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Comparison of Cord Blood Transplantation with Unrelated Bone Marrow Transplantation in Patients Older than Fifty Years

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A B S T R A C T

We retrospectively compared the transplantation outcomes for patients 50 years or older who received umbilical cord blood transplantation (UCBT) with those who received unrelated bone marrow transplantation (UBMT) for hematologic malignancies. A total of 1377 patients who underwent transplantation between 2000 and 2009 were included: 516 received 8/8 HLA allele-matched UBMT, 295 received 7/8 HLA allele-matched UBMT, and 566 received 4/6 to 6/6 HLA-matched UCBT. Adjusted overall survival (OS) was significantly lower in those who underwent UCBT than those who underwent 8/8 HLA–matched UBMT but was similar to that of 7/8 HLA–matched UBMT (the 2-year OS after 8/8 HLA–matched UBMT, 7/8 HLA–matched UBMT, and UCBT were 49% [95% confidence interval (CI), 45% to 55%], 38% [95% CI, 32% to 45%], and 39% [95% CI, 34% to 43%], respectively). However, adjusted OS was similar between 8/8 HLA–matched UBMT and UCBT receiving $\geq .84 \times 10^5$ $CD34^+$ cells/kg among those with acute myeloid leukemia and those with acute lymphoblastic leukemia (the 2-year OS was 49% [95% CI, 43% to 55%], and 49% [95% CI, 41% to 58%], respectively). These data suggest that UCB is a reasonable alternative donor/stem cell source for elderly patients with similar outcomes compared with UBM from 8/8 HLA–matched unrelated donors when the graft containing $\geq .84 \times 10^5$ $CD34^+$ cells/kg is available.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment for patients with high-risk hematologic malignancies. The frequency of adverse cytogenetic abnormalities is higher in elderly patients with acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL) than in younger patients, and overall survival (OS) after intensive chemotherapy in elderly patients is shorter than that in younger patients [1,2]. Inductions of reduced-intensity and nonmyeloablative stem cell transplantations allow elderly patients to receive allogeneic HSCT [3,4], and these patients have increasingly received this type of transplantation [5]. Only approximately 30% of patients

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have an HLA-identical sibling, and some elderly patients have siblings who cannot serve as a donor because of their age or underlying comorbidities; in such cases, an alternative donor is needed.

HLA-matched unrelated bone marrow or peripheral blood stem cells have been used as an alternative to an HLA-identical sibling donor. Umbilical cord blood has been used more frequently over the past decade, and several studies and meta-analyses have compared the outcomes of umbilical cord blood transplantation (UCBT) with that of unrelated bone marrow transplantation (UBMT) or unrelated peripheral blood stem cell transplantation (UPBSCT) [6-15]. However, the findings of those reports varied, and most of those studies included a small number of elderly patients. To the best of our knowledge, there has been no report that compared the outcomes of elderly patients who received UCBT with those who received UBMT or UPBSCT. Therefore, the main objective of this study was to compare the outcomes of patients 50 years or older who received UCBT with those who received UBMT using the Japanese nationwide registry data.

METHODS

Data Collection

Data regarding transplantations were extracted from the Transplant Registry Unified Management Program system of the Japan Society for Hematopoietic Cell Transplantation [16]. A total of 171 transplantation centers performed unrelated HSCT for adults and reported transplantation data to Japan Society for Hematopoietic Cell Transplantation between 2000 and 2009. All patients gave written informed consent at each transplantation center. The trial was conducted in accordance with the Declaration of Helsinki.

Patients with acute leukemia or myelodysplastic syndrome (MDS) who were 50 years or older and who received unrelated HSCT between 2000 and 2009 were included. Because the bone marrow was exclusively harvested from volunteer unrelated donors in Japan, cases of peripheral blood stem cell transplantation were not included in this analysis. Only 7 patients received double UCBT; therefore, these patients were also excluded. For the bone marrow recipients, recipients whose HLA matched 8/8 or 7/8 with their donor at the allelic level for HLA-A, HLA-B, HLA-C, and HLA-DRB1 were included. For UCBT, recipients whose HLA matched 4/6 to 6/6 with their donor at the antigen level for HLA-A and HLA-B and at the allelic level for HLA-DRB1, and who received a single unit of umbilical cord blood containing 2.0×10^7 or more total nucleated cells per kilogram of recipient's body weight at cryopreservation were included. Patients who had previously received autologous or allogeneic transplantation were excluded.

A myeloablative conditioning (MAC) regimen was defined as a total busulfan dose of more than 8 mg/kg, total melphalan dose of more than 140 mg/kg, fractionated total body irradiation (TBI) of 8 Gy or more, or single TBI of 5 Gy or more [17,18]. Other conditioning regimen was defined as reduced-intensity conditioning (RIC). Acute leukemia in the first complete remission (CR), refractory anemia with or without ringed sideroblasts, and refractory cytopenia with multilineage dysplasia for MDS were defined as early phase; acute leukemia in the second or subsequent CR were defined as intermediate phase; and all other statuses were defined as advanced phase. The karyotype at diagnosis for AML, ALL, and MDS were classified as previously reported [2,19,20]. The year of transplantation was divided into 2 groups: 2000 to 2004 was defined as the early period and 2005 to 2009 was defined as the recent period. Neutrophil recovery was defined as the first 3 consecutive days in which absolute neutrophil counts rose to greater than or equal to 500/mm^3. Acute graft-versus-host disease (GVHD) was evaluated based on standard criteria [21]. Chronic GVHD was defined according to the classical classification [22]. Relapse was defined as disease recurrence detected by hematological examination or detected by cytogenetic or molecular examination and requiring any treatment. Patients who did not obtain CR after HSCT were defined as patients who had a relapse the next day after HSCT. Nonrelapse mortality (NRM) was defined as death without relapse. OS was defined as the survival time from the date of transplantation to death from any cause or the last follow-up.

Statistical Analysis

The demographic factors and disease characteristics were compared between patients who underwent transplantation with 8/8 HLA–matched unrelated bone marrow, 7/8 HLA–matched bone marrow, and umbilical cord blood using Fisher's exact test for the categorical data and the Mann-Whitney U test for the continuous variables. OS was calculated from the date of transplantation to death from any cause or last follow-up and was estimated by the Kaplan-Meier method. Cox proportional hazards regression model was used for the multivariate analyses. Adjusted comparison of the stem cell source on OS was performed using the Cox proportional hazards regression model. Gray's test was employed for the comparison of cumulative incidence curves for relapse, NRM, neutrophil and platelet recoveries, and GVHD [23]. NRM and relapse were the competing event for each other. For neutrophil and platelet recovery, death before neutrophil or platelet recovery was the competing event; for GVHD, death without GVHD was the competing event. Fine and Gray's proportional hazard regression model was employed for multivariate analyses with competing risks [24]. Multivariate analyses to compare the effect of stem cell source on transplantation outcomes were performed with the consideration of other significant clinical variables in the final models, which were built with the significant variables ($P < .10$) from the univariate analysis, which were then deleted in a stepwise fashion from the model when a variable was not statistically significant ($P > .05$). The stem cell source was added in the final model. The following variables were considered: patient age at transplantation, sex, primary disease (AML versus ALL versus MDS), karyotype at diagnosis (favorable versus intermediate versus adverse), disease status at transplantation (early phase versus intermediate phase versus advanced phase), year of transplantation (early period versus recent period), conditioning regimen (MAC versus RIC), use of TBI, and GVHD prophylaxis (cyclosporine alone versus cyclosporine and other agent versus tacrolimus alone versus tacrolimus and other agent versus other). All tests were 2-sided, and $P < .05$ was considered to indicate statistical significance. Analyses were performed with EZR version 1.20 (Saitama Medical Center, Jichi Medical University) [25], which is a graphical user interface for R version 3.0.2 (R Development Core Team, Vienna, Austria).

