic activity was tested by measuring the LDH concentration (B). IFN-γ and LDH release depended on the E/T ratio, with equal CTL specificity in each type of mature DCs. Results are presented as the mean ± SD of 3 independent experiments in triplicate.

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portant landmark function of DCs.[23] An immature DC captures antigen in peripheral tissue and processes it to form MHC-peptide complexes at the cell surface. The immature DC then migrates from the terminal tissue to the lymph node and subsequently from the lymph node to the lymphoid tissues to present antigen to T cells through T cell receptors. In this study, we investigated the migration activity of immature DCs derived from freshly isolated CD14$^+$ cells and cryopreserved immature DCs and found that there was no change in the IL-16-induced migratory activity of immature DCs.

Recently, there has been growing interest in the use of DCs for immunotherapy against cancer. For clinical application, large numbers of specific-antigen-pulsed DCs are required to induce potential clinical efficacy. The generation of DCs has been accomplished through the in vitro culture of progenitor cells with combinations of various cytokines including GM-CSF, IL-4, and TNF-α.[10,24] The recent application of this technology to DC therapy has received considerable attention.[25] Most proposed clinical DC therapy protocols are based on sequential injections of antigen-pulsed DCs, in which DCs must be generated from freshly obtained PBMCs for each vaccination. The use of DCs generated from cryopreserved/thawed precursor cells may make it possible to avoid this obstacle and has obvious clinical benefits, only when our observation is confirmed in an experimental system without the use of fetal bovine serum.

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Expansion of α-Galactosylceramide-Stimulated Vα24+ NKT Cells Cultured in the Absence of Animal Materials

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Summary: $V\alpha24^+$ NKT is an innate lymphocyte with potential antitumor activity. Clinical applications of $V\alpha24^+$ natural killer (NK) T cells, which are innate lymphocytes with potential antitumor activity, require their in vitro expansion. To avoid the potential dangers posed to patients by fetal bovine serum (FBS), the authors evaluated non-FBS culture conditions for the selective and efficient expansion of human $V\alpha24^+$ NKT cells. Mononuclear cells (MNCs) and plasma from the peripheral blood of normal healthy donors were used before and after G-CSF mobilization. MNCs and plasma separated from apheresis products were also used. MNCs were cultured for 12 days in AIM-V medium containing α-galactosylceramide (α-GalCer) (100 ng/mL) and IL-2 (100 U/mL) supplemented with FBS, autologous plasma, or autologous serum. The cultured cells were collected and their surface markers, intracellular cytokines, and cytotoxicity were evaluated. The highest expansion ratio for $V\alpha24^+$ NKT cells was obtained from G-CSF-mobilized MNCs cultured in medium containing 5% autologous plasma. Cultures containing MNCs and autologous plasma obtained before and after G-CSF mobilization had approximately 350-fold and 2,000-fold expansion ratios, respectively. These results suggest that G-CSF mobilization conferred a proliferative advantage to $V\alpha24^+$ NKT cells by modifying the biology of cells and plasma factors. Expanded $V\alpha24^+$ NKT cells retained their surface antigen expression and production of IFN-γ and exhibited CD1d-independent cytotoxicity against tumor cells. $V\alpha24^+$ NKT cells can be efficiently expanded from G-CSF-mobilized peripheral blood MNCs in non-FBS culture conditions with α-GalCer and IL-2.

Key Words: NKT cells, G-CSF, α-galactosylceramide

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Murine $V\alpha14^+$ natural killer (NK) T cells express an extremely restricted T-cell receptor (TCR) consisting of a Vα14-Jα281 α chain paired mainly with a Vβ8.2 β chain. Human $V\alpha24^+$ NKT cells are similar to murine $V\alpha14^+$ NKT cells, as $V\alpha24^+$ NKT cells have an invariant Vα24-JαQ α chain preferentially paired with a Vβ11 chain.[1–3] α-Galactosylceramide (α-GalCer) is a specific ligand for human $V\alpha24^+$ NKT cells and murine $V\alpha14^+$ NKT cells.[4] Both types of NKT cells are activated by α-GalCer presented by CD1d. After stimulation with α-GalCer, $V\alpha24^+$ NKT cells exhibit CD1d-dependent cytotoxicity against various types of tumor cells.[5–7] Because CD1d is probably a class I molecule expressed mainly on antigen-presenting cells (APCs) such as dendritic cells, macrophages, and B cells, it is speculated that NKT cells primarily interact with APCs.[6,8,9] NKT cells regulate innate tumor immunity by rapidly producing a large amount of IFN-γ and IL-4.[4,10]

The extremely low frequency of $V\alpha24^+$ NKT cells in human peripheral blood[7,11,12] is an obstacle for their clinical application. To overcome this problem, the establishment of an effective in vitro expansion system for $V\alpha24^+$ NKT cells by stimulation with α-GalCer has been explored. Significant expansion was reported in human $V\alpha24^+$ $V\beta11^+$ NKT cells cultured with a combination of IL-15, IL-7, IL-2, and α-GalCer.[13] Up to a 76-fold expansion of human $V\alpha24^+$ $V\beta11^+$ NKT cells was reported after culture with IL-7, IL-15, and α-GalCer-loaded monocyte-derived dendritic cells.[14] Alternative expansion methods use a combination of IL-2 and IL-15,[15] or α-GalCer and IL-2, with or without APCs.[16] Previously, we observed that $V\alpha24^+$ NKT cells could be expanded 350-fold from human granulocyte-colony stimulating factor (G-CSF)–mobilized peripheral blood cells, upon stimulation with α-GalCer and IL-2 for 12 days.[17] However, in these culture systems, 10% fetal bovine serum (FBS) was used in the medium. To remove the potential risks related to FBS, we developed an efficient non-FBS expansion system for Vα24+ NKT cells.

MATERIALS AND METHODS

Cells, Plasma, and Serum Preparation

Peripheral blood and apheresis products were obtained from normal healthy individuals who were donating peripheral blood stem cells for allogeneic transplants. Written informed consent was obtained from the donors. This study was approved by our institution. Before and after G-CSF mobilization, samples were used immediately and cell fraction and

plasma were separated by centrifugation. The plasma and serum were obtained and cryopreserved at −80°C until use. Plasma and serum samples were heat-inactivated immediately before use. Mononuclear cells (MNCs) were isolated from peripheral blood and apheresis products by Ficoll-Hypaque (Immuno-Biologic Laboratories, Gunma, Japan) density gradient centrifugation. Apheresis plasma was also collected from the apheresis bags and used after heat inactivation.

G-CSF Procedure for Apheresis Donor

The apheresis was indicated for a healthy donor whose relative needed peripheral blood stem cell transplantation. This indication was checked by the clinical team of stem cell transplantation unit in our hospital. G-CSF was administered subcutaneously at a dosage of 300 μg/m^2 divided twice daily for 3 days just before the apheresis procedure. On the day of apheresis, one more dose of G-CSF was administered in the morning before apheresis.

Expansion of Vα24+ NKT Cells

MNCs were cultured in six-well culture plates or culture flasks (Costar, Corning, NY) at 1.0×10^5 cells/mL in medium supplemented with 100 ng/mL α-GalCer (Kirin Brewery Co, Tokyo, Japan) and 100 U/mL recombinant human (rh) IL-2 (R&D Systems, Minneapolis, MN) for 12 to 14 days. The environment for the incubation was under 20% O_2 and 5% CO_2. Cells were cultured in AIM-V (Life Technologies, Rockville, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 10% recombinant human serum albumin (rHSA), 5% or 10% autologous plasma, or 5% or 10% autologous serum. The rHSA was kindly provided by Mitsubishi Welpharma Corporation (Osaka, Japan). Fresh IL-2 was added every 3 days.

