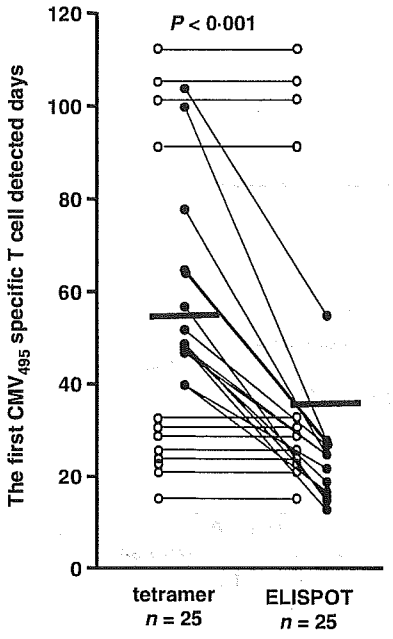


Fig 1. Earlier detection of cytomegalovirus (CMV)-specific T cells with the interferon-γ-enzyme-linked immunospot (IFN-γ-ELISPOT) assay than human leucocyte antigen (HLA) tetramer assay in patients after haematopoietic stem cell transplantations (HSCT). Data points indicate the first detected day of CMV_{495}-specific T cells in each patient (○ and ●) and the mean value (—). CMV-specific T cells was detected significantly earlier with the IFN-γ-ELISPOT assay than the HLA tetramer assay in patients after HSCTs ($P < 0·001$, paired t-test).

Threshold level of CMV-specific T cells for protection from CMV reactivation

To determine the level of CMV-specific T cells sufficient to protect CMV reactivation and infection, correlation between CMV-specific T-cell frequency and CMV antigenaemia was examined (Fig 3A). CMV antigenaemia was not detected in the patients who had absolute CMV-specific T cells over 1×10^7/L in the tetramer or over 1×10^6/L in the ELISPOT assay (Fig 3A), indicating that these threshold levels may be useful in aiding decisions regarding treatment for CMV reactivation and infection. The cumulative rate of patients who reached the threshold level (1×10^6/L) of CMV-specific T cells measured with the ELISPOT after various HSCTs was shown in Fig 3B. The patients who received RIST without ATG reached the threshold level significantly earlier than those who received RIST with ATG, or conventional HSCT.

Discussion

Development of reliable methods for the evaluation of immune status against CMV is clinically important for the management of CMV reactivation in transplant recipients. In this study, using the identified CMV_{495} epitope, which is strongly immunogenic for HLA-A*0201 or HLA-A*0206 patients, we compared various allogeneic HSCTs for the reconstitution of CMV immunity using both IFN-γ-ELISPOT and HLA tetramer assays. The frequency of CMV-specific T cells detected with the tetramer was, on average, 1·5-fold higher than that with the ELISPOT, probably because of the

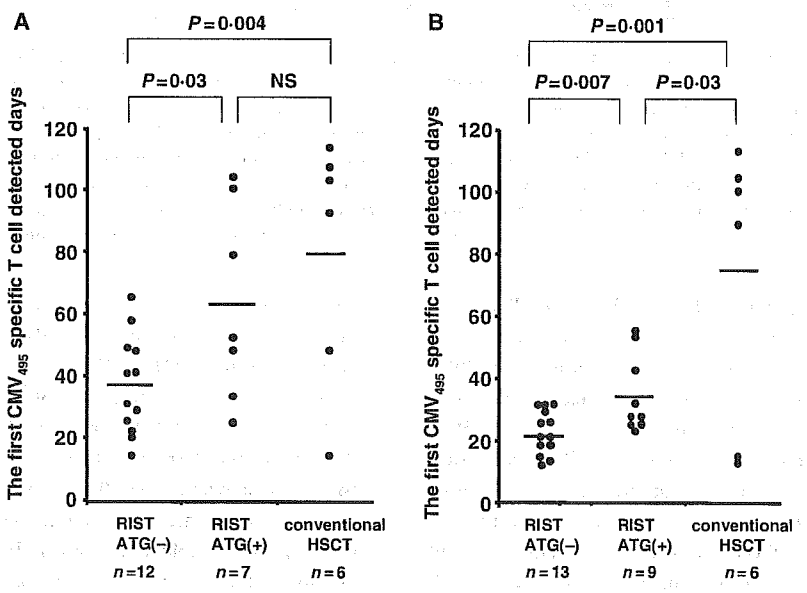
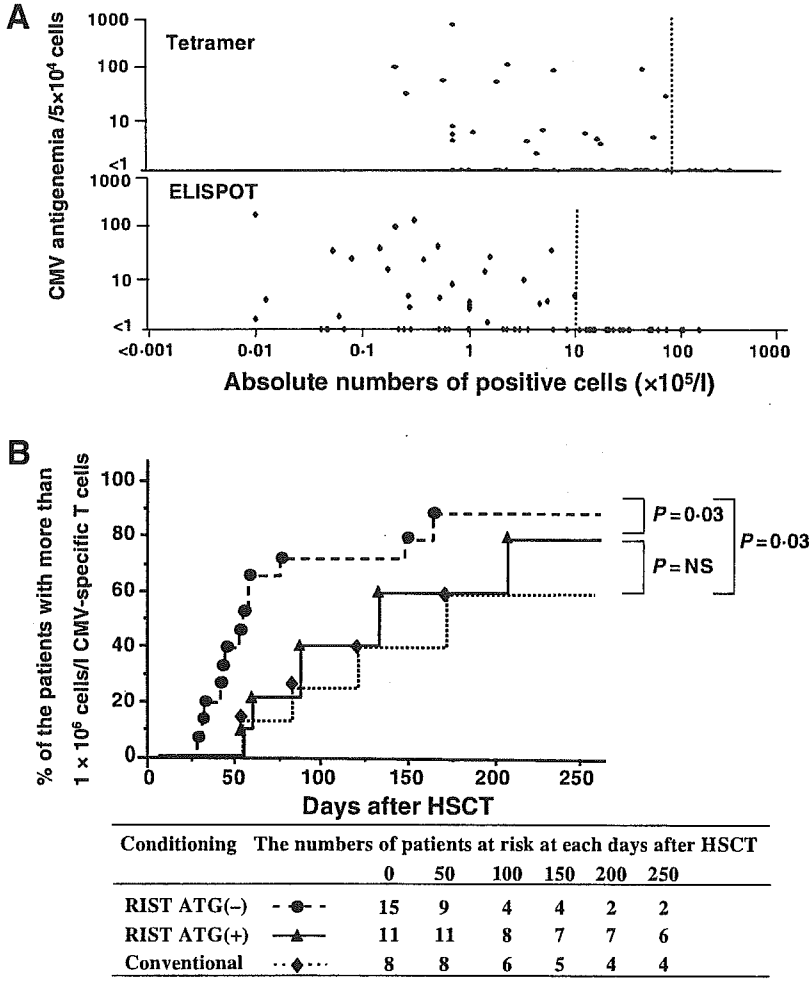


Fig 2. Earlier reconstitution of cytomegalovirus (CMV)-specific T cells in patients who received reduced-intensity transplantation (RIST) without anti-thymocyte globulin (ATG) than those who received RIST with ATG or conventional haematopoietic stem cell transplantation (HSCT). The first detected days of CMV_{495}-specific T cells determined with HLA tetramer assay (A) and interferon-γ-enzyme-linked immunospot assay (B), are shown among patients who received RIST without/with ATG and conventional HSCT. Data points indicate each patient (●) and the mean value (—).



