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

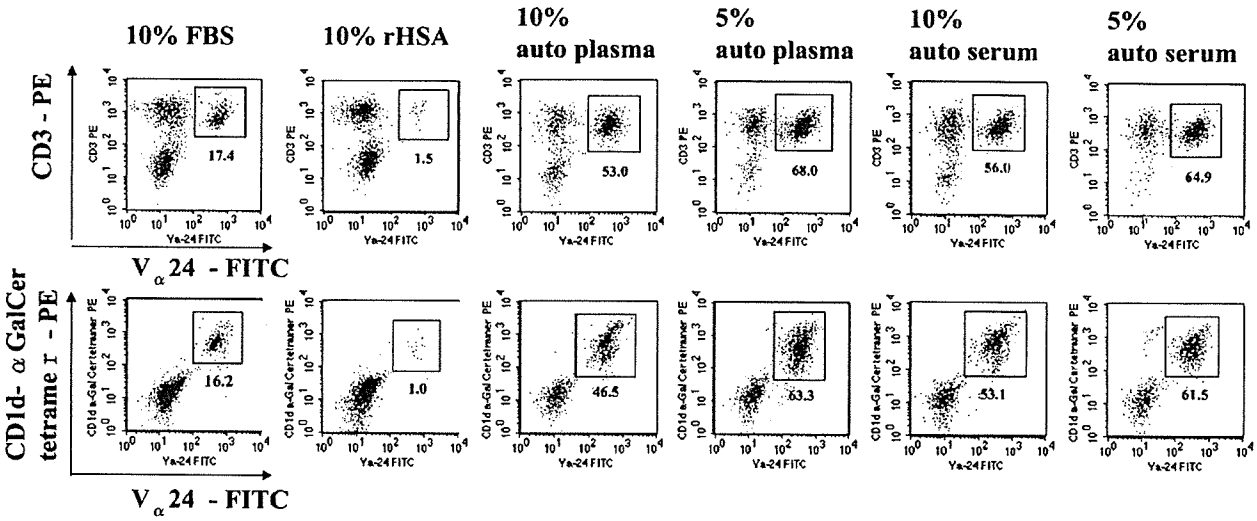


FIGURE 1. Differences in Vα24+ NKT cell expansion according to the type of supplemented protein. PBMCs from normal healthy donors were cultured for 12 to 14 days with α-GalCer and IL-2 in the presence of 10% FBS, 10% rHSA, 5% or 10% autologous plasma, or 5% or 10% autologous serum. Vα24+ CD3+ cells were 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.

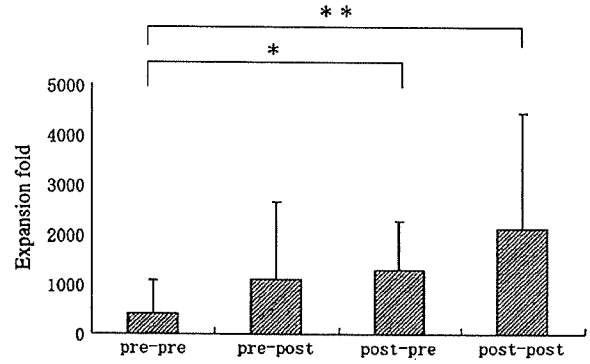
Cell Populations

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

*$P < 0.05$.



* P<0.05 both in t-test and Bonferoni adjustment,
** P<0.05 in t-test but not Bonferoni adjustment

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.

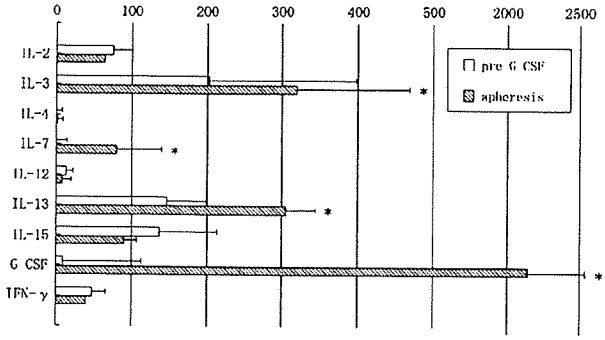


FIGURE 3. Cytokine levels in plasma. Cytokine levels in peripheral blood were measured by ELISA before G-CSF mobilization and in apheresis products from the same normal healthy donors (n = 6). IL-2 levels are plotted in U/mL; all other cytokine levels are plotted in pg/mL. Results are shown as mean values 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 Vα24$^+$ NKT cells produced IFN-γ and 16.2 ± 10.5% produced IL-4. In the comparison of IFN-γ and IL-4 produced by Vα24$^+$ 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. Vα24$^+$ 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 Vα24$^+$ Vβ11$^+$ NKT cells in the peripheral blood within 48 hours of the infusion. This transient decrease was followed by significant increases in Vα24$^+$ Vβ11$^+$ 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 Vα24$^+$ NKT cells requires the development of a highly effective expansion method for Vα24$^+$ 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 Vα24$^+$ 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 Vα24$^+$ 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 Vα24$^+$ 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 Vα24$^+$ NKT cells than did FBS and rHSA. Indeed, there was no significant difference between Vα24$^+$ NKT cell expansion in 5% or 10% autologous plasma or autologous serum. However, the percentage of Vα24$^+$ cells in culture medium was the highest and 87% to 95% of Vα24$^+$ 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 Vα24$^+$ 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 Vα24$^+$ NKT cells (see

