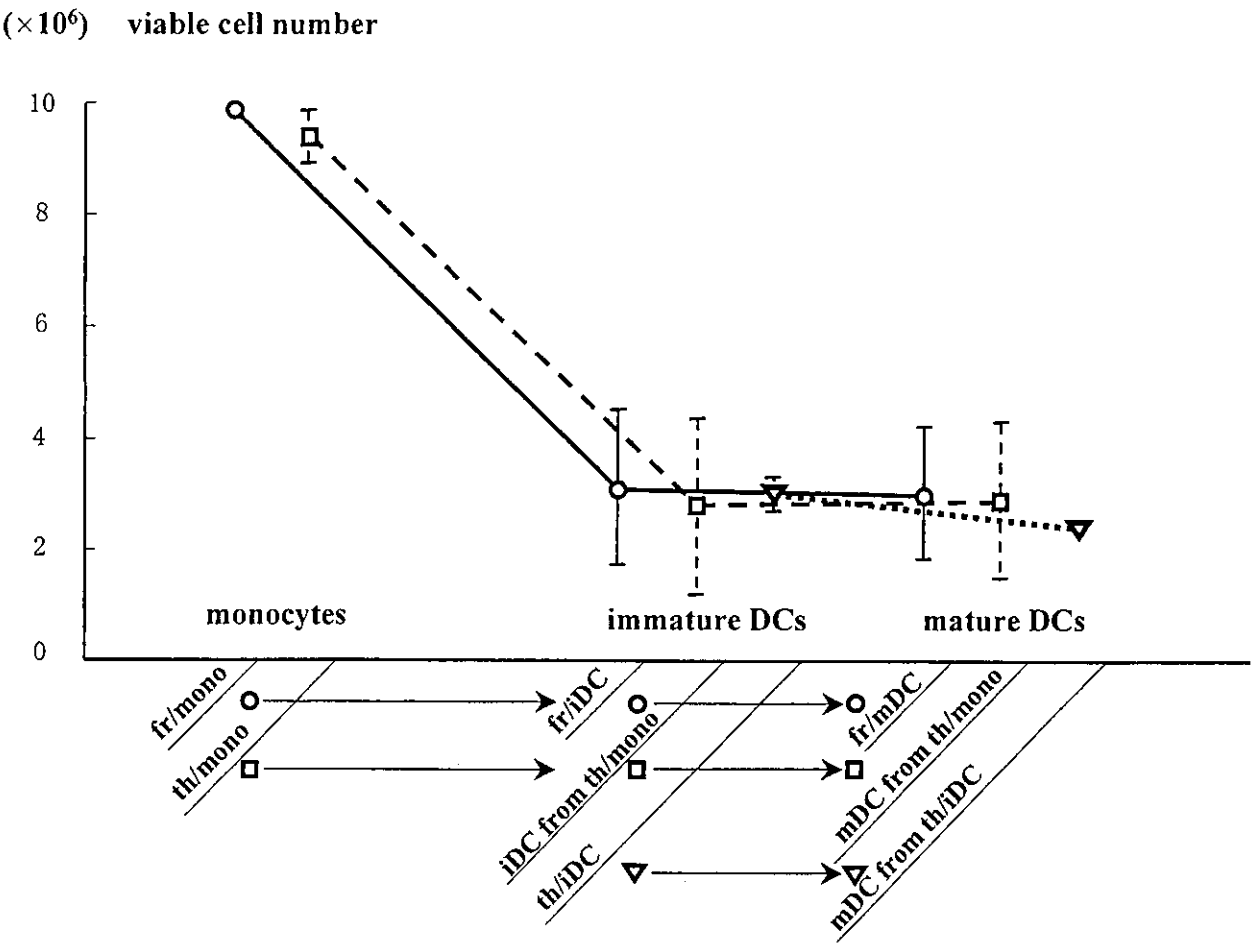

FIGURE 2. Comparison of the number of viable cells at each stage of maturation. Freshly isolated $CD14^+$ cells and cryopreserved $CD14^+$ cells were cultured with GM-CSF and IL-4 for 6 days and then cultured with GM-CSF, IL-4, and TNF-α for an additional 3 days. The initial number of each type of cell 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)]

