Health Organization (WHO)/Revised European-American Lymphoma (REAL) classification was known were described as it is and the cases unclassifiable with WHO/REAL classification were described according to the Working Formulation classification (Table 1) [22]. Patients with complete surgical resection of lymphoma, treated with RT alone and administered CTx other than CHOP regimen were not eligible. An overview of the patients' characteristics according to clinical parameters is given in Table 2.

Minimal staging included chest radiograph, computed tomography (CT) of the abdomen and pelvis, and single percutaneous bone marrow biopsy and blood studies. Gallium scintigram, ^{18}F 2-fluoro-2-deoxy-D-glucose–positron emission tomography (^{18}F-FDG-PET), and barium swallow were optional.

CTx

The treatment consisted of 3–6 cycles of CHOP (day 1 cyclophosphamide, 750 mg/m2; day 1 doxorubicin, 50 mg/m^2, day 1 vincristine, 1.4 mg/m^2 (capped at 2 mg); and days 1–5 oral prednisone, 100 mg) every 3 weeks. We performed 6 cycles for bulky disease more than 5 cm in diameter and 3 cycles for not bulky disease.

RT

RT was performed with a 6-MV X-ray machine. RT delivered with megavoltage equipment began 3–4 weeks after the last cycle of CHOP, confirming the recovery from the toxicity of CTx. The primary tumor and the metastatic lymph nodes were irradiated for a total dose of 30 Gy in 20 fractions over 4 weeks for cases achieving CR after CTx and 40.5 Gy in 27 fractions over 5.4 weeks for cases having a residual lymphoma.

In our institution, RT was confined to the whole neck, supraclavicular region, and Waldeyer's ring for 13 patients with cervical nodal or Waldeyer's disease (Fig. 1).

Toxicity assessment

Patients were observed weekly during the treatment to monitor toxicity. It was graded according to the National Cancer Institute Common Toxicity Criteria (version 2.0).

Table 1 Histopathology characteristics (n=40)

Histopathology	n (%)
Diffuse large B-cell lymphoma	31 (77.5%)
NK/T-cell lymphoma	2 (5%)
Anaplastic large cell lymphoma	2 (5%)
Peripheral T-cell lymphoma	2 (5%)
Diffuse histiocystic lymphoma	1 (2.5%)
Diffuse medium-sized B-cell lymphoma	1 (2.5%)
Diffuse T-cell lymphoma	1 (2.5%)

Table 2 Patient characteristics (n=40)

Patient characteristic	Number
Age	
<60	28
≥60	12
IPI	
Low risk	29
Intermediate-low risk	11
LDH	
High	20
Normal	20
sIL-2R	
High	16
Normal	13
Cell type	
B	33
T	7
Stage	
I	24
II	16
Tumor diameter (cm)	
<5	14
5–7.5	8
7.5–10	3
≥10	4
Mediastinal LNs	
(−)	34
(+)	6
No. of sites	
<3	36
≥3	4
Anatomic distribution	
Head and neck	24
Supra-diaphragmatic	12
Infra-diaphragmatic	4

Late toxicity was graded according to the Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer late radiation morbidity scoring scheme.

Follow-up evaluation

The following evaluations were performed until disease progression every 1 month for the first year after the completion of the protocol treatment, every 2 months for the second year, and every 3–4 months thereafter: physical examination, complete blood count, serum chemistries including soluble interleukin 2 receptor (sIL-2R), β2 microglobuline (β2-MG), and lactate dehydrogenase (LDH). CT scan or FDG-PET was optional.

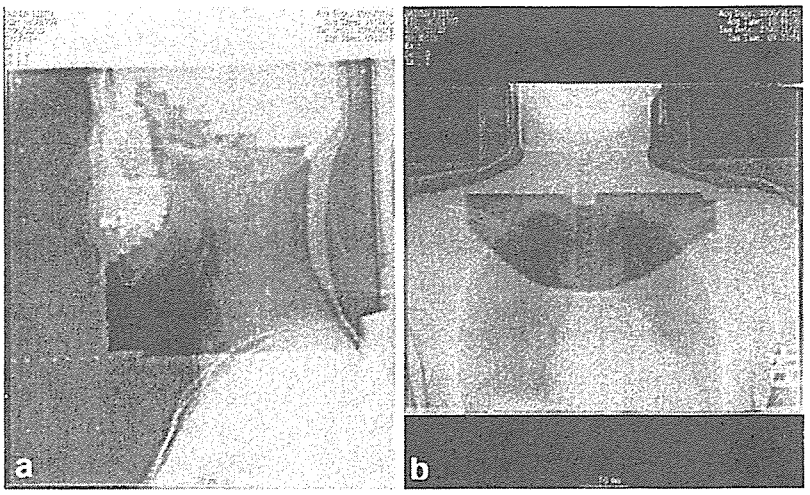

Fig. 1 Linac graphy (LG) for checking RT field for patients with cervical nodal or Waldeyer's disease. *1* upper lateral field. *2* lower anterioposterior field

Response assessment

For the primary tumor and the involved nodes, responses were judged according to the International Workshop Criteria for non-Hodgkin's lymphoma [23]. CR was defined as regression of all palpable nodes (<1×1 cm and of normal consistency) and radiographic disease. Partial response (PR) was defined as ≥50% reduction in the sum of the products of the dimensions of all measurable lesions. Progressive disease (PD) was defined as >50% increase from nadir or the appearance of new lesions. Stable disease (SD) was classified as a response less than PR but not a PD.

Statistical analysis

Statistical analyses were performed using StatView Dataset File version 5.0J for Windows computers (North Carolina, USA). Survival periods were calculated from the start of irradiation. Statistical analysis included the Student *t* test, the Pearson 2 test, and the Fisher exact test as appropriate. The survival functions were estimated with the Kaplan–Meier method estimator, and log-rank tests were used to compare the survival distributions. Univariate Cox proportional hazards models were used to identify the independent factors that predict overall survival.

Results

Patient characteristics

From June 1985 to December 2003, 40 patients were eligible and assessable. The patient characteristics are presented in Tables 1 and 2. Thirty-eight percent of the study population had stage II disease. Extranodal involvement was present at diagnosis in 22 patients (55%), with head and neck sites constituting the vast majority. Few patients had an impaired performance status or systemic symptoms. As expected, approximately 80% of patients had diffuse large-cell lymphoma (N=31). A minority of patients (10%) had three or more Ann Arbor sites of disease. The tumor mass was ≥10 cm in 10%. Mediastinal lymphadenopathy was recorded in six patients (15%). CTx mainly preceded RT (N=35, 88%), although the sequence of RT and CTx was determined by individual physicians and patients' choice.

Total 6 cycles of CTx was performed in 18 cases (45%), 3 cycles in 19 cases (48%). In the other three cases, CTx was called off in 1 cycle (N=2) or 2 cycles (N=1) because lymphomas had a resistance to CTx.

Survival

Complete follow-up was obtained in all patients and the last follow-up was performed in December 2005. The follow-up period of surviving 33 patients ranged from 24.7 to 180 months with a median of 69 and a mean of 72.7 months. The overall survival for all 40 patients at 5 year was 81.2% (Fig. 2).

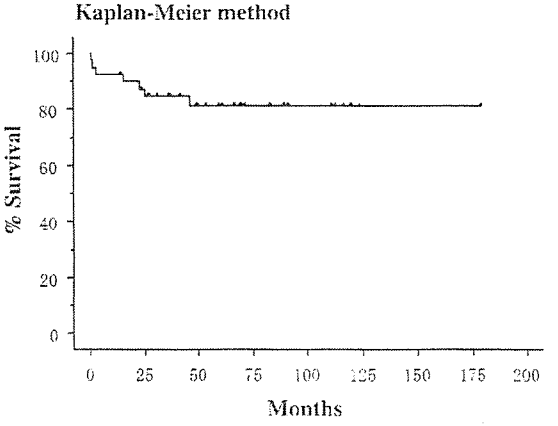

Fig. 2 Overall survival. The estimated 5-year survival rate was 81.2%

Response to the primary therapy

RT preceded CTx in five cases (12%). All the five cases achieved CR at the completion of irradiation before the start of CTx and they were all surviving at this analysis. The survival time was 72, 93, 113, 125, and 180 months respectively. CTx preceded RT in the other 35 cases (88%). In the 35 cases, CR was achieved in 28 cases, PR in two cases, SD in one case, and PD in four cases, after the completion of CTx before the start of RT. As to the seven cases without achieving CR, five cases died in 0.5, 1.2, 3.2, 15.6, and 22.9 months, respectively and the other two cases are surviving at 24.7 and 71.1 months, respectively.

Response to CRT

With application of the above criteria, CR was achieved in 37 patients (93%) and PD in three patients (7%). The three patients died in 0.5, 1.2, and 3.2 months after the completion of irradiation, respectively. All the three patients died of systemic invasion of lymphoma. The first patient died directly of liver dysfunction and sequential multiple organ failure (MOF), the second died of acute liver dysfunction, disseminated intravascular coagulation (DIC), and methicillin-resistant staphylococcus aureus (MRSA) pneumonia, and the third died of infection.

Prognostic factors

A study of relapse patterns after a CR showed that four patients had a first relapse within a radiation field and the other one patient had an extranodal distant relapse with left humeral skin. Significant prognostic factors for overall survival were not identified in age, international prognostic index (IPI), LDH value, sIL-2R value, cell type, stage (Fig. 3), gender (Fig. 4), extent of maximum local disease, with or without mediastinal lymph nodes, number of sites, anatomic distribution, and irradiation dose (Table 3) in the univariate analysis according to Cox proportional hazards models. As to the effect of CTx preceding RT (Fig. 5), CR group (n=28) was a significant better prognosis than non-CR (n=7). Overall survival curves between male and female were a significant difference according to log-rank test (p=0.0296) (Fig. 4). Female group had the significantly more proportion of patients achieving CR from CTx preceding RT (95%) than male group (63%) (p=0.0175). If using modified IPI from a South-west Oncology Group (SWOG) [17] instead of IPI, survival curves between low risk group (n=27) and not low risk group, that is either low-intermediate risk group (n=10) or intermediate-high risk group (n=3), were not of significant difference (p=0.4593). In this study, the number of patients was too small to perform multivariate analysis. The association of other variables with survival was negligible.