RESULTS

Patients and Transplantation Characteristics

Patients and transplantation characteristics are shown in Table 1. A total of 1377 patients were included in this analysis, and of those, 516 patients received 8/8 HLA allele-matched UBMT, 295 patients received 7/8 HLA allelic-matched UBMT, and 566 patients underwent transplantation from 4/6 to 6/6 HLA–matched UCBT. The UCBT recipients were significantly older than the 8/8 or 7/8 HLA–matched UBMT recipients ($P < .001$), and more UCBT recipients underwent RIC or nonmyeloablative transplantation ($P < .001$) and received a TBI-containing conditioning regimen than did the 8/8 or 7/8 HLA–matched UBMT recipients ($P < .001$). More UCBT recipients had advanced phase disease ($P < .001$). Female donor to male recipient transplantation was included in UCBT more than in UBMT ($P < .001$). Compared with those receiving UBMT, more UCBT recipients had AML ($P < .001$) and received GVHD prophylaxis with a single-agent regimen ($P < .001$). The distribution of karyotype at diagnosis was similar (Supplemental Tables 1–3). The distribution of recipients' sex and year of transplantation were similar among the 3 groups. The median duration of follow-up for the surviving patients who underwent transplantation with 8/8 HLA–matched UBMT, 7/8 HLA–matched UBMT, and 4/6 to 6/6 HLA-matched UCBT was 23.7 months (range, 1.8 to 125.2 months), 18.6 months (range, 1.6 to 94.0 months), and 22.3 months (range, .1 to 107.5 months), respectively.

Hematopoietic Recovery

The median time from transplantation to neutrophil recovery in patients who underwent 8/8 HLA–matched UBMT, 7/8 HLA–matched UBMT, and 4/6 to 6/6 HLA–matched UCBT was 17 days (range, 1 to 100 days), 17 days (range, 4 to 169 days), and 24 days (range, 0 to 95 days), respectively. Neutrophil recovery was faster in recipients with early phase disease or intermediate phase disease than in those with advanced phase disease ($P < .001$). MAC was an independent negative predictor for neutrophil engraftment ($P = .007$). The

Table 1
Patients, Disease, and Transplantation Characteristics

Characteristic	Total	8/8 HLA–Matched Bone Marrow	7/8 HLA–Matched Bone Marrow	Umbilical Cord Blood	*P* Value
Number	1377	516	295	566	
Sex (male)	816 (59%)	310 (60%)	188 (64%)	318 (56%)	.091
Age, median (range), yr	57 (50-82)	56 (50-70)	57 (50-71)	58 (50-82)	<.001
50-59	892 (65%)	376 (73%)	198 (67%)	318 (56%)	
60-69	468 (34%)	138 (27%)	96 (33%)	234 (41%)	
70 or older	17 (1%)	2 (<1%)	1 (<1%)	14 (3%)	
Sex matching					<.001
Female donor to male recipient	1030 (75%)	73 (14%)	67 (23%)	153 (27%)	
Others	293 (21%)	443 (86%)	227 (77%)	360 (64%)	
Unknown	54 (4%)	0 (0%)	1 (<1%)	53 (9%)	
Body weight, median (range), kg	56 (32.0-102.4)	58.5 (32.0-102.4)	58.9 (35.1-92.0)	54.0 (32.0-86.0)	<.001
Disease					<.001
AML	902 (65%)	314 (61%)	180 (61%)	408 (72%)	
ALL	244 (18%)	96 (19%)	47 (16%)	101 (18%)	
MDS	231 (17%)	106 (20%)	68 (23%)	57 (10%)	
Disease status at transplantation					<.001
Early phase	471 (34%)	223 (43%)	94 (32%)	154 (27%)	
Intermediate phase	221 (16%)	82 (16%)	58 (20%)	81 (14%)	
Advanced phase	685 (50%)	211 (41%)	143 (48%)	331 (59%)	
Year of transplantation					
2000-2004	343 (25%)	128 (25%)	74 (25%)	141 (25%)	1
2005-2009	1034 (75%)	388 (75%)	221 (75%)	425 (75%)	
Conditioning regimen					
Myeloablative	653 (47%)	291 (56%)	147 (50%)	215 (38%)	<.001
CY + TBI (≥8 Gy)	174 (12%)	79 (15%)	43 (15%)	52 (9%)	
CY + TBI (≥8 Gy) + other	135 (10%)	46 (9%)	19 (6%)	70 (13%)	
BU + CY	110 (8%)	64 (12%)	33 (12%)	13 (2%)	
FLU + BU (>8 mg/kg)	44 (3%)	34 (7%)	5 (2%)	5 (1%)	
FLU + BU (>8 mg/kg) + TBI (<8 Gy)	40 (3%)	14 (3%)	7 (2%)	19 (3%)	
FLU + MEL (>140 mg/m^2)	57 (4%)	28 (5%)	20 (7%)	9 (2%)	
Other TBI-based regimen	66 (5%)	19 (4%)	13 (4%)	34 (6%)	
Other BU-based regimen	27 (2%)	7 (1%)	7 (2%)	13 (2%)	
RIC/NMA	712 (52%)	217 (42%)	145 (49%)	350 (62%)	
FLU + BU (≤8 mg/kg)	25 (2%)	5 (1%)	5 (2%)	15 (3%)	
FLU + BU (≤8 mg/kg) + TBI (<8 Gy)	206 (15%)	91 (17%)	58 (20%)	57 (10%)	
FLU + BU (≤8 mg/kg) + MEL (≤140 mg/m^2)	26 (2%)	13 (3%)	5 (2%)	8 (1%)	
FLU + BU (≤8 mg/kg) + other	33 (2%)	12 (2%)	16 (5%)	5 (1%)	
FLU + MEL (≤140 mg/m^2)	64 (5%)	33 (6%)	16 (5%)	15 (3%)	
FLU + MEL (≤140 mg/m^2) + TBI (<8 Gy)	219 (16%)	33 (6%)	26 (9%)	160 (28%)	
FLU + MEL (≤140 mg/m^2) + TBI (<8 Gy) + other	20 (2%)	3 (1%)	1 (<1%)	16 (3%)	
FLU + CY + TBI (<8 Gy)	56 (4%)	3 (1%)	2 (1%)	51 (9%)	
Other regimen including TBI (<8 Gy)	33 (2%)	13 (3%)	10 (3%)	10 (2%)	
Other regimen not including TBI (<8 Gy)	30 (2%)	11 (2%)	6 (2%)	13 (2%)	
Unknown	12 (1%)	8 (2%)	3 (1%)	1 (<1%)	
TBI-containing conditioning regimen	962 (70%)	306 (59%)	184 (62%)	472 (83%)	<.001
Addition of ATG to conditioning regimen	46 (3%)	17 (3%)	19 (6%)	10 (2%)	.001
GVHD prophylaxis					
CyA + other	370 (27%)	129 (25%)	52 (18%)	189 (33%)	<.001
CyA alone	68 (5%)	5 (1%)	3 (1%)	60 (11%)	
TAC + other	775 (56%)	359 (70%)	226 (76%)	190 (33%)	
TAC alone	138 (10%)	15 (3%)	11 (4%)	112 (20%)	
Others	13 (1%)	7 (1%)	3 (1%)	3 (1%)	
None	13 (1%)	1 (<1%)	0 (0%)	12 (2%)	
Total cell dose (range, ×10^7/kg)				2.56 (2.00-5.62)	
CD34$^+$ cell dose (range, ×10^5/kg)				.83 (.01-14.02)	
HLA-A, B, DR antigen level					
Matched (6/6)		516 (100%)	295 (100%)	46 (8%)	
One-antigen mismatched (5/6)		0	0	159 (28%)	
Two-antigen mismatched (4/6)		0	0	361 (64%)	