Co-Culture and Expansion of Vα24+ NKT Cells

To determine whether G-CSF mobilization conferred any benefits to plasma or cells for the expansion of Vα24+ NKT cells, we tested the following culture conditions: (1) pre-G-CSF peripheral blood mononuclear cells (PBMCs) cultured in AIM-V with 5% pre-G-CSF plasma; (2) pre-G-CSF PBMCs cultured in AIM-V with 5% post-G-CSF plasma; (3) post-G-CSF PBMCs cultured in AIM-V with 5% pre-G-CSF plasma; and (4) post-G-CSF PBMCs cultured in AIM-V with 5% post-G-CSF plasma. After culturing cells with α-GalCer and IL-2 for 12 days, we quantified the expansion of Vα24+ NKT cells.

Cell Surface Antigen Analysis

We used mouse anti-human mAbs conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), or peridinium chlorophyll (PerCP). CD3-PE, CD4-PerCP, CD8-PE, CD14-FITC, CD19-PE, CD25 (IL-2 receptor α chain)-FITC, and CD123 (IL-3 receptor)-PE mAbs were purchased from BD Biosciences (Mountain View, CA). Vα24-FITC, Vα24-PE, Vβ11-PE, CD124 (IL-4 receptor α chain)-PE, and CD127 (IL-7 receptor)-PE mAbs were purchased from Immunotech (Marseille, France). CD161-APC, CD114 (G-CSF receptor)-PE, and CD119 (IFN-γ recep-tor α chain)-PE mAbs were purchased from BD Pharmingen (San Diego, CA). PE-conjugated α-GalCer-CD1d tetramer was produced in our laboratory[18] and used to stain α-GalCer-loaded CD1d-reactive Vα24+ NKT cells. Biotinylated anti-CD1d-mAb, which was originally produced by Dr. Steven A. Porcelli (Albert Einstein College of Medicine, Bronx, NY), was a kind gift from Kirin Brewery Co. The biotinylated mAb was detected using streptavidin-PerCP (BD Biosciences). For cell surface antigen staining, cells were incubated with mAbs for 30 minutes on ice. After staining, cells were washed twice and resuspended in PBS. Propidium iodide (Sigma-Aldrich, St. Louis, MO) staining preceded all experiments to remove dead cells. Data were acquired by flow cytometry (FACSCalibur; BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

Vα24+ NKT Cell Separation

After expansion of Vα24+ NKT cells with α-GalCer and IL-2 in AIM-V with 5% autologous apheresis plasma for 12 days, cells were stained with Vα24-FITC for 20 minutes on ice and washed twice with 5 mM EDTA-PBS. After being incubated with anti-FITC microbeads (Miltenyi Biotec, Gladbach, Germany), Vα24+ NKT cells were sorted by a magnetic cell separation system (Super MACS; Miltenyi Biotec), according to the manufacturer's protocol. After separation, the purity of isolated Vα24+ NKT cells was determined to be more than 95% by flow cytometry. After the cells were re-cultured with 100 U/mL IL-2 for an additional 2 days, they were used for assays of cytotoxic activity against several tumor cell lines.

Intracellular Cytokine Assay

The intracellular cytokine production of cultured cells was measured by flow cytometry. Cells were activated with 10 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich) for 4 hours at 37°C in 10 μg/mL Brefeldin A (Sigma-Aldrich) to prevent cytokine secretion. After activation, cells were stained with Vα24 antigens and permeabilized according to the manufacturer's protocol (BD Biosciences) for staining with IFN-γ-PE or IL-4-PE mAb (BD Biosciences). At least 30,000 gating events per sample were acquired by FACSCalibur, and the data were analyzed using CellQuest software.

Cytotoxicity Assay

The cytotoxicity of isolated Vα24+ NKT cells against tumor cell lines was studied. The following cell lines were purchased from ATCC: Daudi (B-cell lymphoma), K562 (chronic myelogenous leukemia), and Jurkat (T-cell lymphoma).

Target cells were labeled with 50 μCi sodium [^{51}Cr] chromate (NEN Life Science Products, Inc, Boston, MA) per 5×10^5 cells for 1 hour, washed three times, and resuspended at 1×10^5 cells/mL in medium. Next, 100 mL of effector cells and 100 mL of ^{51}Cr-labeled target cells (1×10^4 cells/well) were added to 96-well round-bottomed plates (Nunc, Roskilde, Denmark) at effector-to-target (E/T) ratios of 10:1, 3:1, and 1:1. Plates were incubated for 4 hours at 37°C, and ^{51}Cr release from lysed target cells was measured by a gamma counter. The percentage of specific ^{51}Cr released in each well was analyzed using the following formula: specific lysis (%) = (test cpm − spontaneous cpm)/(total cpm − spontaneous cpm) × 100. "Test cpm" indicates the counts in experimental cultures of target cells and effector cells; "spontaneous cpm" indicates the counts in cultures containing only target cells and medium;

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and "total cpm" indicates the counts obtained by adding 100 mL of 1 N HCl to target cells to lyse all cells. Data are expressed as the mean and standard deviation of triplicate cultures.

ELISA

Levels of IL-2, IL-3, IL-4, IL-7, IL-13, IL-15, IFN-γ, and G-CSF in pre-G-CSF peripheral blood plasma and apheresis plasma were measured by commercial ELISA kits according to the manufacturers' protocols. IL-12 levels were measured by OptEIA (BD Pharmingen), and the other cytokine levels were measured by Immunoassay ELISA kits (BioSource, Camarillo, CA).

Statistical Analysis

The Student *t* test was used to compare groups using the two-tailed method dealing with dependent samples. $P < 0.05$ was considered statistically significant. In multiple group analysis, we adapted Bonferroni adjustment to confirm the significance of *P* values.

RESULTS

Efficient Expansion of Vα24$^+$ NKT Cells in Autologous Plasma

In this study, Vα24$^+$ CD3$^+$ cells were defined as Vα24$^+$ NKT cells. In our preliminary experiments using anti-Vβ11 mAb, we found that expanded Vα24$^+$ NKT cells fully express Vβ11. To search for a suitable non-FBS medium for Vα24$^+$ NKT cell expansion, PBMCs were cultured in medium containing α-GalCer, IL-2, and 10% FBS, 10% rHSA, 5% autologous plasma or serum, or 10% autologous plasma or serum for 12 to 14 days. The percentage of cultured Vα24$^+$ NKT cells increased by 27-fold in 10% FBS, 2-fold in 10% rHSA, 342-fold in 10% autologous plasma, 382-fold in 5% autologous plasma, 315-fold in 10% autologous serum, and 355-fold in 5% autologous serum (n = 5). Representative flow cytometry data are shown in Figure 1. When cells were cultured in medium containing 10% FBS, the percentage of expanded Vα24$^+$ NKT cells was substantially lower than when autologous plasma or autologous serum was used to supplement medium. In medium containing rHSA, the Vα24$^+$ NKT cells were unable to proliferate, whereas CD3$^+$ T cells proliferated. There was no significant difference between Vα24$^+$ cell expansion in 5% or 10% autologous plasma or autologous serum. Additionally, 87% to 95% of Vα24$^+$ NKT cells reacted to the α-GalCer-CD1d tetramer after expansion in 5% autologous plasma. These results suggest that medium containing 5% autologous plasma is suitable for selective expansion of Vα24$^+$ NKT cells with α-GalCer and IL-2 in vitro.