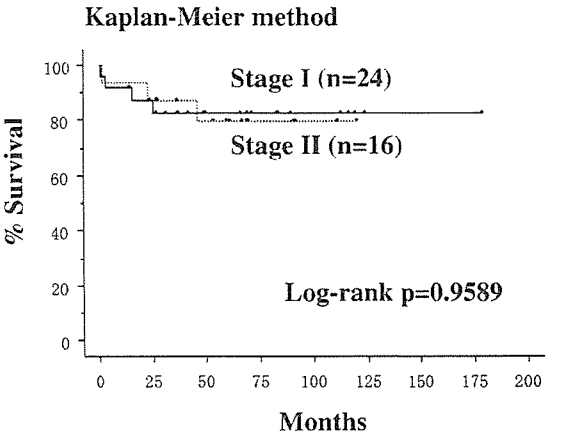

Fig. 3 Overall survival curves of patients between stages I (n=24) and II (n=16)

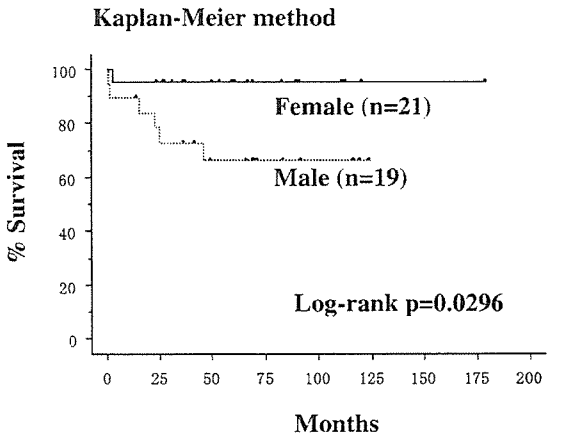

Fig. 4 Overall survival curves of patients between male (n=19) and female (n=21)

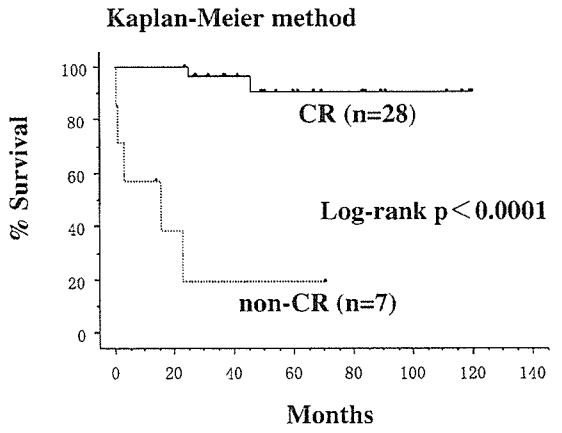

Fig. 5 Overall survival curves of patients between CR (n=28) and non-CR (n=7) of the effect of CTx in patients receiving CTx followed by RT

Treatment-related toxicity

In CTx setting, 32 patients (80%) experienced grade 3 or 4 neutropenia. Grade 3–4 anemia and thrombocytopenia

Table 3 Univariate of relative risks on overall survival in 40 patients

Category	*n*	Univariate analysis		
		Relative risk	95% confidence interval	*p*
Gender				
Male	19	1		
Female	21	0.135	0.016–1.125	0.0641
No. of sites				
<3	36	1		
≥3	4	3.344	0.648–17.24	0.1495
No. of CTx cycle				
3	19	1		
6	18	0.251	0.28–2.252	0.2182
Effect of CTx				
CR	28	1		
Not CR	7	25.64	4.651–142.9	0.0002
RT dose (Gy)				
30	17	1		
40	16	0.859	0.053–13.89	0.9151
Age (years)				
<60	28	1		
≥60	12	0.990	0.192–5.102	0.9909
IPI				
Low	29	1		
Intermediate low	11	1.149	0.22–5.952	0.8685
LDH				
Normal	20	1		
High	20	1.479	0.330–6.622	0.6087
sIL-2R				
Normal	13	1		
High	16	0.492	0.082–2.956	0.4384
Stage				
I	24	1		
II	16	0.784	0.130–4.694	0.9589
Cell type				
B	33	1		
T	7	2.538	0.489–13.16	0.2679
Tumor diameter (cm)				
<5	14	1		
≥5	15	0.621	0.104–3.717	0.6018
Mediastinal LNs				
(−)	34	1		
(+)	6	0.917	0.110–7.634	0.9362
Anatomic distribution				
Supra-diaphragmatic	12	1		
Head and neck	24	2.816	0.329–24.13	0.3448
Infra-diaphragmatic	4	2.713	0.169–43.57	0.4809

occurred in eight patients (5%) and eight patients (5%), respectively. Hematopoietic growth factors were not used routinely.

In RT setting, major toxicity was not seen in the study. All 13 patients, treated with RT field covering over the whole neck, supraclavicular region, and Waldeyer's ring, suffered from grade 1 (mild mouth dryness/may have slightly altered taste such as metallic taste) or grade 2 (moderate to complete dryness/markedly altered taste) acute radiation morbidity on salivary gland. Additionally, those 13 patients experienced grade 1 (slight dryness of mouth; good response on stimulation) or grade 2 (moderate dryness of mouth; poor response on stimulation) late radiation morbidity on salivary gland. No patients developed radiation-induced second malignancy.

The results and the outcome of the subset of patients with T-cell lymphoma were shown in Table 4.

Discussion

The standard therapy for patients with stage IA or contiguous stage IIA and low or low-intermediate grade is the combination of CTx (3–4 cycles of CHOP or R-CHOP) and RT. In a randomized trial by Eastern Cooperative Oncology Group (ECOG)-1484, comparing 8 cycles of CHOP with 8 cycles of CHOP plus RT (30Gy), 10-year disease-free survival was 46 and 57% (p=0.04), and the benefit of tacking on local RT to CTx was proven [24].

SWOG-8736 randomized phase III trial showed a favorable 5-year survival of 82% by 3 cycles of CHOP plus RT (40–55 Gy) compared to that of 72% by 8 cycles of CHOP alone in patients with stage I or IE and nonbulky stage II or IIE localized nodal and extranodal aggressive non-Hodgkin's lymphoma (p=0.02) [21]. The 5-year overall survival in patients with a low-risk IPI and a low-intermediate risk were 82 and 71%, respectively, including both the CHOP plus RT group and the CHOP alone group. After these results, we adopted 3 cycles of CHOP plus RT as a treatment regimen for stage I–II aggressive lymphoma in the present study.

Recently updated analysis of the SWOG trial showed an overlapping curve at 9 years for overall survival as a result of late relapses in the CHOP plus RT group, but it remained the standard treatment for stage I and nonbulky stage II patients based on survival advantages through the first 9 years and less toxicity. Late relapses also suggested that optimal treatment might include more or different systemic CTx. Recent reports suggest that rituximab plus CHOP is more effective than CHOP alone in more advanced stage DLBCL [25, 26] and the standard treatment for early stage DLBCL patients may be changed in the near future [27]. We have also changed CHOP regimen into rituximab plus CHOP (R-CHOP) since February 2004. Since the introduction of rituximab, therapy has changed the following: (1) rituximab has been administered in day 1 of every cycles and subsequently CHOP administered from day 2, and (2) both the number of cycles of CTx and the method of RT have not been changed. Nowadays, a CHOP-RT approach has been used only for patients with either T-cell lymphoma or CD20 negative B-cell lymphoma in our institution.

In the Lymphoma Non-Hodgkinien study 93-4 (LNH 93-4), randomized trial by the Groupe d'Etude des Lymphomes de l'Adulte (GELA) for elderly patients

Table 4 Results and outcome of patients with T-cell lymphoma

Pt No.	Histopathology	Gender	Age	Stage	Modified IPI	Recurrence	Survival time (months)	Outcome
1	Diffuse T cell type	M	49	1A	Low	(+)	0.5	Dead
2	NK/T-cell lymphoma	F	44	1A	Low	(−)	83.3	Alive
3	NK/T-cell lymphoma	M	59	1A	Low	(−)	72.1	Alive
4	Anaplastic large cell lymphoma	M	19	1A	Low	(+)	42.3	Alive
5	Peripheral T cell lymphoma	M	72	1A	Low-intermediate	(+)	14.7	Alive
6	Peripheral T cell lymphoma	F	44	1A	Low	(+)	3.2	Dead
7	Anaplastic large cell lymphoma	M	42	1A	Low	(−)	50.4	Alive

comparing 4 cycles of CHOP plus local radiation (40 Gy) with 4 cycles of CHOP, there was no significant difference in 5-year survival (64 vs 69%, p=0.4) [28]. In the LNH 93-1 for patients under 61 years, dose-intensified doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone (ACVBP) followed by sequential consolidation was superior to 3 cycles of CHOP plus RT for the treatment of low-risk localized lymphoma (5-year overall survival was 90 vs 81%, p=0.001) [29]. The reports, that the meaning of RT for low-risk stage I–II aggressive lymphoma was unclear, continued one after the other.

The possible benefits of a short course of chemotherapy followed by involved-field RT are the potential for eliminating microscopic sites of disease; however, RT alone is still a very important treatment modality for localized low-grade lymphomas [17].

It is well known that CR obtained after first-line treatment is associated with a longer overall survival [30]. In our study, CR obtained after the primary CTx (n=28) was highly associated with a longer overall survival (p<0.0001). A number of clinical variables, among which IPI is the standard approach, predict therapy response, but in our study, there was no significant difference between a low-risk IPI and a low-intermediate risk (p=0.8684). With regard to prognostic factors for survival, clinical variables at presentation appear to lose their predictive significance when response to therapy is taken into account [31].

Among seven cases without achieving CR in the primary CTx preceding RT, RT after CTx may be able to salvage two cases (surviving at 24.7 and 71.1 months with keeping CR, respectively).

Local and systemic-related toxicities were minimal, and patients generally tolerated treatment well [32, 33]. In most series and our experience too, long-term side effects were mild and late toxicities and second malignancies were infrequent.

Our results in the treatment of localized aggressive lymphoma are excellent. A definitive recommendation for treatment of localized aggressive lymphoma is difficult to make based on this study with such a small number of patients, albeit the excellent results.

Conclusion

Stage I–II aggressive lymphoma trend to be localized and to have good outcome. Our limited data suggest that combined CTx and RT is safe, highly effective, and probably curative, with mild and infrequent long-term side effects for the majority of patients with primary localized aggressive lymphoma.

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ORIGINAL ARTICLE: RESEARCH

CD1d expression level in tumor cells is an important determinant for anti-tumor immunity by natural killer T cells

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Abstract

Invariant natural killer T (iNKT) cells are thought to regulate anti-tumor immunity. Human iNKT (i.e. $V\alpha24^+$ NKT) cells have been reported to recognize CD1d on target cells and show cytotoxicity directly on the target cells *in vitro*. However, the anti-tumor effect of mouse iNKT (i.e. $V\alpha14^+$ NKT) cells has been repeatedly reported to be dependent on the activity of natural killer (NK) cells via interferon-γ, with no evidence of direct cytotoxicity. In the present study, we report that *in vitro* cytolysis of EL-4 mouse lymphoblastic lymphoma cells by $V\alpha24^+$ NKT cells and *in vivo* eradication of these cells are both dependent on the level of CD1d expression on the tumor cell surface. These observations possibly suggest that direct cytotoxicity of tumor cells by iNKT cells is common to both humans and mice, and that the high expression level of CD1d may be a predictor whether the tumor is a good target of iNKT cells.

Keywords: *Anti-tumor immunity, CD1d, natural killer T cell, mouse tumor model, T cell lymphoma, immunotherapy, α-galactosylceramide*

Introduction

Natural killer T (NKT) cells are a population of T cells that have natural killer (NK) cell markers such as NK1.1 (NKR-P1C) in mice [1,2]. Sizable populations of NKT cells use an invariant T cell receptor (TCR)-α chain ($V\alpha14$-$J\alpha18$ in mice, $V\alpha24$-$J\alpha18$ in humans) paired with $V\beta8$, $V\beta7$ or $V\beta2$ in mice, or with $V\beta11$ in humans [1,2], and are called invariant NKT (iNKT) cells. iNKT cells are activated through TCR by synthetic glycolipids such as α-galactosylceramide (α-GalCer) in a CD1d-restricted manner [1,2]. iNKT cells produce both Th1 [such as interferon (IFN)-γ and tumor necrosis factor-α] and Th2 [such as interleukin (IL)-4, IL-10 and IL-13] cytokines upon TCR-mediated signaling [1,2]. It has been reported that iNKT cells control immune responses in infections, tumors, autoimmune diseases and transplantation [1–3].

