Table 2. *Comparison of Preengraftment CRP Value Stratified According to the Conditioning Regimen (CST versus RIST) and the Relation to Donor (Related versus Unrelated)*

Patients' Characteristics	CRP Value Median (Range)
All patients	8.9 (0.1-42.7)
CST	10.5 (0.3-31.3)*
Related	9.4 (0.6-30.0)†
Unrelated	10.6 (0.3-31.3)†
RIST	6.2 (0.1-42.7)*
Related	1.6 (0.1-9.7)‡
Unrelated	16.2 (0.5-42.7)‡

CST indicates conventional stem cell transplantation; RIST, reduced-intensity stem cell transplantation.
*P = .017.
†P = .33.
‡P < .001.

before the initiation of the conditioning regimen, and the median pretransplant CRP level was 0.3 mg/dL (range: 0.0-20.5 mg/dL). The median maximum CRP value during neutropenia was 8.9 mg/dL (0.1-42.7, Table 2).

The "maximum CRP level" was determined by measuring both the CRP level and the neutrophil count, as shown in the example in Figure 1A. The average number of levels assessed for each patient was 8 (range: 1-30). The median day of the maximum CRP level was day 10 of HSCT (range: 0-25), with 79% of patients developing this in later days (≥8 days). The patients were categorized according to the maximum CRP level after the threshold CRP level was determined following a preliminary analysis of the maximum CRP level after CST using an ROC curve analysis (data not shown). The "low-CRP" group (CRP <15 mg/dL) included 157 patients and the "high-CRP" group (CRP ≥15 mg/dL) included 67 patients.

Statistical Analyses

The primary endpoint of this study was the occurrence of grade II-IV and grade III-IV aGVHD, according to the Consensus Criteria [25]. The secondary endpoints were overall survival (OS) and nonrelapse mortality (NRM). Standard descriptive

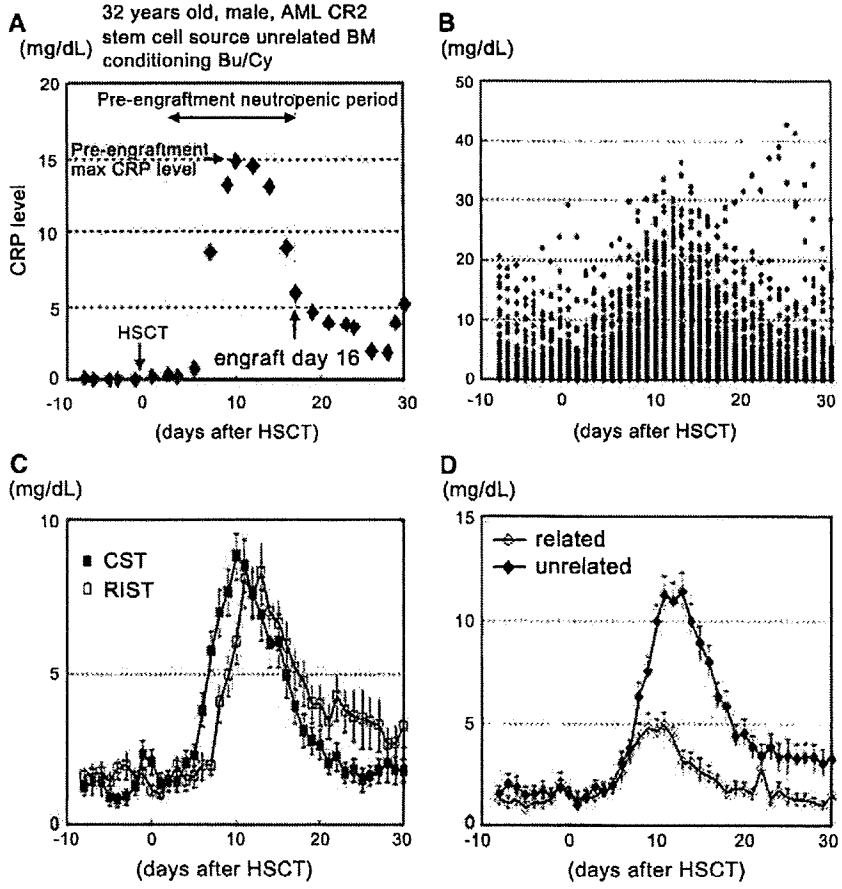

Figure 1. An example of how we measured CRP in a representative patient (A). Dot plot of the CRP level. All patients (B), CST versus RIST (C) and related versus unrelated (D).

statistics were used. Student *t*, chi-square, Fisher's exact test, and Wilcoxon rank-sum tests were used to compare clinical and patient characteristics. To analyze the pretransplant risk factors for a high CRP level, logistic analysis was used. OS was estimated using Kaplan-Meier curves. The cumulative incidence of aGVHD and NRM was estimated based on a Cox regression model for cause-specific hazards by treating progressive disease or relapse as a competing event. Cox proportional hazard models were used for the multivariate analysis of variables in aGVHD, NRM, and OS after HSCT. Clinical factors that were assessed for their association with aGVHD included patient age, patient sex, donor sex, CMV serostatus, conditioning regimen (CST versus RIST), donor (human leukocyte antigen [HLA]-matched versus HLA-mismatched, related versus unrelated), GVHD prophylaxis (cyclosporine-based versus tacrolimus-based, short-term MTX versus no MTX) and disease risk (standard versus high risk). NRM and OS were also assessed for their association with these factors. Factors with $P < .10$ in the univariate analyses were subjected to a multivariate analysis using a multiple logistic analysis and Cox proportional hazard modeling. In Japan, only BM and CB are allowed for unrelated transplantation, and most transplantations with a related donor use PBSC as a stem cell source. Therefore, the stem cell source was not included as a factor in the multivariate analysis. A level of $P < .05$ was defined as statistically significant. All P values are 2-sided. All analyses were made with SPSS ver 10.0 statistical software (Chicago, IL). This analysis was approved by the institutional review board.

RESULTS

Infections

The median duration of follow-up in surviving patients was 965 days (61 to 1432 days) in the high-CRP group and 915 days (76 to 1803 days) in the low-CRP group, and the incidence of total documented infections during neutropenia was, respectively, 23 cases in the high-CRP group (34%) and 27 cases in the low-CRP group (17%, $P = .004$). The incidence of bacteremia was, respectively, 20 cases (30%) and 20 cases (13%, $P = .002$), and the incidence of pneumonia was 7 cases (10%) and 4 cases (3%, $P = .01$). The incidence of central venous catheter infection was, respectively, 4 cases (6%) and 7 cases (4%, $P = .63$).

Serial changes in the CRP level are shown in Figure 1B; in most cases, the CRP level was elevated within 2 weeks of HSCT. Stratified data according to conditioning regimen (CST versus RIST) or relation to donor (related versus unrelated) are shown in Figure 1C and D, respectively.

To clarify the pretransplant risk factors for high CRP values during neutropenia, we performed a logistic regression analysis, which showed that male, unrelated donor, stem cell source with BM or CB transplantation (versus PBSCT), HLA-mismatched donor, and immunosuppression with MTX were associated with high CRP values during neutropenia (Table 1). Factors that showed significant associations ($P < .1$) were subjected to a multiple logistic regression analysis, and the results showed that unrelated donor, HLA mismatch and male sex were associated with high CRP ($P < .001$, $P = .005$, $P = .028$, respectively), as shown in Table 3. The median CRP levels after CST and RIST were 10.5 (0.3-31.3) and 6.2 (0.1-42.7), respectively, with a significant difference ($P = .017$) (Table 2). Notably, within the RIST group, the median CRP level was significantly lower in related than in unrelated transplantation (1.6 mg/dL [0.1-9.7] veruss 16.2 mg/dL [0.5-42.7]: $P < .001$). However, the logistic analysis failed to disclose any overall significant difference between CST and RIST.

Primary Outcomes

The cumulative incidences of aGVHD grade II-IV and grade III-IV are shown, respectively, in Figure 2A and B. Grade II-IV and grade III-IV aGVHD were both more frequent in the high-CRP group than in the low-CRP group ($P = .001$ and $P = .04$, respectively). A Cox proportional hazard model showed that a high CRP level and CMV serostatus were associated with an increased risk of grade II-IV aGVHD (Table 4). Similar results were obtained when we included only the patients who received a myeloablative conditioning regimen (grade II-IV aGVHD 25% in the low-CRP group and 58% in the high-CRP group, $P < .001$, grade III-IV aGVHD 7% in the low-CRP group and 21% in the high-CRP group, $P = .047$).

Secondary Outcomes

OS and NRM are shown, respectively, in Figure 3A and B. OS was significantly worse in the

Table 3. *Multiple Logistic Regression Analysis of Risk Factors for High CRP during Neutropenia*
*Factors with $P < .10$ in a Multivariate Analysis Was Shown**

Outcomes and Variables	Multiple Logistic Regression Analysis		
	Odds	95% CI	P Value
Unrelated donor	4.6	2.2-9.6	<.001
HLA mismatch	2.6	1.3-5.0	.005
Patient sex (male)	2.1	1.1-4.2	.0028

CRP indicates C-reactive protein; CI, confidence interval; HLA, human leukocyte antigen; CMV, cytomegalovirus.
*Factors included in univariate analysis: patient sex, donor sex, CMV serostatus, use of short-term MTX, relation to donor, HLA mismatch, conditioning, GVHD prophylaxis, stem cell source.