G-CSF Mobilization Augmented Vα24$^+$ NKT Cell Expansion

To develop more efficient plasma-based culture conditions for Vα24$^+$ NKT cells, we collected PBMCs and plasma before and after G-CSF mobilization (n = 18) and compared their expansion efficiencies (Table 1). Vα24$^+$ NKT cells significantly expanded to 1,938 (±2,501)-fold in the post-G-CSF condition compared with 346 (±345)-fold in the pre-G-CSF condition ($P = 0.018$). Thus, the Vα24$^+$ NKT cell expansion was 5.6-times greater in the post-G-CSF condition than in the pre-G-CSF condition. As the total cell number including all cell populations was not significantly different between the two cultures, the addition of α-GalCer in the post-G-CSF condition appeared to selectively expand Vα24$^+$ NKT cells.

Characteristics of G-CSF-Mobilized PBMCs and Plasma

To elucidate the contributions of G-CSF-mobilized PBMCs and plasma to Vα24$^+$ NKT cell expansion, different combinations of PBMCs and plasma from pre- and post-GCSF peripheral blood were tested (n = 8) (Fig. 2). Post-G-CSF plasma enhanced Vα24$^+$ NKT cell expansion more than pre-G-CSF plasma. Likewise, more Vα24$^+$ NKT cell proliferation occurred in post-G-CSF PBMCs than in pre-G-CSF PBMCs. The most effective combination was post-G-CSF PBMCs and post-G-CSF plasma. Exogenous G-CSF did not enhance the effective expansion of NKT cells (data not shown). These results suggest that G-CSF mobilization indirectly contributed to both PBMCs and plasma for the expansion of Vα24$^+$ NKT cells.

G-CSF Did Not Increase the Percentage of Vα24$^+$ NKT Cells in Peripheral Blood

We compared the percentages of Vα24$^+$ NKT cells in peripheral blood before and after G-CSF mobilization (n = 10). The percentage of Vα24$^+$ NKT cells in peripheral blood was 0.128% (±0.034) and was reduced to 0.082% (±0.040) by G-CSF mobilization ($P < 0.001$), although the absolute number of Vα24$^+$ NKT cells was similar in pre- and post-G-CSF peripheral blood—4.32 (±2.97) counts/μL and 6.03 (±3.41) counts/μL ($P > 0.05$), respectively. This means that mobilized PBMCs contain a high proportion of monocyte, which resulted in decreasing the percentage of Vα24$^+$ NKT cells relatively. As the total number of Vα24$^+$ NKT cells in peripheral blood did not change, therefore the Vα24$^+$ NKT cells were not mobilized by G-CSF administration.

G-CSF-Induced Changes in Peripheral Blood Cytokine Concentrations

We measured cytokine concentrations in the plasma of pre-G-CSF and apheresis products (n = 6) (Fig. 3). The level of G-CSF increased dramatically after G-CSF administration. There were significant differences between the levels of three cytokines (IL-3, IL-7 and IL-13) between apheresis products and pre-G-CSF plasma. The levels of other cytokines, such as IL-2, IL-12, IL-15, and IFN-γ, which enhance Vα24$^+$ NKT cell function, were not changed by G-CSF mobilization. The concentrations of IL-4 were below the detection limit.

Expression of Cytokine Receptors on T Cells and NKT Cells After G-CSF Mobilization

We evaluated the expression of cytokine receptors for IL-2, IL-3, IL-4, IL-7, G-CSF, and IFN-γ on CD3$^+$ T cells and Vα24$^+$ NKT cells in pre- and post-G-CSF PBMCs (n = 5) (Fig. 4). The expression levels of IL-3, IL-7, and IL-4 receptor (which has IL-13 common receptor[19]) on CD3$^+$ T cells and Vα24$^+$ NKT cells were not affected by G-CSF mobilization, although the corresponding cytokine levels (IL-3, IL-7, and

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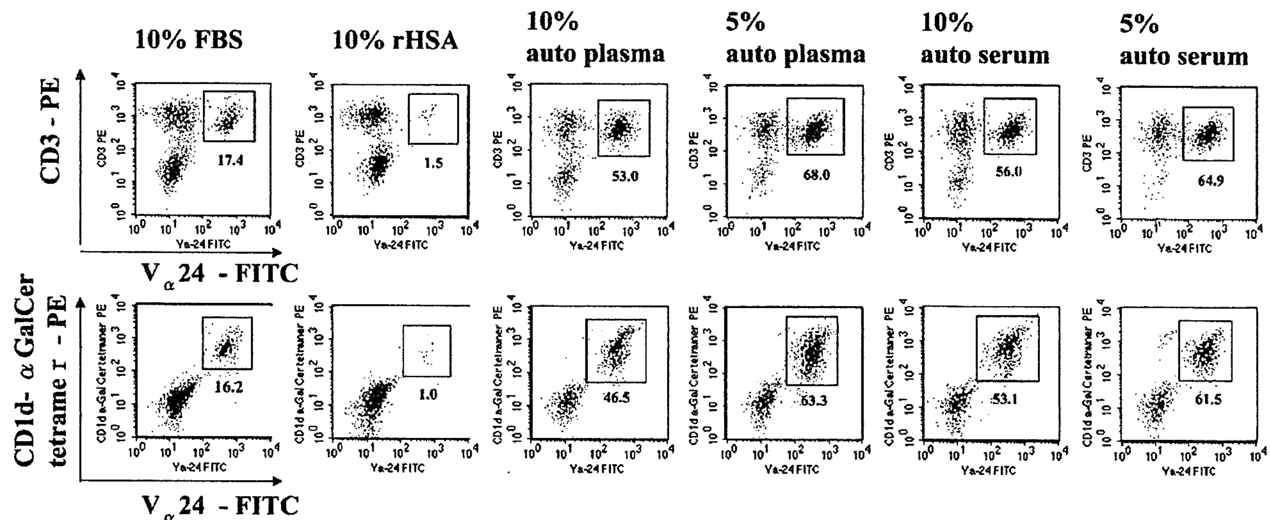

FIGURE 1. Differences in Vα24+ NKT cell expansion according to the type of supplemented protein. PBMCs from normal healthy donors were cultured for 12 to 14 days with α-GalCer and IL-2 in the presence of 10% FBS, 10% rHSA, 5% or 10% autologous plasma, or 5% or 10% autologous serum. Vα24+ CD3+ cells were defined as Vα24+ NKT cells. Vα24+ CD1d-α-GalCer tetramer-positive NKT cells were also stained, and the percentage of the gated population is shown. These flow cytometry results are representative of five independent experiments.

IL-13) were increased by G-CSF mobilization. The IL-7 receptor was expressed on most Vα24+ NKT cells, although some CD3+ T cells showed downregulation of the IL-7 receptor after G-CSF mobilization. There was no obvious tendency for G-CSF mobilization to enhance the expression level of the G-CSF receptor or the α chain of the IL-2 receptor on both CD3+ T cells and Vα24+ NKT cells. Interestingly, only the α chain of the IFN-γ receptor increased after G-CSF mobilization with a significant difference ($P = 0.009$), and this increase occurred on Vα24+ NKT cells but not on CD3+ T cells.

Cell Populations

Table 2 shows mean values and standard deviations for the cell kinetics of apheresis MNCs cultured with autologous apheresis plasma (n = 11). The apheresis procedure did not affect the percentage of Vα24+ NKT cells. On day 0, Vα24+ NKT cells represented only 0.10% (± 0.06) of apheresis MNCs, and the CD4+ to CD8+ T-cell ratio was more than 1.0. Monocytes accounted for approximately 30% of MNCs at day 0, which was substantially higher than the percentage of monocytes (2.7–7.9%) in pre-G-CSF PBMCs. When stimulated with α-GalCer, Vα24+ NKT cells propagated linearly until day 14. CD8+ T cells expanded to become the predominant T-cell population, changing the CD4+ to CD8+ T-cell ratio to less than 1.0. B cells and monocytes almost completely disappeared by day 14 (2.33% and 0.16%, respectively).