In mice, iNKT cells have been demonstrated to have an anti-tumor effect *in vivo*, in which iNKT-dependent activation of NK cells and/or $CD8^+$ cytotoxic T-cell via IFN-γ is considered to be the most important mechanism [2–4]. The direct cytotoxicity of mouse iNKT cells on tumor cells has not been defined to date. A similar mechanism had been suggested for the anti-tumor effect of human iNKT cells through *in vitro* experiments [5]. However, in humans, a small number of recent reports described that iNKT cells directly recognize CD1d on target cells [6–8]. Moreover, some primary leukemia cells express CD1d and are killed by $V\alpha24^+$ iNKT cells in a CD1d-dependent manner [9–11].

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In the present study, we describe that the expression level of CD1d in EL-4 mouse lymphoblastic lymphoma cells correlates with α-GalCer-dependent anti-tumor activity of iNKT cells *in vitro* and tumor growth suppression *in vivo*. Our results potentially support the theory that iNKT cells directly kill the target tumor cells and that the level of CD1d expression on tumor cells is an important indicator for α-GalCer therapy or NKT immunotherapy.

Materials and methods

Mice

C57BL/6 (H-2^b) mice were purchased from Japan Clea (Tokyo, Japan). Female mice, aged 6–12 weeks, were used in all the experiments. Mouse studies were conducted according to the University of Tokyo Animal Experiment Manual.

El-4 T cells and murine CD1d transfection

The murine CD1d expression vector (pSRα neo/CD1d1) was kindly provided by Dr Exley (Beth Israel Deaconess Medical Centre, Boston, MA, USA). The murine T-lineage lymphoblastic lymphoma EL-4 cells were transfected with the plasmids using Superfect (Qiagen GmbH, Hilden, Germany) and then cultured at 3×10^4 cells/well of 24-well plates in RPMI1640 supplemented with 10% FCS (cRPMI) and 1 mg/ml of Geneticin (G418, Invitrogen, Carlsbad, CA, USA). Independent clones with various levels of CD1d expression were obtained. We calculated the growth rate of the clones by daily counting viable cell numbers for 2 weeks.

Phenotypic assay

Anti-CD1d (1B1) and isotype control (A95-1) antibodies were purchased from BD Pharmingen (San Jose, CA, USA). Immunofluorescence staining was performed according to the standard procedures. Cells were analysed by flow cytometry using a FACSCalibur (Becton Dickinson Bioscience, San Jose, CA, USA).

Cytotoxicity assay

Human iNKT cells were established as previously described [6,9]. Briefly, monocytes from healthy human donors were cultured in AIM medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% FCS with recombinant human (rh) IL-4 (500 U/ml, CellGenix, Freiburg, Germany) and rhGM-CSF (500 U/ml, CellGenix) for 5 days, and used as monocyte-derived dendritic cells (Mo-DCs). $V\alpha24^+$ cells, which were isolated from the same donor by positive magnetic bead sorting (Miltenyi Biotec, Bergisch Gladbach, Germany), were cultured with irradiated (50 Gy)-Mo-DCs that were pulsed for 12 h with α-GalCer (100 ng/ml, kindly provided by Kirin Brewery, Gunma, Japan). The cells were maintained in the presence of rhIL-2 (40 U/ml, kindly provided by Shionogi, Osaka, Japan) and restimulated by irradiated-Mo-DCs every 7 days. Identity of the expanded cell populations as iNKT cells was confirmed by cytometric analysis with anti-Vα24 (C15) and Vβ11 (C21) antibodies (Beckman Coulter, Fullerton, CA, USA). Five $\times 10^3$ of ^{51}Cr-labelled (Amersham, Arlington Hights, IL, USA) EL-4 sublines and the effecter iNKT cells at the indicated ratios were seeded onto 96-well round-bottomed microtiter plates in 200 μl of cRPMI with or without 100 ng/ml of α-GalCer. The co-cultured cells were incubated for 4 h, and 100 μl of supernatant was collected from each well and was counted with a Packard COBRA gamma counter (Packard Instrument, Meriden, CT, USA). The percentage of specific ^{51}Cr-release was calculated as: (c.p.m. experimental release − c.p.m. spontaneous release)/(c.p.m. maximal release − c.p.m. spontaneous release) × 100. The ratio of spontaneous release to maximal release was less than 20% in all experiments.

In vitro *iNKT stimulation and cytokine production*

Five $\times 10^4$ $V\alpha24^+$ iNKT cells and 5×10^4 EL-4 clones were suspended in 200 μl cRPMI, with or without 100 ng/ml of α-GalCer, and cultured in 96-well plates. After 24 h, the supernatants were collected from each well and the concentrations of IL-4 and IFN-γ were evaluated by ELISA according to the manufacturer's protocol (CytoSets; Biosource, Camarillo, CA, USA). The cultured cells were cytometrically analysed with anti-TCR Vα24, human CD25 (B1.49.9) and human CD69 (TP1.55.3) antibodies (Beckman Coulter) and analysed by FACSCalbur.

In vivo *tumor-bearing model*

EL-4 cell lines (1×10^5 cells/mouse) were injected to mice intravenously. The resulting tumors were removed from all the dead mice immediately (<24 h) after death and histopathological specimens from tumors were stained with hematoxylin and eosin.

Molecular analysis of iGb3, a natural ligand for TCR on iNKT cells

Total RNA was extracted from EL-4 lines according to the manufacturer's protocol (Tri Reagent LS; Sigma-Aldrich, St Louis, MO, USA) and reverse

transcribed (SuperScript II; Invitrogen). The resulting cDNA was subjected to polymerase chain reaction amplification using the primer pair for iGb3 (5′-ATTATTATCAGGCTCATAGAAGG-3′ and 5′-CTAGTTTCGCACCAGCGTATATTC-3′) [12] or the pair for β-actin (5′-GAGAGGGAAATC GTGCGTGA-3′ and 5′-ACATCTGCTGGAAGG TGGAC-3′).

Statistical analysis

Survival of mice was analysed by the log-rank test. Differences in the amount of secreted cytokines, the percentage specific lysis of EL-4 cells and the proportion of activated iNKT cells were analysed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Establishment of CD1d-transfected EL-4 sublines and cytotoxic activity of NKT cells against the individual sublines

We obtained multiple EL-4 subclones that express various levels of CD1d. Among these, five and two clones with the highest and intermediate CD1d expression levels (EL-4/CD1d^{hi} and EL-4/CD1d^{int}) were used for the following analyses. Three mock-transfected clones were used as controls. The mean ± SD growth rates were 4.0 ± 0.40 divisions per 24 h in mock-transfected clones, 3.8 ± 0.12 in El-4/CD1d^{int} clones and 3.8 ± 0.43 in EL-4/CD1d^{hi} clones, indicating that the expression levels of CD1d did not affect the growth rate. Wild-type and mock-transfected EL-4 expressed a low level of CD1d (Figure 1A). We used human iNKT cells (Figure 1B) as effector cells because *in vitro* expansion of mouse iNKT cells is generally difficult, while the method to expand human iNKT cells has been established [6,9]. Importantly, the mechanism of recognition of CD1d-bound α-GalCer by iNKT cells is conserved through mammalian species [13]. With dependence on the CD1d expression level, iNKT cells exhibited cytotoxicity to EL-4 cells only in the presence of α-GalCer (Figure 1C). We also characterized the activation of the iNKT cells by the different sublines of EL-4 *in vitro* by analysing the surface expression of activation markers (Figure 1D) and the profiles of secreted cytokines (Figure 1E). The proportions of CD69 and CD25 positive cells among iNKT cells were independent of the expression level of CD1d on co-culturing EL-4 clones. Although the amounts of IL-4 and IFN-γ secreted from iNKT cells stimulated by EL-4/CD1d^{hi} tended to be higher than those from iNKT cells stimulated by EL-4/CD1d^{low}, the difference was not always statistically significant. The cytotoxicity of iNKT cells via IFN-γ might not be a major mechanism of anti-tumor activity.

Survival of EL-4 tumor-bearing mice correlated to CD1d expression level on the surface of tumor cells

We next examined whether the CD1d expression level on the surface of tumor cells could affect the survival of the tumor-bearing mice. All the wild-type EL-4-inoculated mice died within 6 weeks, showing multiple lymph node swelling. All the enlarged lymph nodes examined were diffusely infiltrated with morphologically obvious tumor cells. Analysis of the tumor cells by flow cytometry revealed that they had characteristics of T-lineage cells and CD1d expression at levels that were the same as those in the injected EL-4 cells (Figure 2A). Survival of wild-type and mock-transfected EL-4-inoculated mice was not significantly different (data not shown). In contrast, mice inoculated with all of the five EL-4/CD1d^{hi} clones survived significantly longer than those inoculated with EL-4 expressing CD1d at lower levels (Figure 2B). These results suggest that interaction between iNKT cells and CD1d on the tumor cell surface is useful for protecting the host from tumor growth. This anti-tumor immunity should be based on natural ligands. We examined the expression of iGb3, a known natural ligand for TCR on NKT cells [12], and found that all the clones expressed iGb3 (Figure 2C). Therefore, iNKT cells acquired cytotoxic activity against CD1d-expressing EL-4 cells *in vivo* possibly by stimulation with iGb3 that is naturally expressed on EL-4.

Discussion

Anti-tumor activity is one of the attractive functions of iNKT cells. α-GalCer has been tested in phase I clinical trials, either by direct administration of the compound [14] or by administration of α-GalCer-pulsed autologous DCs [15]. *In vitro* expanded iNKT cells might also be used as an immunotherapy. However, despite such progress, very little is known about the tumor-suppressing mechanisms of iNKT cells.

iNKT cells produce both Th1 and Th2 cytokines, suggesting that they also have a certain regulatory function. Some studies reported that NKT cells suppressed tumor immunity [5,16]. Accordingly, there is no definitive information as to whether