Conditioning	The numbers of patients at risk at each days after HSCT					
	0	50	100	150	200	250
RIST ATG(–)	15	9	4	4	2	2
RIST ATG(+)	11	11	8	7	7	6
Conventional	8	8	6	5	4	4

Fig 3. (A) The threshold level (dotted line) of cytomegalovirus (CMV)-specific T cells for efficient protection from CMV antigenaemia. Data points show each patient's status of CMV antigenaemia and absolute numbers of CMV_{495}-specific T cells evaluated with the human leucocyte antigen (HLA) tetramer assay and interferon-γ-enzyme-linked immunospot (IFN-γ-ELISPOT) assay was more sensitive for detection of CMV-specific T cells than the HLA tetramer assay (ELISPOT assay). CMV reactivation was not observed when absolute numbers of CMV_{495}-specific T cells were over 1×10^7/l in the HLA tetramer assay, or over 1×10^6/l IFN-γ-ELISPOT positive cells. (B) Earlier reconstitution of CMV immunity to the threshold protection level in the patients who received reduced-intensity transplantation (RIST) without ATG compared with those who received RIST with ATG or conventional HSCT. Kaplan–Meier analysis was employed to evaluate the difference of days for absolute CMV_{495}-specific T cells to reach the threshold protection level, over 1×10^6/l IFN-γ-ELISPOT positive cells among patients who received RIST without/with ATG and conventional HSCT.

detection of only functional T cells with IFN-γ-ELISPOT assay. Similar observations were reported in CMV and HIV infections (Goepfert *et al*, 2000; Sun *et al*, 2003; Mohty *et al*, 2004). This discrepancy between the results obtained by two assays appears to be exaggerated, particularly when CMV-specific T cells are rapidly expanding in response to CMV antigenaemia. It was reported that ratio of tumour necrosis factor-α-producing T cells, measured by intracellular staining per HLA tetramer positive T cell, was lower in the HSCT patients who experienced CMV antigenaemia than those without CMV antigenaemia. HLA tetramer-positive T cells which do not produce IFN-γ have been reported to have a greater proliferative capacity (Sallusto *et al*, 1999). Detectable cells in this phase may be less differentiated, CCR7-positive, $CD45RA^{low}$ central memory T cells which may later differentiate to CCR7 negative effector memory ($CD45RA^{low}$) and terminally differentiated effector ($CD45RA^{high}$) cells capable of producing IFN-γ.

Sensitivity for the detection of CMV-specific T cells was higher with the ELISPOT (detection limit is $1/1 \times 10^5$ PBMC) than with the tetramer, mainly because of the detection threshold (approximately 0·02% of CD8+ T cells) defined by flow cytometric analysis. We compared the first detected day of CMV-specific T cells after various HSCTs using both assays, and found that the first detectable day was significantly earlier with ELISPOT than the tetramer assay, indicating that IFN-γ-ELISPOT assay is recommended for sensitive measurement of functional CMV-specific T cells, if it can be performed in the institution.

We then compared reconstitution of CMV-specific T cells among various HSCT protocols, including RIST with or without ATG, and conventional HSCT. Although the recovery of CMV-specific T cells and the incidence of CMV infection after RIST have previously been reported from several groups, including ours (Mohty *et al*, 2000; Kanda *et al*, 2001; Chakrabarti *et al*, 2002; Junghanss *et al*, 2002; Nakai *et al*, 2002; Maris *et al*, 2003), detailed comparison for CMV-specific T-cell reconstitution among different HSCT protocols using a single immunodominant peptide, has not been performed. The reconstitution of CMV immunity was significantly earlier in the patients who received RIST without ATG than RIST with ATG or conventional HSCT. Threshold levels of the CMV immunity for effective protection of CMV reactivation were 1×10^6/l with the ELISPOT assay and 1×10^7/l with the tetramer assay as a total CMV immunity, which are consistent with previous reports (Cwynarski *et al*, 2001; Gratama *et al*, 2001).

Anti-thymocyte globulin inhibited the early recovery of functional IFN-γ-producing and HLA tetramer-positive CMV-specific T cells. Eight of eleven patients who received RIST with ATG developed early CMV antigenaemia on day 27, while 6 of 17 patients who received RIST without ATG developed early CMV antigenaemia on day 37. Similar observations were reported in the RIST patients who received ATG using IFN-γ-ELISPOT assay with CMV lysates, although they could not conclusively show a negative effect of ATG on CMV-specific T cells because of small number of samples (Mohty *et al*, 2000). A high CMV infection rate after Campath-1H-based conditioning was also reported (Chakrabarti *et al*, 2002). The use of strongly immunosuppressive ATG or Campath-1H appears to be associated with a high incidence of CMV infections and prolonged delay of CMV immune recovery.

The early reconstitutions in the patients with RIST without ATG may be explained by early recovery of anti-CMV-specific T cells derived from recipients in a mixed chimeric state, although the majority of patients in our HSCT protocols were in a full chimeric state on day 30. Maris *et al* (2003) reported early recovery of CMV-specific T cells after low-dose total body irradiation-based RIST, possibly because of host-derived CMV-specific T cells in a mixed chimeric state. Further investigation is necessary regarding the source of CMV-specific T cells responsible for the early reconstitution of CMV immunity.

In summary, we demonstrated that the measurement of functional CMV-specific T cells with a single immunodominant CMV_{495} peptide using the IFN-γ-ELISPOT assay provided more accurate analysis of CMV immunity in HLA-A*0201 and A*0206 patients after various HSCTs than the HLA tetramer assay. RIST without ATG has an advantage of early recovery of CMV immunity compared with RIST with ATG and conventional HSCT. These results suggest that the IFN-γ-ELISPOT or HLA tetramer assay with the CMV_{495} peptide is useful for aiding decisions regarding treatment for CMV infection and prevention.

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Prospective Trial of High-Dose Chemotherapy Followed by Infusions of Peripheral Blood Stem Cells and Dose-Escalated Donor Lymphocytes for Relapsed Leukemia after Allogeneic Stem Cell Transplantation

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Abstract

To determine whether induction of graft-versus-host disease (GVHD) improves the outcome of acute relapsed leukemia after stem cell transplantation (SCT), we used high-dose cytarabine (ara-C) followed by infusions of donor-derived buffy coats containing peripheral blood stem cells to treat 12 patients with relapsed leukemia. Donor lymphocyte infusion (DLI) was repeated at least twice over a 5-week interval for patients in whom grade II to IV acute GVHD did not develop after the first DLI. Grade II to IV acute GVHD developed in 4 (33%) of the patients. Chronic GVHD developed in 3 patients, 2 of whom had not experienced acute GVHD. Four (67%) of the 6 patients who developed grade II to IV acute and/or chronic GVHD after DLI responded, but none of the other 6 patients responded. Four (33%) of the patients (2 with acute myelogenous leukemia [AML]and 2 with acute lymphoblastic leukemia [ALL]) achieved complete remission lasting longer than 4 months after the first DLI, but 3 of them had relapses in bone sites. Of these 4 patients, 1 patient with AML and 2 with ALL were alive 8 to 27 months after DLI. These findings indicate that high-dose ara-C combined with megadose DLI may produce durable remission of acute leukemia that has relapsed after SCT when GVHD is induced. The low induction rate of GVHD and extramedullary relapse after remission is achieved with DLI are problems yet to be solved.
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Key words: Megadose donor lymphocyte infusion; Peripheral blood stem cells; Graft-versus-leukemia effect; High-dose ara-C; Acute leukemia relapse

1. Introduction

Acute leukemia relapse after allogeneic stem cell transplantation (SCT) has a high mortality rate and has posed a serious challenge for SCT physicians. Donor lymphocyte infusion (DLI) can exert a graft-versus-leukemia effect (GVLE) in the treatment of the molecular, cytogenetic, and chronic phase relapses of chronic myeloid leukemia (CML) with a remission induction rate as high as 80% [1,2]. When DLI is used to treat other types of leukemia, such as acute-phase CML, acute leukemia, and advanced myelodysplastic syndrome (MDS), far lower response rates have been observed, and even when remission occurs, it is usually transient [1,2].