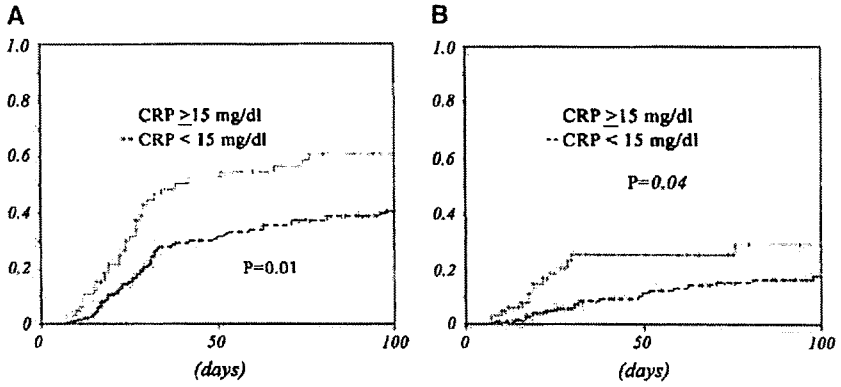

Figure 2. Cumulative incidence of grade II-IV aGVHD (A) and grade III-IV aGVHD (B) stratified according to the maximal CRP level during neutropenia.

high-CRP group than in the low-CRP group (1-year OS 47% versus 75%, $P = .001$). NRM was significantly higher in the high-CRP group than in the low-CRP group (1-year NRM 47% versus 13%, $P < .001$). Similar results were obtained when we included only patients who received a myeloablative conditioning regimen (1-year NRM 8% in the low-CRP group and 38% in the high-CRP group, $P = .007$). A Cox proportional hazard model showed that the risk factors for poor OS were high CRP ($P = .002$, hazard ratio [HR] 2.0, 95% confidence interval [CI] 1.3-3.1) and high-risk disease ($P = .015$, HR 2.2, 95% CI 1.2-4.0), whereas those for high NRM were high CRP ($P < .001$, HR 4.0, 95% CI 2.0-8.0) and high-risk disease ($P = .029$, HR 2.6, 95% CI 1.1-6.2), as shown in Table 4. When the threshold was set at 15 mg/dL, the sensitivity and specificity of the CRP level for prediction of grade II-IV aGVHD, NRM, or OS were 37% and 75%, 59% and 79%, and 40% and 78%, respectively. The relapse rate was significantly lower in the high-CRP group than in the low-CRP group (1-year relapse 21% versus 33%, $P = .02$).

Causes of death are summarized in Table 5. A total of 57 patients (36%) in the low-CRP group and 39 patients (58%) in the high-CRP group died ($P = .002$, OR 2.4 [1.4-4.4]). Six patients (4%) in the low- and 5 (7%) in the high-CRP group died because of aGVHD, for example, death because of infectious diseases associated with aGVHD and its treatment. Seven patients (4%) in the low- and 11 (16%) in the high-CRP group ($P = .003$, OR 4.2 [1.6-11.4]) died because of chronic GVHD (cGVHD), including death because of infectious diseases associated with cGVHD and its treatment. No patient (0%) in the low- and 5 (7%) in the high-CRP group ($P = .002$) died because of infectious diseases excluding infectious disease concomitant with GVHD. No patient in the low-CRP group and 4 (6%) in the high-CRP group ($P = .008$) died because of multiple-organ failure (MOF) excluding MOF because of GVHD and infectious disease.

DISCUSSION

The results of this retrospective study suggested that higher CRP values during the neutropenic period may reflect net inflammation secondary to tissue damage because of the conditioning regimen, infection, and subsequent allogeneic immune reactions, all of which lead to aGVHD/cGVHD and ultimate NRM. In a mouse model, the concept that the production of inflammatory cytokines plays an important role in the development of aGVHD, by affecting the afferent and effector phase [12,13], has been accepted. Cooke et al. [26] showed that LPS antagonism reduced aGVHD in a mouse model, as indicated by Ferrara et al. [4]. However, in human studies, the value of determining individual levels of cytokines to monitor aGVHD has not been fully explored, because this approach is very costly and requires sophisticated techniques, which impedes its universal applicability. On the other hand, CRP is already being widely used

Table 4. *Multiple Variate Analysis for aGVHD, NRM, and OS**

Outcomes and Variables	Hazard Ratio	95% CI	P value
Grade II-IV aGVHD			
High CRP	1.7	1.1-2.6	.02
CMV positivity	3.1	1.0-9.8	.5
Disease risk (high)	1.6	0.9-2.7	.10
NRM			
High CRP	4.0	2.0-8.0	<.001
Age (≥40 years old)	1.9	0.9-3.9	.07
Disease risk (high)	2.6	1.1-6.2	.03
OS			
High CRP	2.0	1.3-3.1	.002
Disease risk (high)	2.2	1.2-4.0	.02

CRP indicates C-reactive protein; CI, confidence interval; CMV, cytomegalovirus; GVHD, graft-versus -host disease; TBI, total body irradiation; NRM, nonrelapse mortality; OS, overall

*Factors included in univariate analysis: patient sex, donor sex, CMV serostatus, use of short-term MTX, relation to donor, HLA mismatch, conditioning, GVHD prophylaxis, stem cell source

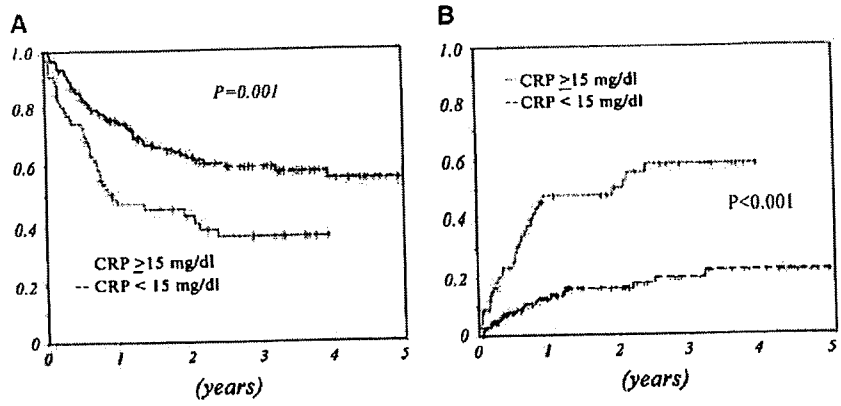

Figure 3. OS stratified according to the maximal CRP level during neutropenia (A). Cumulative incidence of TRM stratified according to the maximal CRP level during neutropenia (B).

worldwide, especially in Japan, to distinguish bacterial infections from other causes of fever [15-19]. Based on this practice, we reviewed the value of the CRP level after HSCT, and our data suggest that it might be useful to monitor the CRP value as a net surrogate marker for produced cytokines, and for predicting the subsequent development of aGVHD and NRM.

Our patients had various interacting backgrounds, and it is still difficult to predict whether a patient with a high CRP level is destined to suffer from GVHD or major infectious complications. Infectious diseases were previously reported to be a primary cause of elevated CRP [8,20], which might, in turn, affect the severity of aGVHD. In this study, we made every effort, including intense culture studies, to exclude infection as a primary cause of increased CRP, and showed that there were significantly more documented infections in the high-CRP group than in the low-CRP group. Current practice for the prevention of infection mostly focuses on the effective control of Gram-negative bacteria, considering the potent immediate pathologic effect of the organisms. However, if the hypothesis that decreasing the net production of cytokines is important for the prevention of subsequent GVHD is correct, more effort should be paid to broadly cover other types of organisms or even clinically less significant infection, that is, stomatitis, at least during the early period of neutropenia, particularly in patients carrying risk factors for high CRP, which included unrelated donor, HLA mismatch, BM, and CB transplantation in this study. The addition of other markers, such as procalcitonin, may be useful for identifying the risk of major infectious complications [24].

Table 5. *Causes of Death Stratified According to CRP Value during Neutropenia*

Causes of death	Low CRP Group CRP < 15 mg/dL n = 157	High CRP Group CRP ≥ 15 mg/dL n = 67	*P* Value
Total	57 (36%)	39 (58%)	.002
Relapse/progressive disease	34 (22%)	8 (12%)	.09
acute GVHD (total)	6 (4%)	5 (7%)	.25
acute GVHD	5 (3%)	3 (5%)	.63
acute GVHD + infection	1 (1%)	2 (3%)	.16
chronic GVHD (total)	7 (4%)	11 (16%)	.003
chronic GVHD	3 (2%)	7 (10%)	.005
chronic GVHD + infection	4 (3%)	4 (6%)	.21
Infection*	0 (0%)	5 (7%)	.002
MOF†	0 (0%)	4 (6%)	.008
Respiratory failure‡	3 (2%)	4 (6%)	.11
Others	Stroke 2 VOD 2 Secondary cancer 1 Unknown 2	VOD 1 Myocardial infarction 1	

CRP indicates C-reactive protein; GVHD, graft-versus-host disease; TBI, total-body irradiation; MOF, multiple organ failure; VOD, veno-occlusive disease.

*Excluding infection during GVHD or GVHD treatment.

†Excluding MOF due to GVHD, infection.

‡Excluding respiratory failure because of GVHD, infection, and MOF.