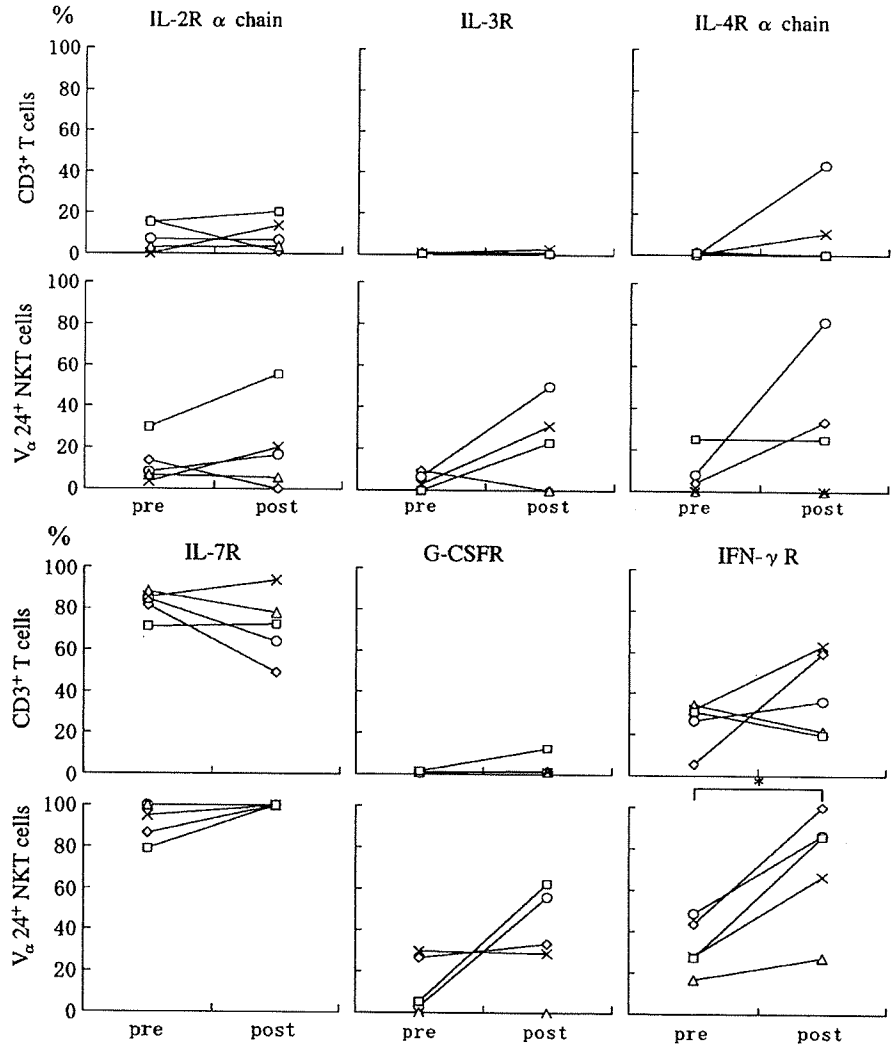


FIGURE 4. Cytokine receptor expression. Changes in the cytokine receptor expression of CD3+ T cells and Vα24+ NKT cells in peripheral blood before and after G-CSF mobilization are shown as five independent experiments. Peripheral blood before and after G-CSF-mobilization was obtained from the same healthy 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

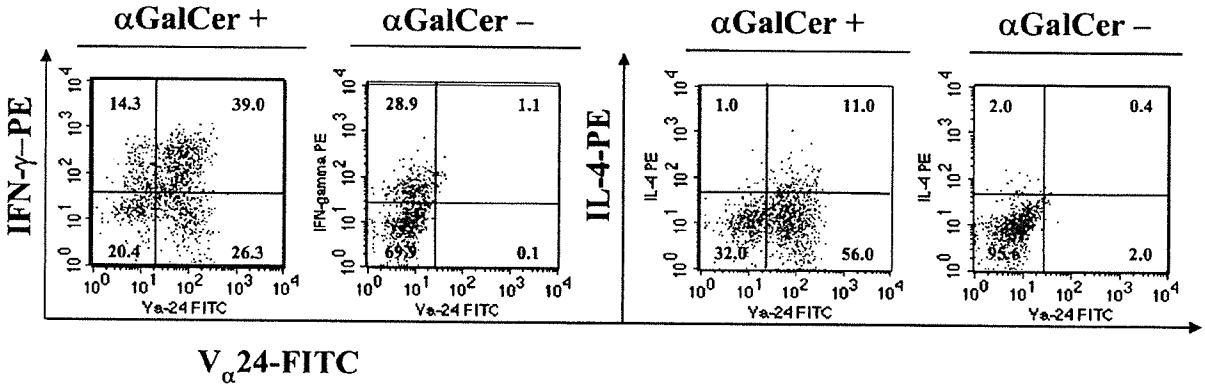


FIGURE 5. Intracellular cytokines in cultured Vα24⁺ NKT cells. Intracellular IFN-γ and IL-4 were stained in whole cells after culture with or without α-GalCer. Cells were activated with phorbol 12-myristate 13-acetate and ionomycin for 4 hours. Representative data from 1 of 10 independent experiments 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