HLA indicates human leukocyte antigen; TBI, total body irradiation; GVHD, graft-versus-host disease; CY, cyclophosphamide; BU, busulfan; FLU, fludarabine; MEL, melphalan; NMA, nonmyeloablative; ATG, antithymocyte globulin; CyA, cyclosporine A; TAC, tacrolimus.

probability of neutrophil recovery by day 50 was significantly lower in recipients of 4/6 to 6/6 HLA–matched UCBT (72% [95% confidence interval (CI), 68% to 75%]) than in those of 8/8 HLA–matched UBMT (95% [95% CI, 92% to 96%]) or 7/8 HLA–matched UBMT (90% [95% CI, 85% to 93%]). On multivariate analysis, the 4/6 to 6/6 HLA–matched UCBT was an independent negative predictor for neutrophil engraftment when compared with the 8/8 HLA–matched UBMT (hazard ratio [HR], .43 [95% CI, .38 to .50]; $P < .001$) and the 7/8 HLA–matched UBMT (HR, .47 [95% CI, .40 to .56]; $P < .001$) (Table 2).

The probability of platelet recovery by day 180 was also significantly lower in the 4/6 to 6/6 HLA–matched UCB recipients (54% [95% CI, 50% to 58%]) than in those who received the 8/8 HLA–matched UBMT (83% [95% CI, 79% to 86%]) or the 7/8 HLA–matched UBMT (75% [95% CI, 70% to 80%]). The median times from transplantation to platelet recovery in the recipients of 8/8 HLA–matched

Table 2
Multivariate Analysis of Transplantation Outcomes

Outcome	HR (95% CI)	*P* Value
Overall survival*	Overall	<.001
4/6-6/6–Matched UCB versus 8/8 HLA–matched UBM	1.47 (1.24-1.74)	<.001
4/6-6/6–Matched UCB versus 7/8 HLA–matched UBM	1.03 (.86-1.24)	.75
Relapse†	Overall	.02
4/6-6/6–Matched UCB versus 8/8 HLA–matched UBM	1.35 (1.05-1.74)	.02
4/6-6/6–Matched UCB versus 7/8 HLA–matched UBM	1.18 (.89-1.56)	.26
NRM‡	Overall	.013
4/6-6/6–Matched UCB versus 8/8 HLA–matched UBM	1.32 (1.06-1.64)	.013
4/6-6/6–Matched UCB versus 7/8 HLA–matched UBM	.98 (.77-1.25)	.88
Neutrophil recovery§	Overall	<.001
4/6-6/6–Matched UCB versus 8/8 HLA–matched UBM	.42 (.37-.48)	<.001
4/6-6/6–Matched UCB versus 7/8 HLA–matched UBM	.47 (.40-.55)	<.001
Platelet recovery‖	Overall	<.001
4/6-6/6–Matched UCB versus 8/8 HLA–matched UBM	.36 (.30-.42)	<.001
4/6-6/6–Matched UCB versus 7/8 HLA–matched UBM	.44 (.37-.53)	<.001
Grade II-IV acute GVHD¶	Overall	.36
4/6-6/6–Matched UCB versus 8/8 HLA–matched UBM	1.10 (.89-1.36)	.38
4/6-6/6–Matched UCB versus 7/8 HLA–matched UBM	.69 (.56-.87)	.001
Extensive chronic GVHD#	Overall	.022
4/6-6/6–Matched UCB versus 8/8 HLA–matched UBM	.65 (.46-.92)	.015
4/6-6/6–Matched UCB versus 7/8 HLA–matched UBM	.56 (.38-.82)	.003

UCB indicates umbilical cord blood; UBM, unrelated bone marrow.

* For overall survival, hazard ratio is adjusted with recipient age, sex, primary disease, disease status at transplantation, and year of transplantation.

† For relapse, hazard ratio is adjusted with primary disease, the use of TBI, the use of antithymocyte globulin, and disease status at transplantation.

‡ For NRM, hazard ratio is adjusted with recipient sex, the use of TBI, and year of transplantation.

§ For neutrophil recovery, hazard ratio is adjusted with disease status at transplantation, conditioning regimen, the use of TBI, and GVHD prophylaxis.

‖ For platelet recovery, hazard ratio is adjusted with recipient sex, disease status at transplantation, the use of TBI, year of transplantation, and GVHD prophylaxis.

¶ For grade II to IV acute GVHD, hazard ratio is adjusted with age, disease status at transplantation, and the use of TBI.

\# For extensive chronic GVHD, hazard ratio is adjusted with recipient sex.

UBMT, 7/8 HLA–matched UBMT, and 4/6 to 6/6 HLA–matched UCBT were 29 days (range, 1 to 228 days), 32 days (range, 1 to 323 days), and 66 days (range, 8 to 230 days), respectively. Platelet recovery was also faster in recipients with early phase disease or intermediate phase disease than in those with advanced phase disease in ($P < .001$). A 4/6 to 6/6 HLA–matched UCBT was a strong independent negative predictor for platelet engraftment within the multivariate analysis (versus 8/8 HLA–matched UBMT, HR, .36 [95% CI, .30 to .42]; $P < .001$, versus 7/8 HLA–matched UBMT, HR, .44 [95% CI, .37 to .53]; $P < .001$, respectively) (Table 2). MAC was not a negative predictor for platelet engraftment.

GVHD

The cumulative incidence of grade II to IV acute GVHD by 100 days after transplantation was lower in recipients of an 8/8 HLA–matched UBMT (34% [95% CI, 30% to 39%]) than in recipients of a 7/8 HLA–matched UBMT (50% [95% CI, 44% to 56%]) or a 4/6 to 6/6 HLA–matched UCBT (41% [95% CI, 36% to 45%]). More recipients who received a TBI-containing regimen experienced grade II to IV acute GVHD by day 100 than did those who received a non-TBI regimen (43% [95% CI, 40% to 46%] versus 34% [95% CI, 29% to 39%], $P = .001$). The 4/6 to 6/6 HLA–matched UCBT recipients had a similar risk of grade II to IV acute GVHD to the 8/8 HLA–matched UBMT recipients within the multivariate analysis (HR, 1.10 [95% CI, .89 to 1.36]; $P = .38$) (Table 2). However, the 4/6 to 6/6 HLA–matched UCBT recipients had a significantly lower risk of grade II to IV acute GVHD than did the 7/8 HLA–matched UBMT recipients (HR, .69 [95% CI, .56 to .87]; $P = .001$) (Table 2).

The cumulative incidence of the extensive type of chronic GVHD by 2 years after transplantation was lower in recipients of the 4/6 to 6/6 HLA–matched UCB (15% [95% CI, 11% to 19%]) than in those who received the 8/8 HLA–matched UBMT or 7/8 HLA–matched UBMT (23% [95% CI, 19% to 27%] and 25% [95% CI, 20% to 32%], respectively). The same relationship was observed when performing the multivariate analysis (versus 8/8 HLA–matched UBMT, HR, .65 [95% CI, .46 to .92]; $P = .015$, versus 7/8 HLA–matched UBMT, HR, .56 [95% CI, .38 to .82]; $P = .003$, respectively) (Table 2).