Tissue damage caused by the conditioning regimen, complicated infections, and allogeneic immune reactions are the primary factors that are associated with the initial elevation of CRP early in the course of allogeneic HSCT. Consequently, it can be speculated that a reduced-intensity conditioning regimen results in decreased cytokine release and a resultant lower CRP value, which may lead to less chance of developing GVHD. Although the RIST regimens we used were relatively dose-intense, in this retrospective review we still found that CRP levels tended to be decreased after RIST compared to conventional myeloablative transplantation, particularly in a related compared to an unrelated transplantation setting. Because augmentation of allogeneic immune and inflammation reactions may induce a higher CRP value, we speculate that the benefit of RIST is diminished when a strong allogeneic reaction is induced, as in cases of unrelated transplantation.

To further evaluate the relationship between a higher CRP value during neutropenia and common risk factors associated with transplantation, we performed a multivariate analysis and showed that unrelated donor, HLA mismatch, and male sex were associated with higher CRP values. Additionally, from the finding in the multivariate analysis that unrelated donor and HLA mismatch were independently associated with high CRP, we surmised that the degree of genetic disparity might be associated with higher CRP during neutropenia. Based on a consideration of these findings together, we think that a higher CRP value may reflect the degree of tissue damage because of the transplant regimen and the subsequent magnitude of allogeneic immune reactions. Nevertheless, our analysis was hampered, because in Japan only BM and CB are allowed for unrelated transplantations, and most transplantations with a related donor use PBSC as a stem cell source. In these settings, a theoretically longer neutropenic period after unrelated BM or CB transplantation might be associated with a higher risk of infection, which could lead to higher CRP, as shown in this study.

In this study, the primary causes of death in the low-CRP group were mainly relapse and progression, whereas in the high-CRP group this was NRM. Notably, the observation that the relapse rate was higher in the low-CRP group than in the high-CRP group, as previously suggested by Min et al. [23], may further support our hypothesis that serum CRP values represent overall inflammation and cytokine production, which paves the way to GVHD and related graft-versus-leukemia (GVL) effects. A possible reason for this finding is that a low CRP level resulted in a lower incidence of GVHD and a resultant decrease in the GVL effect, or the high-CRP group developed earlier and more-frequent death from NRM compared to the low-CRP group, which left fewer patients for evaluation of the later occurrence of relapse.

In conclusion, our results suggest that the CRP value in the neutropenic period before engraftment in patients undergoing allogeneic HSCT may be a net surrogate marker of early inflammation that leads to the development of aGVHD/cGVHD and subsequent NRM, as has been proposed in mouse models. The intensity of the conditioning regimen, infectious diseases, and degree of allogeneic immune response attributed to HLA compatibility and the stem cell source may be the major factors that predict higher CRP values. Based on the results of this retrospective study, future clinical studies to evaluate the feasibility of earlier intervention and adjustment of the procedure for preventing GVHD and NRM based on monitoring of the early CRP value are warranted.

ACKNOWLEDGMENTS

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Interleukin (IL)-4 promotes T helper type 2-biased natural killer T (NKT) cell expansion, which is regulated by NKT cell-derived interferon-γ and IL-4

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Summary

CD1d-restricted natural killer T (NKT) cells can rapidly produce T helper type 1 (Th1) and Th2 cytokines and also play regulatory or pathological roles in immune responses. NKT cells are able to expand when cultured with α-galactosylceramide (α-GalCer) and interleukin (IL)-2 in a CD1d-restricted manner. However, the expansion ratio of human NKT cells is variable from sample to sample. In this study, we sought to determine what factor or factors are responsible for efficient *in vitro* expansion of NKT cells from various inbred mouse strains. Although the proportion of NKT cells in the spleen was nearly identical in each mouse strain, the growth rates of NKT cells cultured *in vitro* with α-GalCer and IL-2 were highly variable. NKT cells from the B6C3F1 and BDF1 mouse strains expanded more than 20-fold after 4 days in culture. In contrast, NKT cells from the strain C3H/HeN did not proliferate at all. We found that cell expansion efficiency correlated with the level of IL-4 detectable in the supernatant after culture. Furthermore, we found that exogenous IL-4 augmented NKT cell proliferation early in the culture period, whereas interferon (IFN)-γ tended to inhibit NKT cell proliferation. Thus, the ratio of production of IL-4 and IFN-γ was important for NKT cell expansion but the absolute levels of these cytokines did not affect expansion. This finding suggests that effective expansion of NKT cells requires Th2-biased culture conditions.

Keywords: natural killer T cell; interleukin-4; interferon-γ; glycolipid

Introduction

Mouse natural killer T (NKT) cells were initially identified as a T-cell subset that expresses NK cell receptors such as NK1·1, CD94 and Ly49.[1,2] The majority of NKT cells have the invariant T-cell receptor (TCR) α-chain rearrangement Vα14-Jα18 and recognize antigens presented by CD1d, a non-classical major histocompatibility complex (MHC) class I molecule.[3,4] NKT cells are continuously sensitized by endogenous antigens so that they display an effector-memory phenotype (such as $CD62L^{low}$ $CD44^{high}$)[5–7] and rapidly produce large amounts of T helper type 1 (Th1) and Th2 cytokines when stimulated with lipid antigens such as α-galactosylceramide (α-GalCer) in a CD1d-dependent manner.[2,8] NKT cells are regarded as immunoregulatory because of their cytokine profile. Moreover, NKT cells are thought to play an important role in response to infectious agents and in pathological responses such as allergies or autoimmune disease. NKT cells are cytotoxic to various tumour cell lines via Fas-ligand-, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)- and/or perforin-dependent pathways,[9–12] and play a role in tumour surveillance.[13] NKT cells activated by interleukin (IL)-12 or α-GalCer sequentially activate natural killer (NK) cells by producing interferon (IFN)-γ and induce antitumour immune responses. This in turn inhibits tumour metastasis and can suppress solid tumour growth. In some studies, it has been suggested that this ability helps to induce tumour antigen-specific CD8 T cells, thereby making an additional contribution to the immune response to cancer.[14]

In humans, counterparts of mouse NKT cells have also been found to be responsive to α-GalCer, which induces them to secrete IL-4 and IFN-γ. In addition, they have been shown to be cytotoxic to tumour cells via two different mechanisms, a CD1d-dependent and a CD1d-independent mechanism.[15] Human NKT cells have the

potential to induce antitumour responses *in vivo*. However, in patients with malignancies,[16,17] NKT cells are reduced in number and activity, and *in vivo* activation by α-GalCer leads to transient activation and long-term unresponsiveness of NKT cells.[18,19] For that reason, adaptive transfer of *in vitro* expanded and/or activated NKT cells is expected to induce effective antitumour responses.

To date, several combinations of cytokines with α-GalCer have been reported to expand NKT cells isolated from peripheral mononuclear cells. However, NKT cells present a diverse range of expansion ratios even among healthy individuals.[20,21] Although a previous study suggested that differences in NKT cell proliferation are associated with the age of the donor,[22] there is still much that remains to be determined concerning additional factors that influence NKT cell proliferation.

In this study, we used inbred mouse strains as an experimental system in which to reveal factors that affect variation in proliferation rates among individuals. Previously, we found that *in vitro* expanded NKT cells from C57BL/6 mice retained an effector-memory-like phenotype and retained the ability to produce cytokines.[23] In addition, we found that there was a marked difference in the NKT cell expansion ratio among various mouse strains and that the differences were closely related to the bias in production of Th1 or Th2 cytokines by NKT cells. Finally, we report that a relatively low rate of proliferation can be enhanced by the addition of IL-4, which creates Th2-biased culture conditions.

Materials and methods

Mice

Female C57BL/6N, BALB/cA, C3H/HeN, DBA/2N (C57BL/6 × DBA/2)F_1 (BDF1), (C57BL/6 × C3H/HeN)F_1 (B6C3F1), and SJL/J mice were purchased from Charles River Japan (Kanagawa, Japan). All mice, which were maintained in our animal facilities, were 8–11 weeks of age at the time of the experiment. All animal protocols for this study were reviewed and approved by the committee for ethics of animal experimentation at the National Cancer Center of Japan prior to the beginning of the study.

Monoclonal antibodies and reagents

Anti-IL-4 (clone 11B11) and anti-IFN-γ (clone R4-6A2) monoclonal antigen-neutralizing antibodies (mAbs) were obtained from the supernatant of a hybridoma culture maintained in serum-free medium in a CELLine CL-1000 flask (BD Biosciences, San Jose, CA) and purified by Protein G Sepharose (GE Healthcare Amersham Biosciences AB, Uppsala, Sweden) affinity column chromatography. Anti-CD16/32 (clone 2·4G2) was obtained from a hybridoma supernatant. Fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (clone 145-2C11), allophycocyanin (APC)-conjugated anti-IL-4 (11B11), anti-IFN-γ (XMG1·2), and a rat immunoglobulin G1 (IgG1) isotype control (clone R3-34) and Golgi Stop™ were obtained from BD Biosciences. α-Galactosylceramide (α-GalCer) was kindly provided by the Pharmaceutical Research Laboratory, KIRIN Brewery Co., Ltd (Gunma, Japan). The phycoerythrin (PE)-conjugated CD1d/α-GalCer tetramer was prepared using a baculovirus expression system as previously described.[24] Human recombinant IL-2 (rIL-2) was kindly provided by Takeda Chemical Industries Ltd (Osaka, Japan). Mouse rIL-4 was obtained from PeproTech EC Ltd (London, UK).