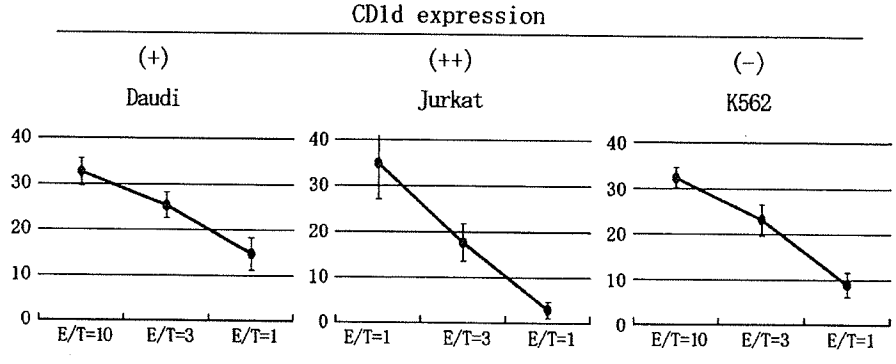


FIGURE 6. Cytotoxicity of purified Vα24⁺ NKT cells after culture. Vα24⁺ NKT cell-mediated cytotoxicity against tumor cells was measured with effector-to-target ratios of 10:1, 3:1, and 1:1. Cell lines were classified into the following four groups based on the expression level of CD1d: (-), 0–3%; (±),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

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 $V\alpha24^+$ NKT cell expansion through the use of G-CSF-mobilized peripheral blood. We also featured the possible clinical applications of $V\alpha24^+$ NKT cells in adoptive immunotherapy, both in autologous and allogeneous settings. Further research needed to achieve this goal is underway.

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ORIGINAL STUDY

Efficient *ex vivo* expansion of Vα24$^+$ NKT cells derived from G-CSF-mobilized blood cells

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

Key Words: Vα24$^+$ NKT cells, α-galactosylceramide, CD14$^+$ cells, CD56$^+$ NK cells

(*J Immunother* 2006;00:000–000)

INTRODUCTION

NKT cells are lymphocyte lineage and show characteristics of both T cells and NK cells.[1] NKT cells coexpress T cell receptors (TCRs) and NK cell markers, and display an extremely restricted TCR repertoire, consisting of Vα24 chain preferentially paired with Vβ11 chain. Upon activation by a specific ligand, NKT cells produce high levels of interferon-gγ (IFN-γ) and interleukin-4 (IL-4), and yield a strong immune response against several types of tumor cells.[2] Therefore, these invariant NKT cells are considered key effector cells, and play critical roles in immunity against microbial infection, tumor and autoantigens.

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

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

MATERIALS AND METHODS

Cells and Plasma Preparation

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

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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 Vα24$^+$ NKT cells to refer to Vα24$^+$ 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 Vα24$^+$ 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.

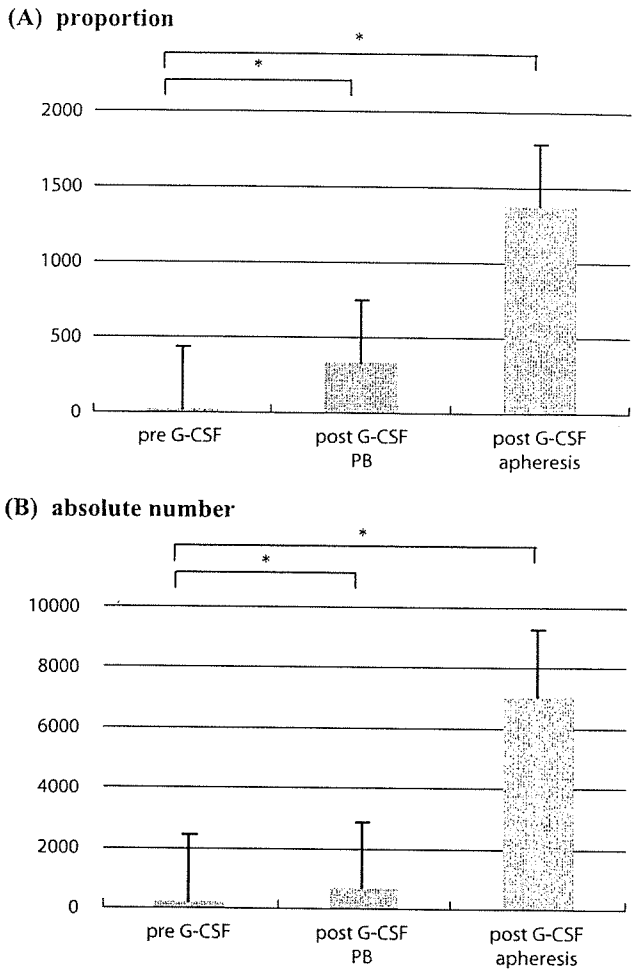

FIGURE 1. Proportion and absolute number of Vα24$^+$ NKT cells on day 12 The proportion (A) and absolute number (B) of Vα24$^+$ NKT cells increased 18(SD±23)- and 182(±158)-fold at the end of 12 days of culture for cells harvested before G-CSF administration, whereas these values were 333(±347)- and 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,

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 $V\alpha24^+$ NKT cells, which are difficult to obtain in high purity because of their rarity in PB. Although $V\alpha24^+$ NKT cells had a low purity (20% at most) after isolation by MACS, they were still considered enriched $V\alpha24^+$ NKT cells. On the other hand, contamination by $CD14^+$, $CD\ 56^+$, $CD34^+$, or $V\alpha24^+$ cells in their respective negative fractions was less than 10%.