The effect of omitting graft-versus-host disease (GVHD) prophylaxis after allogeneic transplantation has been examined in several studies [3,4]. In a 2000 report by Fassas et al [3], all 12 patients with high-risk hematologic malignancies developed grade III or IV acute GVHD within 14 days after allogeneic peripheral blood SCT without prophylactic immunosuppression. Early and aggressive treatment of GVHD with high doses of corticosteroids and either

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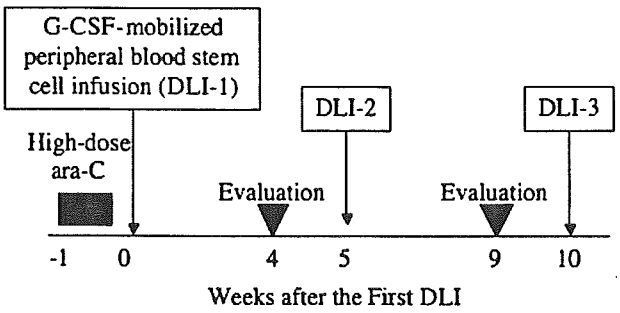

Figure 1. Treatment protocol. G-CSF indicates granulocyte colony-stimulating factor; DLI, donor lymphocyte infusion.

tacrolimus in 10 patients or cyclosporine in 2 patients helped to control severe GVHD. Two of the 3 patients with acute leukemia refractory to chemotherapy achieved durable remission. These findings suggest that acute GVHD induced by DLI may produce durable remission of relapsed leukemia only if acute GVHD is controlled with intensive immunosuppressive regimens. Repeated infusions of large numbers of T-cells within short intervals may raise the incidence of acute GVHD [5].

To test these hypotheses, we conducted a prospective trial in which a high-dose DLI regimen was repeated to induce acute GVHD in patients with acute leukemia that had relapsed after transplantation. We used a buffy coat collected after granulocyte colony-stimulating factor (G-CSF) mobilization as the first DLI to enhance hematologic recovery after intensive chemotherapy. The results of this study confirmed the importance of GVHD in obtaining durable remission of acute leukemia after DLI and revealed the limits of adoptive immunotherapy using donor lymphocytes.

2. Patients and Methods

2.1. Eligibility Criteria

The study was approved by the institutional review boards at all participating centers, and informed consent was obtained from all patients and donors. Eligibility criteria were diagnosis of acute-phase CML [6], acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), or MDS in hematologic relapse after allogeneic SCT from a related donor and the absence of acute or chronic GVHD at the time of relapse. Lymphocytes were apheresed from original stem cell donors.

2.2 Study Design

The primary end point of this study was treatment-related mortality irrelevant to leukemia within 20 weeks after the initiation of cytoreductive chemotherapy (Figure 1). The secondary end point included the incidence and severity of GVHD and remission duration. Cytarabine (ara-C) (2 g/m^2 twice a day for 5 days) was administered as the cytoreductive chemotherapy. The first DLI comprised a buffy coat collected after G-CSF mobilization to minimize aplasia after chemotherapy. Donors received G-CSF at 10 μg/kg daily subcutaneously for 6 days and underwent apheresis on days 5 and 6. The target CD34$^+$ cell dose was 2.0 × 10^6/kg of recipient body weight. No CD3$^+$ lymphocyte target dose was set for the first DLI. All of the collected cells were intravenously transferred 48 hours after completion of the tenth administration of ara-C. Post-DLI immunosuppression was not given. When the patient platelet count surpassed 50,000/μL, the patient received 1 dose of intrathecal methotrexate at 15 mg to prevent leukemia relapse in the central nervous system (CNS). For patients who did not develop grade II to IV GVHD or chronic GVHD within 4 weeks after the first DLI, 5 × 10^7 to 10^8/kg CD3$^+$ lymphocytes were administered at week 5 after the first DLI. The remaining cells were cryopreserved for subsequent infusion. Patients who did not develop GVHD within 4 weeks after the second DLI underwent the third DLI at week 5 after the second DLI. Grade II to IV GVHD was treated with intravenous methylprednisolone, 2 mg/kg, plus tacrolimus, 0.03 mg/kg. Acute and chronic GVHD was graded according to the standard criteria [7,8].

The usefulness of treatment was assessed 12 weeks after the first DLI. Deaths due to causes other than leukemia relapse were defined as adverse events, and survival in complete remission (CR) was defined as a response. Chimerism was assessed by determining the variable number of tandem repeats by polymerase chain reaction analysis [9] or fluorescence in situ hybridization analysis.

2.3. Statistical Analysis

The survival curves and probability of GVHD were plotted by the Kaplan-Meier method and compared by log-rank test. The Fisher exact test for categorical data was used to compare variables in 2-by-2 tables. Unless otherwise stated, a 2-tailed test was performed.

3. Results

3.1. Patients

A total of 12 patients took part in the study. Patient characteristics are listed in Table 1. Eleven patients had undergone SCT from a matched related donor and 1 patient from a 2-locus– mismatched related donor. Six patients had ALL, 5 had AML, and 1 patient had MDS. The median time from SCT to relapse was 185 days (range, 61-2240 days).

3.2. Donor Lymphocyte Infusion

The median doses of CD3$^+$ cells and CD34$^+$ cells in the first DLI were 2.2 × 10^8/kg (range, 1.0-4.2 × 10^8/kg) and 2.9 × 10^6/kg (range, 0.9-6.4 × 10^6/kg), respectively (Table 2). Of the 12 patients, 8 received the second DLI, and 5 of those 8 received the third DLI because of failure of the preceding DLI to induce GVHD or GVLE. The median follow-up time from the first DLI was 206 days (range, 35-810 days)

Table 1.
Patient Characteristics*

Patient	Age, y/Sex	Diagnosis	Cytogenetics	Donor	First SCT	Prior aGVHD Grade	Prior cGVHD	Time from SCT to Relapse, d
1	44/M	AML, M1	t(5;6),del(20)	Matched sibling	PBSCT	0	Yes	162
2	23/F	ALL, L2	1p+,5q−,6q−,7p−,12p+	Matched sibling	BMT	0	No	1960
3	55/F	MDS-RAEB	Normal	Mismatched sibling†	PBSCT	0	No	454
4	56/M	ALL, L2	add(3)(p11),add(8)(p11),add(9)(p13), del(9)(p?),−17,+mar	Matched son	PBSCT	0	No	84
5	52/M	AML, M2	Normal	Matched sibling	PBSCT	I	No	2240
6	22/F	ALL, L2	Normal	Matched sibling	PBSCT	0	Yes	570
7	53/M	AML, M2	Normal	Matched sibling	PBSCT	0	No	100
8	39/F	ALL, L2	Normal	Matched sibling	PBSCT	0	No	574
9	29/F	AML, M2	Normal	Matched sibling	PBSCT	0	No	208
10	25/M	ALL, L2	Ph+	Matched sibling	PBSCT	II	No	61
11	50/F	AML, M4	Normal	Matched sibling	PBSCT	0	No	118
12	45/F	ALL, L2	Normal	Matched sibling	PBSCT	0	No	101

*SCT indicates stem cell transplantation; aGVHD, acute graft-versus-host disease; cGVHD, chronic GVHD; AML, acute myelogenous leukemia; PBSCT, peripheral blood SCT; ALL, acute lymphoblastic leukemia; BMT, bone marrow transplantation; MDS-RAEB, myelodysplastic syndrome–refractory anemia with excess of blasts; Ph, Philadelphia chromosome.
†HLA 2-locus mismatched.