TABLE 1. Comparison of Expansion Efficiencies

	Vα24+ NKT Cells			Whole Cells
	Day 0 (%)	Day 12–14 (%)	Expansion Fold	Day 12–14 (expansion fold)
Pre-G-CSF	0.19	10.45 ± 8.53	×345.96 ± 345	×5.33
Post-G-CSF	0.11	21.97 ± 11.70*	×1,938.11 ± 2,501*	×4.62

*$P < 0.05$.

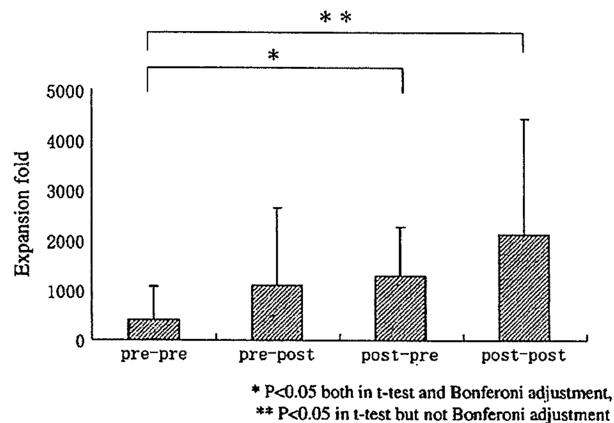

FIGURE 2. Differences in Vα24+ NKT cell expansion influenced by a combination of PBMCs and plasma. The expansion of Vα24+ NKT cells was analyzed in several co-culture combinations of PBMCs and 5% plasma before and after G-CSF mobilization. Cells were cultured for 14 days in the presence of α-GalCer and IL-2. Values are the mean and standard deviation of the Vα24+ NKT cell expansion fold. Samples were obtained from the same donor (n = 8), and the following co-culture conditions were examined: (1) pre-G-CSF PBMCs and pre-G-CSF plasma (pre-pre); (2) pre-G-CSF PBMCs and post-G-CSF plasma (pre-post); (3) post-G-CSF PBMCs and pre-G-CSF plasma (post-pre); and (4) post-G-CSF PBMCs and post-G-CSF plasma (post-post). *$P < 0.05$. P values were determined using the Student t test and Bonferroni adjustment.

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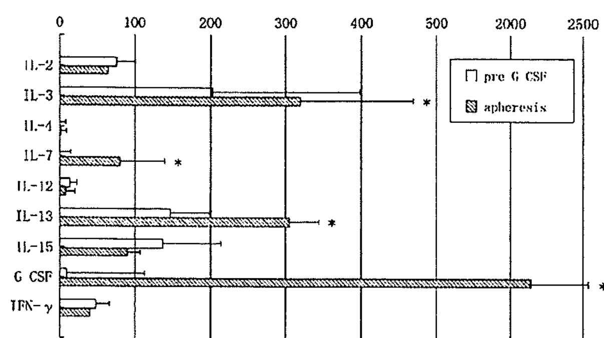

FIGURE 3. Cytokine levels in plasma. Cytokine levels in peripheral blood were measured by ELISA before G-CSF mobilization and in apheresis products from the same normal healthy donors (n = 6). IL-2 levels are plotted in U/mL; all other cytokine levels are plotted in pg/mL. Results are shown as mean values with standard deviations. *$P < 0.05$ vs. pre-G-CSF peripheral blood and apheresis product.

NK cells were also remarkably reduced after day 7, although they grew rapidly in the first 7 days of culture.

Cytokine Production

We measured IFN-γ and IL-4 production in apheresis MNCs (n = 10) that were cultured with or without α-GalCer for 14 days. Representative flow cytometry data are shown in Figure 5. The percentage of IFN-γ-producing MNCs was 58.7 ± 13.9% when cultured with α-GalCer and 44.8 ± 15.6% when cultured without α-GalCer. The percentage of IL-4-producing MNCs was 8.6 ± 8.5% when cultured with α-GalCer and 5.0 ± 2.9% when cultured without α-GalCer. When cultured with α-GalCer, 75.7 ± 12.2% of $V\alpha24^+$ NKT cells produced IFN-γ and 16.2 ± 10.5% produced IL-4. In the comparison of IFN-γ and IL-4 produced by $V\alpha24^+$ NKT cells, IFN-γ was significantly dominant ($P = 0.023$).

Cytotoxicity Assays

Three tumor cell lines were used as target cells in the cytotoxic assay. CD1d expression on the target tumor cells was evaluated using CD1d mAb. CD1d was expressed on 87% of Jurkat cells and 13% of Daudi cells. K-562 did not express CD1d. $V\alpha24^+$ NKT cells purified from MNCs stimulated with α-GalCer mediated strong cytotoxic effects against all of these hematologic cell lines (Fig. 6). The cytotoxicities were unrelated to CD1d expression on the target cells.

DISCUSSION

NKT cells help regulate a variety of immune responses, including the immune responses associated with autoimmune diseases,[20] including inflammatory bowel disease,[21] graft-versus-host disease,[22] and tumor rejection.[23] Two main strategies have been devised to use the specific ligand for NKT cells, α-GalCer, in therapeutic settings: the in vivo use of α-GalCer to enhance an immune response and the ex vivo use of α-GalCer to expand NKT cells for adoptive transfer. When the former approach was tested in patients with various solid tumors,[24] there were short-term elevations in IL-12 and GM-CSF levels and NK cell activity, and a slight elevation in serum IFN-γ and IL-4 levels occurred in some patients. Interestingly, the NKT cells disappeared from peripheral blood within 24 hours of α-GalCer injection. Although no adverse events were associated with this approach, no therapeutic benefits were apparent either. In murine models, high doses of α-GalCer showed significant liver toxicity.[25]

Nieda et al[16] studied the alternative approach of the infusion of α-GalCer-pulsed dendritic cells. They reported a transient decrease in the number of $V\alpha24^+$ $V\beta11^+$ NKT cells in the peripheral blood within 48 hours of the infusion. This transient decrease was followed by significant increases in $V\alpha24^+$ $V\beta11^+$ NKT cells and the serum levels of IFN-γ and IL-12, in addition to the activation of NK cells and T cells. No significant adverse events were reported in a clinical trial of this approach.[26]

The clinical use of $V\alpha24^+$ NKT cells requires the development of a highly effective expansion method for $V\alpha24^+$ NKT cells ex vivo. Previous reports of ex vivo cell expansion for clinical applications have focused on T cells,[27,28] NK cells,[29] or dendritic cells[30] rather than NKT cells. A few reports have found that the expansion of human NKT cells from steady-state peripheral blood cells or cord blood cells can be mediated by α-GalCer and several cytokines.[13–16] However, the expansion ratios of these NKT cells were limited. Our previous study showed that G-CSF-mobilized peripheral blood cells, whether from normal donors or cancer patients, had a significantly higher expansion potential for $V\alpha24^+$ NKT cells in a combination culture of α-GalCer and IL-2.[17] These results provide a realistic rationale for performing adoptive transfer of α-GalCer-expanded $V\alpha24^+$ NKT cells in combination with high-dose chemotherapy and G-CSF treatment or in combination with autologous or allogeneic hematopoietic stem cell transplantation including G-CSF mobilization. Nevertheless, these approaches are seriously limited by the use of FBS, and the development of a non-FBS culture system is critical.