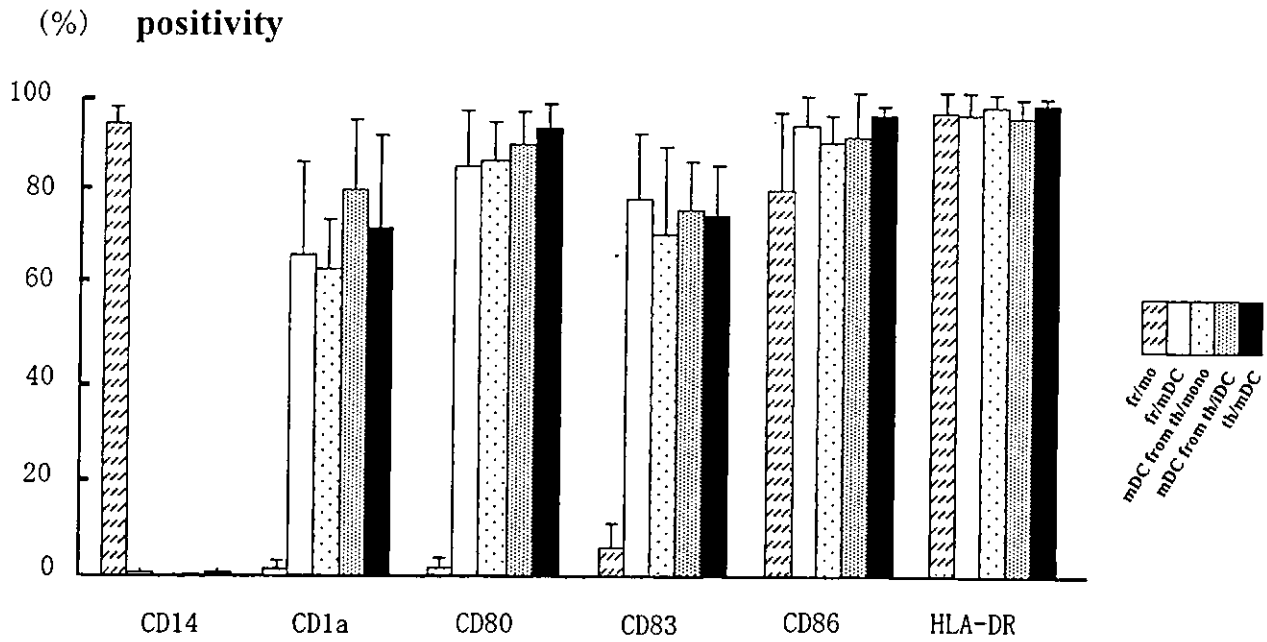

FIGURE 3. Surface markers on cells cultured for 9 days. Characteristics of mature DCs, ie, down-regulation of CD14 and up-regulation of CD1a, CD83, and HLA-DR, were observed on cells cultured for 9 days. Each marker for mature DCs was expressed similarly on cells generated from cryopreserved $CD14^+$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