(Table 3). The median of total $CD3^+$ cells infused was 3.1×10^8/kg (range, $1.0\text{-}6.2 \times 10^8$/kg).

3.3. Toxicity and GVHD

Four patients died within 10 weeks of treatment owing to sepsis in 1 case, sepsis associated with acute GVHD in 1 case, multisystem organ failure associated with regimen-related toxicities (RRT) in 1 case, and cerebral hemorrhage associated with RRT in 1 case (Table 3). Acute GVHD of grade II or higher developed in 4 patients who received 1.0 to 4.2×10^8/kg of $CD3^+$ cells (median, 3.0×10^8/kg) a median of 35 days after the first DLI (range, 30-49 days). Chronic GVHD occurred de novo in 2 patients. One patient developed chronic GVHD after acute GVHD. The probabilities of developing grade II to IV acute GVHD, chronic GVHD, and either acute or chronic GVHD were 40%, 37%, and 58%, respectively (Figure 2A). All but 1 (patient 4) of the patients with GVHD responded to standard immunosuppressive therapy with corticosteroids and tacrolimus. The 2-year probability of treatment-related mortality was 33% (Figure 2B). There was no correlation between the development of GVHD and the dose of $CD3^+$ cells infused. The total dose of $CD3^+$ cells infused was $3.2 \pm 1.4 \times 10^8$/kg in patients with GVHD development. The total dose was $4.0 \pm 2.5 \times 10^8$/kg in patients without GVHD development ($P = .68$).

Table 2.
Donor Lymphocyte Infusion (DLI) Doses*

Patient	First DLI $CD34^+$, $\times 10^6$/kg	First DLI $CD3^+$, $\times 10^8$/kg	Second DLI $CD3^+$, $\times 10^8$/kg	Third DLI $CD3^+$, $\times 10^8$/kg	Total $CD3^+$, $\times 10^8$/kg
1	6.4	1.1	ND	ND	1.1
2	5.4	3.8	0.7	1.4	5.9
3	4.9	4.2	0.5	ND	4.2
4	4.0	2.8	ND	ND	2.8
5	4.8	1.9	1.1	ND	3.0
6	2.5	2.4	1.0	3.8	7.2
7	1.8	1.8	ND	ND	1.8
8	1.8	1.9	0.8	0.6	3.2
9	2.6	3.2	1.0	2.0	6.2
10	3.0	1.1	1.4	ND	2.5
11	0.9	1.0	ND	ND	1.0
12	2.9	2.7	1.2	1.4	5.3

*ND indicates not done.

3.4. Disease Responses and Response Durability

Table 3 shows the clinical course and present status of all 12 patients treated. Four (33%) of the patients with ALL (2 patients) and AML (2 patients) remained in CR 12 weeks after the first DLI. Of these 4 patients, 1 ALL patient remained in CR with a follow-up period of 810 days. Three of the 4 patients had relapses at an extramedullary site. Patient 5 had a relapse of ALL in the thoracic vertebrae on day 270 and underwent retransplantation with stem cells from the original donor on day 399. The patient died of grade IV acute GVHD 120 days after retransplantation. Patients 8 and 11 developed bilateral isolated relapse of leukemia in the femoral bones on days 240 and 134, respectively. Patient 8 was treated with an additional DLI containing 1.6×10^7/kg of $CD3^+$ cells on day 307 and local irradiation of 15 Gy and was alive and in good condition without evidence of disease 4 months after treatment. The third patient (patient 11) responded to local radiotherapy and was free of AML for 3 months. The 2-year probabilities of progression-free survival and overall survival were 14% and 29%, respectively (Figure 3A). The overall survival of patients with or without GVHD is illustrated in Figure 3B. Two (50%) of the 4 patients who developed grade II to IV acute GVHD

Table 3.
Clinical Outcome and Development of Graft-versus-Host Disease (GVHD) after Donor Lymphocyte Infusion (DLI)*

	Acute GVHD		Chronic GVHD			
Patient	Grade	Days after DLI	Type	Days after DLI	Response	Outcome after DLI
1	0		NA		No	Died with disease of multisystem organ failure on day 35
2	0		Ext, c/oc	90	CR	Disease-free >27 months
3	II, c	49	No		No	Died of sepsis on day 64
4	II, c/h/g	30	No		No	Died of sepsis and acute GVHD on day 43
5	0		Ext, c/h	48	CR	Relapse on day 270,† died on day 519
6	0		No		No	Disease-free >8 mo after chemotherapy
7	0		No		No	Disease-free >5 mo after chemotherapy
8	II, g	38	No		CR	Relapse on day 240,‡ disease-free >15 mo
9	0		No		No	Received chemotherapy but did not respond, lived >10 mo
10	I, c	11	No		No	Died of cerebral hemorrhage on day 41
11	III, g	31	Ext, g	84	CR	Relapse on day 134,§ disease-free >8 mo
12	0		No		No	Died of pneumonia on day 171

*NA indicates not assessable; ext, extensive; c, cutaneous; oc, oral cavity; CR, complete response; h, hepatic; g, gut.

†Patient 5 developed bone relapse on day 270 preceding bone marrow involvement, underwent retransplantation from the original donor on day 399, and died of grade IV acute GVHD 120 days after retransplantation.

‡Patient 8 developed an isolated relapse of the bilateral femoral bones on day 240 and responded to additional DLI on day 307 plus local radiotherapy.

§Patient 11 developed isolated relapse of the bilateral femoral bones on day 134 and responded to local radiotherapy.

responded, and all 3 patients who developed chronic GVHD responded. In contrast, none of the 6 patients who did not develop grade II to IV acute GVHD or chronic GVHD responded (Figure 3C).

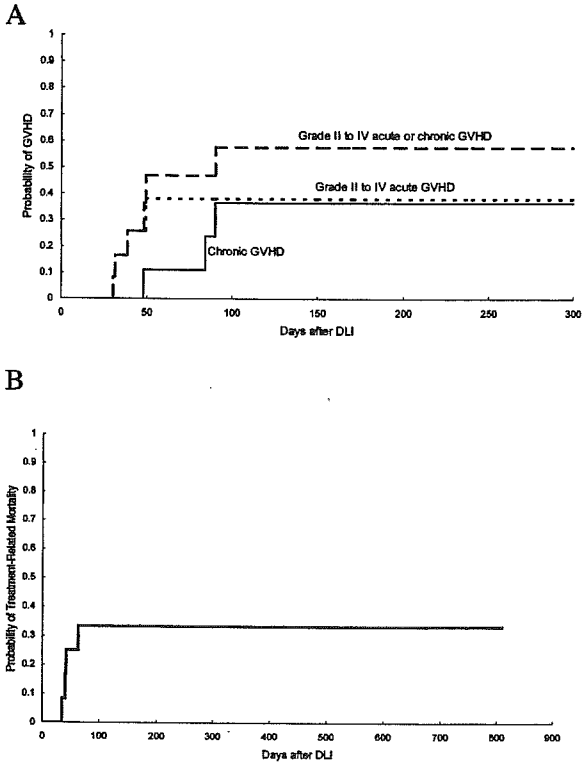

Figure 2. Probability of graft-versus-host disease (GVHD) in patients with advanced leukemia relapse treated with donor lymphocyte infusion (DLI) measured from the time of first DLI (A) and the probability of treatment-related mortality (B).