Relapse

The cumulative incidence of relapse by 2 years was significantly higher in patients receiving the 4/6 to 6/6 HLA–matched UCBT (26% [95% CI, 22% to 30%]) than in those who received the 8/8 HLA–matched UBMT (18% [95% CI, 15% to 22%]) or those who received the 7/8 HLA–matched UBMT (21% [95% CI, 16% to 26%]). However, according to disease status at transplantation, the relapse rate by 2 years after the 8/8 HLA–matched UBMT, 7/8 HLA–matched UBMT, and 4/6 to 6/6 HLA–matched UCBT were not statistically different regardless of disease status at transplantation (8/8 HLA–matched UBMT, 7/8 HLA–matched UBMT, and 4/6 to 6/6 HLA–matched UCBT; early phase disease, 11% [95% CI, 7% to 16%], 15% [95% CI, 8% to 23%], and 19% [95% CI, 12% to 26%]; intermediate phase disease, 22% [95% CI, 13% to 32%], 26% [95% CI, 15% to 39%], and 17% [95% CI, 10% to 27%]; advanced phase disease, 35% [95% CI, 28% to 42%], 36% [95% CI, 28% to 44%], and 43% [95% CI, 38% to 49%], respectively) (Figure 1A–C). On multivariate analysis, the 4/6 to 6/6 HLA–matched UCBT recipients had a significantly higher risk of relapse than did the recipients of the 8/8 HLA–matched UCBT (HR, 1.35 [95% CI, 1.05 to 1.74]; $P = .02$) and had a similar risk to that of the 7/8 HLA–matched UBMT recipients (HR, 1.18 [95% CI, .89 to 1.56]; $P = .26$) (Table 2).

According to primary disease, the cumulative incidence of relapse after the 4/6 to 6/6 HLA–matched UCBT was higher than that after the 8/8 HLA–matched UBMT only in MDS patients and was similar both in AML patients and in ALL patients (Supplemental Table 4).

According to conditioning regimen, the cumulative incidence of relapse after the 4/6 to 6/6 HLA–matched UCBT was higher than that after the 8/8 HLA–matched UBMT only in recipients of MAC (Supplemental Table 5). Among the patients who received RIC, the cumulative incidence of relapse after the 4/6 to 6/6 HLA–matched UCBT was significantly higher than that after the UBMT in recipients without extensive chronic GVHD. However, the cumulative incidence of relapse after the 4/6 to 6/6

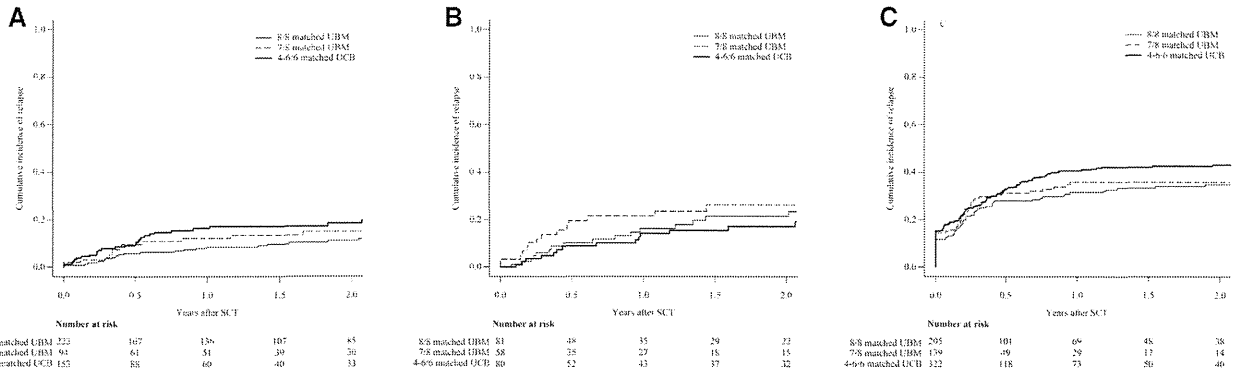

Figure 1. Cumulative incidence of relapse in patients with early phase disease, in those with intermediate phase disease, and in those with high-risk disease according to hematopoietic stem cell source and donor-recipient HLA match. (A) The cumulative incidences of relapse in patients with early phase disease by 2 years after an 8/8 HLA–matched unrelated bone marrow transplantation (UBMT), a 7/8 HLA–matched UBMT, and a 4/6 to 6/6 HLA–matched umbilical cord blood transplantation (UCBT) were 11% (95% CI, 7% to 16%), 15% (95% CI, 8% to 23%), and 19% (95% CI, 12% to 26%), respectively. (B) The cumulative incidences of relapse in patients with intermediate phase disease by 2 years after an 8/8 HLA–matched UBMT, a 7/8 HLA–matched UBMT, and a 4/6 to 6/6 HLA–matched UCBT were 22% (95% CI, 13% to 32%), 26% (95% CI, 15% to 39%), and 17% (95% CI, 10% to 27%), respectively. (C) The cumulative incidences of relapse in patients with intermediate phase disease by 2 years after an 8/8 HLA–matched UBMT, a 7/8 HLA–matched UBMT, and a 4/6 to 6/6 HLA–matched UCBT were 35% (95% CI, 28% to 42%), 36% (95% CI, 28% to 44%), and 43% (95% CI, 38% to 49%), respectively.

HLA–matched UCBT was not statistically different from that after UBMT among the recipients of MAC (Supplemental Figure 1).

NRM

The 2-year cumulative incidences of NRM after the 8/8 HLA–matched UBMT, 7/8 HLA–matched UBMT, and 4/6 to 6/6 HLA–matched UCBT were 32% (95% CI, 27% to 36%), 40% (95% CI, 33% to 46%), and 38% (95% CI, 34% to 43%), respectively. Among patients with early phase disease, the cumulative incidence of NRM at 2 years after the 8/8 HLA–matched UBMT was significantly lower than that after the 7/8 HLA–matched UBMT or 4/6 to 6/6 HLA–matched UCBT (25% [95% CI, 19% to 32%], 35% [95% CI, 25% to 45%], and 37% [95% CI, 29% to 46%]) (Figure 2A). Among patients with intermediate phase disease or advanced phase disease, NRM by 2 years was not statistically different among 3 groups (8/8 HLA–matched UBMT, 7/8 HLA–matched UBMT, and 4/6 to 6/6 HLA–matched UCBT; intermediate phase disease; 32% [95% CI, 31% to 43%], 27% [95% CI, 16% to 40%], and 28% [95% CI, 18% to 38%]; advanced phase disease, 34% [295% CI, 7% to 41%], 41% [95% CI, 32% to 50%], and 36% [95% CI, 30% to 41%], respectively) (Figure 2B,C). On multivariate analysis, the 4/6 to 6/6 HLA–matched UCBT recipients had a higher risk of NRM than the 8/8 HLA–matched UBMT recipients (HR, 1.32 [95% CI, 1.06 to 1.64]; $P = .013$); however, they had a similar risk to the 7/8 HLA–matched UBMT recipients (HR, .98 [95% CI, .77 to 1.25]; $P = .88$) (Table 2). According to primary disease, NRM by 2 years after the 4/6 to 6/6 HLA–matched UCBT was likely higher than that after the 8/8 HLA–matched UBMT only among patients with MDS; however, the difference was not significant regardless of primary diseases (Supplemental Table 4). On multivariate analysis of subgroup analysis according to conditioning regimen, NRM after the 8/8 HLA–matched UBMT was significantly lower than that after the 7/8 HLA–matched UBMT and 4/6 to 6/6 HLA–matched UCBT only among recipients of RIC (Supplemental Table 5).