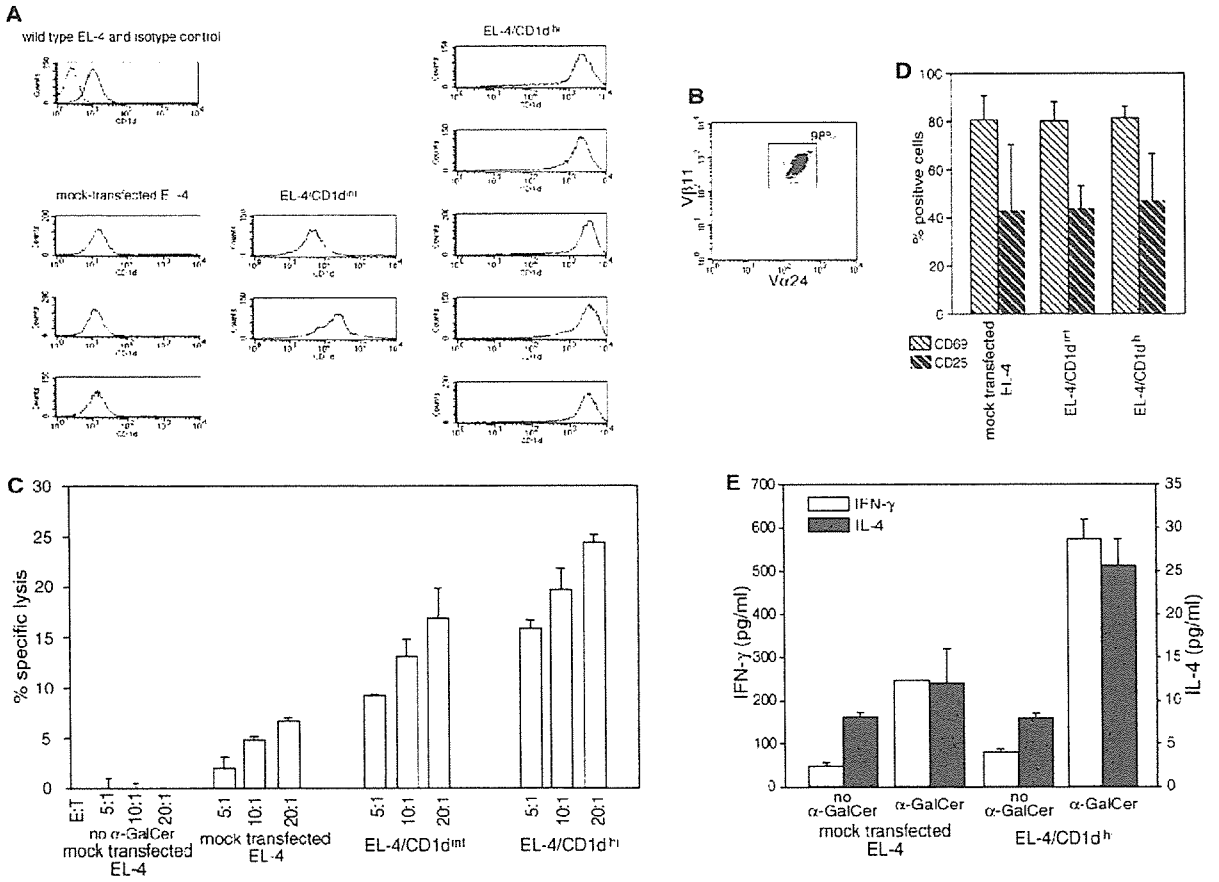

Figure 1. *In vitro* cytotoxic activity of CD1d-transfected EL-4. (A) Expression levels of CD1d in transfected EL-4 clones. The pattern of CD1d expression on the wild-type EL-4 cell surface is overlayed on an isotype-control (dashed line). EL-4/CD1d^{hi} and EL-4/CD1d^{int} are clones with the highest and intermediate CD1d expression level, respectively. (B) Flow cytometric analysis of the expanded iNKT cells. (C) Cytotoxic assay for iNKT cells. ^{51}Cr-labelled mock-transfected EL-4, EL-4/CD1d^{int} and EL-4/CD1d^{hi} were used as the target cells. Comparing each E : T ratio, there are significant differences in the percentage of specific lysis among the three groups of EL-4 sublines classified by the expression level of CD1d. Data are shown as the mean ± SD. Very similar results were obtained using other clones from each group. (D) Expression of activation markers of iNKT cells by each subline of EL-4. The results shown are the sum of seven independent experiments. (E) Cytokine profile of activated iNKT cells by each subline of EL-4. The levels of cytokines secreted by mock-transfected EL-4 and EL-4/CD1d^{hi} were not significantly different. Similar results were obtained using other clones from each group.

iNKT cells act as a cytotoxic effector or a regulatory effector for individual tumors. The results of the present study raise the possibility that the CD1d expression level is the determinant in an *in vivo* animal model and that such an effect is mainly mediated through the direct killing activity of iNKT cells rather than secretion of Th1 cytokines.

Information about the physiological ligands for the invariant TCR, which should play a significant role in anti-tumor function of iNKT cells in EL-4-bearing mice, is limited. Unfortunately, examination of tumor-infiltrating lymph nodes in the present study failed to delineate the mechanisms of tumor eradication by *in situ* iNKT cells present in the lymph nodes. The fraction of iNKT cells in the EL-4 CD1d^{hi} tumor ($0.24 \pm 0.14\%$, $n=9$) was significantly ($P=0.04$) larger than in the mock-transfected EL-4 tumor ($0.12 \pm 0.05\%$, $n=5$). However, interpretation of this finding is difficult because we could not standardize the sizes of lymph nodes, the cellularity of infiltrating EL-4 cells and various infiltrating immune cells other than iNKT cells (data not shown).

Nevertheless, CD1d expression on the tumor surface may be a predictor of whether the tumor is a good target for iNKT cells. The results of the present study raise the possibility of NKT cell therapy being used as a treatment modality for CD1d-expressing tumors. Direct evidence showing that iNKT cells kill the tumor cells *in vivo* should

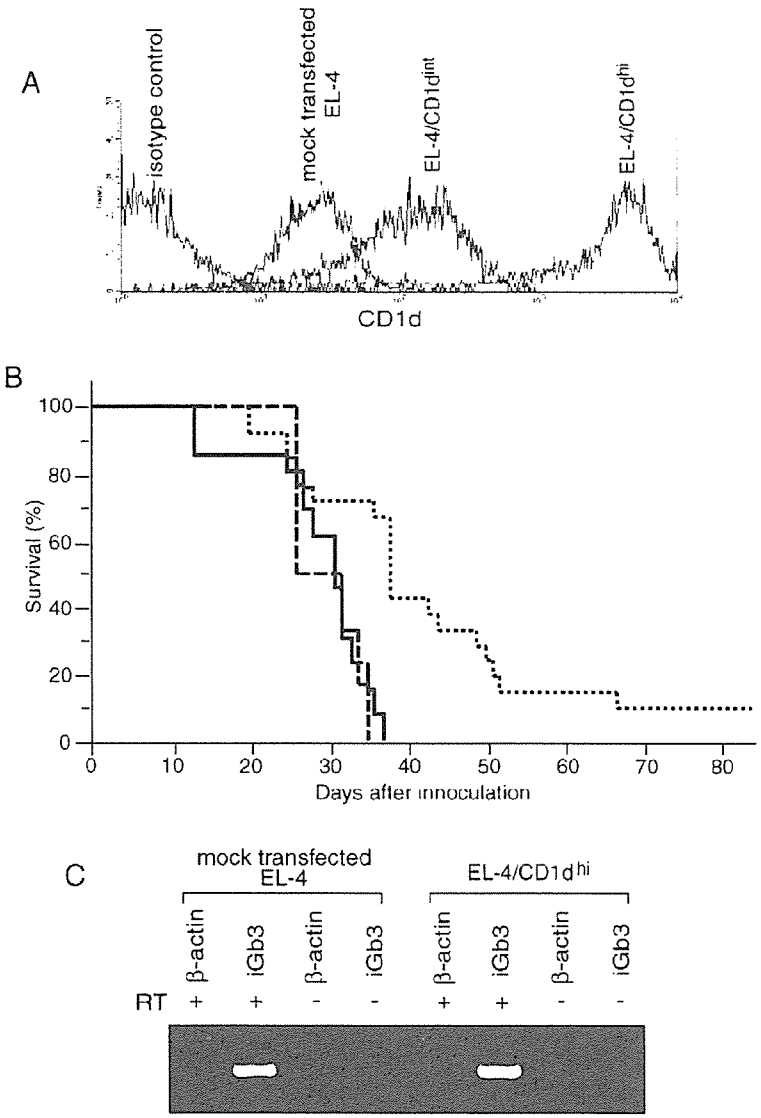

Figure 2. Survival of mice inoculated with various EL-4 sublines. (A) Flow cytometric analysis of the infiltrating lymphoma cells. (B) Survival of mock-transfected (solid line, $n = 21$) EL-4-, EL-4/CD1d^{int}-(dashed line, $n = 6$) and EL-4/CD1d^{hi}-(dotted line, $n = 13$) inoculated mice. EL-4/CD1d^{hi}-inoculated mice survived significantly longer ($P = 0.0005$). The result is the sum of six independent experiments. (C) RT-PCR assay showing that the EL-4 cell lines expressed iGb3. Very similar results were obtained using all the clones.

be warranted, which might be obtained after the technical improvements have been implemented.

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Key Words. AC133 antigen • Hematopoietic stem cells • Notch • Stem cell expansion

ABSTRACT

Ex vivo expansion of hematopoietic stem cells (HSCs) has been explored in the fields of stem cell biology, gene therapy, and clinical transplantation. Here, we demonstrate efficient ex vivo expansion of HSCs measured by long-term severe combined immunodeficient (SCID) repopulating cells (SRCs) from human cord blood CD133-sorted cells using a soluble form of Delta1. After a 3-week culture on immobilized Delta1 supplemented with stem cell factor, thrombopoietin, Flt-3 ligand, interleukin (IL)-3, and IL-6/soluble IL-6 receptor chimeric protein (FP6) in a serum- and stromal cell-free condition, we achieved approximately sixfold expansion of SRCs when evaluated by limiting dilution/transplantation assays. The maintenance of full multipotency and self-renewal capacity during culture was confirmed by transplantation to nonobese diabetic/SCID/γc^{null} mice, which showed myeloid, B, T, and natural killer cells as well as $CD133^+CD34^+$ cells, and hematopoietic reconstitution in the secondary recipients. Interestingly, the CD133-sorted cells contained approximately 4.5 times more SRCs than the CD34-sorted cells. The present study provides a promising method to expand HSCs and encourages future trials on clinical transplantation. STEM CELLS *2006;24: 2456–2465*

INTRODUCTION

Umbilical cord blood (CB) is an established stem cell source for hematopoietic stem cell (HSC) transplantation. In many cases, however, CB transplantation is unavailable to patients with relatively high body weight because of the insufficient number of HSCs obtained from a single CB unit [1–3]. Recently, transplantation of multiple units of CB in adult patients was reported in an experimental attempt to infuse a higher number of HSCs or hematopoietic progenitor cells (HPCs), but the effectiveness of this novel trial needs further investigation [4–7].

Another possibility to acquire a higher number of stem cells is ex vivo expansion of HSCs. Although many reports have described potential methods to increase HSCs ex vivo, only a few of them have clearly demonstrated the expansion of long-term severe combined immunodeficient (SCID) repopulating cells (SRCs), currently the only reliable measure of HSCs [8–10]. According to one of these reports, combined use of soluble interleukin (IL)-6 receptor (sIL-6R) and IL-6 together with stem cell factor (SCF), thrombopoietin (TPO), and flt-3 ligand (FL) appeared to be helpful for the successful expansion

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of HSCs, probably because gp130-mediated signals play a critical role in stem cell proliferation and combined use of sIL-6R and IL-6 can transmit signals through gp130 in HSCs, which express gp130 but lack IL-6R [10]. Based on these findings, an artificially generated IL-6/sIL-6R fusion protein, named FP6, which could more efficiently transmit gp130 signals in hematopoietic cells [11], might be a promising agent for ex vivo expansion of HSCs.

Another method that is potentially useful for stem cell expansion is the use of Notch signaling. It is mediated by interactions between transmembrane receptors (Notch1, -2, -3, and -4) and their membrane-bound ligands (Delta and Jagged family molecules). The signaling pathway is known to have differentiation-inhibitory effects in different stem cell systems, including hematopoiesis [12–14]. It has been reported that Notch signaling might play a role in the bone marrow niche, in which Notch ligands are presented by osteoblasts, main components of the niche [15]. In fact, soluble forms of the Notch ligands have been shown to increase immature hematopoietic cells [16–18]. These findings strongly prompt us to use Notch ligands in combination with FP6, for stem cell expansion.