Flow cytometry

NKT cells were detected by multicolour flow cytometry as previously described.[23] Briefly, cells were preincubated with anti-CD16/32 mAb to block non-specific FcRγ binding and then stained with FITC-conjugated anti-CD3 and PE-conjugated CD1d/α-GalCer tetramer. Dead cells were excluded by propidium iodide staining and electronic gating. For detection of intracellular cytokines, cells were stimulated for 3 hr with phorbol 12-myristate 13-acetate (PMA) (25 ng/ml) and ionomycin (1 μg/ml), with the last 1 hr of stimulation in the presence of Golgi block, in a 37°, 5% CO_2 incubator, and then washed and incubated with anti-CD16/32 mAb, followed by incubation with FITC-conjugated anti-CD3 and PE-conjugated CD1d/α-GalCer tetramer. Cells were then permeabilized using Cytofix/Cytoperm (BD Biosciences) and IL-4 or IFN-γ was detected using APC-conjugated mAbs. Cells were analysed by flow cytometry (FACSCalibur; BD Biosciences).

NKT cell proliferation assay

Preparation of splenic mononuclear cells and *in vitro* expansion of NKT cells were performed as previously described.[23] Briefly, spleens of each mouse strain were macerated aseptically and pushed through a nylon mesh to obtain single-cell suspensions, and erythrocytes were lysed in ammonium chloride buffer. Mononuclear cells (1×10^6 cells/ml) were cultured with α-GalCer (50 ng/ml) and rIL-2 (100 IU/ml) in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO) supplemented with 8% fetal calf serum (JRH Biosciences, Lenexa, KS), 2-mercaptoethanol (5×10^{-5} M) 100 U/ml penicillin and 100 μg/ml streptomycin for 4 days in a 37°, 5% CO_2 incubator. After 4 days in culture, the absolute number of living cells was counted using a microscope after staining of cells with 0·2% trypan blue, and the relative percentages of NKT cells were determined by flow cytometry.

Cytokine production

The cell culture supernatant was collected after 24 hr or 4 days in culture and stored at −20°. The concentrations

of IL-4 and IFN-γ were determined by enzyme-linked immunosorbent assay (ELISA) (OptEIA ELISA set; BD Biosciences).

Results

α-GalCer-induced expansion of NKT cells from various mouse strains

Mouse NKT cells show a similar variation in expansion ratios to that observed for human NKT cells. We found that the expansion ratios were different for different mouse strains (Fig. 1). Before culture, spleen cell suspensions contained a small percentage (0·8–1·5%) and a small number (7–18 × 10^3 cells/ml) of NKT cells in each mouse strain. As shown in Fig. 1, culture of spleen cells with α-GalCer and IL-2 induced expansion of NKT cells, except for C3H/HeN mice. After 4 days of culture, NKT cells constituted 6·4–40·7% of cells in the culture and had expanded 7–25-fold in BALB/c, C57BL/6, DBA/2, B6C3F1 and BDF1 mice. The CD1d-restricted TCR α-chain Vα14 dominantly associates with the high-affinity TCR β-chain Vβ8·2, or the lower affinity chain Vβ8·3, Vβ7 or Vβ2, and a genetic defect in Vβ8 is reportedly the cause of the low responsiveness of NKT cells. We next asked if the TCR-β status of NKT cells had an effect on expansion. However, we found no significant differences among the six strains that were tested, and selective proliferation did not occur (data not shown).

NKT cell proliferation ratio correlates with amount of IL-4 in supernatant from a 4-day culture

Previously, a high concentration of IL-4 and IFN-γ in supernatant from a 4-day culture was observed.[23] Firstly, we measure amounts of IL-4 and IFN-γ in the culture supernatant.

An increase in the number of NKT cells was positively correlated with the production of IL-4 in the 4-day culture (Fig. 2a). However, high levels of IFN-γ were observed in all of the mouse strains, independent of an increase in either NKT cell number or IL-4 production. Almost all CD8 T cells acquired the ability to produce IFN-γ when activated indirectly via NKT cells by α-GalCer (data not shown), so it appears that, in C3H/HeN mice, NKT cells do not proliferate. Instead, it seems reasonable that a large amount of IFN-γ might be produced by the activated NK cells and CD8 T cells.[25,26]

A previous study reported cytokine secretion of NKT cells prior to their proliferation.[2,27] Thus, we harvested culture supernatants at 24 hr, before NKT cell expansion,[27] to determine the status of cytokine production at this early stage, which is the stage at which NKT cells initially respond to culture and initiate production of IL-4. This initial response positively correlated with NKT cell expansion to some degree, although the response was weaker than that observed for cells in culture for 4 days. It is notable that IL-4 production by C3H/HeN was more robust than that observed for C57BL/6, and IFN-γ

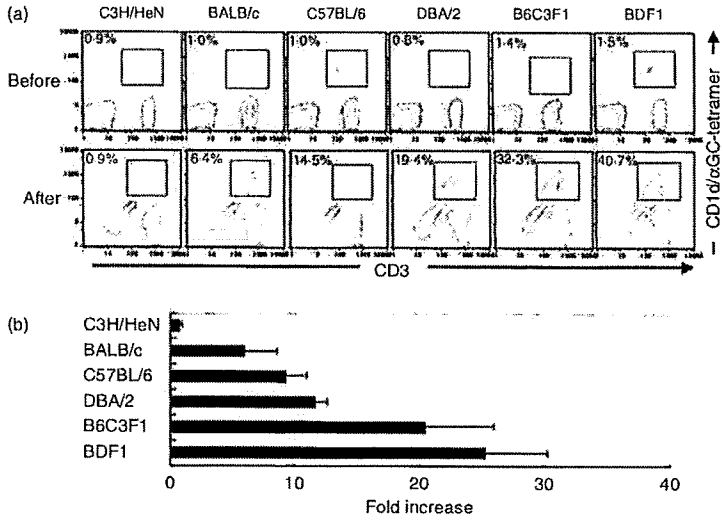

Figure 1. Expansion of natural killer T (NKT) cells *in vitro*. (a) Mouse spleen cells (1 × 10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 for 4 days. Cells were stained with anti-CD3 monoclonal antibody (mAb) and CD1d/α-GalCer tetramer and analysed by flow cytometry. The percentage of NKT cells was determined for both fresh (upper row) and cultured (lower row) cells. Representative results from replicate experiments are shown. (b) The fold increase in NKT cells after culture was calculated based on living cell counts and the percentage of NKT cells in the total cell population. Data are shown as mean ± standard error of the mean (n = 9 for C3H/HeN, BALB/c and C57BL/6; n = 4 for DBA/2, B6C3F1 and BDF1).

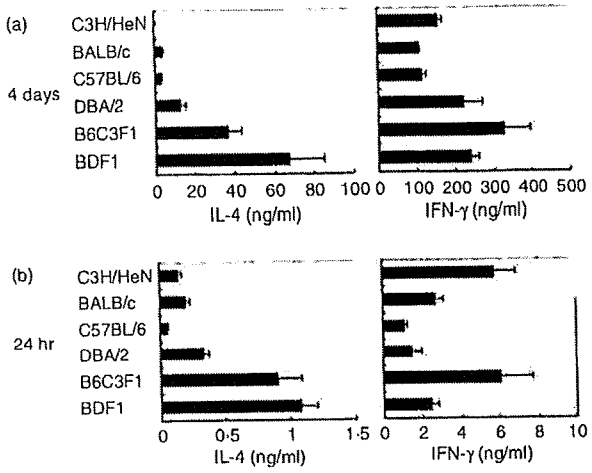

Figure 2. Production of interleukin (IL)-4 and interferon (IFN)-γ in expansion cell culture supernatants. Mouse spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml IL-2 for 4 days. Supernatants were collected after 24 hr (b) or 4 days (a). The levels of IFN-γ and IL-4 in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA). Data are shown as mean ± standard error of the mean ($n = 9$ for C3H/HeN, BALB/c and C57BL/6; $n = 4$ for DBA/2, B6C3F1 and BDF1).

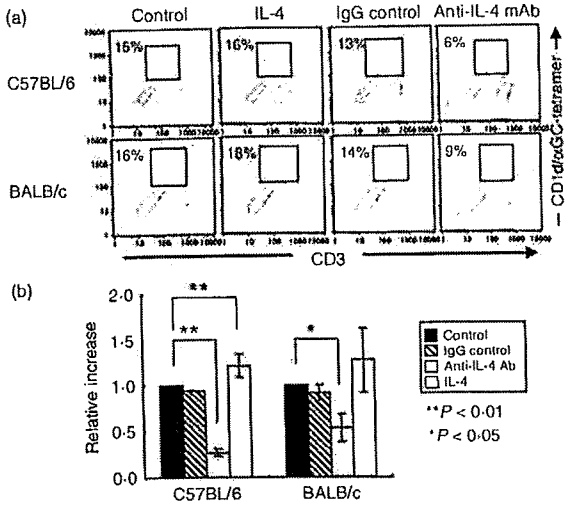

Figure 3. Expansion of natural killer T (NKT) cells in the presence or absence of interleukin (IL)-4. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml IL-2 for 4 days with IL-4 (10 ng/ml) or anti-IL-4 monoclonal antibody (mAb) (1 mg/ml). The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with control expansion culture. Data are shown as mean ± standard deviation for five independent experiments. A paired two-tailed Student's t-test was used for statistical analysis ($^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$).

production of C3H/HeN mice was much higher than that of other strains (Fig. 2b). These observations lead us to speculate that IL-4 and IFN-γ produced by NKT cells work as promoting and suppressing factors, respectively, during NKT cell proliferation.