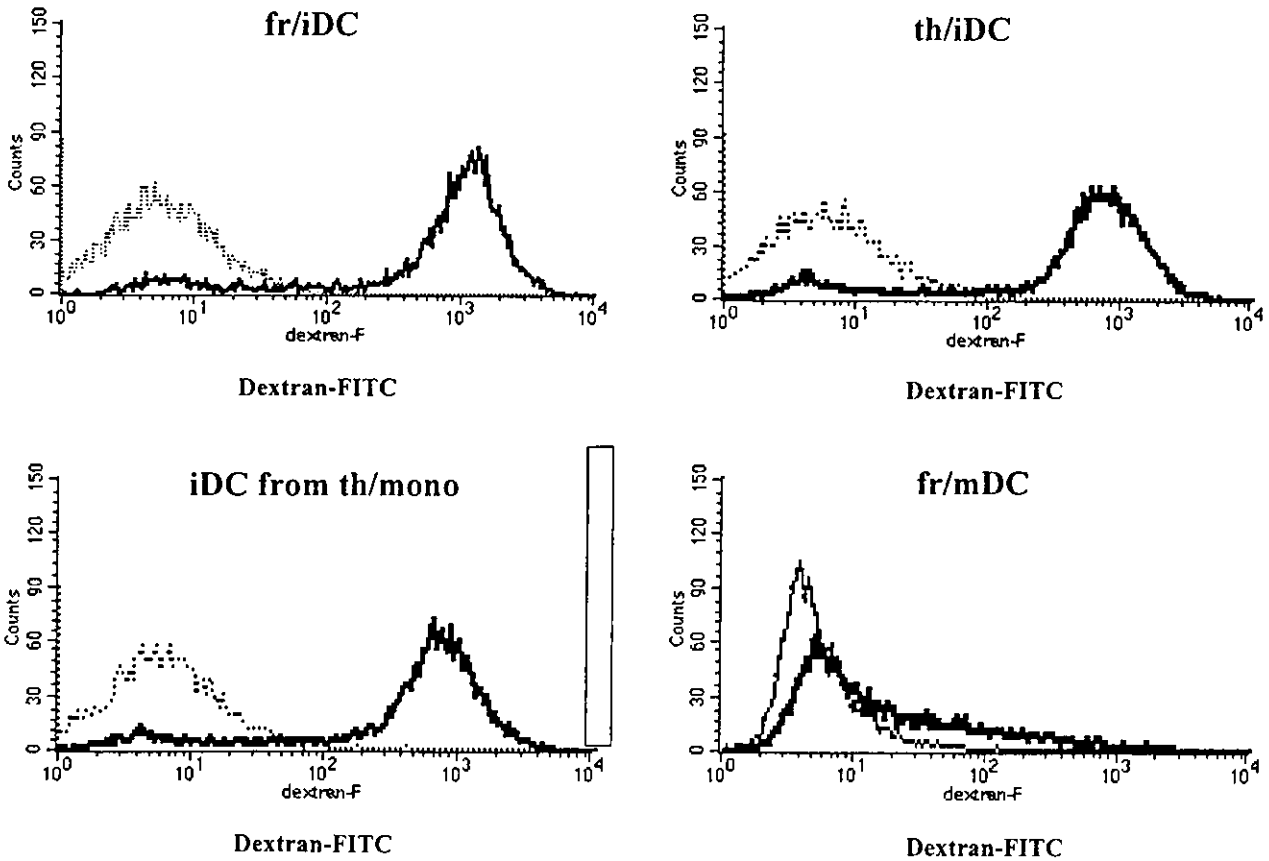

FIGURE 4. Endocytic capacity for dextran in each type of immature DCs. Dextran was added at a final concentration of 1 mg/mL to each type of immature DCs, and the cells were then incubated for 45 minutes at either 37°C (thick lines) or 4°C (thin lines) as controls. The values shown in the flow cytometry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