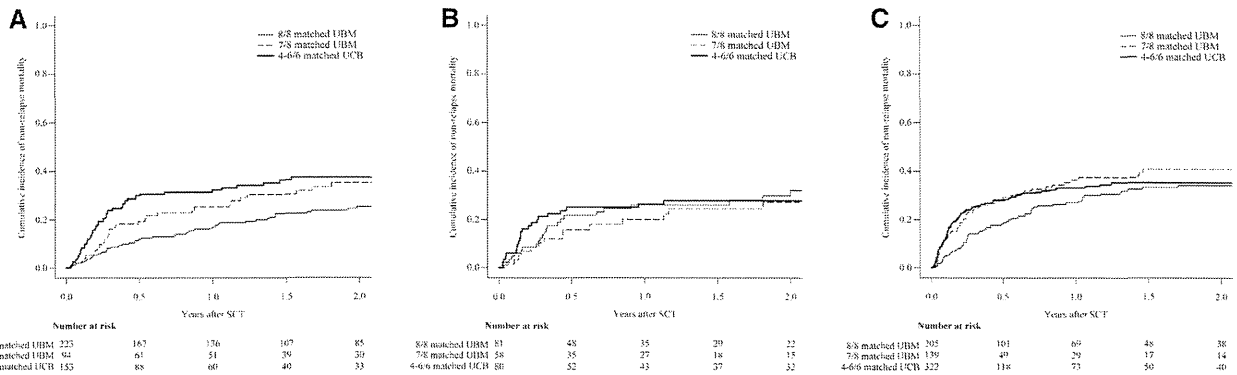

Figure 2. Cumulative incidence of NRM in patients with early phase disease, in those with intermediate phase disease, and in those with advanced phase disease according to hematopoietic stem cell source and donor-recipient HLA match. (A) The cumulative incidences of NRM in patients with early phase disease by 2 years after an 8/8 HLA–matched unrelated bone marrow transplantation (UBMT), a 7/8 HLA–matched UBMT, and a 4/6 to 6/6 HLA–matched umbilical cord blood transplantation (UCBT) were 25% (95% CI, 95% CI, 19% to 32%), 35% (95% CI, 95% CI, 25% to 45%), and 37% (95% CI, 29% to 46%), respectively. (B) The cumulative incidences of NRM in patients with intermediate phase disease by 2 years after an 8/8 HLA–matched UBMT, a 7/8 HLA–matched UBMT, and a 4/6 to 6/6 HLA–matched UCBT were 32% (95% CI, 31% to 43%), 27% (95% CI, 16% to 40%), and 28% (95% CI, 18% to 38%), respectively. (C) The cumulative incidences of NRM in patients with advanced phase disease by 2 years after an 8/8 HLA–matched UBMT, a 7/8 HLA–matched UBMT, and a 4/6 to 6/6 HLA–matched UCBT were 34% (95% CI, 27% to 41%), 41% (95% CI, 32% to 50%), and 36% (95% CI, 30% to 41%), respectively.

Survival

The 2-year unadjusted probabilities of OS after the 8/8 HLA–matched UBMT (51% [95% CI, 46% to 56%]) were significantly higher than those of the 7/8 HLA–matched UBMT (39% [95% CI, 32% to 45%]) and 4/6 to 6/6 HLA–matched UCBT (35% [95% CI, 31% to 39%]) recipients, respectively. The adjusted probabilities of OS at 2 years were also significantly better in recipients of the 8/8 HLA–matched UBMT than in the recipients of the 7/8 HLA–matched UBMT or 4/6 to 6/6 HLA–matched UCBT (49% [95% CI, 44% to 54%], 38% [95% CI, 32% to 45%], 39% [95% CI, 35% to 44%], respectively). This finding was also observed in the subgroup analysis for disease status (at early phase: the adjusted probabilities of OS at 2 years after the 8/8 HLA–matched UBMT, 7/8 HLA–matched UBMT, and 4/6 to 6/6 HLA–matched UCBT were 69% [95% CI, 62% to 76%], 54% [95% CI, 44% to 66%], and 46% [95% CI, 38% to 56%]; at intermediate phase: 53% [95% CI, 42% to 67%], 55% [95% CI, 42% to 72%], and 62% [95% CI, 52% to 74%], respectively; at advanced phase: 31% [95% CI, 24% to 39%], 24% [95% CI, 17% to 33%], and 25% [95% CI, 21% to 31%], respectively) (Figure 3).

According to the multivariate analysis, the 4/6 to 6/6 HLA–matched UCBT recipients had a significantly higher risk of overall mortality than did the 8/8 HLA–matched UBMT recipients (HR, 1.47 [95% CI, 1.24 to 1.74]; $P < .001$) (Table 2). However, the 4/6 to 6/6 HLA–matched UCBT recipients had a similar risk of overall mortality when compared with the 7/8 HLA–matched UBMT recipients (HR, 1.03 [95% CI, .86 to 1.24]; $P = .75$) (Table 2). The adjusted probabilities of OS at 2 years after 8/8 HLA–matched UBMT were superior to those after 4/6 to 6/6 HLA–matched UCBT, regardless of primary disease and conditioning regimen, especially in the patients with MDS (Supplemental Figure 2, Supplemental Tables 4 and 5).

To identify the population of UCBT recipients who had a similar OS to those of 8/8 HLA–matched UBMT, we evaluated the impact of cell dose, HLA matching, and GVHD prophylaxis on the OS of UCBT recipients. The 2-year unadjusted OS of UCBT recipients who received $\geq .84 \times 10^5$ CD34$^+$ cells/kg, which was median cell dose, was significantly higher than those who received $< .84 \times 10^5$ CD34$^+$ cells/kg (Supplemental Figure 3A). HLA matching did not have an effect on OS (Supplemental Figure 3B). GVHD prophylaxis with calcineurin inhibitor (CNI) and other agents improved OS compared with that with CNI alone (Supplemental Figure 3C). Therefore, we compared the OS of 4/6 to 6/6 HLA–matched UCBT recipients who received umbilical cord blood units containing $\geq .84 \times 10^5$ CD34$^+$ cells/kg with 8/8 HLA–matched UBMT recipients, among those with AML and those with ALL who received GVHD prophylaxis with CNI and other agent. The unadjusted 2-year OS after 8/8 HLA–matched UBMT was higher than 4/6 to 6/6 HLA–matched UCBT in patients with early phase disease. Among those with intermediate phase disease, the unadjusted 2-year OS after 4/6 to 6/6 HLA–matched UCBT was likely higher than 8/8 HLA–matched UBMT. Among those with advanced phase disease, the 2-year OS were similar between 2 groups (8/8 HLA–matched UBMT versus 4/6 to 6/6 HLA–matched UCBT; the unadjusted OS of early phase disease, 67% [95% CI, 59% to 74%] versus 55% [95% CI, 40% to 67%], $P = .044$; the unadjusted OS of intermediate disease, 52% [95% CI, 39% to 64%] versus 77% [95% CI, 56% to 89%], $P = .08$; the unadjusted OS of advanced phase disease, 25% [95% CI, 17% to 33%] versus 26% [95% CI, 16% to 36%], $P = .82$) (Figure 4A,C). The adjusted probability of OS were similar between 2 groups (8/8 HLA–matched UBMT versus 4/6 to 6/6 HLA–matched UCBT; the adjusted OS, 49% [95% CI, 43% to 55%] versus 49% [95% CI, 41% to 58%], $P = .74$, respectively) (Figure 4D).