NKT cell proliferation partially depends on IL-4 and is enhanced by Th2 cytokines

We next examined the influence of IL-4 on NKT cell proliferation *in vitro*. Proliferation of these cells was accelerated by addition of IL-4 at the start of the culture period, an effect that could be partially suppressed by neutralization of IL-4 (Fig. 3). In the C3H/HeN strain, where proliferation of NKT cells was not robust, a more significant induction of proliferation by IL-4 was observed (Fig. 4). In addition, neutralization of IFN-γ using antibodies did not significantly change the proportion of NKT cells in the total cell population. However, this did appear to up-regulate the total number of living cells and lead to a concomitant increase in the total number of NKT cells (Fig. 4b). Only NKT cells can produce IL-4 when cultured with α-GalCer and IL-2,[23] so IL-4 must act as an autocrine growth factor in the expansion of NKT cells in this context.

The proportion of intracellular IFN-γ high positive NKT cells is reduced by addition of IL-4

Exogenous IL-4 promoted NKT cell expansion in C3H/HeN mice, as shown in Figs 3 and 4. We next examined

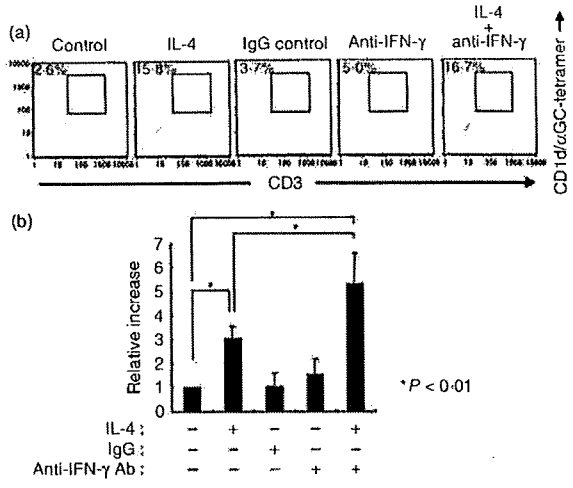

Figure 4. Expansion of natural killer T (NKT) cells from C3H/HeN strain mice in conditions that favour production of T helper type 2 (Th2)-biased cytokines. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 and with IL-4 (10 ng/ml) and/or anti-interferon (IFN)-γ monoclonal antibody (mAb) (1 mg/ml) for 4 days. The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with the control expansion culture. Data are shown as mean ± standard deviation for seven independent experiments. A paired two-tailed Student's t-test was used for statistical analysis ($^*P < 0{\cdot}01$).

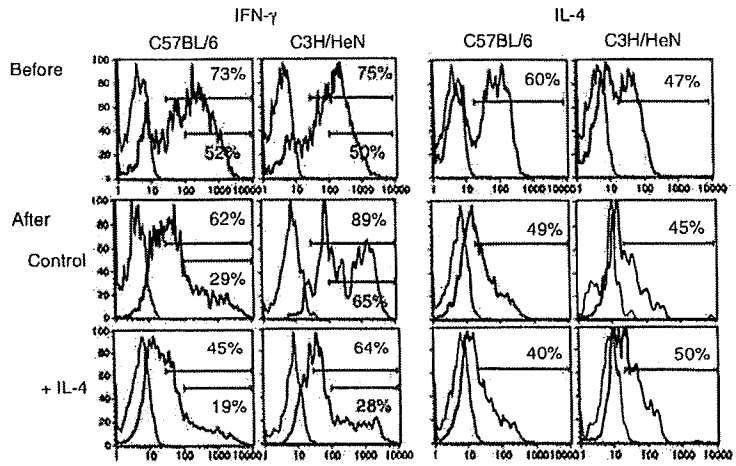

Figure 5. Cytokine production profile of natural killer T (NKT) cells treated with interleukin (IL)-4. Intracellular cytokine staining for interferon (IFN)-γ and IL-4 in NKT cells that were fresh (upper), cultured (middle), or cultured with additional IL-4 (lower) is shown. The cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 3 hr, stained with anti-CD3 monoclonal antibody (mAb), CD1d/α-galactosylceramide (α-GalCer) tetramer and anti-IFN-γ, anti-IL-4, or an isotype control mAb, and then detected and sorted via flow cytometry. Histogram panels for CD1d/α-GalCer-tetramer$^+$ CD3$^+$ cells are shown. Closed histograms indicate isotype controls. The percentage of total positive and high positive cells are indicated in the histograms. Data are representative of replicate experiments.

whether NKT cells cultured in Th2 conditions produced IFN-γ and IL-4. After 4 days of culture with α-GalCer and IL-2, intracellular IFN-γ- and IL-4-positive NKT cells were observed in both strains of mice. However, the proportion of intracellular IFN-γ high positive NKT cells was reduced when the cells were cultured with additional IL-4 (Fig. 5). In contrast to IFN-γ, the proportion of IL-4-positive NKT cells did not differ between cultures with and without IL-4. Therefore, NKT cells expanding as a result of induction with additional IL-4 displayed a polarized Th2 phenotype.

NKT cell expansion is accelerated by Th2-biased cytokine conditions

The SJL/J mouse strain is defective in cytokine production by NKT cells, as a consequence of a loss of high-affinity TCR to CD1d, which results from a deletion of the TCR Vβ8 subfamily genomic loci.[28,29] The proportion of NKT cells in the spleens of these mice was lower than that observed for other strains (Fig. 6a), and IFN-γ and IL-4 production after α-GalCer stimulation was also lower than that observed for other strains tested in this study (data not shown). NKT cells from SJL/J mice proliferated even in the absence of additional IL-4, as was observed for NKT cells from C57BL/6 mice. Moreover, similar to findings for NKT cells from C3H/HeN mice, the NKT cell proliferation effect could be enhanced by addition of IL-4 and further enhanced by addition of IL-4 combined with neutralization of IFN-γ (Fig. 6b).

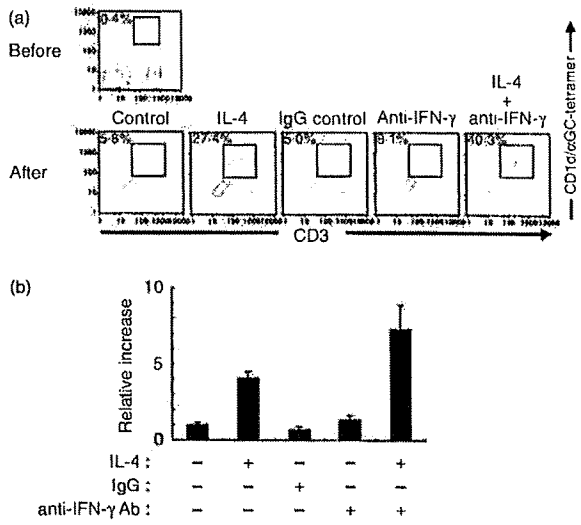

Figure 6. Expansion of natural killer T (NKT) cells from SJL/J mice *in vitro*. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 for 4 days with IL-4 (10 ng/ml) and/or anti-interferon (IFN)-γ monoclonal antibody (mAb) (1 mg/ml). The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with the control expansion culture. Data are shown as the mean of three wells ± standard deviation. Similar results were obtained in two independent experiments.

Discussion

In a previous study in which we induced expansion of NKT cells collected from human peripheral blood, we

observed wide variation in the efficiency of NKT cell expansion.[21] Similarly, when mouse NKT cells were induced to proliferate using similar methods in the present study, the ratios of expanding cell types were distinctly different in cells obtained from different mouse strains (Fig. 1). This suggests that genetic background influences or controls the difference in proliferation efficiency observed in humans and mice. However, we could not rule out the alternative possibility that the effect was a result of bipolar expansion of the cells, rather than originating from genetic variation in one or a few loci.

In this study, we have shown that the amount of IL-4 in the culture supernatant was related to the efficiency of NKT cell expansion induced by α-GalCer and IL-2. Previous studies revealed that addition of exogenous IL-2, IL-7 and IL-15 was able to augment NKT cell expansion by α-GalCer.[30–34] Similarly, in the present study we found that exogenous IL-2 augmented α-GalCer-induced NKT cell expansion in various mouse strains, with the exception of C3H/HeN mice. Moreover, addition of exogenous IL-4 promoted α-GalCer-induced NKT cell expansion in spleen cells from C3H/HeN mice. It has been shown that only NKT cells have the ability to produce IL-4 in this culture.[23] IL-4 might therefore be an autocrine or paracrine growth factor in α-GalCer-induced NKT cell expansion.

NKT cells, NK cells and some T cells when cultured with α-GalCer and IL-2 produce IFN-γ.[23] In contrast to IL-4, the amount of IFN-γ did not correlate with the efficiency of NKT cell expansion. Furthermore, we found that NKT cell proliferation in C3H/HeN mice was slightly increased by neutralization of IFN-γ in the culture. These results suggest that IFN-γ partially inhibits NKT cell expansion by α-GalCer. Interestingly, we found an inverse correlation between the IFN-γ:IL-4 ratio in the culture supernatant after 24 hr of culture and the efficiency of NKT cell proliferation (data not shown). Although higher amounts of IL-4 were detected in the culture of cells from C3H/HeN mice than in the culture of cells from C57BL/6 mice after 24 hr of culture, α-GalCer stimulated spleen cells from C3H/Ne mice produced higher amounts of IFN-γ and exhibited the highest IFN-γ:IL-4 ratio of all mouse strains tested. These results may explain the failure of NKT cell expansion in spleen cells from C3H/HeN mice.