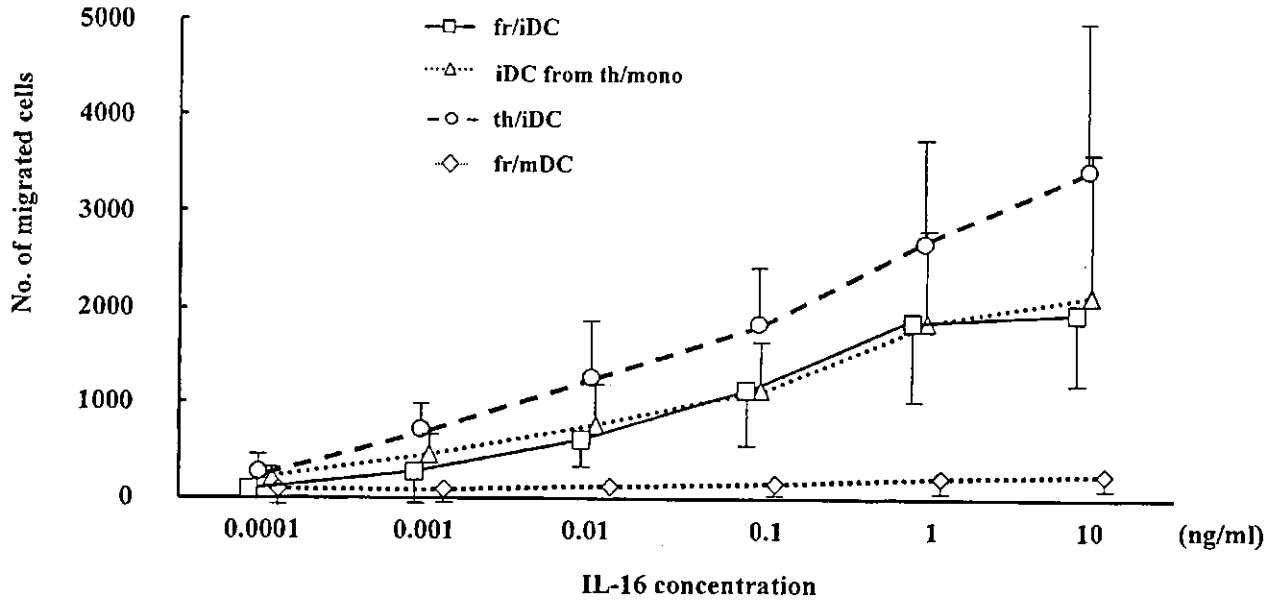

FIGURE 5. Chemotactic migratory capacity of each type of immature DCs. Each type of immature DCs showed similar dose-dependent migration activity upon stimulation with IL-16, while fresh mature DCs did not. Data are mean values of triplicate runs and represent the mean ± SD of 5 independent experiments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

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 ($HLA\text{-}A24^+$, 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 ($HLA\text{-}A24^-$) 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 $HLA\text{-}A24^+$ and showed that the CTL-induction ability of DCs at any stage of maturation was preserved after cryopreservation/thawing. Migration activity is another im-

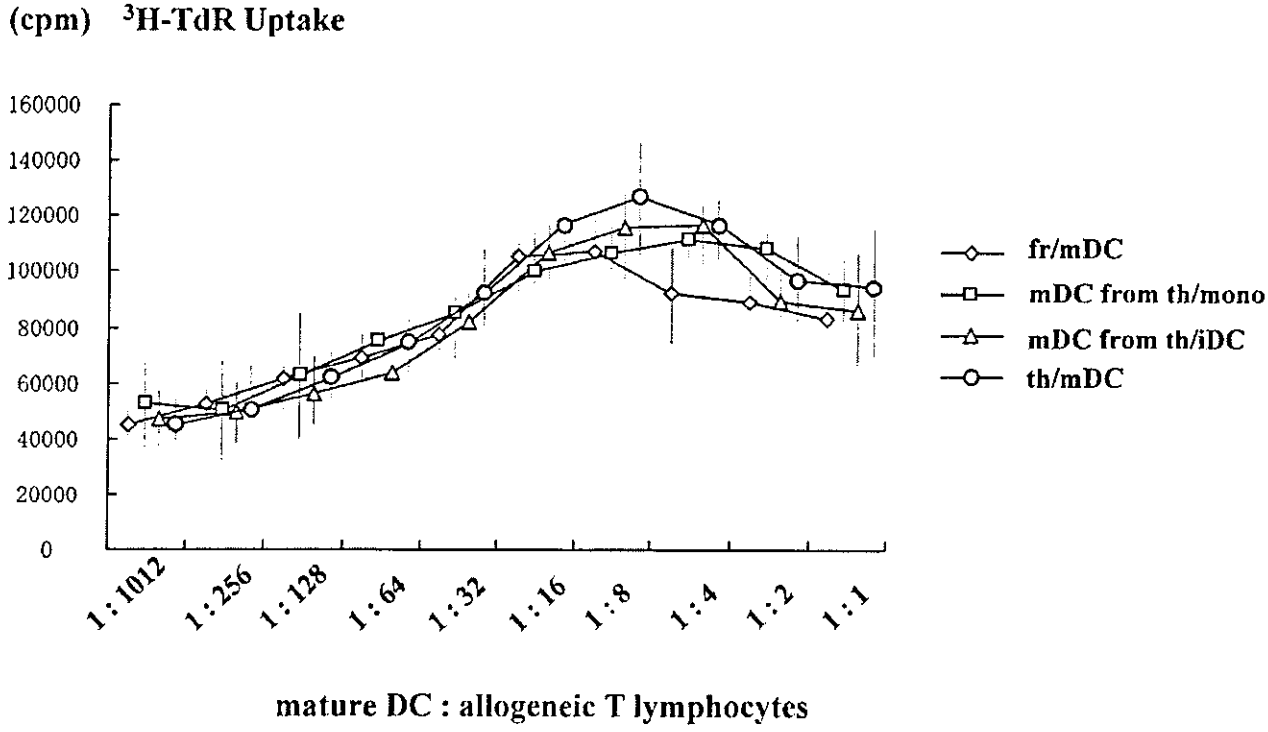

FIGURE 6. Allogeneic T lymphocyte stimulatory capacity of mature DCs. Allogeneic T lymphocytes, as a responder, were plated at 5×10^4 per well with increasing numbers of each type of irradiated (30 Gy) mature DCs as a stimulator. After 5 days of culture, the proliferative response was shown to depend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