DISCUSSION

The primary objectives of this study were to compare OS after 4/6 to 6/6 HLA–matched UCBT with those after 8/8 and 7/8 HLA–matched UBMT in patients with hematologic malignancies ages 50 years or older and to provide useful data for the selection of an appropriate unrelated stem cell source for those patients who do not have an available HLA-identical sibling. Our findings suggested that an 8/8 HLA allele–matched unrelated donor is the best alternative to a HLA-identical sibling donor. Four of 6 to 6/6 HLA–matched UCBT had a similar OS to 8/8 HLA–matched UBMT for patients with AML and for those with ALL when the umbilical cord blood unit containing $\geq .84 \times 10^5$ CD34$^+$ cells/kg is available.

Neutrophil and platelet recovery were significantly slower after the 4/6 to 6/6 HLA–matched UCBT than after the

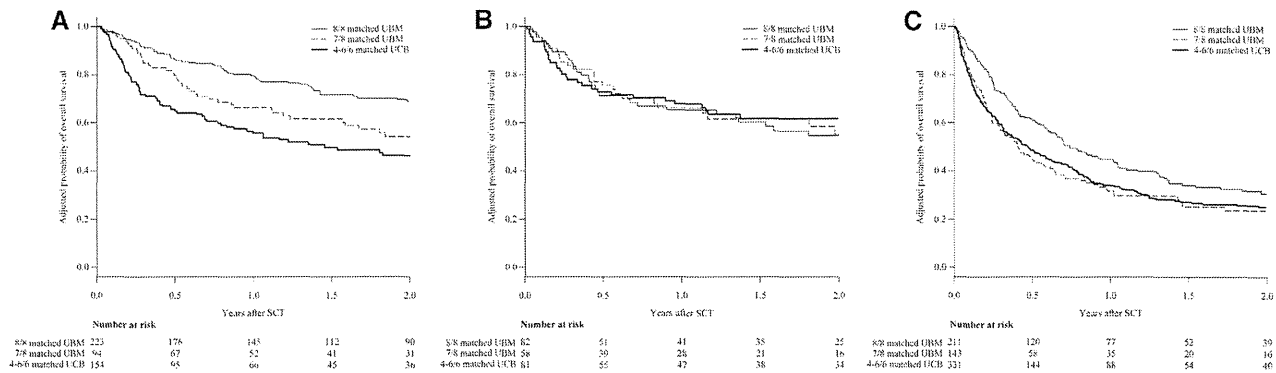

Figure 3. Adjusted probabilities of OS in patients with early phase disease, in those with intermediate phase disease, and in those with advanced phase disease according to hematopoietic stem cell source and donor-recipient HLA match. (A) The adjusted probabilities of the 2-year OS after transplantation in patients with early phase disease who received an 8/8 HLA–matched unrelated bone marrow transplantation (UBMT), a 7/8 HLA–matched UBMT, and a 4/6 to 6/6 HLA–matched umbilical cord blood transplantation (UCBT) were 69% (95% CI, 62% to 76%), 54% (95% CI, 44% to 66%), and 46% (95% CI, 38% to 56%), respectively. (B) The adjusted probabilities of the 2-year OS after transplantation in patients with intermediate phase disease who received an 8/8 HLA–matched UBMT, a 7/8 HLA–matched UBMT, and a 4/6 to 6/6 HLA–matched UCBT were 53% (95% CI, 42% to 67%), 55% (95% CI, 42% to 72%), and 62% (95% CI, 52% to 74%), respectively. (C) The adjusted probabilities of the 2-year OS after transplantation in patients with intermediate phase disease who received an 8/8 HLA–matched UBMT, a 7/8 HLA–matched UBMT, and a 4/6 to 6/6 HLA–matched UCBT were 31% (95% CI, 24% to 39%), 24% (95% CI, 17% to 33%), and 25% (95% CI, 21% to 31%), respectively.