In the present study, we tested a culture system that uses autologous plasma for the expansion of $V\alpha24^+$ NKT cells in the presence of α-GalCer and IL-2. We also evaluated the sustained usefulness of G-CSF-mobilized specimens. We found that autologous serum and autologous plasma had greater capacities to expand $V\alpha24^+$ NKT cells than did FBS and rHSA. Indeed, there was no significant difference between $V\alpha24^+$ NKT cell expansion in 5% or 10% autologous plasma or autologous serum. However, the percentage of $V\alpha24^+$ cells in culture medium was the highest and 87% to 95% of $V\alpha24^+$ NKT cells reacted to the α-GalCer-CD1d tetramer after expansion in 5% autologous plasma. Additionally, plasma can easily be obtained in the process of PBMC preparation from peripheral blood samples and in the process of apheresis. Thus, we selected plasma as a medium supplement. We also found that G-CSF-mobilized PBMCs and G-CSF-mobilized plasma, which were used instead of steady-state PBMCs and plasma, yielded the highest expansion ratio for $V\alpha24^+$ NKT cells. When we comparatively analyzed cells and plasma before and after G-CSF mobilization, we found that both G-CSF-mobilized PBMCs and G-CSF-mobilized plasma had the capability to support expansion of $V\alpha24^+$ NKT cells (see

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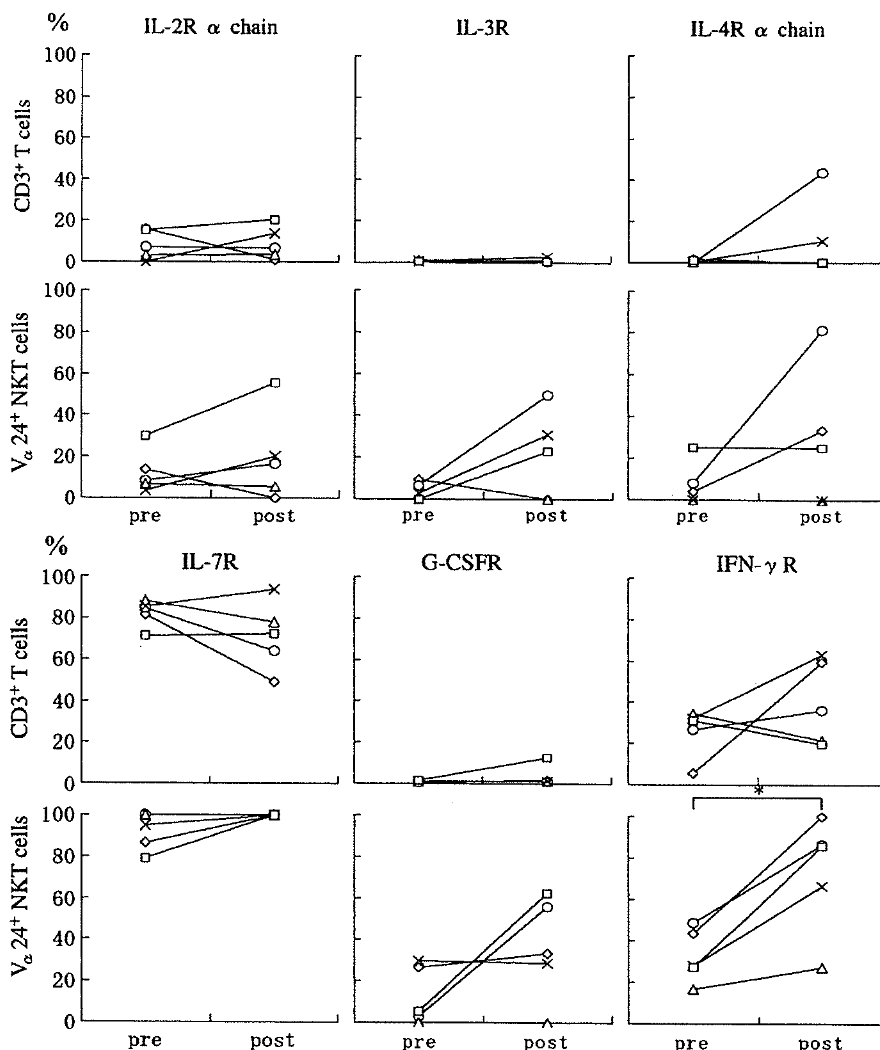

FIGURE 4. Cytokine receptor expression. Changes in the cytokine receptor expression of CD3+ T cells and Vα24+ NKT cells in peripheral blood before and after G-CSF mobilization are shown as five independent experiments. Peripheral blood before and after G-CSF-mobilization was obtained from the same healthy donors. Figure symbols indicate individual donors. *$P < 0.05$.

Fig. 2). In the clinical setting, we plan to use mobilized PBMCs and apheresis product derived from cancer patients in the autologous setting or derived from a healthy donor in the allogeneic setting. The clinical application of ex vivo expanded NKT cells has a possibility of wide modification, including combination therapy with stem cell transplantation.

TABLE 2. Cell Kinetics of Apheresis MNCs Cultural with Autologous Apheresis Plasma

Cell Population	Day 0	Day 7	Day 14
Vα24+ CD3+ (NKT)	0.10 ± 0.06	12.90 ± 15.15	21.77 ± 21.68
CD3− CD161+ (NK)	3.41 ± 2.08	26.03 ± 15.47	8.79 ± 6.85
CD161− Vα24− CD4+ (CD4 T)	18.57 ± 7.53	18.07 ± 7.02	16.91 ± 12.28
CD161− Vα24− CD8+ (CD8 T)	12.42 ± 3.42	26.71 ± 12.28	23.69 ± 12.20
CD19+ (B cell)	7.40 ± 4.30	5.62 ± 3.27	2.33 ± 2.06
CD14+ (monocyte)	29.39 ± 15.58	0.93 ± 1.12	0.16 ± 0.16

Data are given as percentages ± SD.

Contrary to our expectations, our flow cytometry data revealed that the percentage of Vα24+ NKT cells in vivo decreased after G-CSF mobilization. As the absolute number of Vα24+ NKT cells did not change by G-CSF mobilization, the decreased percentage of it was caused by the increment of other cell populations after G-CSF mobilization. That means that G-CSF does not mobilize Vα24+ NKT cells directly. Also, ex vivo supplementation of G-CSF did not enhance the expansion of Vα24+ NKT cells (data not shown), which suggests an indirect contribution of G-CSF in the expansion of NKT cells, contrary to a previous report.[31] On the other hand, the post-G-CSF PBMCs (see Table 2) and apheresis products contained a high percentage of monocytes, which include APCs capable of presenting α-GalCer. This observation indicates that the number of CD1d-expressing PBMCs also increased after G-CSF mobilization and might be one factor responsible for the significant expansion of Vα24+ NKT cells in post-G-CSF

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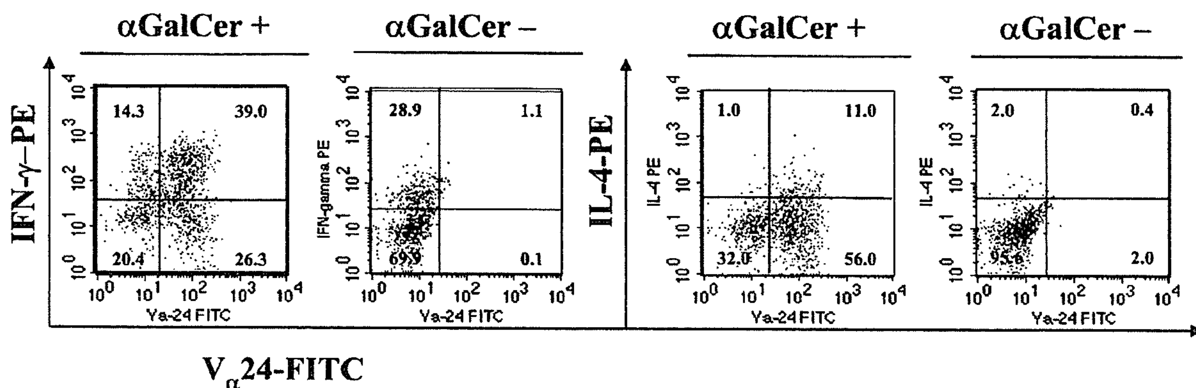


FIGURE 5. Intracellular cytokines in cultured Vα24⁺ NKT cells. Intracellular IFN-γ and IL-4 were stained in whole cells after culture with or without α-GalCer. Cells were activated with phorbol 12-myristate 13-acetate and ionomycin for 4 hours. Representative data from 1 of 10 independent experiments are presented. *$P < 0.05$, difference between the production of IFN-γ and IL-4, Student t test.