The initial stem cell source is also an important issue for obtaining the maximum efficiency of stem cell expansion. Whereas many investigators use the CD34-sorted cells as a source of stem cell expansion, recent reports suggested that CD133 sorting can concentrate SRCs more efficiently than CD34 sorting [19, 20], and it is still open to question which population is more suitable for stem cell expansion.

In this study, we first addressed the issue of stem cell sources, demonstrating that the CB CD133-sorted cells contained an approximately 4.5-fold greater absolute number of SRCs than CD34-sorted cells. We next evaluated the integrated effect of Notch and gp130 signalings using soluble Delta1 and FP6 in combination with SCF, TPO, FL, and IL-3 and found that this combination could expand human CB CD133-sorted SRCs by 5.8-fold in a serum- and stromal cell-free condition.

Materials and Methods

Separation of CD133- and CD34-Enriched Cells from Human CB

Human CB samples were collected from normal full-term deliveries after informed consent was obtained. Mononuclear cells (MNCs) were separated by density gradient centrifugation (Lymphoprep; Axis-shield, Oslo, Norway, http://www.axis-shield.com) after depletion of phagocytes with Silica (Immuno-Biological Laboratories Co., Takasaki, Gunma, Japan, http://www.ibl-japan.co.jp). CD133- and CD34-enriched cells were separated from MNCs by using magnetic cell sorting (MACS) CD133 MicroBead Kit or MACS Direct CD34 Progenitor Cell Isolation Kit (hereafter CD133-MACS and CD34-MACS, respectively; Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com), respectively. In some experiments, separated cells were examined by flow-cytometric analyses using FcR Blocking Reagent, fluorescein isothiocyanate (FITC)-conjugated anti-human CD34, allophycocyanin (APC)-conjugated anti-human CD133 (clone 293C3) (Miltenyi Biotec), phycoerythrin (PE)-conjugated anti-human CD38 antibodies (BD Pharmingen, San Diego, http://www.bdbiosciences.com/pharmingen), and 7-amino-actinomycin D (7-AAD) (Via-Probe; BD Pharmingen). The yield of the target cells was calculated as follows: [(number of cells after separation) × (purity of the target cells after separation defined by flow-cytometric analysis)] / [(number of MNCs before separation) × (frequency of the target cells among MNCs before separation defined by flow-cytometric analysis)] × 100 (%).

Cytokines

Recombinant human SCF, TPO, IL-3, and IL-6/sIL-6R chimeric protein FP6 were generated by Kirin Brewery Co., Ltd. (Tokyo, http://www.kirin.co.jp/english), and the recombinant Delta1-Fc chimeric protein was generated as previously described [17]. These reagents were certified as free from endotoxin (<0.28 EU/mg protein). Recombinant human IL-6 and FL were purchased from Wako Pure Chemicals (Osaka, Japan, http://www.wako-chem.co.jp/english) and R&D Systems Inc. (Minneapolis, http://www.rndsystems.com), respectively.

Cell Culture

Plates or dishes not treated with tissue culture were precoated with 10 μg/ml Delta1-Fc or control Fc fragment of human IgG (IgG-Fc) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, http://www.jacksonimmuno.com) followed by 10 μg/ml human fibronectin (Boehringer Ingelheim GmbH, Ingelheim, Germany, http://www.boehringer-ingelheim.de). Cells were cultured in serum-free medium composed of Iscove's modified Dulbecco's medium (IMDM) supplemented with 10 mg/ml bovine serum albumin, 10 μg/ml human insulin, 200 μg/ml human transferrin, 2 mM L-glutamine, 0.1 mM 2-Mercaptoethanol, 4.7 μg/ml linoleic acid, 4.7 μg/ml oleic acid, and 8 μg/ml cholesterol (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, http://www.kyokutoseiyaku.co.jp) at 37°C in a humidified atmosphere flushed with 5% CO_2 in air. Cytokines were added at concentrations of 100 ng/ml for SCF, 10 ng/ml for TPO, 100 ng/ml for FL, 100 ng/ml for FP6, 100 ng/ml for IL-6, and 10 ng/ml for IL-3. Cell culture was initiated in 24-well plates and serially transferred to six-well plates and 10-cm dishes to avoid overgrowth of the cells. Half of the culture medium was changed every 2 or 3 days.

Colony Assays

At the indicated time points, cultured cells were harvested and plated in a semisolid medium, Methocult GF H4434, containing IMDM with 30% fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml human SCF, 10 ng/ml human granulocyte-macrophage colony stimulating factor, 10 ng/ml human IL-3, and 3 units/ml human erythropoietin (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) and incubated at 37°C. Colony-forming ability was assessed after 15–16 days of culture.

Transplantation to Nonobese Diabetic/SCID or Nonobese Diabetic/SCID/γc^{null} Mice

To assess the in vivo repopulating capacity of isolated cells and their cultured progeny, we used nonobese diabetic (NOD)/SCID (NOD/Shi-scid; CLEA Japan, Inc., Tokyo, http://www.clea-japan.com) and NOD/SCID/γc^{null} (NOG) mice [21] (Central Institute for Experimental Animals, Kanagawa, Japan, http://www.ciea.or.jp/English/eindex.htm) as xenotransplantation recipients. Cells separated by CD133-MACS and their cultured

progeny were transplanted intravenously into sublethally irradiated (2.5 Gy using an x-ray irradiator), 8–10-week-old NOD/SCID or NOG mice. When transplanting cells into NOD/SCID mice, we injected intraperitoneally 20 μl of anti-asialo GM1 antibody (Wako Pure Chemicals) diluted in phosphate-buffered saline (PBS) to a total volume of 420 μl immediately before transplantation and on days 11, 22, and 33 after transplantation to reduce the natural killer (NK) cell activity in NOD/SCID mice [10]. Because NOG mice lack intrinsic NK cell activities, administration of anti-asialo GM1 antibody to NOG mice was not needed [21]. Mice were fed with autoclaved acidified water and sterilized food. At 10–13 weeks after transplantation, mice were sacrificed, and cells were harvested from both femurs, peripheral blood, spleen, and thymus. In the indicated experiments, analyses were performed 24 weeks after transplantation. In the limiting dilution transplantation analyses, we transplanted cells into six to 12 recipient mice in each limiting dose for reliable estimation.

In the serial transplantation experiment, we isolated bone marrow cells from the primary NOG recipient mouse 24 weeks after the first transplantation, and MNCs were separated by density gradient centrifugation (Histopaque-1083; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). The MNCs were divided into three aliquots and injected intravenously into secondary NOG recipients. Ten weeks after the second transplantation, bone marrow cells were harvested and analyzed.

Flow-Cytometric Analysis of Transplanted NOD/SCID and NOG Mice

Engraftment of human cells was examined by analyzing human surface antigens using BD LSR2 (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). Cells harvested from the bone marrow, peripheral blood, spleen, and thymus of recipient mice were treated with ammonium chloride red blood cell lysis buffer (Sigma-Aldrich) and blocked with PBS containing 2% FBS, anti-mouse CD16/32 antibody (BD Pharmingen), and FcR Blocking Reagent (Miltenyi Biotec). Then, they were stained with FITC-conjugated anti-human CD45 (clone HI30; BD Pharmingen) and anti-human CD3 (Beckman Coulter, Inc., Fullerton, CA, http://www.beckmancoulter.com), PE-conjugated anti-human CD13, CD33, CD56, CD4 (Beckman Coulter, Inc.), and CD133 (clone 293C3; Miltenyi Biotec), APC-conjugated anti-murine CD45 (clone 30-F11; BD Pharmingen), anti-human CD3, CD19, CD8 (Beckman Coulter, Inc.), and CD34 (Miltenyi Biotec), and 7-AAD (Via-Probe; BD Pharmingen). Successful engraftment of human hematopoietic cells was determined by detection of greater than 0.1% of human $CD45^+$ cells in recipient bone marrow cells.

Limiting Dilution Analysis

The frequencies of SRCs capable of repopulating in NOD/SCID mice were quantified by a limiting dilution analysis by applying Poisson statistics to the single-hit model as described previously [8, 22, 23]. The frequencies of SRCs and statistical comparison between individual populations were calculated by using L-Calc software (StemCell Technologies).

Statistical Analysis

Data are presented as mean ± SEM. Analysis of statistical significance was determined by paired *t* test.

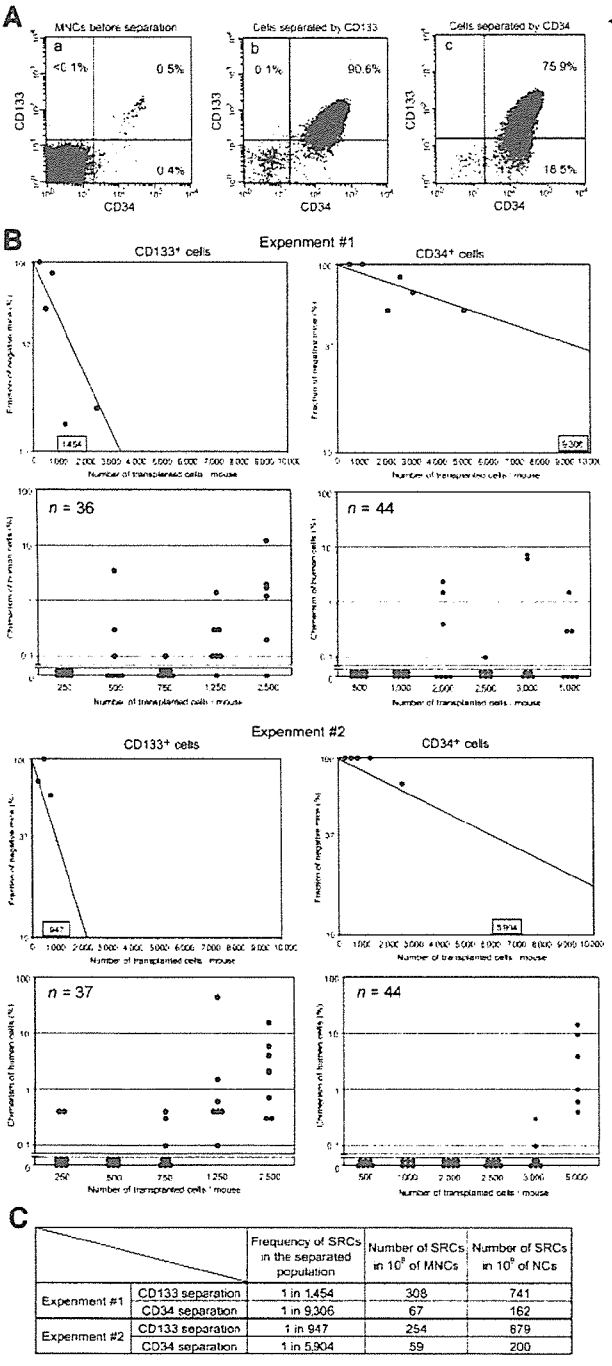
RESULTS

Stem Cell Isolation by the CD133-MACS Recovers a Higher Number of SRCs than by CD34

Because several investigators have suggested that SRCs are more concentrated in $CD133^+$ cells than in $CD34^+$ cells [19, 20], we directly compared the frequency of SRCs contained in the populations sorted by CD133-MACS and CD34-MACS. Flow-cytometric analyses of four CB samples showed that 0.2%–1.4% (mean 0.8%) and 0.8%–3.0% (mean 1.9%) of MNCs were positive for CD133 and CD34, respectively. More than 98% of $CD133^+$ cells were $CD34^+$, and approximately 43% (25%–56%) of $CD34^+$ cells were $CD133^+$ (Fig. 1A, a).