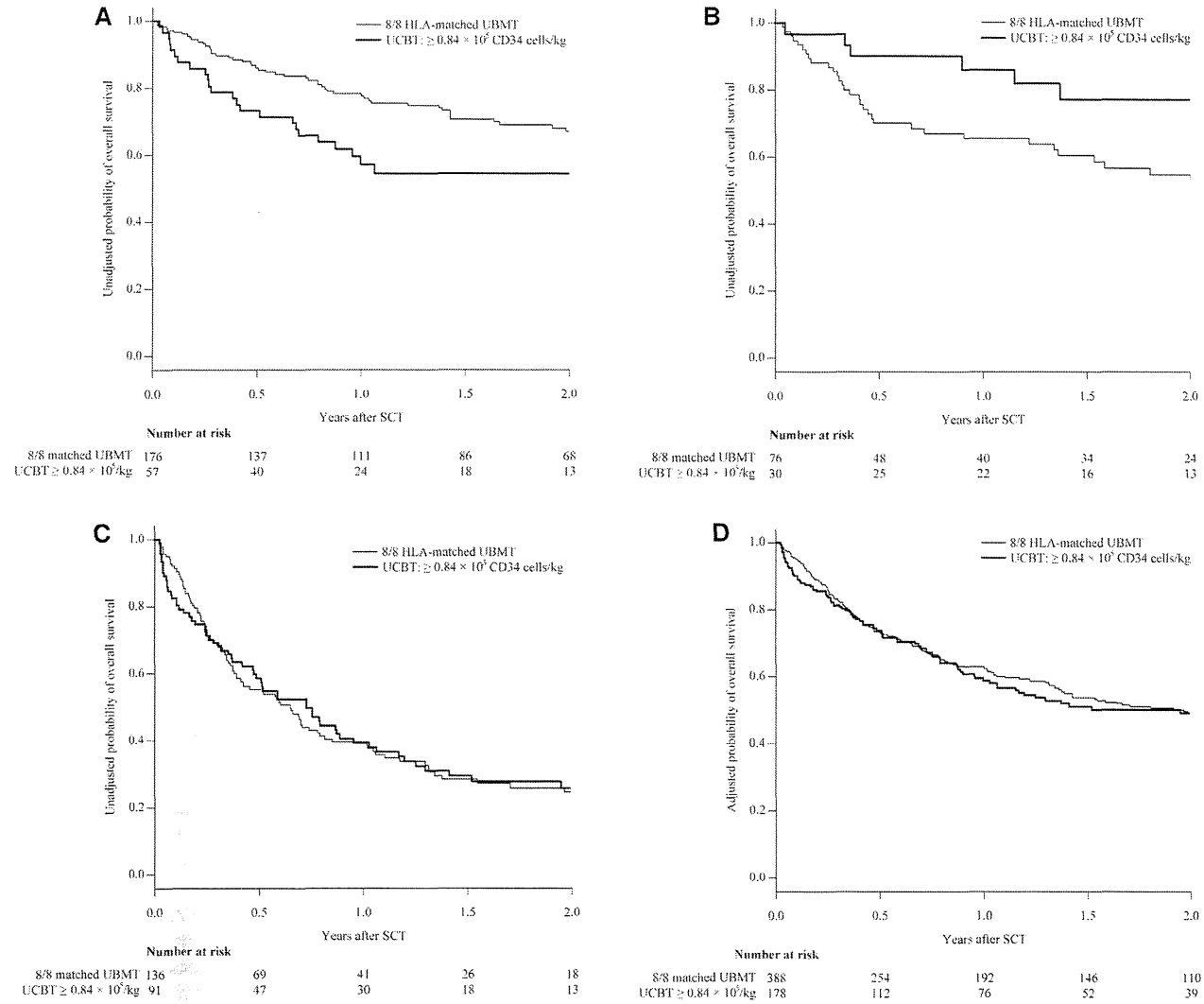

Figure 4. OS in UCBT recipient who received ≥ .84 × 10^5/kg CD34 cells compared with 8/8 HLA–matched UBMT recipients, among those with AML and ALL who prevented graft-versus-host disease with CNI and other agents. (A) The unadjusted probabilities of the 2-year OS after transplantation in patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) at early phase disease and prevented GVHD with CNI and other agent who received an 8/8 HLA–matched unrelated bone marrow transplantation (UBMT) and a 4/6 to 6/6 HLA–matched umbilical cord blood transplantation (UCBT) receiving ≥.84 × 10^5/kg CD34 cells were 67% (95% CI, 59% to 74%) and 55% (95% CI, 40% to 67%), respectively, $P = .044$. (B) The unadjusted probabilities of the 2-year OS after transplantation in patients with AML and ALL at intermediate phase disease and prevented GVHD with CNI and other agent who received an 8/8 HLA–matched UBMT and a 4/6 to 6/6 HLA–matched UCBT receiving ≥.84 × 10^5/kg CD34 cells were 52% (95% CI, 39% to 64%) and 77% (95% CI, 56% to 89%), respectively, $P = .08$. (C) The unadjusted probabilities of the 2-year OS after transplantation in patients with AML and ALL at advanced phase disease and prevented GVHD with CNI and other agent who received an 8/8 HLA–matched UBMT and a 4/6 to 6/6 HLA–matched UCBT receiving ≥.84 × 105/kg CD34 cells were 25% (95% CI, 17% to 33%) and 26% (95% CI, 16% to 36%), respectively, $P = .82$. (D) The adjusted probabilities of the 2-year OS after transplantation in patients with AML and ALL prevented GVHD with CNI and other agent who received an 8/8 HLA–matched UBMT and a 4/6 to 6/6 HLA–matched UCBT receiving ≥.84 × 10^5/kg CD34 cells were 49% (95% CI, 43% to 55%) and 49% (95% CI, 41% to 58%), respectively, $P = .74$.

8/8 and 7/8 HLA–matched UBMT, which was consistent with findings from previous studies [6-9,11,15]. Neutrophil recovery in patients with early phase disease and intermediate phase disease at transplantation was significantly faster than in those with advanced phase disease, which was consistent with the findings in allogeneic peripheral blood stem cell transplantation that had been previously reported [26]. This may be associated with the fact that patients with advanced phase disease were likely pretreated more heavily than those with early phase disease and intermediate phase disease and that they had damage in the microenvironment of the bone marrow.

UCBT recipients had a lower risk of extensive chronic GVHD and a higher risk of relapse compared with 8/8 HLA–matched UBMT recipients. These findings suggested that the graft-versus-leukemia effect in the UCBT recipients was lower than that in the recipients of 8/8 HLA–matched UBMT.

Several studies comparing transplantation outcomes after UBMT versus after UCBT have been reported [6-9]. In some studies, serological HLA class I typing was used for UBMT [6-8]. In another study, UCBT recipients were significantly younger than UBMT recipients, and all patients received a MAC regimen. As a result, only a small number of patients aged 50 years or older were included [9], so direct comparisons of our findings with previous studies are difficult. We had previously demonstrated that HR of overall mortality after a 4/6 to 6/6 HLA–matched UCBT was significantly

higher than that after an 8/8 HLA–matched UBMT among AML patients but not among ALL patients [15]. By contrast, this study showed that the overall survival after an 8/8 HLA–matched UBMT was superior to that after a 4/6 to 6/6 HLA–matched UCBT for patients with AML and for patients with ALL. The present study included patients 50 years or older who received HSCT between 2000 and 2009 regardless of intensity of the conditioning regimen, whereas our previous study had included the recipients of MAC between 2000 and 2005 ages 16 years or older. Therefore, 20% of the 8/8 HLA–matched UBMT recipients and 10% of the 4/6 to 6/6 HLA–matched UCBT recipients in the present study were also included in our previous study. The discrepancy of the results for ALL may be partly due to differences in conditioning regimens (only recipients of MAC regimens were described in our previous report, whereas more than one half of the patients in this study received RIC regimen). Older patients with ALL had a higher risk of relapse and tended to receive RIC when compared with younger patients [27]; therefore, these patients would need a strong graft-versus-leukemia effect. In addition, short-term methotrexate improved OS in the UCBT recipients [28]. In our cohort, approximately 30% of UCBT recipients received GVHD prophylaxis with cyclosporine or tacrolimus alone, and this reduced OS in UCBT recipients. As previously described [29], UCBT recipients receiving higher $CD34^+$ cells had a higher OS than those receiving lower $CD34^+$ cells. For patients with AML and for patients with ALL, UCBT recipients receiving $\geq .84 \times 10^5$ $CD34^+$ cells/kg had a similar adjusted and unadjusted OS to 8/8 HLA–matched UBMT recipients. These findings suggest that the outcomes of UCBT may improve with graft selection based on $CD34^+$ cell dose. The HR of overall mortality after a 4/6 to 6/6 HLA–matched UCBT was similar to that after a 7/8 HLA–matched UBMT, regardless of disease status at transplantation. To the best of our knowledge, this is the first report to compare transplantation outcomes in patients 50 years or older who received a 4/6 to 6/6 HLA–matched UCBT with those who received a 7-8/8 HLA–matched UBMT in a large cohort.

This study had several limitations. Although we adjusted for known risk factors using multivariate analysis, we could not exclude selection bias because this was a retrospective study based on registry data. Further, donor selection was influenced by several factors that were not statistically adjustable. Some patients with urgent disease who could not wait for the preparation of UBMT received UCBT; in other cases, a suitable UCB unit with enough cell doses was not available, and these patients therefore received UBMT. Patients who planned to receive UBMT and could not receive transplantation because of disease progression during the donor coordination were not included in this analysis. In addition, only 5% of recipients of UBMT received GVHD prophylaxis using only a CNI; on the other hand, approximately 30% of UCBT recipients employed the same protocol, which may have influenced the occurrence of GVHD and overall survival. A randomized controlled trial comparing UCBT with UBMT is needed to validate the findings from the present study; however, a study of that design is very difficult to conduct. Clinical decision analysis may help to address any selection bias caused by the donor search process. From 2000 onwards, UPBSCT was more common than UBMT [5]; however, we could not compare the transplantation outcomes of the 4/6 to 6/6 HLA–matched UCBT with the UPBSCT because more than 99% of the unrelated donors from Japan Marrow Donor Program were harvested bone marrow. A randomized controlled trial comparing UPBSCT with UBMT had shown similar outcomes for OS, NRM, and relapse rate [30]. Taken together, UCBT may also be an alternative stem cell source when a HLA-matched peripheral blood stem cell donor is not available.