The balance between the production of IFN-γ and the production of IL-4 by NKT cells is influenced by microenvironmental factors such as cytokines and antigen-presenting cells.[20,35–38] IL-7 and IL-12 selectively enhance IL-4 production by NKT cells.[35,36] Antigen-presenting cells such as α-GalCer-pulsed B cells selectively elicit weak IL-4 but not IFN-γ production from NKT cells.[37] There is a high IFN-γ:IL-4 ratio in cultures of spleen cells from C3H/HeN mice, which is caused by splenic NKT cells (A. Iizuka *et al.*, unpublished data) Moreover, it has been reported that the balance of IFN-γ:IL-4 production by NKT cells is developmentally controlled.[39,40] At immature stages, NKT cells predominantly produce IL-4, whereas IFN-γ secretion increases during the course of development.[39] Moreover, immature NKT cells have the ability to proliferate as compared with mature NKT cells.[39] Therefore, NKT cells in the spleen of C3H/HeN mice may be more mature than those of C57BL/6 mice, or contain only a few immature NKT cells. We assume that the failure of proliferation and the high IFN-γ:IL-4 cytokine production ratio of NKT cells in the spleen of C3H/HeN mice were attributable to their maturation stage.

Although IL-4 has opposite effects to IFN-γ and suppresses the Th1 immune response, IL-4 induces proliferation of human IL-13$^+$ NK cells[41] and CD8$^+$ T cells.[42] We found that Th2 culture conditions (in the presence of IL-4 and anti-IFN-γ mAb) facilitated NKT cell expansion induced by α-GalCer and IL-2 even in C3H/HeN and SJL/J mice. IL-4 also induces IFN-γ production by NK and NKT cells *in vivo*.[43] However, the proportion of IFN-γ-positive, but not IL-4-positive, NKT cells decreased when cells were cultured in the presence of IL-4. As in human immature IL-13$^+$ NK cells,[41] IL-4 may induce expansion of developmentally immature NKT cells which have a Th2-biased phenotype.

NKT cell maturation is controlled by the transcription factor T-bet.[44,45] Terminally differentiated NKT cells acquire a strong ability to produce IFN-γ and elicit cytotoxicity.[44] Assuming that expanded Th2-biased NKT cells after culture with α-GalCer, IL-2 and IL-4 are immature cells, it will be possible to induce terminally differentiated Th1-biased NKT cells for Th1 cell immunotherapy, such as cancer cell therapy.

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Insufficient *ex vivo* expansion of Vα24$^+$ natural killer T cells in malignant lymphoma patients related to the suppressed expression of CD1d molecules on CD14$^+$ cells

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Background

Vα24$^+$ natural killer T (NKT) cell is a human counterpart of mice Vα14$^+$ NKT cell that has a regulatory role for innate and acquired potential antitumor activity. The efficient expansion of NKT cells is an obstacle to the clinical application of Vα24$^+$ NKT cells for immunotherapy.

Methods

We used mononuclear cells (MNC) obtained from the peripheral blood (PB) of normal healthy donor (HD) and malignant lymphoma (ML) patients before and after granulocyte colony-stimulating factor (G-CSF) treatment. MNC were cultured for 12 days with α-galactosylceramide (100 ng/mL) and interleukin-2 (IL-2, 100 U/mL).

Results

The fold expansion of Vα24$^+$ NKT cells was higher in HD than in ML patients (208 versus 0.00), despite comparable numbers of Vα24$^+$ NKT cells before culture. G-CSF administration enhanced the predominance of Vα24$^+$ NKT cell fold expansion in HD compared with ML patients (1935 versus 1.95). After treatment with G-CSF, the expression of CD1d molecules was up-regulated in CD14$^+$ cells from HD but not ML patients. The fold expansion of Vα24$^+$ NKT cells and CD1d expression on CD14$^+$ cells was strongly correlated in both HD and ML patients ($r^2 = 0.84$). However, replacement of a patient's CD14$^+$ cells with HD cells did not increase the efficacy of Vα24$^+$ NKT cell expansion.

Discussion

G-CSF-mobilized PB from ML patients has inhibitory characteristics for Vα24$^+$ NKT cell expansion as a result of both monocytes and Vα24$^+$ NKT cells. Multiple procedures would be needed for the expansion of patients' Vα24$^+$ NKT cells.

Keywords

CD1d, α-galactosylceramide, granulocyte colony-stimulating factor, malignant lymphoma, natural killer T cells.

Introduction

Vα24$^+$ natural killer T (NKT) cells are the human counterpart of murine Vα14$^+$ NKT cells. Human NKT cells express a CD1d-restricted, invariant T-cell receptor (TCR) chain, the Vα24-JαQ α-chain, which is preferentially coupled with Vβ11 chains; human NKT cells share some common immunobiologic characteristics with murine NKT cells [1]. NKT cells recognize and respond to glycolipid antigen (Ag) presented by the CD1d molecule and produce large amounts of cytokines, such as interferon (IFN)-γ and interleukin (IL)-4, which exert strong cytotoxicity for various cancer cells and regulate autoimmunity, respectively [2,3]. The production of these cytokines has a critical and crucial role for initial immune

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responses and tumor rejection directly or indirectly via dendritic cells (DC), activated T cells and natural killer (NK) cells [4].

NKT cells are reactive to the non-classical class I Ag-presenting molecule CD1d and they recognize glycolipid Ag [2,5]. The CD1d family of MHC-unlinked class Ib molecules is conserved through mammalian species [6,7]. The CD1d molecule is necessary for immune responses to several microbial infections in mice [8]. With regard to tumor immunology, two major subpopulations of NKT cells, CD1d-restricted and CD1d-unrestricted, have been identified; CD1d-restricted NKT cells are mainly involved in tumor immunity but little is known about the characteristics of CD1d-unrestricted NKT cells [9]. What is known is that no NKT cells develop in the absence of CD1d [10].

α-Galactosylceramide (α-GalCer) is a specific ligand for human $V\alpha24^+$ NKT cells and murine $V\alpha14^+$ NKT cells [2]. Both types of NKT cells are activated by α-GalCer presented by CD1d. Because CD1d is a class I molecule expressed mainly on Ag-presenting cells (APC), such as DC, macrophages and B cells, it is speculated that NKT cells interact primarily with APC [5,11]. After stimulation with α-GalCer, $V\alpha24^+$ NKT cells exhibit CD1d-dependent cytotoxicity against various types of tumor cells [11,12]. In contrast, some researchers report that $V\alpha24^+$ NKT cells are cytotoxic against CD1d-negative cells; this observation suggests that α-GalCer is not essential for CD1d-independent cytotoxicity. Thus it is possible that $V\alpha24^+$ NKT cells involved in tumor immunity are activated through the recognition of the α-GalCer–CD1d complex, although the CD1d molecule is not necessary in some killing stages.

We are attempting to use NKT cells for adaptive immunotherapy [11,13]. However, the extremely low frequency of $V\alpha24^+$ NKT cells in human peripheral blood (PB) [2,14,15], which is even lower in cancer patients than in healthy individuals [12,16,17], 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 by several research groups, including ours. Previously, we observed that $V\alpha24^+$ NKT cells could be expanded effectively from human granulocyte colony-stimulating factor (G-CSF)-mobilized PB cells upon stimulation with α-GalCer and IL-2 [18], and we established an efficient non-fetal bovine serum (FBS) expansion system for $V\alpha24^+$ NKT cells to remove the potential risks related with FBS [19]. Consequently, we reported the essential effect of $CD14^+$ cells for *ex vivo* expansion of human NKT cells [20]. In the present report, we show that one mechanism of reduced expansion of $V\alpha24^+$ NKT cells in mononuclear cells (MNC) obtained from malignant lymphoma (ML) patients is the suppressed expression of the CD1d molecule on $CD14^+$ cells, and we discuss the important roles of the CD1d molecule on monocytes in the *ex vivo* expansion of human NKT cells.

Methods

Cells and plasma derived from healthy donors and ML patients

This study was approved by the National Cancer Center Institutional Review Board and written informed consent was obtained from the healthy donor (HD) volunteers and ML patients. PB and apheresis products were obtained from normal healthy individuals who donated PB stem cells for allogeneic transplants and from consecutive patients (from July 2004 to December 2004) with ML who would undergo autologous stem cell transplantation. In using apheresis products, leftover blood was used for healthy and autologous donors. Before and after G-CSF mobilization (pre- and post-G-CSF), the samples were manipulated immediately, and the cell fraction and plasma were separated by centrifugation at 3000 r.p.m. for 15 min. Plasma samples from both the PB and apheresis products were heat-inactivated immediately after separation and stored at −80°C before use. MNC were isolated from PB and apheresis products by Ficoll–Hypaque (Immuno-Biological Laboratories, Gunma, Japan) density-gradient centrifugation.

G-CSF procedure for apheresis donors

Apheresis was indicated for HD whose related patients needed PB stem cell transplantation or patients who would receive autologous stem cell transplantation after high-dose chemotherapy. This indication was determined by the clinical team of the stem cell transplantation unit in our hospital (National Cancer Center). G-CSF was administered subcutaneously at a dose of 300 $\mu g/m^2$ divided twice a day for 3 days just before the apheresis procedure. On the morning of the apheresis day, additional G-CSF was administered.