Then, we prepared two identical CB MNC aliquots and isolated CD133- and CD34-enriched cells by CD133- and CD34-MACS. Flow-cytometric analyses after isolation showed that the purities of separated cells were variable among samples (53.1%–93.5% for CD133 and 53.9%–96.3% for CD34), but there was no significant difference between the two separation methods ($p = .12$). Calculated recovery rates of the target cells (see Materials and Methods) were 66% ± 10% for CD133 and 46% ± 10% for CD34, showing a tendency of better recovery of CD133 cells by CD133-MACS than recovery of CD34 cells by CD34-MACS, but the difference was not significant ($p = .06$). After separation, approximately 75% of the CD34-sorted cells were $CD133^+$, whereas virtually all of the CD133-sorted cells were $CD34^+$ with only rare (0.1%) $CD34^-$ cells in most of the samples (Fig. 1A, b and c). Based on the comprehensive calculation, the recovery rates of $CD133^+CD34^+$ cells (i.e., the major SRC-containing population) in the individual samples were 66% ± 10% and 83% ± 8% by CD133- and CD34-MACS separation, respectively. The recovery efficiency of this most immature fraction by CD34-MACS tended to be superior to the one by CD133-MACS, but again it was not significantly different ($p = .14$).

Then, to compare the number of SRCs contained in the populations separated by CD133- and CD34-MACS, we transplanted cells of each population into irradiated NOD/SCID mice intravenously and examined their in vivo hematopoietic repopulating capacity. To evaluate the number of SRCs quantitatively, we transplanted serially reduced numbers of cells. Frequencies of SRCs in the CD133-sorted population were one of 1,454 and 947 in samples 1 and 2, respectively. In contrast, those in the CD34-sorted population were one of 9,306 and 5,904 in samples 1 and 2, respectively (Fig. 1B, 1C). This means that SRCs were sixfold more concentrated in the CD133-sorted population than in the CD34-sorted one. Converting this frequency into the absolute number of SRCs obtained from the same number of primary MNCs, CD133 sorting recovered 308 (sample 1) and 254 (sample 2) SRCs, and CD34 sorting recovered 67 (sample 1) and 59 (sample 2) SRCs from 10^8 of total MNCs (Fig. 1C). Therefore, despite the similar recovery rate of $CD133^+CD34^+$ cells by CD133- and CD34-sorting procedures, CD133 sorting provides 4.3–4.6-fold greater absolute numbers of SRCs than CD34 sorting. Thus, for our subsequent SRC expansion experiments, we used the CD133-sorted population as the culture-initiating cells.



C

		Frequency of SRCs in the separated population	Number of SRCs in 10^5 of MNCs	Number of SRCs in 10^5 of NCs
Expenment #1	CD133 separation	1 in 1,454	308	741
	CD34 separation	1 in 9,306	67	162
Expenment #2	CD133 separation	1 in 947	254	679
	CD34 separation	1 in 5,904	59	200

Figure 1. Separation of CD133- or CD34-enriched cells from CB MNCs and comparison of their in vivo repopulating capacity. **(A):** Expression profiles of CD133 and CD34 on CB MNCs (a) and cells separated by CD133- (b) and CD34-MACS (c). Representative data among several samples are shown. **(B):** The repopulating ability of CD133- and CD34-sorted cells isolated from the same CB samples (1 and 2). Frequencies of SRCs estimated by limiting dilution analyses are shown. The lower panels show chimeric proportion of human $CD45^+$ cells in the bone marrow of recipient mice, and the number of transplanted mice is shown in the upper-left margin of the panels. **(C):** Estimated frequencies and numbers of SRCs in the transplanted samples. In both experiments, CD133-sorted cells contain higher frequencies of SRCs than CD34-sorted cells, and CD133 sorting provides higher numbers of SRCs from the same volume of original MNCs or NCs than CD34 sorting. Abbreviations: CB, cord blood; MACS, magnetic cell sorting; MNC, mononuclear cell; NC, nucleated cell; SRC, severe combined immunodeficient repopulating cell.

Immobilized Delta1-Fc Chimeric Protein Can Expand Immature CB Hematopoietic Precursors in the Presence of Cytokines

Before evaluating methods for HSC expansion ex vivo, we first explored optimal culture conditions to expand immature hematopoietic precursors by using various combinations of hematopoietic cytokines and soluble Notch ligands. Because immobilization of Notch ligands has been demonstrated to be important for their efficient activity [24, 25], and it has been suggested that immobilized fibronectin fragment CH-296 along with Notch ligands augmented the effect of Notch ligands [24], we immobilized Delta1-Fc chimeric protein on the bottom of the culture plates along with human fibronectin prior to starting culture. We included SCF, TPO, and FL in the culture system as a basal cytokine combination (designated hereafter three growth factors, 3GFs) because these cytokines have been repeatedly shown to be effective for immature HSC/HPC expansion [26].

We cultured CB CD133-sorted cells in four cytokine combinations of (a) 3GFs + IL-6, (b) 3GFs + IL-6 + IL-3, (c) 3GFs + FP6, and (d) 3GFs + FP6 + IL-3, plus additional conditions with Delta1-Fc or IgG-Fc in each cytokine combination, and compared the expansion rate of total cells, $CD133^+CD34^+CD38^-$ immature hematopoietic cells, and mixed colony-forming cells (CFU-Mix). All culture conditions increased the number of total cells and $CD133^+CD34^+CD38^-$ cells during 3-week culture (Fig. 2A, 2B). Addition of IL-3 or replacement of IL-6 with FP6 gave greater expansion of total cells and $CD133^+CD34^+CD38^-$ cells. However, Delta1-Fc had very little effect on the expansion of these cells (Fig. 2A, 2B).

In contrast, addition of IL-3 was always required for the consistent expansion of CFU-Mix until 3 weeks (Fig. 2C). In the presence of IL-3, addition of FP6 increased the number of CFU-Mix significantly better than IL-6 ($p < .01$), recapitulating the previous findings of the lack of IL-6R on immature HPCs [10, 22] and of the requirement of gp130 signaling for the optimal expansion of these immature cells [10, 11]. Regarding the effect of soluble Notch ligands, Delta1-Fc remarkably increased the number of CFU-Mix for a period of 3 weeks, particularly when combined with IL-3 and FP6. Ultimately, the maximum expansion of CFU-Mix was achieved when cells were cultured with 3GFs + FP6 + IL-3 + Delta1-Fc for 3 weeks ($p < .05$) (Fig. 2C).

Effects of IL-6-gp130, IL-3, and Notch Signalings on SRC Expansion in the Serum-Free Culture

We have found that the number of CFU-Mix was continuously increased until 3 weeks in several conditions (Fig. 2C) and declined thereafter (data not shown). And although a previous report demonstrated that serum-containing culture with 3GFs and IL-6/sIL-6R for 1 week increased the number of SRCs by fourfold, no increase of human blood cell chimerism in recipient mice was observed when we cultured cells for 1 week in the serum-free conditions with either 3GFs + FP6 or 3GFs + FP6 + IL-3 + Delta1-Fc (data not shown). Based on these obser-

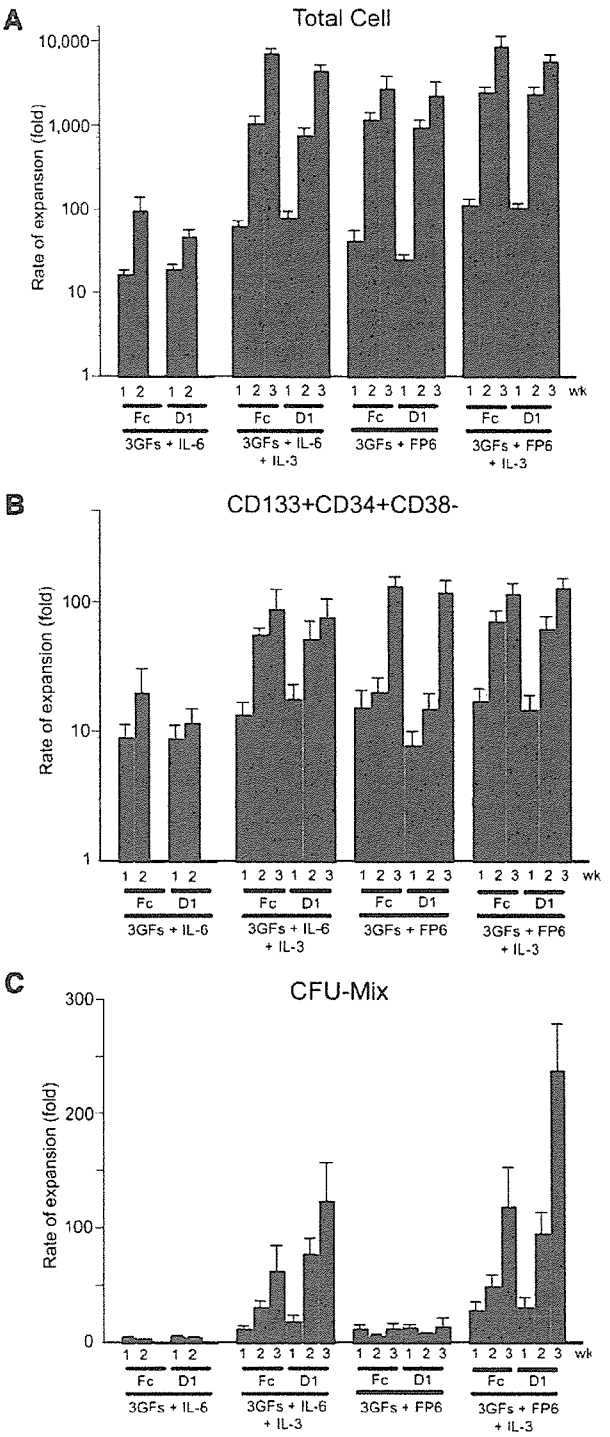


Figure 2. The expansion rates of total cells, $CD133^+CD34^+CD38^-$ immature cells, and CFU-Mix. CB CD133-sorted cells were cultured in the presence of indicated cytokines and Notch ligands for 1, 2, and 3 weeks. **(A, B):** The numbers of total cells **(A)** and $CD133^+CD34^+CD38^-$ immature hematopoietic cells **(B)** were counted, and the expansion rates are shown. **(C):** After indicated periods of culture, cells were replated in a semisolid medium and the number of CFU-Mix was evaluated. The expansion rate of the number of CFU-Mix is shown. Abbreviations: 3GFs, three growth factors; CFU-Mix, mixed colony-forming cells; D1, Delta1-Fc; Fc, IgG-Fc; FP6, interleukin-6/soluble interleukin-6 receptor chimeric protein; IL, interleukin; wk, week.

vations, we determined to culture cells for 3 weeks to evaluate SRC expansion.