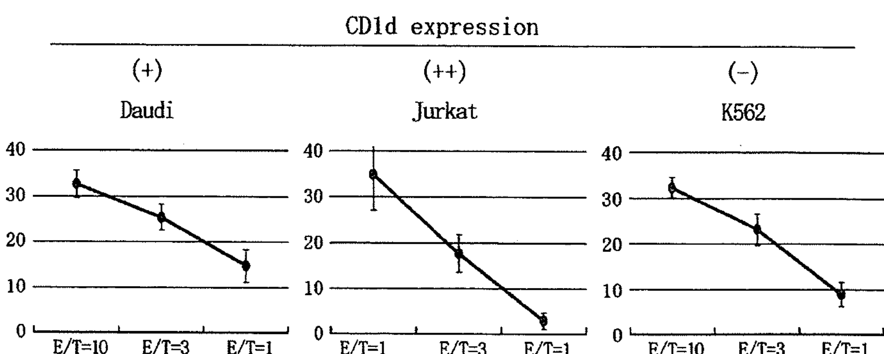
PBMCs. We previously reported that cell-to-cell contact with CD14⁺ cells was needed for the expansion of NKT cells.[17]

The plasma collected after G-CSF mobilization also had an enhanced capacity for Vα24⁺ NKT cell expansion. IL-2, IL-7, IL-12, IL-15, IL-18, and IFN-γ directly induce proliferation and activation of NKT cells.[13,14,32,33] However, none of these cytokines, with the exception of IL-7, was increased in the plasma of G-CSF-mobilized peripheral blood. When Vα24⁺ NKT cells were cultured with α-GalCer and increased levels of cytokines (IL-3, IL-7, IL-13, and G-CSF) in medium containing pre-G-CSF plasma, the expansion efficiency of Vα24⁺ NKT cells was not enhanced to the level achieved with post-G-CSF plasma (data not shown). These results suggest that IL-3, IL-7, IL-13, and G-CSF do not directly contribute to the proliferation of Vα24⁺ NKT cells. The identification of these unknown factors in post-G-CSF plasma, which promote the proliferation of Vα24⁺ NKT cells, would increase the effectiveness of Vα24⁺ NKT cell expansion.

To determine whether the characteristics of cells were changed by G-CSF mobilization, we evaluated the expression of several cytokine receptors on CD3⁺ T cells and Vα24⁺ NKT cells isolated from peripheral blood before and after G-CSF mobilization. A significant increase was observed in the expression of the IFN-γ receptor α chain on Vα24⁺ NKT cells after G-CSF mobilization ($P = 0.009$). This increased α-chain expression may be partially responsible for the proliferative advantage of Vα24⁺ NKT cells after G-CSF mobilization. However, the variability of response between individuals is essential issue, especially in the evaluation of receptor intensity. This variation suggests that the ex vivo expansion of NKT cells is controlled by mutual change, which exists in cellular and humoral factor.

We found that expanded Vα24⁺ NKT cells predominantly produced IFN-γ. The expanded Vα24⁺ NKT cells exhibited augmented cytotoxicity against CD1d⁺ tumor cell lines (Daudi and Jurkat) as well as CD1d⁻ tumor cell line (K562). In CD1d-blocking experiments, we found that expanded Vα24⁺ NKT cells mediated cytotoxic activity against CD1d-blocked Jurkat cells that was comparable to the cytotoxic activity against CD1d-unblocked Jurkat cells (data not shown). Thus, the expanded Vα24⁺ NKT cells yielded lytic activity against tumor cells in a CD1d-independent manner. Although the mechanism of CD1d-related cytotoxicity mediated by Vα24⁺ NKT cells has not been clarified, other recent studies of NKT cells suggest that CD1d expression on the target tumor cells is not essential for cytotoxicity.[34–36] The Vα24⁺ NKT cells

FIGURE 6. Cytotoxicity of purified Vα24⁺ NKT cells after culture. Vα24⁺ NKT cell-mediated cytotoxicity against tumor cells was measured with effector-to-target ratios of 10:1, 3:1, and 1:1. Cell lines were classified into the following four groups based on the expression level of CD1d: (-), 0–3%; (±), 3–10%; (+), 10–60%; (++), 60–100%. Cytotoxicity was evaluated with ⁵¹Cr release assays. The means and standard deviations of triplicate culture are shown in representative result of four independent experiments.



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obtained in our culture system appear to be T_H1-type NKT cells that have strong antitumor activity through direct and indirect mechanisms.

In the present study, we developed an effective method for Vα24$^+$ NKT cell expansion through the use of G-CSF-mobilized peripheral blood. We also featured the possible clinical applications of Vα24$^+$ NKT cells in adoptive immunotherapy, both in autologous and allogeneous settings. Further research needed to achieve this goal is underway.

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3 # Efficient *ex vivo* expansion of Vα24+ NKT cells derived from 5 G-CSF-mobilized blood cells

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Summary: Natural killer T (NKT) cells are involved in the 15 function of innate immune systems and also play an important role in regulating acquired immune responses. In previous 17 reports, we showed that Vα24+ NKT cells proliferated more efficiently from granulocyte-colony stimulating factor (G-CSF)- 19 mobilized peripheral blood mononuclear cells (PBMC) than from non-mobilized PBMC. However, the mechanism of this 21 enhanced NKT cell expansion is not yet clear. The goal of this research was to develop culture conditions for the more efficient 23 ex vivo expansion of NKT cells. G-CSF-mobilized PBMC was cultured in AIM-V medium supplemented with 10% auto- 25 plasma, 100 ng/mL α-galactosylceramide (α-GalCer) and 100 IU/mL recombinant human (rh) interleukin (IL)-2. The 27 efficiency of the expansion of Vα24+ NKT cells was evaluated on day 12. The expansion-fold of Vα24+ NKT cells was 29 augmented depending on the proportion of CD14+ cells at the beginning of culture. The depletion of Vα24+ NKT cells 31 abrogated the expansion of Vα24+ NKT cells. Depletion of CD56+ NK cells from mobilized PBMC enhanced, and add- 33 back of purified CD56+ NK cells suppressed the expansion of Vα24+ NKT cells. Experiments with different timings for the 35 addition of cells, IL-2 and α-GalCer suggested that follow-up supplementation with IL-2 or CD14+ cells should be avoided 37 for the efficient expansion of Vα24+ NKT cells. These results should be useful for the development of an efficient and practical 39 expansion protocol for adoptive immunotherapy with Vα24+ NKT cells.