Cell-surface Ag analysis

We used mouse anti-human monoclonal antibodies (MAb) conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinium chlorophyll (PerCP). CD3–PE, CD4–PerCP, CD8–PE, CD14–FITC, CD19–PE and CD1d–PE MAb were purchased from BD Biosciences (Mountain View, CA, USA). Vα24–FITC, Vα24–PE and Vβ11–PE MAb were purchased from BD Pharmingen (San Diego, CA, USA). Lineage cocktail 1 (lin 1; CD3, CD14, CD16, CD19, CD20 and CD56)–FITC MAb were purchased from BD Immunocytometry Systems (San Jose, CA, USA). For cell-surface Ag staining, cells were incubated with MAb for 30 min on ice. After staining, cells were washed twice and resuspended in phosphate-buffered saline (PBS). Propidium iodide (Sigma-Aldrich, St Louis, MO, USA) staining preceded all experiments in order to remove dead cells. Data were acquired by flow cytometry (FACSCalibur; BD Biosciences) and analyzed using CellQuest software (BD Biosciences). A measurement of CD1d intensity in fresh CD14$^+$ cells was performed.

CD14$^+$ cell separation

Apheresis cells were collected from the bag and stained with CD14–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), CD14$^+$ 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 CD14$^+$ cells was determined to be >95% by flow cytometry, and low purity (less than 95%) samples were discarded. Both the CD14$^+$ and CD14$^-$ fractions were used for cross-culture experiments.

Expansion of Vα24$^+$ NKT cells

MNC were cultured in 6-well culture plates (Costar, Corning, NY, USA; product 3335) or culture flasks (Costar; product 3815) at 1.0×10^5 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, USA) for 9–12 days. The incubation environment was maintained at 37°C and contained 20% O_2 and 5% CO_2. Cells were cultured in AIM-V (Life Technologies, Rockville, MD, USA) supplemented with 5% autologous plasma. Fresh IL-2 was added every 3 days during culture to maintain its activity. After 12 days of culture, incubated cells were collected from flasks or plates and washed with PBS three times; differential markers were then analyzed by FACSCalibur (BD Biosciences). In the present report, Vα24$^+$ Vβ11$^+$ double-positive cells are defined as human NKT cells and denoted as Vα24$^+$ NKT cells.

Effect of CD14$^+$ cells on Vα24$^+$ NKT cell expansion

To evaluate the contribution of repeated CD14$^+$ cell supplements to Vα24$^+$ NKT cell expansion, we added CD14$^+$ cells to the CD14$^-$ fraction on day 0, 3, 6 or 9, or every 3 days. CD14$^+$ cells were collected from MNC by MACS (described below). The collected CD14$^+$ cells (4.0×10^5 cells) were added to the CD14$^+$-depleted fraction (CD14$^-$ fraction). We also tested several CD14$^-$ cell/CD14$^+$ cell ratios to evaluate the CD14$^+$ contribution to NKT cell proliferation.

Vα24$^+$ NKT cell expansion in co-culture with alternative CD14$^+$ cells

To determine whether CD14$^+$ cells derived from HD with G-CSF mobilization sustained the expansion efficacy for the patients' Vα24$^+$ NKT cells, we mixed G-CSF-mobilized HD CD14$^+$ cells and patient CD14$^-$ cells. A mixture of G-CSF-mobilized patient CD14$^+$ cells and HD CD14$^-$ cells was also tested. We compared the efficacy of the fold expansion under the following conditions: (1) a CD14$^+$ fraction with a CD14$^-$ fraction both from HD; (2) a CD14$^+$ fraction from a HD with a CD14$^-$ fraction from an ML patient; (3) a CD14$^+$ fraction from an ML patient with a CD14$^-$ fraction from a HD; and (4) a CD14$^+$ fraction with a CD14$^-$ fraction both from ML. After 12 days of culture with α-GalCer and IL-2, the expansion of Vα24$^+$ NKT cells was quantified.

Statistic analysis

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

Results

Impaired expansion of Vα24$^+$ NKT cells in ML patients

The group of HD consisted of six men and four women (median age 28 years, range 24—42). The group of ML patients consisted of three men and three women who had

Table 1. Patients' characteristics for their clinical status and course. Six consecutive apheresis patients for autologous stem cell transplantation were included in this study

UNP	age	sex	histology	ongoig therapy	length of time in CR (months)
#1	36	M	FL	EPOCH	1
#2	54	M	FL	ESHAP	2
#3	42	M	FL	EPOCH	10
#4	63	M	FL	EPOCH	2
#5	44	F	FL	EPOCH	6
#6	51	F	DLBCL	ESHAP	3

M, male; F, female; FL, follicular lymphoma; DLBCL, diffuse large B-cell lymphoma; EPOCH is a salvage regimen for refractory ML consisting of etoposide, methylprednisolone, vincristine, cyclophosphamide and doxorubicin, as is ESHAP, consisting of etoposide, methylprednisolone, high-dose cytarabine and cisplatin.

non-Hodgkin's lymphoma (median age 48 years; range 36–63) and were in a second complete remission (CR). Patients' characteristics are summarized in Table 1. The frequency of $V\alpha24^+$ NKT cells in PB was comparable in HD and ML patients before (Figure 1a,c) and after (Figure 1b,d) G-CSF administration. The fold expansion of cell number before G-CSF treatment was 208 in the HD group but only 1.00 in the ML patients, with a statistical

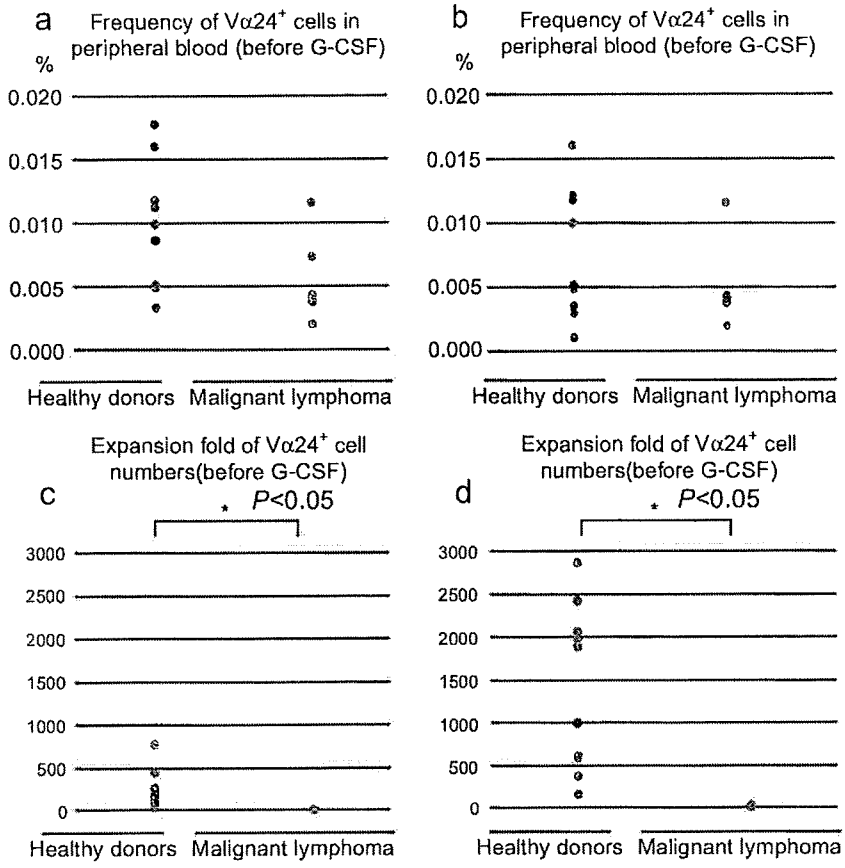

*Figure 1. Impaired expansion of $V\alpha24^+$ NKT cells in ML patients. The frequency of $V\alpha24^+$ $V\beta11^+$ NKT cells in PB was equivalent in HD (n = 10) and ML patients (n = 6) (a) before and (b) after G-CSF administration. However, the $V\alpha24^+$ NKT cells from the ML patients had a significantly lower fold expansion compared with the cells from HD both (c) before (0.00 versus 208) and (d) after G-CSF treatment (1.95 versus 1935). *P < 0.05. P-values were determined by using the Student's t-test.*

significance of $P=0.005$ (Figure 1c); however, the difference between the two groups after G-CSF treatment was more (1935 and 1.95, respectively), with a statistical significance of $P=0.036$ (Figure 1d). The fold expansion was calculated as the ratio of the absolute number of Vα24$^+$ NKT cells before and after culture (Figure 1c,d) [20].

Augmentation of CD1d expression on CD14$^+$ cells by G-CSF mobilization

The proportion of CD14$^+$ cells in PB was not significantly different between the HD and the ML patients; however, a higher proportion of monocytes was observed in HD compared with ML patients after G-CSF stimulation (60% versus 43%, respectively). In a steady state (without G-CSF mobilization), the expression of CD1d molecules on CD14$^+$ cells, CD20$^+$ cells, CD56$^+$ CD3$^-$ cells and CD3$^+$ CD4$^+$ cells in PB was low for both the HD and the ML patients. After *in vivo* administration of G-CSF in HD, the expression of CD1d molecules was up-regulated in CD14$^+$ cells (monocytes) and CD20$^+$ cells (B lymphocytes) (Figure 2a) but not in other cells, including CD56$^+$ CD3$^-$ cells (NK cells) and CD3$^+$ CD4$^+$ cells (CD4$^+$ T lymphocytes). However, in the ML patients the up-regulation of CD1d molecules was not detected in any population of mobilized PB cells (Figure 2b).