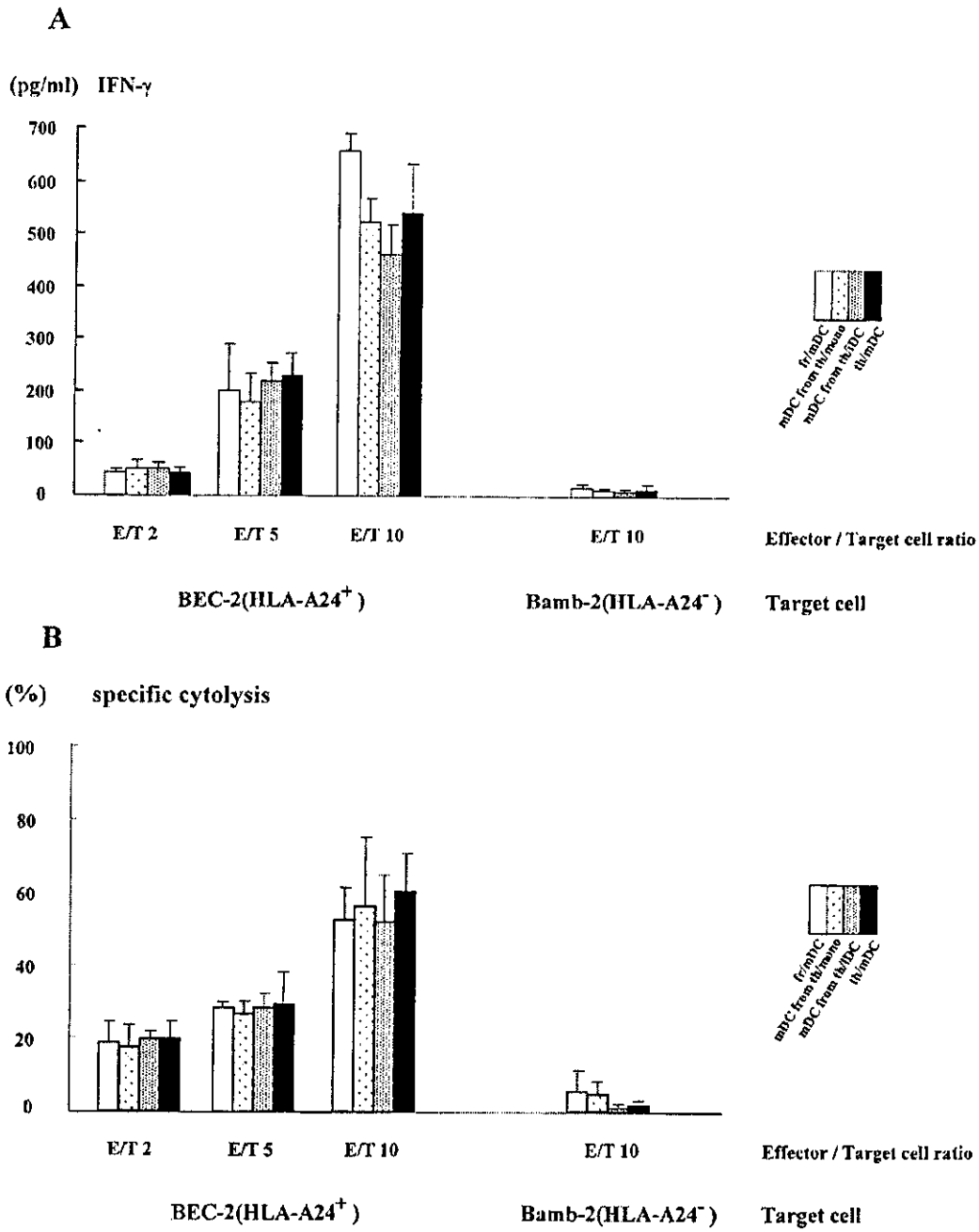

FIGURE 7. Specific CTL induction capacity of each type of mature DCs. Mature DCs prepared from HLA-A24+ donors were pulsed with HLA-A24-restricted EBV peptides for 2 hours. Pulsed mature DCs were cultured with autologous CD8+ cells as an effector cell with 100 IU/ mL IL-2 for 10 days. Stimulated effector 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.