As shown in Figure 3A, all the mice transplanted with more than 5,000 fresh CD133-sorted cells showed engraftment, but fewer than 2,500 cells failed to engraft in some of the mice. The frequency of SRCs in this sample was calculated as one of 1,020 (95% confidence interval [CI], 1/548–1/1,899) CD133-sorted cells. Progeny of the CD133-sorted cells grown for 3 weeks with 3GFs + FP6 contained SRCs at a frequency of equivalent to one of 640 (95% CI, 1/414–1/988) culture-initiating cells, and there was no statistical difference between the frequencies of SRCs in these populations, indicating that the addition of FP6–3GFs does not expand SRCs in the serum-free condition ($p = .11$; Fig. 3B), unlike in the serum-containing condition [10].

In contrast, when immobilized Delta1-Fc was present in the same cytokine combination (i.e., 3GFs + FP6), the frequency of SRCs increased to the equivalent to one of 361 (95% CI, 1/218–1/596) culture-initiating cells, indicating 2.8-fold SRC expansion compared with the SRC number before culture ($p = .005$, Fig. 3C). The addition of IL-3 to this condition further augmented the expansion efficiency, achieving the SRC frequency of equivalent to one of 175 (95% CI, 1/109–1/279) culture-initiating cells, indicating 5.8-fold expansion ($p = .0001$, Fig. 3D). To our knowledge, this ranks with the highest human SRC expansion efficiency ever reported. It is of note that two of six mice transplanted with cultured progeny equivalent to 60 culture-initiating cells showed human blood cell chimerism. To further compare the effects of IL-6 and FP6, we replaced FP6 with IL-6. In this condition, SRC frequency was equivalent to one of 266 (95% CI, 1/159–1/446) culture-initiating cells. The expansion rate was reduced from 5.8-fold to 3.8-fold, although significant expansion was still achieved ($p = .0006$; Fig. 3E).

Taken together, significant SRC expansion was realized in all three conditions with immobilized Delta1-Fc chimeric protein. Among these, combination of Delta1-Fc, IL-3, and IL-6/sIL-6R chimeric protein, FP6, in addition to 3GFs, provided the most significant expansion in the serum-free condition. It is noteworthy that IL-3 showed a positive effect in this condition, in contrast to the negative impact in the serum-containing condition without Notch signaling [10].

SRCs Cultured for 3 Weeks in the Serum- and Stromal Cell-Free Condition with 3GFs, FP6, IL-3, and Delta1-Fc Normally Contribute to Myeloid, B, T, and NK Cell Lineages in NOG Recipient Mice and Repopulate Recipients of Secondary Transplantation

To examine the long-term in vivo myeloid and lymphoid repopulating capacity of the cells cultured with 3GFs, FP6, IL-3, and Delta1-Fc, we transplanted these cells into NOG mice, which were generated by intercrossing NOD/SCID mice with IL-2 receptor common γ chain-knockout (γc^{null}) mice. These mice, unlike NOD/SCID mice, are known to allow transplanted human HSCs/HPCs to differentiate even into the T-cell lineage [21], and therefore we could examine the in vivo differentiation capacity of the ex vivo expanded HSCs most efficiently. These mice also have the advantage of higher engraftment of transplanted human cells. We cultured 10,000 CB CD133-sorted cells for 3 weeks and transplanted them into NOG mice. After 12 weeks, we observed 53%–67% human $CD45^+$ cells in the

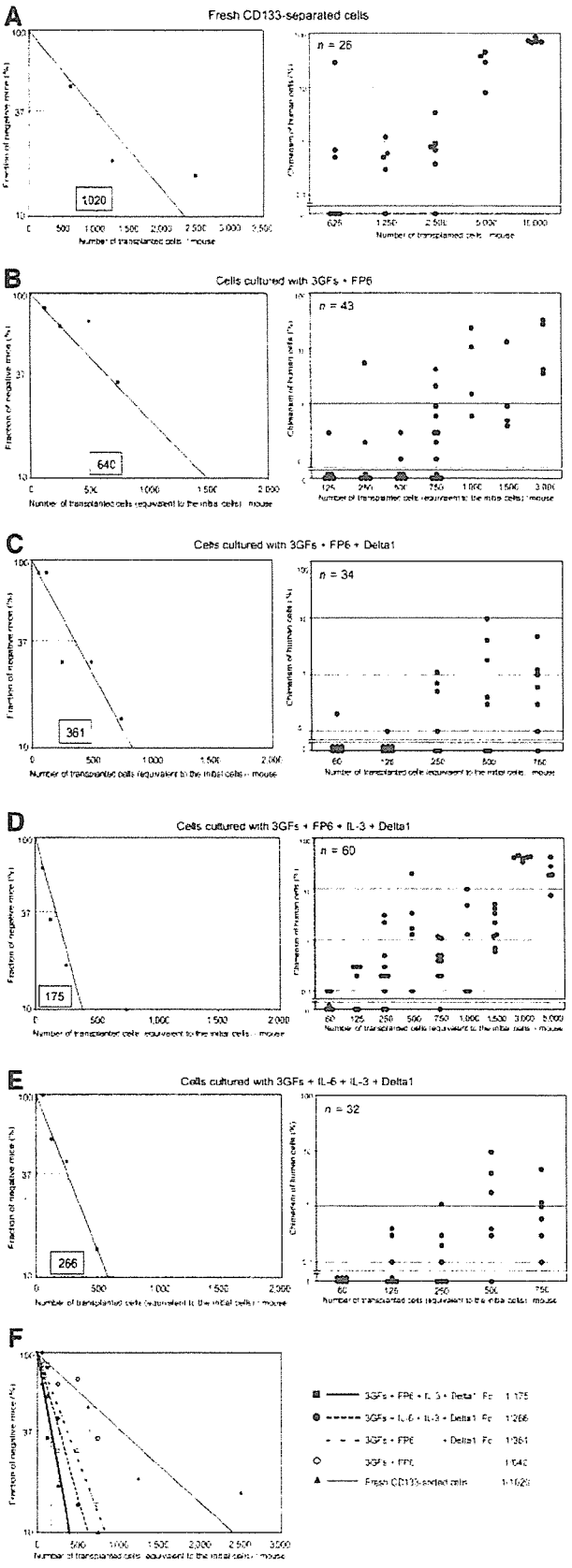


Figure 3. The repopulating ability of fresh CB CD133-sorted cells and their progenies after the culture with various combinations of cytokines for 3 weeks. **(A–E):** The frequencies of SRCs in fresh CD133-sorted cells **(A)**, cells cultured with SCF + TPO + FL (3GFs) + FP6 **(B)**, 3GFs + FP6 + Delta1-Fc **(C)**, 3GFs + FP6 + IL-3 + Delta1-Fc **(D)**, and 3GFs + IL-6 + IL-3 + Delta1-Fc **(E)**. They were estimated as 1/1,020 **(A)**, 1/640 **(B)**, 1/361 **(C)**, 1/175 **(D)**, and 1/266 **(E)**, respectively, by limiting dilution analyses. The right panels show chimeric proportion of human $CD45^+$ cells in the bone marrow of recipient mice, and the number of transplanted mice is shown in the upper-left margin of the panels. **(F):** Integrated representation of **(A–E)**. Correspondence of the symbols and lines is noted in the right. Abbreviations: 3GFs, three growth factors; FL, flt-3 ligand; FP6, interleukin-6/soluble interleukin-6 receptor chimeric protein; IL, interleukin; SCF, stem cell factor; TPO, thrombopoietin.

recipient bone marrow. Further analyses of the bone marrow, peripheral blood, spleen, and thymus of recipient mice revealed that human hematopoietic cells differentiated into myeloid ($CD13^+$ or $CD33^+$), B ($CD19^+$), T ($CD3^+$), and NK ($CD56^+$) cell lineages (Fig. 4A). In addition, in the bone marrow of recipient mice, we detected $CD133^+CD34^+$ immature hematopoietic cells at frequencies of 0.5%–1.1% of human cells. In the thymus, human cells represented virtually all the $CD3^+$ cells (data not shown), and among the $CD3^+$ cells, the patterns of differentiation to CD4/CD8 double-positive, CD4 single-positive, and CD8 single-positive cells were very similar to that of normal thymocytes (Fig. 4A). Robust human hematopoietic repopulation was confirmed in another recipient mouse 24 weeks after transplantation (Fig. 4B). In this mouse, more definite reconstitution of $CD3^+$ mature T cells was observed in the peripheral blood and spleen.

To confirm that transplanted HSCs still retain their self-renewal capacity after primary transplantation, we collected bone marrow cells 24 weeks after the transplantation from a primary recipient, which had been transplanted with the progeny of 1×10^4 CB CD133-sorted cells ex vivo expanded, and injected them into three secondary NOG mice. Ten weeks after the secondary transplantation, we observed engraftment of human $CD45^+$ cells (0.1%) in the bone marrow of two recipient mice (Fig. 5A), and human hematopoietic cells differentiated into myeloid ($CD13^+$ or $CD33^+$) and lymphoid ($CD19^+$) cells (Fig. 5B). These findings strongly indicate that cells cultured with 3GFs, FP6, IL-3, and Delta1-Fc for 3 weeks retain long-term repopulating capacity and normal differentiation capacity in vivo.

DISCUSSION

Efficient Ex Vivo Expansion of SRCs

In this study, we demonstrated successful expansion of SRCs by approximately sixfold, by culturing human CB CD133-enriched cells with SCF, TPO, FL, FP6, IL-3, and Delta1-Fc. SRCs have now been widely accepted as the most immature human hematopoietic cells and are regarded as surrogates for HSCs [26]. In many reports, expansion of SRCs has been discussed by comparison of human blood cell chimerism in recipient mice [16–18, 27–31]. However, to quantify the number of SRCs, limiting dilution/transplantation analyses are essential. Moreover, because the human blood cell chimerism in the bone marrow of recipient NOD/SCID mice typically stabilizes at 10–12 weeks