4. Discussion

The best way to manage acute leukemia relapse after allogeneic SCT is unclear. Attempts to induce GVLE in this setting using conventional DLI have been unsuccessful [1,10-14]. In this study, 33% of patients achieved CR with vigorous attempts to induce GVHD by repeated megadoses of DLI after cytoreduction. Progression-free survival at 2 years was 14%, and overall survival was 57% at 1 year and 29% at 2 years. The probability of developing GVHD was 58% at 1 year, and overall treatment-related mortality was 33% at 2 years.

The occurrence of GVHD after DLI translated into an improved response as expected. All 4 patients who achieved durable remission developed at least either acute or chronic GVHD or both. Development of acute GVHD and subsequent remission of relapsed leukemia were induced by megadose DLI in a previous study by Verfaillie et al [5]. Those authors treated 5 patients who had refractory AML with 3 consecutive infusions of as high a dose as approximately 2×10^8 donor lymphocytes/kg each over 5 days. Acute GVHD developed in 3 of 5 AML patients, and all 3 patients developing GVHD attained durable remission.

The European Blood and Marrow Transplantation Group (EBMT) retrospectively analyzed the cases of 108 patients with relapsed AML and MDS who received DLI. The investigators found that 66% of patients with acute GVHD of grade II or higher after DLI responded, whereas only 18% of the patients with acute GVHD of grade I or without acute GVHD responded [15]. The high response rate to DLI led to improved overall patient survival with grade II to IV acute GVHD. More recently, Kim et al [16] reported the beneficial effects of acute GVHD on the survival of 12 patients with

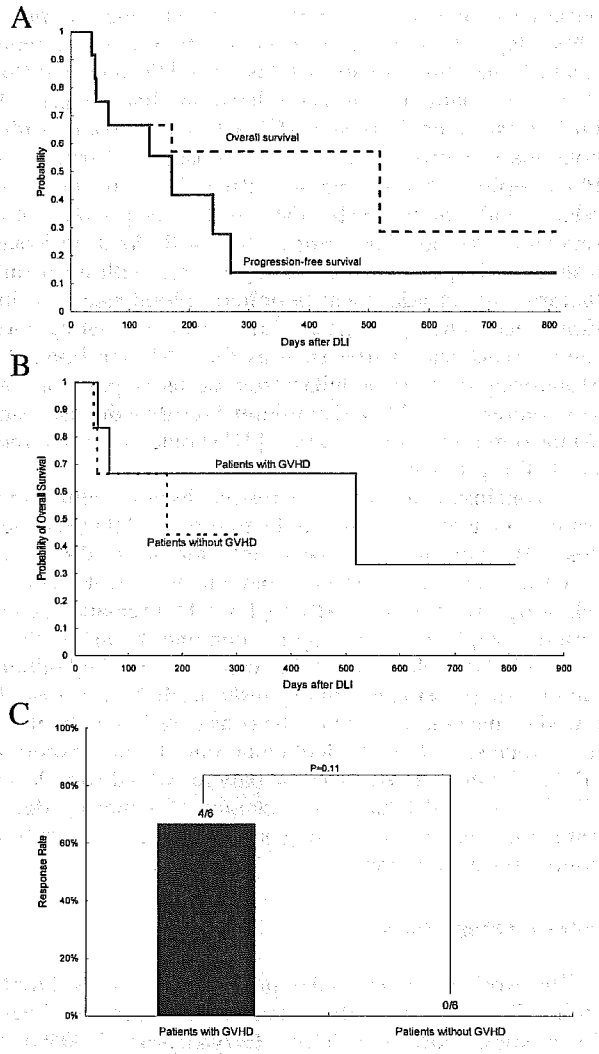

Figure 3. Progression-free survival and overall survival in patients with advanced leukemia relapse treated with donor lymphocyte infusion (DLI) measured from the time of first DLI infusion (A), overall survival with or without graft-versus-host disease (GVHD) (B), and response rate (C) with respect to development of grade II to IV acute GVHD or chronic GVHD after DLI.

acute leukemia relapses. In their analysis, the 1-year overall survival rate for 6 patients with acute GVHD greater than grade II (33%) was better than those without acute GVHD (0%). According to unpublished Japanese Marrow Transplant Registry (JMTR) data on 25 ALL patients, there was a better survival trend in patients who developed grade II to IV acute GVHD after DLI (9 [25%] of the patients survived) than in patients without such GVHD (16 [18%] of the patients survived).

In contrast to the aforementioned findings, the findings of Levine et al [14] did not demonstrate a beneficial effect of GVHD after DLI. Levine et al treated relapsed acute leukemia patients with cytoreductive chemotherapy followed by G-CSF–primed DLI, as we did in this study. In the study by Levine et al, the development of acute GVHD was associated with a lower probability of survival rather than a good response. Although response rates were similar among 32 patients with GVHD (50%) and 25 patients without GVHD (44%), 5 patients with GVHD died in CR from GVHD-related toxicity. This finding explains the lower survival rate among patients with GVHD. The report did not describe the GVHD treatment in detail. GVHD in our 4 patients who later attained CR was successfully treated with corticosteroids plus tacrolimus, and 3 of the 4 patients were in durable bone marrow remission for more than 8 months. These findings suggest that our treatment method is useful in controlling GVHD while preserving GVLE.

The incidence of grade II to IV acute GVHD after conventional DLI was 34% in the JMTR study [2]. This finding was comparable with the 46% incidence in the North American BMT group [11] and 41% in the EBMT group [10]. To increase the incidence of GVHD, we repeated DLI every 5 weeks on the basis of the finding in the North American BMT group that the median time to development of acute GVHD was 32 days [11]. However, only 40% of patients in this study developed acute GVHD, and 32% of patients developed neither acute nor chronic GVHD. Two possible reasons for the lower incidence of GVHD are that 92% of patients received DLI from a matched related donor and 69% had no history of GVHD. In patients in whom engraftment of donor stem cells occurred without the development of GVHD, it appears difficult to break down tolerance in recipient minor histocompatibility antigens, even when intensive chemotherapy such as high-dose ara-C is given to reset tolerance.

In our study, the rate of achieving durable remission tended to depend on the time from BMT to relapse, as reported previously [14,17]. The rate of CR was 17% in patients who had early (less than 6 months) relapses after SCT and 50% in patients who had late (more than 6 months) relapses after BMT ($P = .27$). Therefore DLI appears to have a small role in inducing durable remission for patients who have relapses within 6 months after allogeneic SCT. Conversely, it is notable that 3 of 4 patients whose initial post-SCT remission duration was more than 12 months responded to this approach. These findings are consistent with the results of a prospective study [14] showing that the most important predictive factor associated with a higher likelihood of response to chemotherapy followed by G-CSF–primed DLI was posttransplantation remission lasting more than 6 months before relapse; 44% of such patients were alive 1 year after treatment.

DLI alone in advanced myeloid leukemia reportedly has a response rate of 0% to 25%, and there is no expectation of durable remission [1,2,10-12]. Although these data suggest that the results from combined chemotherapy and DLI are superior to those with DLI alone, these results remain to be clarified because no comparative trials have been conducted. Likewise, little is known about whether more intensive chemotherapy can be applied prior to DLI. However, this approach may be worth considering because results with an experimental model [18] and clinical experience [19] show that most kinds of immunotherapy work best when tumor burden is low.

The efficacy of the current approach incorporating high-dose ara-C followed by megadose DLI needs to be compared with that of second SCT, especially reduced-intensity SCT. Our approach differs from second SCT in terms of the intensity of conditioning and with respect to the use of post-transplantation prophylactic immunosuppression. For patients with acute leukemia who have a relapse after the first SCT, reports demonstrated probabilities of disease-free survival at 2 years ranging from 2% to 44% with relapse rates of 25% to 75% and treatment-related mortality rates of 28% to 56% [20-28]. In this study, the 2-year survival rate was 29% with a treatment-related mortality of 33%. Second SCT is usually administered to patients who achieve remission by induction chemotherapy for relapse, and this selection may bias the comparison in favor of second SCT.