To evaluate the influence of each cell population on $V\alpha24^+$ NKT cell expansion, we depleted and/or added back $CD34^+$ cells, $V\alpha24^+$ 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 $V\alpha24^+$ NKT Cell Expansion

To evaluate the contribution of $CD14^+$ cells to $V\alpha24^+$ 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 $V\alpha24^+$ 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

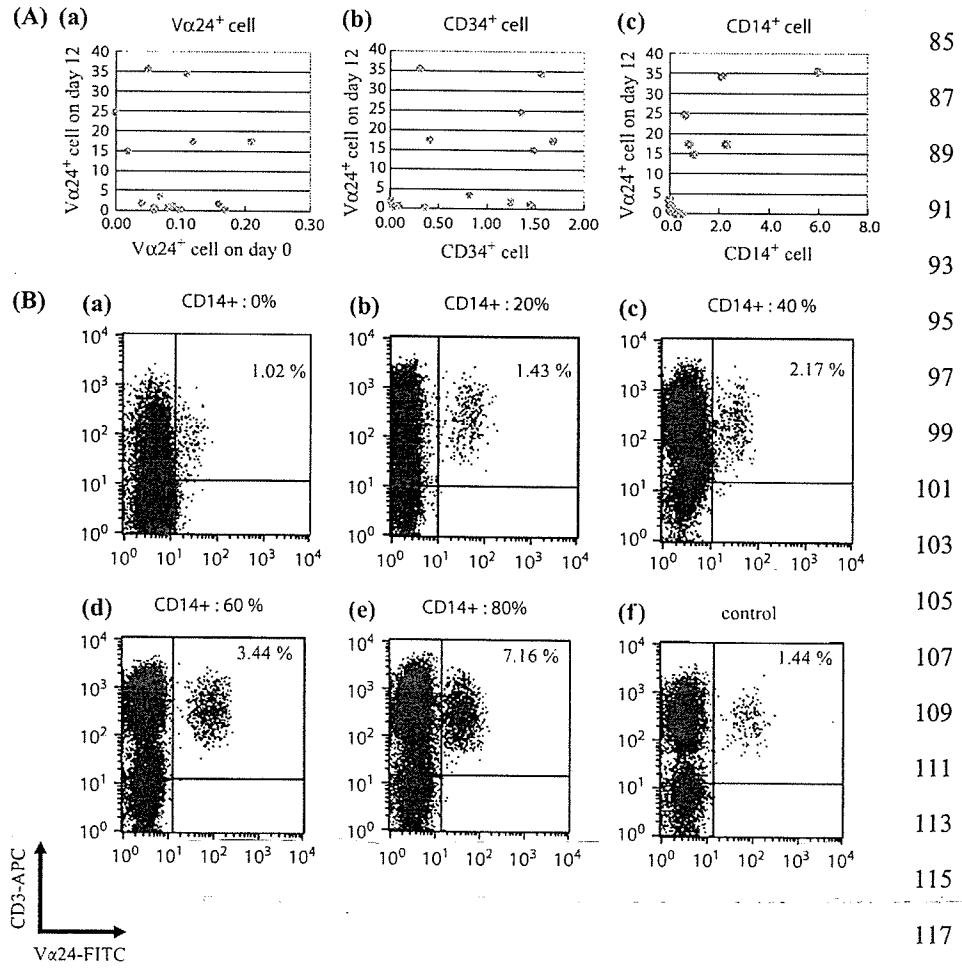

FIGURE 2. Effect of $CD34^+$, $V\alpha24^+$, and $CD14^+$ cells on expansion of $V\alpha24^+$ NKT cells (A) The proportion of (a) $CD34^+$, (b) $V\alpha24^+$ on day 0 were not associated with the expansion efficacy of $V\alpha24^+$ NKT cells ($r^2 = 0.171$, 0.016, respectively). Only $CD14^+$ cells (c) in the initial cell mixture had a relatively strong correlation ($r^2 = 0.545$) with the proliferation of cultured $V\alpha24^+$ NKT cells. These results were analyzed in 16 healthy donors. (B) The efficacy of $V\alpha24^+$ 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.

contribution of $CD14^+$ cells in the different timing of culture process and by the different proportion.

Modification of IL-2 Supplementation Schedule

In our original protocol established by Mikami and Harada, we added IL-2 to the cell culture medium every 3 days to maintain its biologic activity. However, in this study, we modified the schedule of IL-2 administration to determine the suitable culture conditions for $V\alpha24^+$ NKT expansion as follows: addition of IL-2 i) only on day 0, ii) days 0 & 3, iii) days 0, 3 & 6, and iv) days 0, 3, 6 & 9. Each supplementation of IL-2 was oriented to 100 IU/ml as a final concentration. The cell numbers and their phenotypes were analyzed on day 12. α-GalCer was also supplemented at final concentration 100 ng/ml.