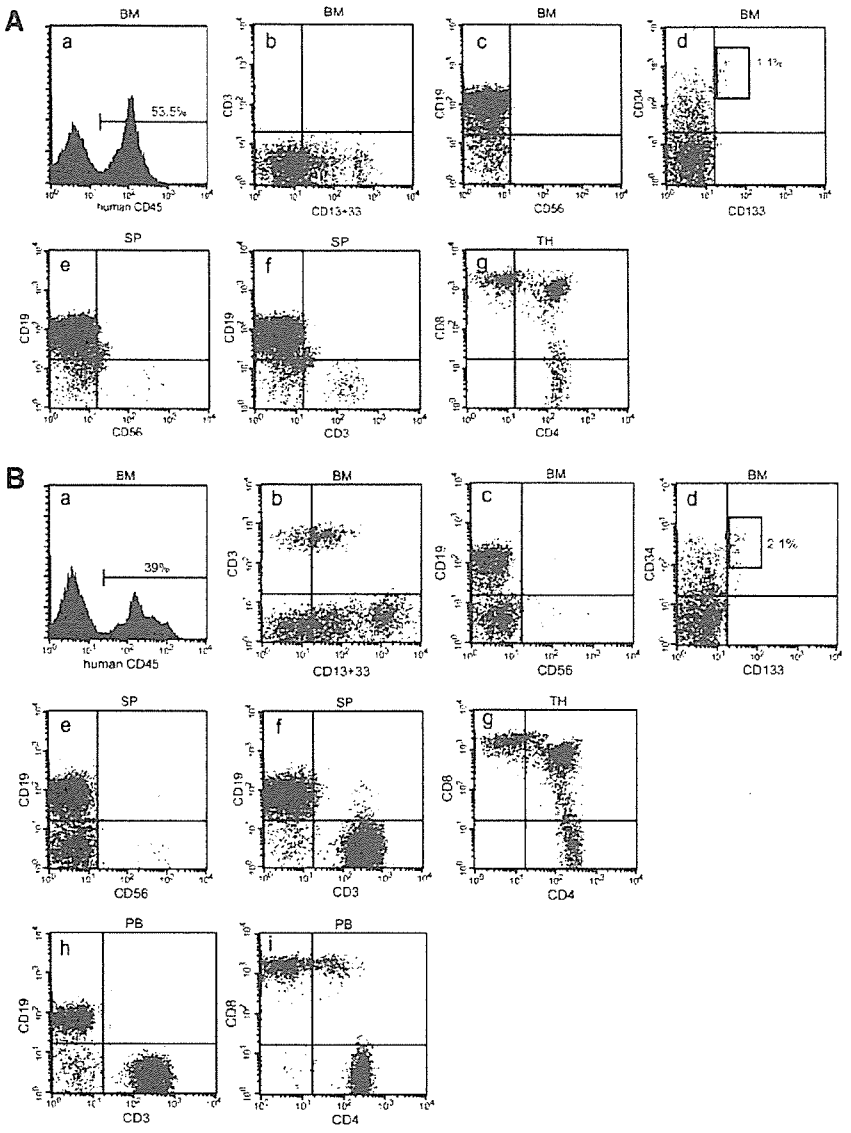


Figure 4. In vivo repopulating and differentiation capacity of the cells cultured with 3GFs + FP6 + IL-3 + Delta1-Fc. Cord blood CD133-sorted cells were cultured with 3GFs + FP6 + IL-3 + Delta1-Fc for 3 weeks and were transplanted into NOG mice. The BM, PB, SP, and TH of recipient mice were collected 12 weeks **(A)** and 24 weeks **(B)** after transplantation, and contribution of human cells to various hematopoietic lineages was examined by flow-cytometric analyses. **(A):** Representative data of recipient mice examined 12 weeks after transplantation. Human $CD45^+$ cells accounted for 53.5% of total BM cells (a), and a substantial number of human $CD3^+$ (b, f), $CD13^+$ (b), $CD33^+$ (b), $CD19^+$ (c, e), and $CD56^+$ (c, e) cells were detected in the BM and spleen. $CD133^+CD34^+$ immature hematopoietic cells were also clearly identified (1.1%) in the BM (d). In the thymus, $CD3^+$ cells expressed CD4 and/or CD8 (g) showing a solid development of human T cells. (b–g) represent data gated by human $CD45^+$ cells. **(B):** Flow-cytometric data from a mouse examined 24 weeks after transplantation. A high level of engraftment (a) (39%), reconstitution of $CD133^+CD34^+$ immature cells (d) (2.1%), and contribution to myeloid (b), B-cell (c, e, f, h), T-cell (b, f–i) and NK-cell (c, e) lineages were confirmed in the BM, spleen, thymus, and peripheral blood. (b–i) represent data gated by human $CD45^+$ cells. Abbreviations: 3GFs, three growth factors; BM, bone marrow; FP6, interleukin-6/soluble interleukin-6 receptor chimeric protein; IL, interleukin; NK, natural killer; PB, peripheral blood; SP, spleen; TH, thymus.

after transplantation [32–34], observation for at least 8–10 weeks is optimal to evaluate SRC numbers accurately, and only a few reports have fulfilled these conditions [8–10]. Our analysis, satisfying these criteria, revealed expansion of SRCs, which ranks as the most efficient one. Our method also enabled a 240-fold expansion of CFU-Mix, demonstrating its surprisingly strong effect on expanding immature progenitors.

We also demonstrated that the cultured cells can differentiate in vivo into myeloid, B, T, and NK cell lineages in the bone marrow, peripheral blood, spleen, and thymus in NOG mice. Human cells transplanted into NOG mice can engraft at significantly higher levels than NOD/SCID mice, and transplanted cells can differentiate even to the T-cell lineage. Based on these features, NOG mice are increasingly used as recipients of human stem cells as well as NOD/SCID/β2-microglobulin null mice [21, 35, 36]. We found immature $CD133^+CD34^+$ human cells in the bone marrow of recipient NOG mice at a substantial frequency, and after serial transplantation, progeny of the cultured cells engrafted most of the secondary recipients. These findings suggest that the culture system preserves normal stem cell functions.

Positive Effects of Notch Signaling on SRC Expansion

A positive effect of soluble Notch ligands on human SRCs was previously suggested by two groups, although they did not confirm the increase of SRCs quantitatively [16–18]. In the current study, we have provided clear data that demonstrate that the soluble Notch ligand can truly increase the number of human SRCs ex vivo. There are increasing lines of evidence suggesting that the Notch signaling pathway physiologically plays an important role in maintaining HSCs in the bone marrow niche [15, 37], where the Notch signal might inhibit differentiation of HSCs [15, 37]. Recently, the negative effects of reactive oxygen species for HSC maintenance were discovered [38], and the importance of the low oxygen environment in the HSC niche has been highlighted [39]. Interestingly, maintenance of the undifferentiated state by hypoxia-induced hypoxia-inducible

A

Primary transplant		Secondary transplants			
Cell dose	Human engraftment (%)	Mouse	Transplanted Cell dose	Transplanted Human Cell dose	Human engraftment (%)
cultured cells from 1×10^4 $CD133^+$ cells	24.2	S1	1.1×10^6	2.6×10^5	0.1
		S2	1.1×10^6	2.6×10^5	0
		S3	1.1×10^6	2.6×10^5	0.1

B

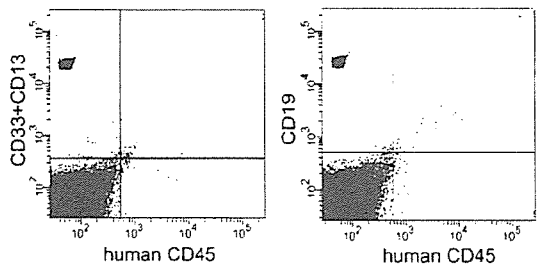


Figure 5. Cells cultured with three growth factors (3GFs) + interleukin (IL)-6/soluble receptor chimeric protein (FP6) + IL-3 + Delta1-Fc retain long-term repopulating capacity after serial transplantation into secondary nonobese diabetic/severe combined immunodeficient/γc^{null} (NOG) recipients. Ten-thousand cord blood CD133-sorted cells were cultured with 3GFs + FP6 + IL-3 + Delta1-Fc for 3 weeks and were transplanted into a primary NOG mouse. Twenty-four weeks after transplantation, bone marrow (BM) cells were harvested and serially transplanted into three secondary NOG recipients. **(A):** Ten weeks after secondary transplantation. BM cells of recipient mice were harvested and chimerism of human cells was analyzed. Two of the three secondary recipients showed substantial human engraftment. **(B):** Representative flow-cytometric data of BM cells in a secondary recipient (mouse S1). Human myeloid ($CD13^+$ or $CD33^+$) and lymphoid ($CD19^+$) cells can be identified. Data with isotype controls are shown as insets in the upper-left margin of the figures.

factor 1α (HIF1α) activation requires Notch signaling, and conversely, Notch signaling is enhanced by activation of HIF1α [39]. It is thus interesting to combine our system with hypoxic conditions for further better efficiency of ex vivo HSC expansion.

Effects of IL-3 and gp130 Signaling Pathways on SRC Expansion

We found that IL-3 exerts positive effects on amplifying SRCs at least in the presence of SCF, TPO, FL, FP6, and Delta1-Fc in a serum-free condition. To date, many researchers have examined the effects of IL-3 on HSCs, but the results have been controversial: some reports showed maintenance of HSCs, whereas others showed negative effects [40]. This discrepancy may depend on the addition of serum, the difference of coexisting cytokines, and the culture-initiating cells. Our result may suggest that IL-3 has additive or synergistic effects with Delta1-Fc on HSCs in the absence of serum.

We also found that replacement of IL-6 with FP6 had some superior effects on SRC expansion. Unlike the addition of IL-3, however, the effects of FP6 were marginal in the presence of Delta1-Fc and IL-3. This could be because the combination of Delta1-Fc and IL-3 could transmit nearly optimal growth signals in HSCs. Or the difference of the cell source (i.e., CD133- vs. CD34-sorted cells) might explain the results [10, 11].

Stem Cell Source for Transplantation and Ex Vivo Culture

To obtain the maximum efficiency of stem cell expansion, the isolation method for culture-initiating cells is also very important. CD34 sorting has been most widely used for positive selection of HSCs in the clinical practice. Recently, feasibility of the CD133-sorted cell transplantation has been evaluated in several clinical trials [41, 42]. There has been, however, no direct comparison of the SRC numbers obtained by these two methods. To the best of our knowledge, most of the CB SRCs are present in the $CD133^+CD34^+$ population [19, 20], and thus, the isolation methods are expected to provide a similar number of SRCs if the separation efficiencies are the same. Surprisingly, however, we found that the absolute SRC numbers were approximately 4.5-fold greater in the CD133-sorted population than in the CD34-sorted one, despite the fact that the recovery efficiency of $CD133^+CD34^+$ cells was very similar. One explanation to this apparently unexpected result could be that CD34, a cell-surface sialomucin protein, might be interfered with by the anti-CD34 antibody used for isolation. Given that accumulating evidence suggests that CD34 regulates homing of the cells to the proper microenvironment after i.v. injection by inhibiting inappropriate cell adhesion [43–45], the anti-CD34 antibody might interfere with the proper homing of the cells by modulating the adhesion capacity. Specifically, we used MACS Direct CD34 Progenitor Cell Isolation Kit for enrichment of $CD34^+$ cells, and this system uses monoclonal antibody QBend10, which recognizes the Class II epitope of the CD34 antigen. The QBend10 and other ClassII antibodies have been shown to induce actin polymerization and enhance cytoadhesiveness of KG-1 cells and primary bone marrow $CD34^+$ cells [46, 47], and this biological property may have reduced the SCID repopulating capacity of the CD34-sorted cells.

Recently, a small population of $CD133^+CD34^-$(Lineage$^-$ [Lin^-]) hematopoietic cells was identified. Because these cells give rise to $CD34^+$ SRCs during culture, $CD133^+CD34^-$ cells might represent precursors of SRCs [48–50]. Therefore, although these cells account for no more than 0.1% of the CD133-sorted cells, the use of CD133-sorted cells as the culture-initiating cells may help increase the absolute number of HSCs after culture.

All these considerations imply that CD133-sorted cells are more advantageous as a direct source for HSC transplantation and as a culture-initiating source for ex vivo HSC expansion than CD34-sorted cells to obtain a greater number of HSCs. According to our results, we can estimate that culturing CD133-sorted cells with Delta1-Fc yields as many as approximately 25-fold greater numbers of HSCs compared with the fresh CD34-sorted cells. Currently, clinical devices for CD34 sorting which use QBend10 or other Class II anti-CD34 antibodies are widely used. Our findings suggest that CD133 sorting might be a better way to collect and enrich HSCs than CD34 sorting by QBend10 or other ClassII antibodies. Future studies that directly compare the clinical outcome of CD133 and CD34 sortings may deepen our understandings for effective enrichment of HSCs.

Conclusion

In this report, we have demonstrated that serum-free culture of the CD133-sorted human CB cells in the presence of SCF, TPO, FL, FP6, IL-3, and Delta1-Fc is an optimized condition to obtain