Our results with DLI for ALL patients were an improvement on the outcomes of previous studies. Two (33%) of the 6 ALL patients treated with megadose DLI therapy achieved durable remission. One of the 2 patients was in remission more than 2 years after the first DLI. The other patient developed bone relapse on day 240. The bone lesion responded to additional DLI and local radiotherapy, and the patient was disease-free more than 5 months after the second relapse. These observations are in contrast to those in a previous report in which only 3 (7%) of 44 ALL patients treated with DLI attained remission of 1 year or more [12]. In addition, in the EBMT survey DLI was found to have only a limited role in the treatment of recurrent ALL [1,10]. A possible reason for the poor outcome of conventional DLI in ALL is the fast pace of disease progression and an intrinsic resistance to immune attack, such as a lack of costimulatory molecules [1,13]. The treatment strategy for inducing GVHD using megadose DLI may produce persistent remission, although mechanisms of such GVLE are unclear.

The patients received G-CSF–mobilized peripheral blood stem cells after chemotherapy as the first DLI instead of non-primed DLI for the treatment of relapsed leukemia to minimize chemotherapy-induced cytopenia. This approach has been used successfully to avoid prolonged cytopenia after chemotherapy-preceded DLI [14,16]. The results of the current study also showed rapid recovery of hematopoiesis in all patients except patient 1, who died with disease progression early after treatment. Our study included too few patients treated to answer the question whether use of G-CSF–mobilized peripheral blood stem cells is as effective and safe as unprimed DLI in the treatment of relapse after SCT. However, the observation that collection of G-CSF–mobilized peripheral blood stem cells was safely carried out in each original stem cell donor as well as donors of lymphocytes for subsequent DLI in some cases may be encouraging.

In the present study 3 patients had relapses at extramedullary sites while signs of bone marrow involvement were undetectable. In a pilot study with 2 AML patients performed prior to initiation of the current trial, CR was achieved by both patients after the development of acute GVHD after repeated infusions of $CD3^+$ cells at a dose of 1×10^8/kg every 3 to 4 weeks. Relapse was discovered in the meninges before bone marrow relapse, and both patients died of systemic relapse of leukemia (unpublished observations). Therefore the current study incorporated scheduled intrathecal injection of methotrexate (15 mg) to prevent CNS relapse. Despite the absence of CNS relapse, patients 6, 9, and 12 had bone relapses. Glass et al [17] reported that 3 (50%) of 6 patients with acute leukemia had relapses after DLI at extramedullary sites (CNS, testis, and skin) without bone marrow involvement. Of the 3 patients, 1 patient with Philadelphia chromosome–positive ALL in meningeal relapse and 1 patient with AML in skin relapse died of disseminated disease. The third patient with AML in isolated testicular relapse was successfully treated with local radiotherapy and an additional peripheral blood stem cell infusion. These findings suggest that GVLE-mediating T-cells cannot reach sanctuaries such as the CNS and bones [29]. Monitoring of extramedullary relapse using positron emission tomography [30] and of minimal residual disease using a Wilms tumor gene (WT1) assay [31] should be considered to tackle this problem.

In conclusion, given the aggressive disease features in the patient population treated, induction of GVHD with megadose DLI appears to translate into long-term disease-free survival or cure in a subset of patients with acute leukemia relapsing late after SCT. Although GVHD remains the most serious complication of adoptive immune therapy with DLI [1,13], GVHD-related mortality can be avoided by initiating immunosuppression in a timely fashion. High-dose ara-C followed by megadose DLI may be considered for patients who have relapses of acute leukemia but do not experience GVHD before relapse. Because patients who do not develop GVHD after DLI have little chance of achieving durable remission, second SCT using stem cells from a different donor appears necessary.

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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

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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.

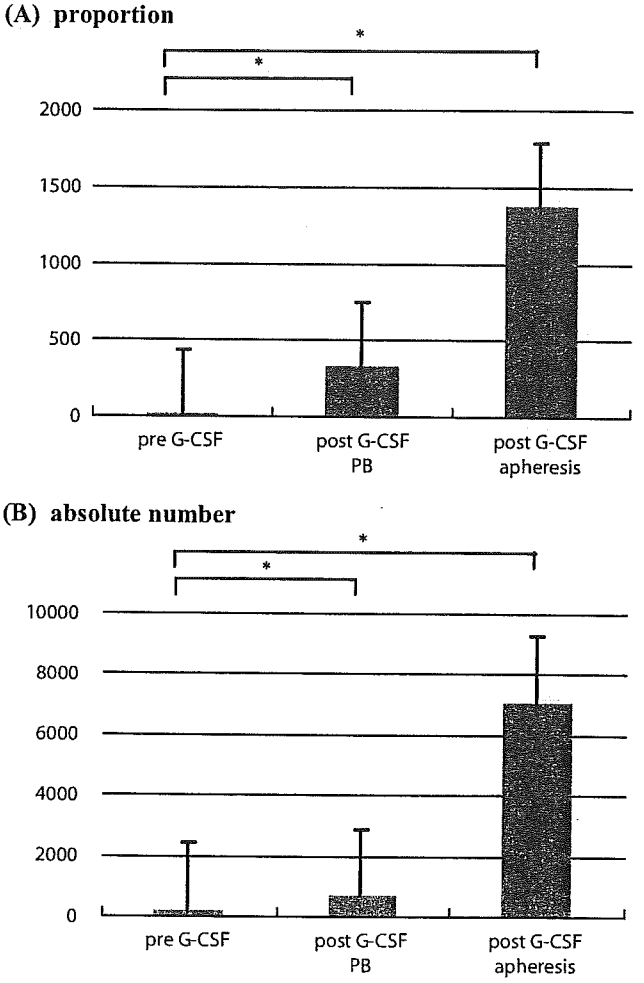

FIGURE 1. Proportion and absolute number of Vα24$^+$ NKT cells on day 12 The proportion (A) and absolute number (B) of Vα24$^+$ NKT cells increased 18(SD±23)- and 182(±158)-fold at the end of 12 days of culture for cells harvested before G-CSF administration, whereas these values were 333(±347)- and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