Correlation of the effective expansion of Vα24$^+$ Vβ11$^+$ NKT cells and CD1d expression on CD14$^+$ cells

We analyzed the relationship between the expansion efficacy of Vα24$^+$ NKT cells and CD1d expression on CD14$^+$ cells in 16 samples derived from HD ($n=10$) and ML patients ($n=6$). We analyzed G-CSF-mobilized

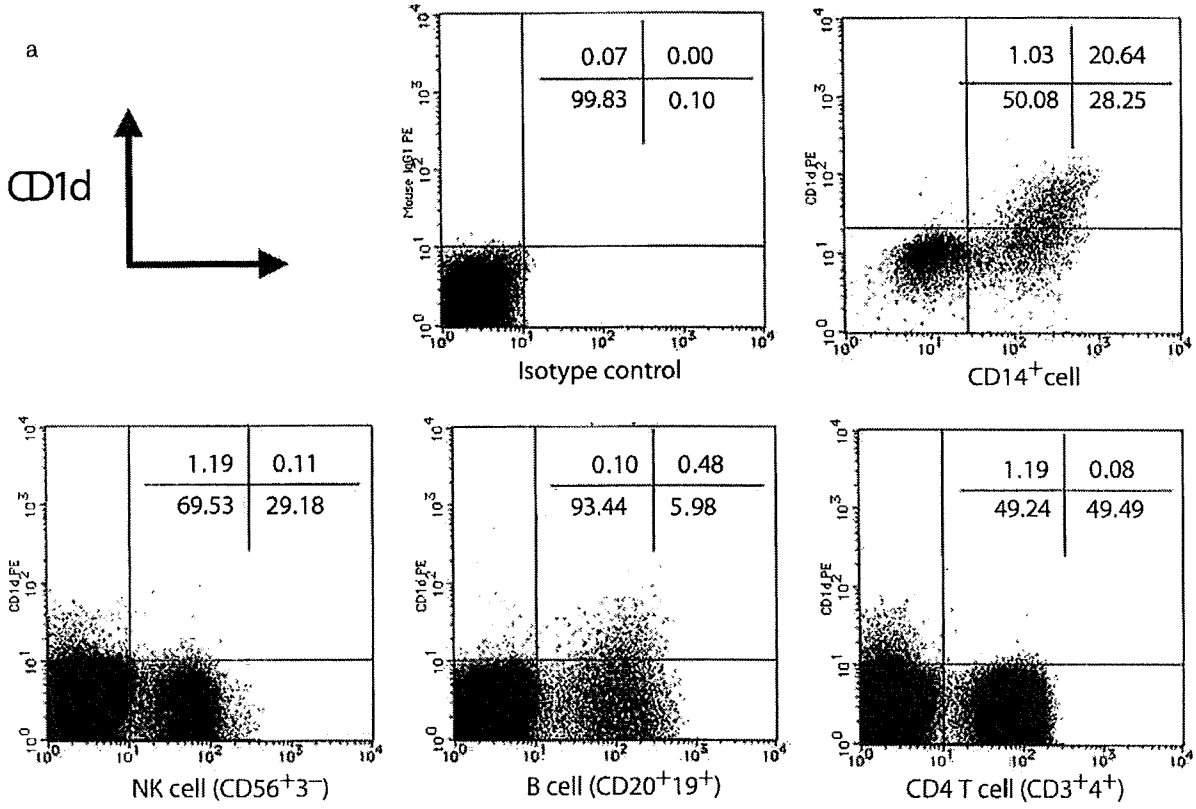

Figure 2. Induction of CD1d molecules on CD14$^+$ cells after G-CSF mobilization. (a) After G-CSF treatment in HD, the expression of the CD1d molecule was up-regulated in CD14$^+$ cells and CD20$^+$ cells but not in other cells, including CD56$^+$ CD3$^-$ cells and CD3$^+$ CD4$^+$ cells. (b) In ML patients, the up-regulation of CD1d molecules was not observed in any subpopulation of mobilized PB cells. The proportion of CD1d-positive CD14$^+$ cells was up-regulated to 25.88% in HD, while no apparent up-regulation was observed in ML patients (from 2.79%). The ratio of CD1d positivity 9.27 times (25.88/2.79). The numbers in each dot plot panel indicate the quadrant proportional status (%) of the gated cells.

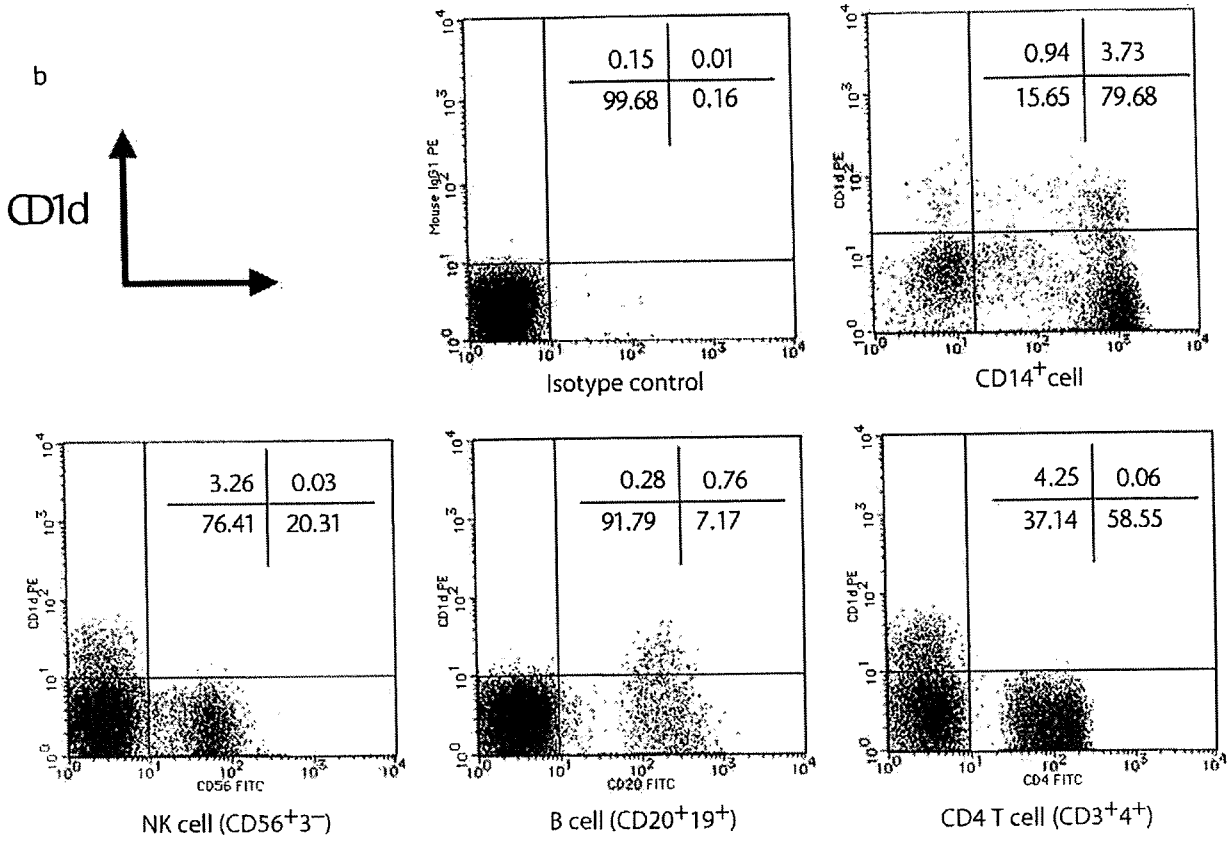

Figure 2 (*Continued*)

samples and found a strong correlation between these two factors in HD ($r^2 = 0.84$) but not in ML patients (Figure 3). These differences were not observed in MNC before G-CSF mobilization because the expression of CD1d on HD $CD14^+$ cells was similar to that of cells from ML patients (data not shown).

Extended survival of $CD14^+$ cells in the culture process of patients' MNC

The kinetics of cell populations from 10 HD and six ML patients were evaluated. The initial absolute number of CD1d-positive $CD14^+$ cells was lower in ML patients than HD but the $CD14^+$ cells derived from ML patients survived longer than HD (Figure 4a). The percentage of CD1d-positive $CD14^+$ cells in the total $CD14^+$ cells before culture was 25.25 $\pm$10.50% and 1.40 $\pm$1.56% in HD and ML patients, respectively. As presented in Figure 4b, the proportion of $CD14^+$ cells from ML patients was maintained at higher levels in culture medium than $CD14^+$ cells from HD.

Stimulation by $CD14^+$ cells rescued the NKT cell expansion in accordance with the intensity of CD1d expression

To test the influence of repeated stimulation by $CD14^+$ cells, we added $CD14^+$ cells to the culture system every 3 days or on day 0, 3, 6 or 9. In the case of HD, with an expression frequency of the CD1d molecule on $CD14^+$ cells of 25.00%, the repeated addition of $CD14^+$ cells promoted the expansion of $V\alpha24^+$ NKT cells (Figure 5a). However, in ML patients, with an expression frequency of the CD1d molecule on $CD14^+$ cells of 1.96%, repeated initiation of $CD14^+$ cells suppressed the expansion of $V\alpha24^+$ NKT cells (Figure 5b). These results were confirmed in four cases from each population.

$CD14^+$ cells derived from HD did not augment the expansion of $V\alpha24^+$ NKT cells from an ML patient

It is possible that the low level of CD1d expression on $CD14^+$ cells from ML patients was the origin of suppressed NKT expansion. If so, the replacement of