Statistical Analysis

Student's t test was used to compare 2 groups and P values of < 0.05 were considered statistically significant. Correlation was estimated by the ordinary least squares method. Correlation coefficients are shown as squared values (r^2).

RESULTS

Efficient Expansion of $V\alpha24^+$ NKT Cells Derived from G-CSF-Mobilized PBSCT of Normal Healthy Donors

We compared the expansion-fold of $V\alpha24^+$ NKT cells in PBSCT before and after G-CSF mobilization in 20 healthy donors. The expansion fold of percentage and absolute number of $V\alpha24^+$ NKT cells increased, respectively, 18(SD ± 23)- and 182(± 158)-fold in PBMC before G-CSF mobilization, whereas these were 333(± 347)- and 669(± 925)-fold in G-CSF-mobilized PBMC. Apheresis products from collection bags showed more efficient expansion capacities, from 1384(± 1434)- to 7091(± 2160)-fold (Figure 1A,B). Thus, G-CSF mobilization significantly increased the capacity for $V\alpha24^+$ NKT cell expansion.

Relationship Between the Concentration of $CD34^+$, $V\alpha24^+$ and $CD14^+$ Cells on V α 24^+ NKT Expansion

To analyze the contribution of $CD34^+$, $V\alpha24^+$ and $CD14^+$ cells on the proliferation of $V\alpha24^+$ NKT cells in apheresis product, we compared the percentage of $CD34^+$, $V\alpha24^+$ and $CD14^+$ cells on day 0 and $V\alpha24^+$ NKT expansion efficacy on day 12. The results suggested only $CD14^+$ cells showed the correlation with the expansion of $V\alpha24^+$ NKT cells. (Figure 2A).

Contribution of $CD14^+$ Cells to the Ex Vivo Expansion of $V\alpha24^+$ NKT Cells

It has been reported that $CD14^+$ cells, dendritic cells and monocytes play a critical role in the initiation of proliferation of $V\alpha24^+$ NKT cells.[9] In PB after G-CSF treatment, the absolute number of $CD14^+$ cells significantly increased (from 350 ± 81 to 2353 ± 1220/μL),

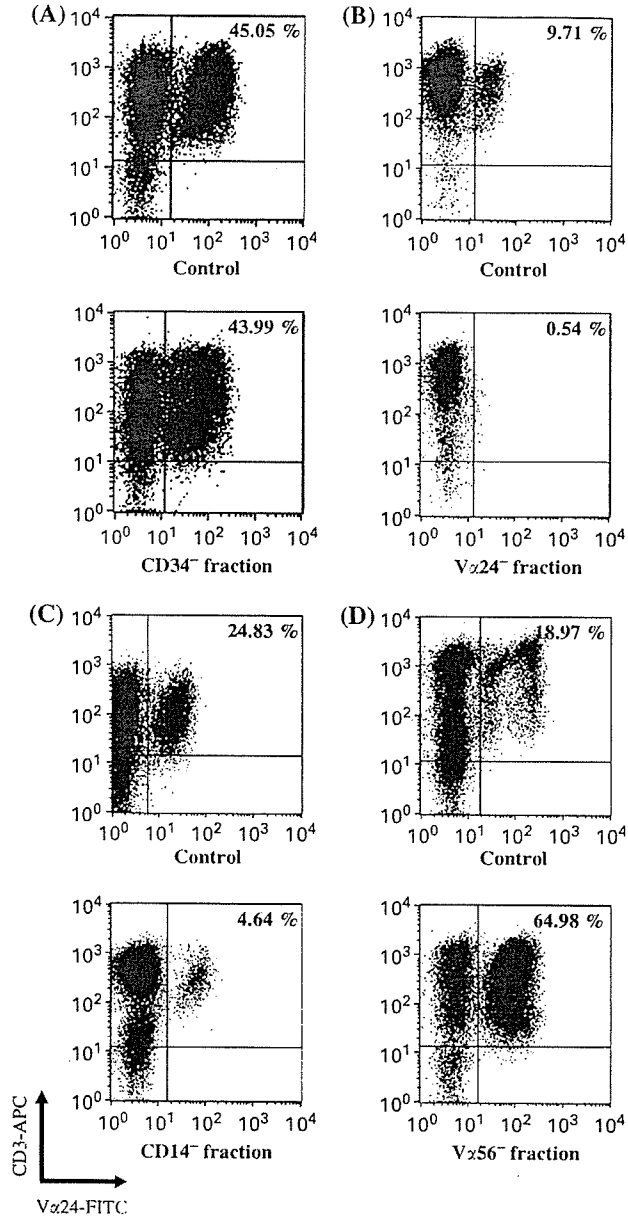

FIGURE 3. Effects of $CD34^+$, $V\alpha24^+$ NKT, $CD14^+$ and $CD56^+$ NK cell depletion on the expansion of $V\alpha24^+$ NKT cells $CD34^+$, $V\alpha24^+$ NKT, $CD14^+$, and $CD56^+$ NK cells were depleted using a MACS sorting system. (A) When $CD34^+$ cells were depleted, $V\alpha24^+$ NKT cells proliferated the same as in culture without $CD34^+$ cell-depletion. When (B) $V\alpha24^+$ NKT cells or (C) $CD14^+$ cells were depleted, $V\alpha24^+$ NKT cells did not expand. (D) When $CD56^+$ NK cells were depleted, the expansion efficiency of $V\alpha24^+$ NKT cells improved. These are each representative results from four experiments. The control in this experiment means the result by using apheresis product without target cell depletion.