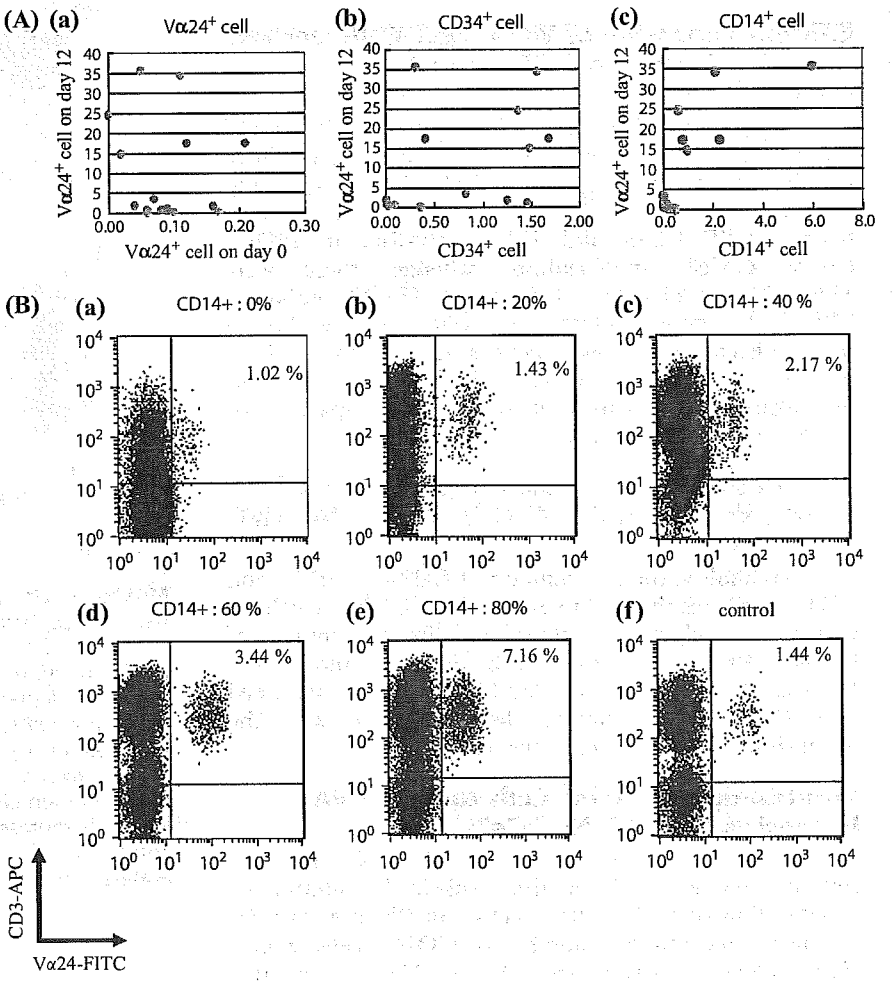

FIGURE 2. Effect of $CD34^+$, $V\alpha24^+$, and $CD14^+$ cells on expansion of $V\alpha24^+$ NKT cells (A) The proportion of (a) $CD34^+$, (b) $V\alpha24^+$ on day 0 were not associated with the expansion efficacy of $V\alpha24^+$ NKT cells ($r^2 = 0.171$, 0.016, respectively). Only $CD14^+$ cells (c) 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),

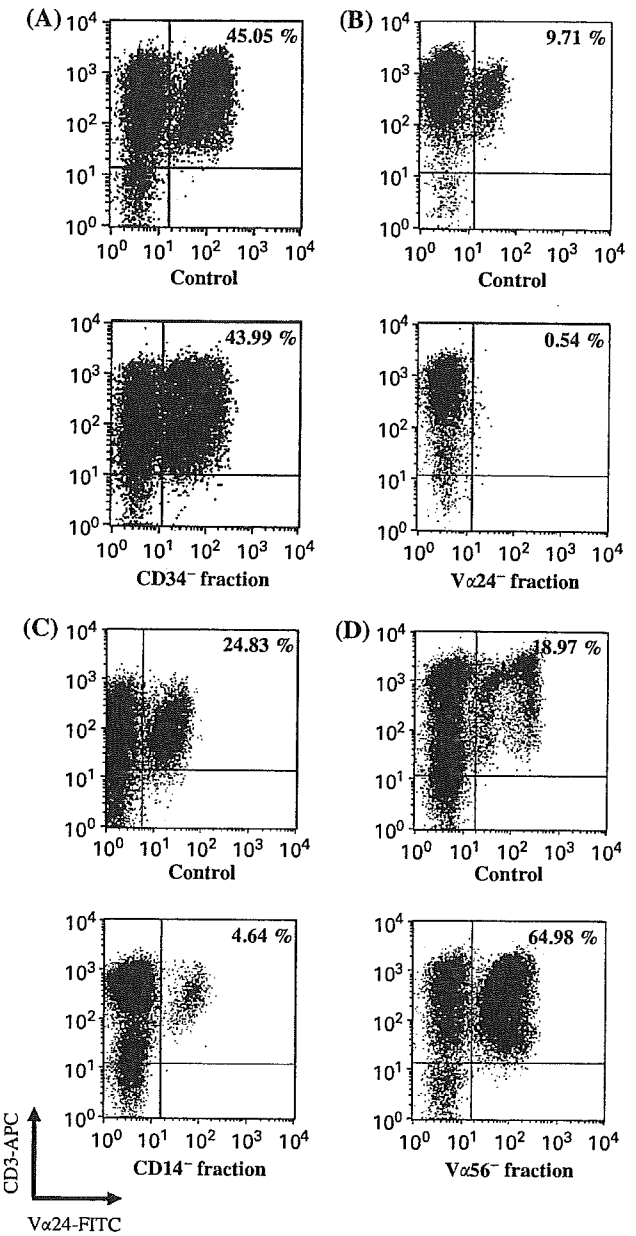

FIGURE 3. Effects of $CD34^+$, $V\alpha24^+$ NKT, $CD14^+$ and $CD56^+$ NK cell depletion on the expansion of $V\alpha24^+$ NKT cells $CD34^+$, $V\alpha24^+$ NKT, $CD14^+$, and $CD56^+$ NK cells were depleted using a MACS sorting system. (A) When $CD34^+$ cells were depleted, $V\alpha24^+$ NKT cells 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

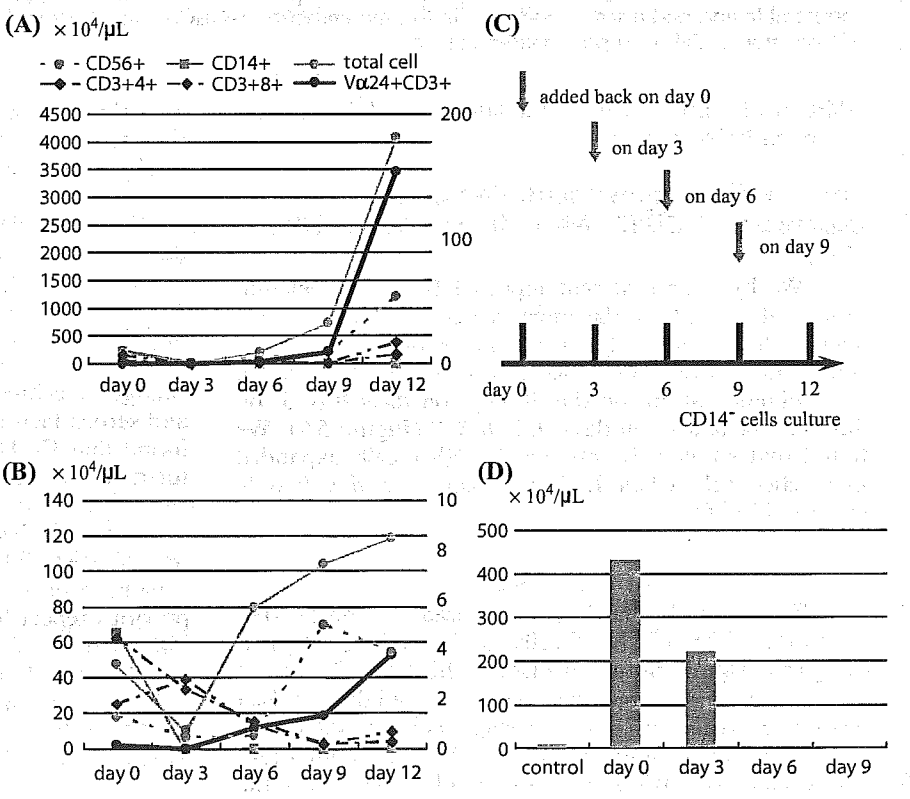

FIGURE 4. Cell kinetics of $V\alpha24^+$ NKT cells and $CD56^+$ NK cells in good and poor expanders (A) In a good-expanding donor, both $CD56^+$ NK cells and $V\alpha24^+$ NKT cells continued to proliferate without decline (representative results from four experiments). The right hand y-axis is used 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.