41 **Key Words:** Vα24+ NKT cells, α-galactosylceramide, CD14+ cells, CD56+ NK cells

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INTRODUCTION

NKT cells are lymphocyte lineage and show 61 characteristics of both T cells and NK cells.[1] NKT cells coexpress T cell receptors (TCRs) and NK cell markers, 63 and display an extremely restricted TCR repertoire, consisting of Vα24 chain preferentially paired with $V_{\beta}11$ 65 chain. Upon activation by a specific ligand, NKT cells produce high levels of interferon-gγ (IFN-γ) and inter- 67 leukin-4 (IL-4), and yield a strong immune response against several types of tumor cells.[2] Therefore, these 69 invariant NKT cells are considered key effector cells, and play critical roles in immunity against microbial infection, 71 tumor and autoantigens.

The marine sponge-derived glycosphingolipid α- 73 galactosylceramide (α-GalCer) specifically activates human and mouse invariant NKT cells[3](4). In vivo 75 activation of NKT cells by α-GalCer induced strong cytotoxicity and the production of several cytokines in 77 mice,[5] and it is well known that NKT cells differentiate efficiently with the in vitro administration of α-GalCer to 79 acquire cytotoxic activities.[6] Therefore, this glycolipid agent may be able to effectively expand and activate NKT 81 cells, and thus may be a useful tool for clinical immunotherapy. 83

For the clinical application of NKT cells in cancer immunotherapy, efficient expansion of the cells is very 85 important. We previously reported that granulocyte colony-stimulating factor (G-CSF)-mobilized PBMC 87 showed a higher efficacy of expansion of NKT cells,[7] and a fetal bovine serum (FBS)-free culture system has 89 been developed.[8] In this study, we further attempted to improve the culture system by evaluating the effects of 91 other cell components and interleukin (IL)-2.

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MATERIALS AND METHODS

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Cells and Plasma Preparation

Peripheral blood (PB) or apheresis products were 97 obtained from normal healthy donors for allogeneic peripheral blood stem cell transplantation (PBSCT) after 99 written informed consent was obtained. Healthy donors were administered G-CSF (filgrastim) 10 μg/kg subcuta- 101 neously for 4 continuous days, and leukapheresis was performed on the 4th day. PB was collected in a heparin- 103 containing collection tube before and after G-CSF mobilization. The plasma was separated from cell 105 components by centrifugation at 3,000 rpm for 15

minutes. The cells were loaded on lymphocyte separation medium (Ficoll-Conray, Immuno-Biologic Laboratories, Gunma, Japan), and centrifuged at 2,000 rpm for 20 minutes. PBMC were collected from the intermediate layer of Ficoll-Conray density gradient centrifugation and washed twice with PBS. The plasma was subjected to heat-inactivation and stored at -20°C until use. A cell separator (COBE-Spectra, GANBRO, Stockholm, Sweden) was used for leukapheresis. Any residual mononuclear cells were collected from apheresis tubes and bags by washing with PBS after cells were collected for clinical transplantation, and separated by Ficoll-Conray density gradient centrifugation. The apheresis plasma was also collected from the collection bags.

Expansion of Vα24⁺ NKT Cells

In this manuscript, we use the term $V\alpha24^+$ NKT cells to refer to $V\alpha24^+$ $CD3^+$ double-positive NKT cells and confirmed the co-expression of $V_{\beta}11$ chain. Isolated PBMC were cultured in 6-well culture plates (Costar, Corning, NY) at 2.0×10^5 cells/mL (each well filled with 4 mL media) in AIM-V media (Life Technologies, Rockville, MD) containing 10% autologous plasma, supplemented with 100 ng/mL α-galactosylceramide (α-GalCer, supplied by Kirin Brewery Co., Tokyo, Japan) and 100 IU/mL recombinant human (rh) IL-2 (R&D Systems, Minneapolis, MN) for 12 days. IL-2 was freshly added every 3 days to maintain its biologic activity. In the first experiment to define the efficacy for $V\alpha24^+$ NKT cells expansion between before and after G-CSF mobilization, we used steady-state autologous plasma before G-CSF administration (pre-G-CSF), autologous plasma derived from PB after G-CSF administration (post-G-CSF PB) and autologous plasma obtained from apheresis product after G-CSF administration (post-G-CSF apheresis). In other experiments, we uniformly used autologous plasma obtained from apheresis product.

Monoclonal Antibodies

For flow cytometry analysis, anti-CD3-APC, anti-CD14-FITC, anti-CD16-PE, anti-CD56-FITC, anti-CD161-PE, anti-CD20-FITC and anti-CD19-PE monoclonal antibodies (mAbs) were purchased from BD Biosciences (Mountain View, CA). IgG1-FITC & IgG1-PE (cocktail), anti-Vα 24-FITC, anti-Vα24-PE, anti-$V_{\beta}11$-PE and anti-$CD4^-$FITC & anti-$CD8^-$PE (cocktail) mAbs were from Immunotech (Marseilles, France). Anti-CD3-FITC mAb was from BD Pharmingen (San Diego, CA). For cell separation, anti-CD34-FITC, anti-CD56-FITC and anti-CD14-FITC mAbs were purchased from BD Biosciences (Mountain View, CA). Anti-Vα24-FITC mAb was from Immunotech (Marseilles, France). Anti-CD3-FITC mAb was from BD Pharmingen (San Diego, CA).

Cell Surface Antigen Analysis

For cell surface antigen staining, cells were incubated with FITC-, PE- or APC- conjugated mouse anti-human mAbs for 30 minutes on ice. After staining, cells were washed twice and re-suspended in PBS. Staining with propidium iodide (PI; Sigma-Aldrich, St. Louis, MO) preceded all experiments to remove dead cells. Data were acquired by flow cytometry (FACSCalibur; BD Biosciences) and analyzed using CellQuest software (BD Biosciences). In this manuscript, we considered "$CD56^+$ cells" as NK cells and use the phrase "$CD56^+$ NK" cells.