although their percentage in PB did not change (from 7.24 ± 5.07 to 5.53 ± 2.10%) due to an overwhelming increase in granulocytes. In apheresis products, the proportion of $CD14^+$ cells in nuclear cells also increased 5.7- to 38-fold compared with before G-CSF mobilization, because the apheresis products included low granulocyte contaminations, less than 20%. We obtained $CD14^+$ cells using the MACS system with a purity of > 95%, and made a $CD14^+$ cell gradation (0%, 20%, 40%, 60%, 80% and 100%) under a fixed total cell count of 2.0×10^5 cells/mL/well. The efficacy of $V\alpha24^+$ NKT expansion was related to the initial proportion of $CD14^+$ cells, and the percentage of $V\alpha24^+$ NKT after expansion was increased in $CD14^+$ cell dose dependent manner (Figure 2B).

Effect of Depletion of Cells, Including $CD34^+$, $V\alpha24^+$ NKT, $CD14^+$ and $CD56^+$ Cells, on $V\alpha24^+$ NKT Cell Expansion

To determine the origin of $V\alpha24^+$ NKT cells and the contribution of each cell population on $V\alpha24^+$ NKT cell expansion, we tested the following cell culture conditions with apheresis products: 1) $CD34^+$ cell-depleted, 2) $V\alpha24^+$ NKT cell-depleted, 3) $CD14^+$ cell-depleted, and 4) $CD56^+$ cell-depleted culture. When $CD34^+$ cells were depleted, $V\alpha24^+$ NKT cells proliferated the same as in non-depleted culture (Figure 3A). However, the depletion of $V\alpha24^+$ NKT cells completely abrogated the expansion of $V\alpha24^+$ NKT cells (Figure 3B). Depletion of $CD14^+$ cells also abrogated $V\alpha24^+$ NKT cell expansion to result in the complete disappearance of $V\alpha24^+$ NKT cells on day 12 (Figure 3C). Interestingly, when $CD56^+$ NK cells were depleted, a remarkable improvement in V $\alpha24^+$ NKT cell proliferation was observed (Figure 3D). In experiments with $CD56^+$ NK cells separated from $CD56^-$ fraction using a 3.0 μm-pore membrane, the proliferation of $V\alpha24^+$ NKT cells was maintained in $CD56^-$ fractions. The mixed culture of $CD56^+$ NK cells with $CD56^-$ fraction in the same wells resulted in the suppressed proliferation of $V\alpha24^+$ NKT cells, even though there were 1.0×10^5 $CD14^+$ cells (data not shown).

Add-Back of Cells, Including $CD14^+$ Cells, to $V\alpha24^+$ NKT Cell Cultures

The analysis of cell kinetics during culture suggested that $CD14^+$ cells gradually decreased in the early phase (days 0–3), whereas $V\alpha24^+$ NKT cells gradually increased in the latter phase of culture (days 9–12). With regard to $CD56^+$ NK cell kinetics, cell numbers continued to increase during culture in good responders (Figure 4A), whereas they peaked on day 9 in poor responders (Figure 4B). To evaluate the effects of $CD14^+$ NK cells in the early phase and late phase of $V\alpha24^+$ NKT cell expansion, we depleted and added back $CD14^+$ cells to the $CD14^-$ cell population, which included $V\alpha24^+$ NKT cells, on days 0, 3, 6 and 9, respectively (Figure 4C). Figure 4D shows that add-back of $CD14^+$ cells on day 0 induced the highest expansion of $V\alpha24^+$ NKT cells, whereas the