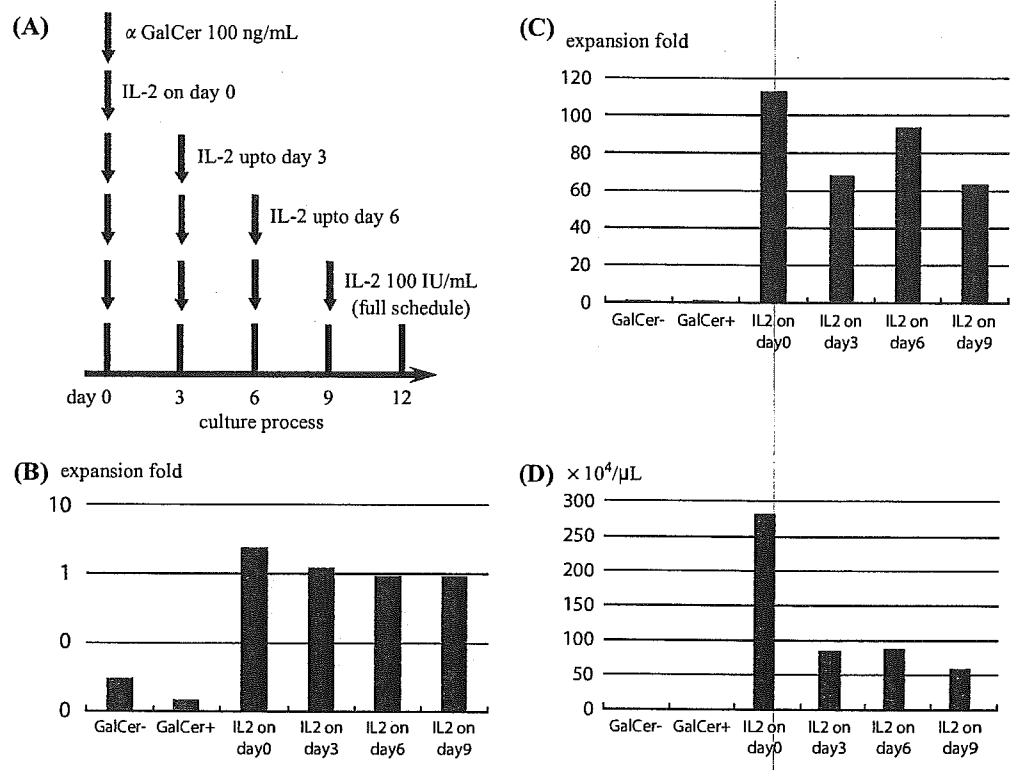

FIGURE 5. Effects of treatment with IL-2 on the expansion of Vα24⁺ NKT cells (A) We tested different schedules for the administration of IL-2, as follows: on day 0 only, on days 0 & 3, on days 0, 3 & 6, and on days 0, 3, 6 & 9. We found that (B) the expansion-fold of whole cells, and the expansion-fold 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 $V\alpha24^+$ NKT Cells

We hypothesized that repeated IL-2 supplementation could result in the enhancement of $CD56^+$ NK activity to suppress the proliferation of $V\alpha24^+$ 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 $V\alpha24^+$ 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 $V\alpha24^+$ 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 $V\alpha24^+$ 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 $V\alpha24^+$ NKT cells. Only the number of pre-cultured $CD14^+$ cells affected the magnitude of the expansion of $V\alpha24^+$ 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 $V\alpha24^+$ NKT cells[17](18). Additionally, we showed that 1) depletion of $CD14^+$ cells resulted in the loss of $V\alpha24^+$ NKT cell expansion, and 2) the expansion efficacy of $V\alpha24^+$ 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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Stem Cell Transplantation

High incidence of cytomegalovirus reactivation in adult recipients of an unrelated cord blood transplant

This retrospective analysis for cytomegalovirus (CMV)-seropositive adult transplant recipients showed that CMV antigenemia occurred after transplantation in 10/10 (100%) recipients of unrelated cord blood, 17/39 (43%) recipients of a related matched donor graft, 16/23 (79%) recipients of an unrelated matched donor graft, and 8/12 (67%) recipients of a mismatched related donor graft. These results suggest that unrelated cord blood transplantation itself may be correlated with a high incidence of CMV reactivation.

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Cytomegalovirus (CMV) infection is still a major concern following allogeneic hematopoietic transplantation because CMV pneumonia is fatal in 70% of patients, even when treated with a combination of antiviral therapies and CMV hyperimmune immunoglobulin.[1] Allogeneic cord blood transplantation, especially from unrelated donors, has progressively gained favor as treatment for patients with both malignant and non-malignant disorders.[2-4] As compared to allogeneic bone marrow transplantation (BMT) and peripheral blood stem cell transplantation (PBSCT), advantages of unrelated cord blood transplantation (UCBT) include ease and safety of cell collection, low risk of transmitting viral infections, prompt availability of stem cells, and reduced incidence and severity of graft-versus-host disease (GVHD).[2-4] The reduction of GVHD after UCBT is likely due to the naïve state of cord blood lymphocytes and the low cytotoxic capacity of cord blood T cells.[5] However, such immunological immaturity after UCBT can place a patient at risk

Table 1. Patient characteristic.

Characteristics	Stem cell donor: HLA identical sibling	HLA matched unrelated donor	HLA mismatched relative	Unrelated CB
No. of patients	39	23	12	10
Sex, male/female	23/16	10/13	5/7	6/4
Median age (range), years	53 (14-69)	36 (17-54)	43 (15-58)	61 (15-69)
Disease				
Acute myelogenous leukemia	9	6	0	2
Acute lymphoblastic leukemia	4	6	6	2
Chronic myeloid leukemia	3	4	2	0
Myelodysplastic syndrome	6	2	0	1
Non-Hodgkin's lymphoma	7	3	3	1
Sever aplastic anemia	4	2	1	1
Myelofibrosis	1	0	0	0
Renal cell carcinoma	4	0	0	3
Osteosarcoma	1	0	0	0
Standard risk/advanced risk*	18/21	14/9	3/9	1/9
Stem cell source				
PBSC/BM	33/6	0/23	10/2	0/0
HLA disparity				
0/1/2/3	39/0/0/0	23/0/0/0	0/2/6/3/1	3/1/6/0
CMV-seropositive donor	35	22	11	0
Prior transplantation	4	2	1	5
Conditioning regimen				
Myeloablative/Reduced-intensity	15/24	18/5	7/5	1/9
GVHD prophylaxis				
CSP-based/FK506-based	38/1	11/11	7/5	7/3
Use of ATG	5	3	2	1
Use of steroids	13	4	8	6
Use of MMF	3	0	5	5
Survival >100 days, %	92	87	75	78
Survival >365 days, %	82	83	40	56

*PBSC, peripheral blood stem cell; BM, bone marrow; CB, cord blood; CSP, cyclosporine; FK506, tacrolimus; MMF, mycophenolate mofetil. *Acute leukemia in first remission, chronic myeloid leukemia in the first chronic phase, myelodysplastic syndrome with refractory anemia or refractory anemia with ringed siderblasts, malignant lymphoma in any remission, and aplastic anemia were defined as standard-risk diseases. All other patients were classified as having advanced disease.*

of early infectious complications, accounting for most transplant-related deaths, especially in adults.[1,6] We have observed that patients undergoing UCBT appear to be at increased risk of CMV infection. Ninety-one consecutive adult patients who were CMV-seropositive and received non-T-cell-depleted allogeneic transplants at the Kanazawa University Hospital between April 1999 and April 2004 were eligible for inclusion. Written informed consent was obtained from all patients. Six patients died of regimen-related toxicities before engraftment and one developed primary graft rejection followed by autologous hematopoietic recovery. The remaining 84 patients had