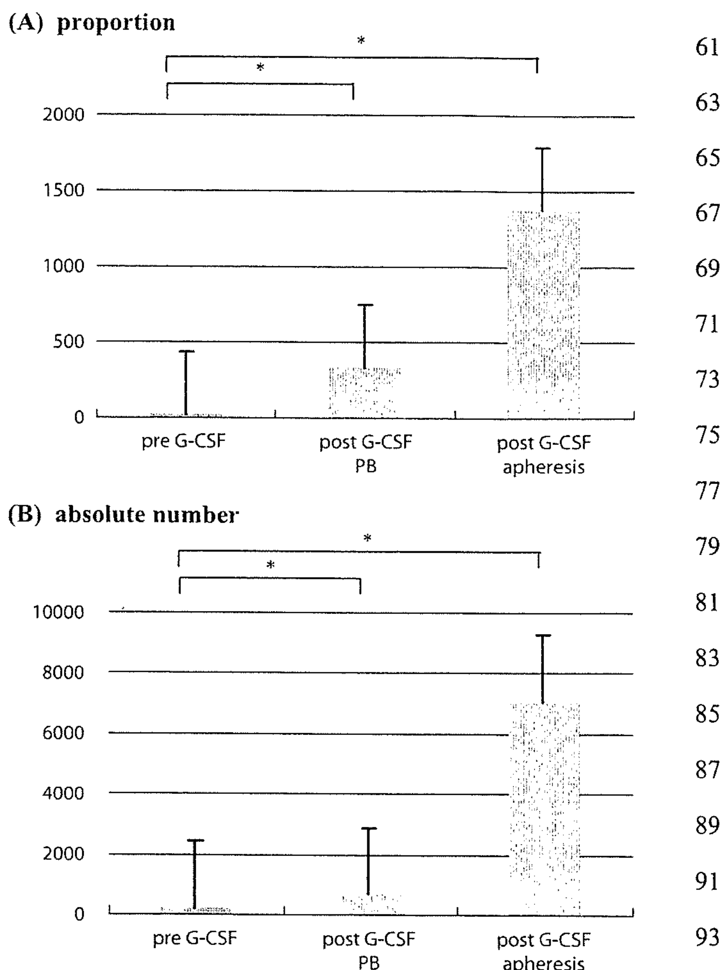

FIGURE 1. Proportion and absolute number of $V\alpha24^+$ NKT cells on day 12 The proportion (A) and absolute number (B) of $V\alpha24^+$ NKT cells increased 18(SD ± 23)- and 182(± 158)-fold at the end of 12 days of culture for cells harvested before G-CSF administration, whereas these values were 333(± 347)- and 669(± 925)-fold in cells harvested after treatment with G-CSF. The highest increase was observed with apheresis product, which showed values of 1384(± 1434)- to 7091(± 2160)-fold respectively. The results were based on data obtained from 20 healthy donors. The bar means standard deviation. (*; $P<0.05$)

Cell Separation and Coculture

PBSC Obtained from apheresis products were stained with FITC-conjugated mAbs against CD34,

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Vα24, CD14, and CD56 for 20 minutes at 4°C and washed once with 5 mM EDTA-PBS. Anti-FITC-microbeads (Miltenyl Biotec, Gladbach, Germany) were then added to PBSC. After target cells were reacted with anti-FITC-microbeads, they were sorted by a magnetic cell separation system (Super MACS; Miltenyl Biotec), according to the manufacturer's protocol. The purity of isolated cells in the positive fraction was monitored and assured to be higher than 90% by flow cytometry, except for Vα24$^+$ NKT cells, which are difficult to obtain in high purity because of their rarity in PB. Although Vα24$^+$ NKT cells had a low purity (20% at most) after isolation by MACS, they were still considered enriched Vα24$^+$ NKT cells. On the other hand, contamination by CD14$^+$, CD 56$^+$, CD34$^+$, or Vα24$^+$ cells in their respective negative fractions was less than 10%.

To evaluate the influence of each cell population on Vα24$^+$ NKT cell expansion, we depleted and/or added back CD34$^+$ cells, Vα24$^+$ NKT cells, CD14$^+$ cells or CD56$^+$ NK cells, and evaluated the results on days 3, 6, 9 and 12. To evaluate the direct cell-cell interaction between CD56$^+$ NK cells and others, we used a Cell Culture Insert System with a 3 μm-pore membrane (Transwell, Corning, NY), and placed the CD56$^+$ NK fraction in the upper chamber and the CD56$^-$ fraction in the lower chamber. On day 12, the cells in the lower chamber were analyzed.

Contribution of CD14$^+$ Cells to Vα24$^+$ NKT Cell Expansion

To evaluate the contribution of CD14$^+$ cells to Vα24$^+$ NKT cell expansion and to optimize the CD14$^+$ cell conditions in our culture system, we depleted and added back CD14$^+$ cells to CD14$^-$ cells on day 0, on day 3, on day 6 or on day 9. CD14$^+$ cell was depleted by MACS (described above) and each added-back cells were 4.0×10^5 cells with optimized medium to maintain final concentration of IL-2 and autologous plasma. We also evaluated changes of concentration of CD14$^+$ cells before and after G-CSF administration and also evaluated the effects of them between different CD14$^+$ cell/CD14$^-$ cell ratio on Vα24$^+$ NKT cell expansion using the following culture conditions. The whole cell number was adjusted to 2.0×10^5 cells/ml in all wells, and the ratio of CD14$^+$ cells: CD14$^-$ cells was 0:5, 1:4, 2:3, 3:2, 4:1 or 5:0. The purpose of these manipulation was to detect the

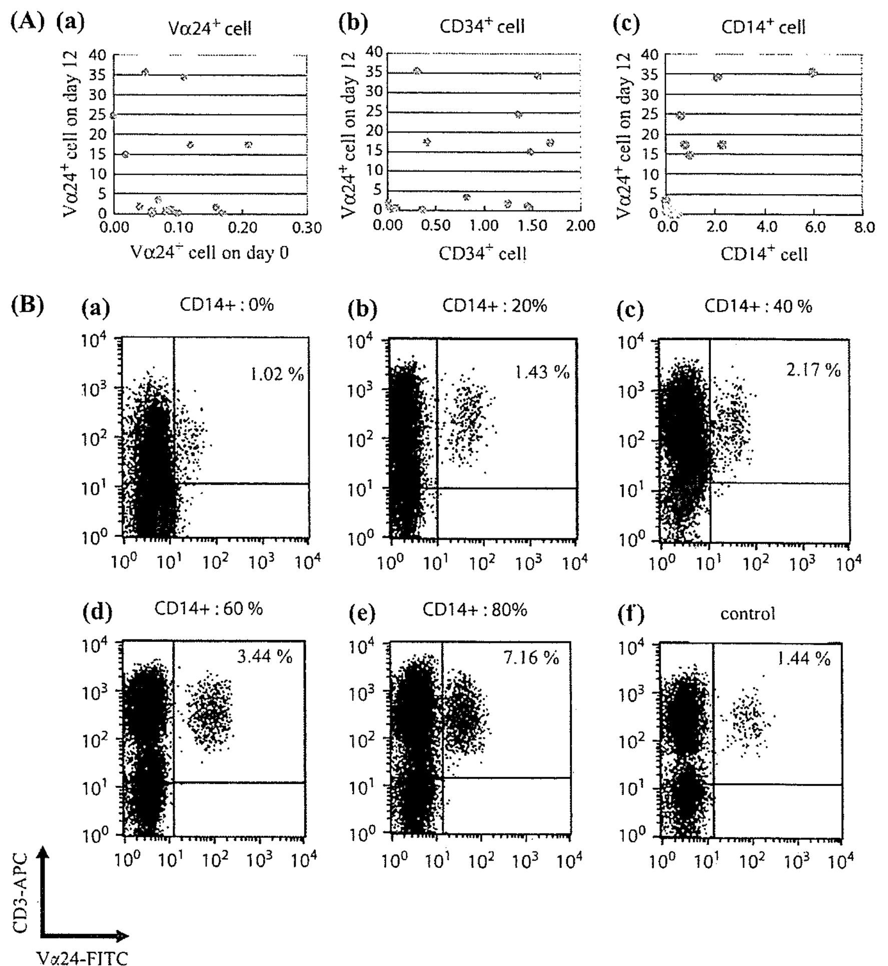

FIGURE 2. Effect of CD34$^+$, Vα24$^+$, and CD14$^+$ cells on expansion of Vα24$^+$ NKT cells (A) The proportion of (a) CD34$^+$, (b) Vα24$^+$ on day 0 were not associated with the expansion efficacy of Vα24$^+$ NKT cells ($r^2 = 0.171$, 0.016, respectively). Only CD14$^+$ cells (c) in the initial cell mixture had a relatively strong correlation ($r^2 = 0.545$) with the proliferation of cultured Vα24$^+$ NKT cells. These results were analyzed in 16 healthy donors. (B) The efficacy of Vα24$^+$ NKT expansion depended on the proportion of CD14$^+$ cells in apheresis products. The proportion of CD14$^+$ cells was as follows: (a) 0, (b) 20, (c) 40, (d) 60 and (e) 80% with a fixed total cell number of 2.0×10^5 cells/ml. The control means the result by using apheresis product without manupiration. These results are representative data from four experiments.

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