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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Abstract

Objective: Vα24+ NKT is an innate lymphocyte with potential antitumor activity. Clinical applications of Vα24+ 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), we evaluated non-FBS culture conditions for the selective and efficient expansion of human Vα24+ NKT cells.

Materials and Methods: We used mononuclear cells (MNCs) and plasma from the peripheral blood of normal healthy donors 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.

Results: The highest expansion ratio for Vα24+ 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 2000-fold expansion ratios, respectively. Our results suggest that G-CSF mobilization conferred a proliferative advantage to Vα24+ NKT cells by modifying biology of cells and plasma factors. Expanded Vα24+ NKT cells retained their surface antigen expression and production of IFN-γ. They exhibited CD1d-independent cytotoxicity against tumor cells.

Conclusion: Vα24+ NKT cells can be efficiently expanded from G-CSF-mobilized peripheral blood MNCs in non-FBS culture conditions with α-GalCer and IL-2.

(241 words)

Introduction

Murine Vα14+ 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 Vα24+ NKT cells are similar to murine Va14+ NKT cells, as Vα24+ 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 Vα24+ NKT cells and murine Vα14+ NKT cells [4]. Both types of NKT cells are activated by α-GalCer presented by CD1d. After stimulation with α-GalCer, Vα24+ 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 interferon (IFN)-γ and interleukin (IL)-4 [4, 10].

The extremely low frequency of Vα24+ 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 Vα24+ NKT cells by stimulation with α-GalCer has been explored. Significant expansion was reported in human Vα24+ Vβ11+ 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 Vα24+ Vβ11+ 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 Vα24+ 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 (pre- and post-G-CSF), gained samples were used immediately and cell fraction and plasma were separated by the 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-Biological 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 healthy donor, whose related patient needed the 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 by the dose of 300 $\mu g/m^2$ divided twice daily for 3 days just before the apheresis procedure. On the apheresis day, one more dose of G-CSF was administered in the morning before apheresis.

Expansion of Vα24+ NKT cells

MNCs were cultured in 6-well culture plates or culture flasks (Costar, Corning, NY) at 1.0 x 10^5 cells/ml in media 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 12-14 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 if 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 monoclonal antibodies (mAbs) that were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), or peridinium chloropphyll (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 receptor 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 min on ice. After staining, cells were washed twice and re-suspended in PBS. Propidium iodide (Sigma-Aldrich, St. Louis, MO) staining preceded all experiments in order to remove dead cells. Data were acquired by flow cytometry (FACS Calibur; 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

min on ice and washed twice with 5 mM EDTA-PBS. After being incubated with anti-FITC-microbeads (Miltenyl Biotec, Gladbach, Germany), Vα24+ NKT cells were sorted by a magnetic cell separation system (Super MACS; Miltenyl Biotec), according to the manufacturer's protocol. After separation, the purity of isolated Vα24+ NKT cells was determined to be >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 h 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); Jurkat (T-cell lymphoma)

Target cells were labeled with 50 μCi of sodium [^{51}Cr] chromate (NEN Life Science Products, Inc., Boston, MA) per 5 x 10^5 cells for 1 h, washed three times, and re-suspended at 1 x 10^5 cells/ml in medium. Next, 100 ml of effector cells and 100 ml of ^{51}Cr-labeled target cells (1 x 10^4 cells/well) were added to 96-well round-bottom plates (Nunc, Roskilde, Denmark) at an effector to target (E/T) ratio of 10:1, 3:1 and 1:1. Plates were incubated for 4 h 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)] x 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; and "total cpm" indicates the counts obtained by adding 100 ml of 1N HCL to target cells in order 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).

Statistic analysis

The Student's t test was used to compare groups in two-tailed method dealing with dependent samples. P values <0.05 were considered statistically significant. In

multiple group analysis, we adapted Bonferoni adjustment for confirmation the significance of P value.

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 either 10% FBS, 10% rHSA, 5% autologous plasma or serum, or 10% autologous plasma or serum for 12-14 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%-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 1938(±2501)-fold in the post-G-CSF condition compared to 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 appears 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. Exogeneous 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 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 contains 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, which means 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 was significant differences between the levels of 3 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 lower than the detection limit.