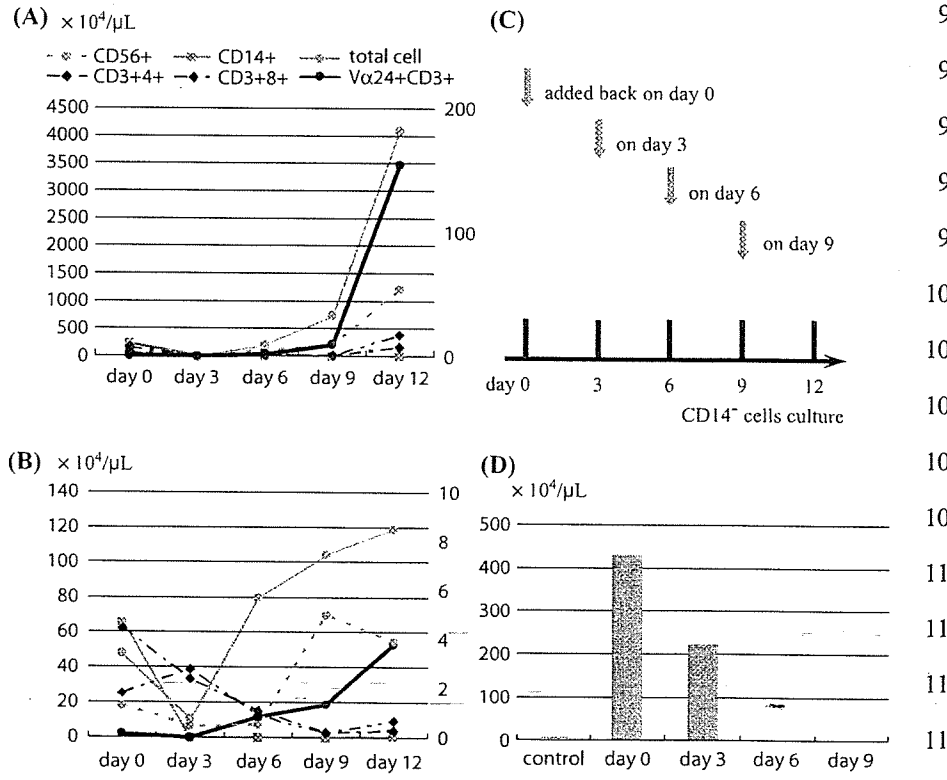

FIGURE 4. Cell kinetics of $V\alpha24^+$ NKT cells and $CD56^+$ NK cells in good and poor expanders (A) In a good-expanding donor, both $CD56^+$ NK cells and $V\alpha24^+$ NKT cells continued to proliferate without decline (representative results from four experiments). The right hand y-axis is used for the cell number of $V\alpha24^+$ NKT cells. (B) In a poor-expanding donor, $CD56^+$ NK cells proliferated more efficiently than $V\alpha24^+$ NKT cells, with a peak on day 9, concomitant with a suppression of $V\alpha24^+$ NKT cell proliferation on day 12 (representative results from four experiments). The right hand y-axis is used for the cell number of $V\alpha24^+$ NKT cells. (C) We added-back $CD14^+$ cells to $CD14^-$ cells on days 0, 3, 6, and 9. (D) Add-back of $CD14^+$ cells before day 3 enhanced the proliferation of $V\alpha24^+$ NKT cells. These are representative results from four independent experiments. All of four experiments were comparable and had a same tendency.

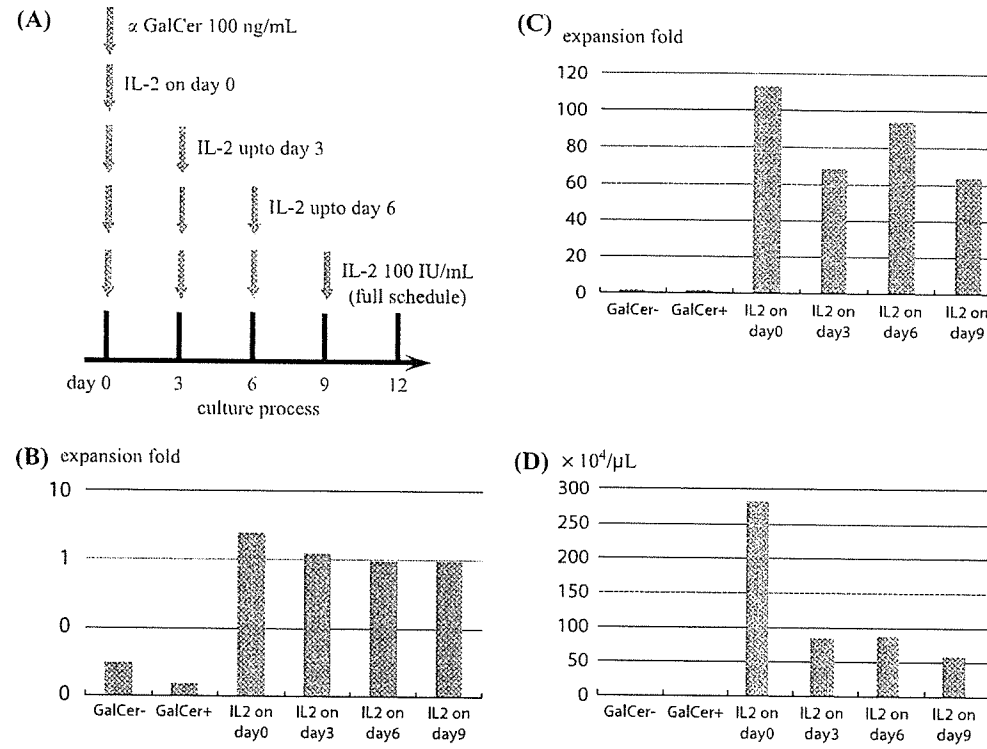

FIGURE 5. Effects of treatment with IL-2 on the expansion of Vα24$^+$ NKT cells (A) We tested different schedules for the administration of IL-2, as follows: on day 0 only, on days 0 & 3, on days 0, 3 & 6, and on days 0, 3, 6 & 9. We found that (B) the expansion-fold of whole cells, and the expansion-fold of the proportion (C) and absolute number (D) of Vα24$^+$ NKT cells were higher when IL-2 was supplemented on day 0 only (representative results from four experiments). All of four experiments were comparable and had a same tendency. In this experiments, α-GalCer was also supplemented at the concentration of 100 ng/mL without non-α-GalCer supplemented control.

addition of CD14$^+$ cells in the late phase did not show any remarkable benefit.

Effect of IL-2 Supplementation on the Expansion of CD56$^+$ NK Cells and Vα24$^+$ NKT Cells

We hypothesized that repeated IL-2 supplementation could result in the enhancement of CD56$^+$ NK activity to suppress the proliferation of Vα24$^+$ NKT cells.[10] In Figure 5, we tested four different schedules of IL-2 administration: on day 0 only, on days 0 & 3, on days 0, 3 & 6, and on days 0, 3, 6 & 9 (Figure 5A). We found that whole cells and Vα24$^+$ NKT cells expanded most effectively when IL-2 was added on day 0 only (Figure 5B,C,D).

DISCUSSION

The methods that have been used for the ex vivo expansion of human NKT cells can be divided into two categories: simple culture of PBMC with α-GalCer,[12] and a two-step culture method that uses α-GalCer-pulsed monocytes as feeder cells[15],. A single culture system has the benefit of simplicity and a low risk of contamination, and a major obstacle in a two-step culture system is the availability of a large number of feeder cells. Hence, in this study of the former type, we intended to improve and establish culture conditions for realistic clinical application. Previously, we used a single stimulation of α-GalCer on the initial day, and then administered IL-2 every 3 days to obtain satisfactory expansion of human Vα24$^+$ NKT cells.[7] We have also reported that the addition of 5% autologous plasma was also effective.[8] G-CSF mobilization increased the efficacy of Vα24$^+$ NKT cell expansion, and our data suggested that this was due to a change in cellular component including CD14$^+$ cells[16] and serous factors in the blood. In our present study, we found that CD14$^+$ cells, which are effectively mobilized together with CD34$^+$ cells by G-CSF,[16] are one of the candidates hat contribute to the effective ex vivo expansion of Vα24$^+$ NKT cells. Only the number of pre-cultured CD14$^+$ cells affected the magnitude of the expansion of Vα24$^+$ NKT cells, and this agreed with a previous report by van der Vliet et al that dendritic cells (DC) derived from monocytes including CD14$^+$ cells could efficiently mediate the expansion of Vα24$^+$ NKT cells[17](18). Additionally, we showed that 1) depletion of CD14$^+$ cells resulted in the loss of Vα24$^+$ NKT cell expansion, and 2) the expansion efficacy of Vα24$^+$ NKT

cells depended on the ratio of $CD14^+$ cells at the initiation of culture. Based on these observations, we speculated that the initial presence of $CD14^+$ cells plays an important role in the subsequent effective expansion of $V\alpha24^+$ NKT cells. We observed that the intensity of CD1d molecules on $CD14^+$ cells, which is critical for interaction with α-GalCer for the expansion of $V\alpha24^+$ NKT cells,[19] increased after G-CSF mobilization (data not shown). Hence, it is reasonable to speculate that more $CD14^+$ cells with a high intensity of CD1d molecules plays a key role in NKT cell expansion. The higher expansion efficiency in apheresis products compared with G-CSF-mobilized PB may be secondary to a higher concentration of $CD14^+$ cells.

The removal of $V\alpha24^+$ NKT cells before culture resulted in the loss of $V\alpha24^+$ NKT cell proliferation, and this supported previous reports that *ex vivo*-expanded $V\alpha24^+$ NKT cells were neither committed nor supported by $CD34^+$ cells, but were derived from peripheral circulating V α 24^+ NKT cells.[17] Whereas $CD34^+$ cells do not appear to be directly involved in the expansion of $V\alpha24^+$ NKT cells, they might make the circumstances suitable for $V\alpha24^+$ NKT cell expansion, through the secretion of unidentified soluble factors from bone marrow-derived stromal cells, as suggested by Johnston et al.[20] Although the presence of $V\alpha24^+$ NKT cells on day 0 is critical for the expansion of $V\alpha24^+$ NKT cells, no correlation was found between the proportion of $V\alpha24^+$ cells before culture and the proportion of $V\alpha24^+$ NKT cells at the end of culture. This suggests that some other factor(s) might regulate the expansion kinetics of $V\alpha24^+$ NKT cells. The inhibition of cell expansion by $CD56^+$ NK cells was restored when direct cell-to-cell contact was interrupted, which suggests that direct interaction between $V\alpha24^+$ NKT cell and $CD56^+$ NK cells plays a role. This hypothesis was indirectly supported by the phenomina that IL-2 supplementation in every 3 days suppressed expansion of $V\alpha24^+$ NKT cells. Indeed, NK cell-mediated interference of NKT cells is well known to be a primary immune regulatory mechanism.[21] Another possibility is indirect inhibition through the modulation of DC functions. It has been reported that NK cells could yield cytolytic activity against DC during their expansion.[22–24] NKT cells were also activated by DC, resulting in the suppression and killing of DC[25](26) in the same manner as NK cells.

In conclusion, for the efficient ex vivo expansion of $V\alpha24^+$ NKT cells, the presence of $V\alpha24^+$ cells and $CD14^+$ cells at the initiation of culture is critical. NK cells may interact with antigen presenting cells (APC) and interfere with the expansion of NKT cells by hindering the function of antigen presentation or providing direct cytotoxicity against APC. We believe that these findings may be useful for the development of an efficient system for the expansion of NKT cells for future adaptive immunotherapy.

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