In conclusion, UCB is a reasonable alternative donor/stem cell source for elderly patients with AML and for those with ALL with similar outcomes compared with UBM from a 8/8 HLA–matched unrelated donor when UCB unit containing $\geq .84 \times 10^5$ $CD34^+$ cells/kg is available. If urgently needed or if there is no 8/8 HLA–matched unrelated donor, a 4/6 to 6/6 HLA–matched UCBT is an acceptable treatment.

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Supplementary information is available at Leukemia's website.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbmt.2014.11.685

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Dexamethasone Palmitate Ameliorates Macrophages-Rich Graft-versus-Host Disease by Inhibiting Macrophage Functions

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Abstract

Macrophage infiltration of skin GVHD lesions correlates directly with disease severity, but the mechanisms underlying this relationship remain unclear and GVHD with many macrophages is a therapeutic challenge. Here, we characterize the macrophages involved in GVHD and report that dexamethasone palmitate (DP), a liposteroid, can ameliorate such GVHD by inhibiting macrophage functions. We found that host-derived macrophages could exacerbate GVHD in a mouse model through expression of higher levels of pro-inflammatory TNF-α and IFN-γ, and lower levels of anti-inflammatory IL-10 than resident macrophages in mice without GVHD. DP significantly decreased the viability and migration capacity of primary mouse macrophages compared to conventional dexamethasone *in vitro*. DP treatment on day 7 and day 14 decreased macrophage number, and attenuated GVHD score and subsequent mortality in a murine model. This is the first study to provide evidence that therapy for GVHD should be changed on the basis of infiltrating cell type.

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Introduction

Macrophages are recruited by chemokines including CCL2 to the inflammatory site and, primarily, play an indispensable role in both innate and acquired immunity [1]. Macrophage phenotypes and functions can vary with different external stimuli, and macrophages are divided into two major classifications: classically activated, i.e. inflammatory, and alternatively activated, i.e. anti-inflammatory macrophages [1]. Persistence of activated macrophages can occasionally be harmful to the host [2,3].

Graft-versus-host (GVHD) is often a prominent complication after allogeneic stem cell transplantation (allo-SCT) and can be fatal despite aggressive interventions including corticosteroids [4]. It has been reported that GVHD can be divided into 3 subtypes based on the number of macrophages and T lymphocytes infiltrated in the skin, and that GVHD with many $CD163^+$ macrophages was refractory with poor prognosis and a therapeutic challenge [5]. However, those macrophages were CD163 positive, a member of the scavenger receptor cysteine-rich superfamily, which was one of anti-inflammatory macrophage markers [6]. Thus, the pathogenesis between macrophage infiltration and refractory GVHD is currently unclear. These facts prompted us to characterize the phenotypes of macrophages related to refractory GVHD.

Corticosteroids inhibit functions of inflammatory cells via glucocorticoid receptors in the cytoplasm [7]. Therefore, efficient delivery of corticosteroids into the cytoplasm could enhance their therapeutic effect. It is known that a dexamethasone palmitate emulsion (DP) is readily taken up by macrophages via phagocytosis and is strongly retained in the cytoplasm [8]. Here, we report that the macrophages increased in fatal GVHD are inflammatory and that DP treatment efficiently attenuated such GVHD by inhibiting macrophage functions.

Materials and Methods

1. Mice

Male 6- to 8-week-old male C57BL/6J mice and female BALB/c mice were purchased from Chubu Kagaku Shizai (Nagoya, Japan). The animal GVHD experiments using spontaneous death

as an endpoint were approved by the Institutional Ethics Committee for Laboratory Animal Research, Nagoya University School of Medicine (protocol 24298), and were performed according to the guidelines of the institute. Animals were maintained at constant ambient temperature (22±1°C) under a 12-h light/dark cycle (lights on between 9:00 and 21:00), with food and water available ad libitum.

2. Cells and reagents

A murine macrophage cell line, RAW264.7, was purchased from American Type Culture Collection (Manassas, VA, USA). Primary peritoneal macrophages and skin macrophages were obtained from the peritoneal lavages of C57BL/6J mice and from the ears of mice after BMT, respectively, as described elsewhere with slight modification [9,10]. Briefly, peritoneal lavages were collected 3 days after intraperitoneal injection of 1 mL of 2% thioglycolate (Kanto Chemical Co., Inc., Tokyo, Japan) and macrophages were positively selected from the lavages by AutoMACS system with anti CD11b immunomagnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). To prepare single cell suspensions from dermis, the ears were split into dorsal and ventral halves with removal of subcutaneous tissues such as cartilage and the dermal sheets were incubated in RPMI containing 2% Liberase (Liberase TL Research Grade, Roche Applied Science) for 2 hours at 37°C. After digestion, residual tissue was minced in RPMI and disaggregated by using a tissue homogenizer (Medimachine; Becton Dickinson, San Jose, CA). Dermal macrophages were positively selected from the suspension cells by AutoMACS as described above. FACS analysis using a monoclonal antibody (mAb) (F4/80: BM8, BioLegend, San Diego, CA, USA) showed that the purity of isolated macrophages were > 90%. Primary T cells were positively isolated from splenocytes of BALB/c mice by using anti CD90.2 immunomagnetic microbeads (Miltenyi Biotec). T cell-depleted (TCD) donor bone marrow (BM) cells was obtained from BALB/c mice by negative selection by using CD90.2 microbeads.

Dexamethasone sodium phosphate (DSP) and Dexamethasone palmitate emulsion (DP) were from MSD K.K. (Tokyo, Japan) and Mitsubishi Tanabe Pharma (Tokyo, Japan), respectively.

3. Induction and assessment of GVHD

A fatal murine GVHD model was established by allogeneic BM transplantation. Lethally irradiated C57/BL6 recipient mice (5 Gy×2; days -2 and -1) were co-transplanted with TCD-BM (5×10^6) and T lymphocytes (1×10^7) from BALB/c donor mice via tail vein without anesthesia. DP or DSP (10 mg/kg as dexamethasone) were administrated intravenously into the mice on day 7 and 14 after transplantation (control: n = 9, DSP: n = 9, DP: n = 10). The conditions and survival of animals after BMT were monitored daily with all efforts to alleviate pain and suffering, and the degree of GVHD was evaluated clinically (3 times/week) for 28 days (until day 42) after the last administration of DP or DSP (day 14) because our preliminary experiments showed that GVHD-related complications were neither worsen nor cause of death after 28 days of DSP treatment. The reasons why we set spontaneous death as an endpoint are as follows. GVHD also possesses an antitumor effect; so-called graft-versus-leukemia (GVL), and 'mild' GVHD confers a survival benefit [11][12]. Thus, physicians try to modulate the GVL-GVHD balance by immune-suppressants such as steroid and cyclosporine A. However, GVHD, once became refractory to conventional therapies, could cause high mortalities [13]. To determine whether DP can improve overall survival outcomes or not in a GVHD mouse model brings a lot of useful information to physicians. The animals survived by DP treatment were humanely euthanized by overexposure to carbon dioxide after day 42. Mice treated with no steroid or with DSP had all died of GVHD before day 42. The detailed clinical GVHD scoring system by using 5 parameters is as follows: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10) [14]. Acute GVHD was also assessed in a blind fashion by detailed histopathologic analysis in hematoxylin and eosin-stained tissue sections (the skin from the interscapular region, ears and descending colon). Skin sections were scored on the basis of the following criteria: epidermis (0, normal; 1, foci of interface damage in <20% of section with occasional necrotic keratinocytes; 2, widespread interface damage in >20% of section); dermis (0, normal; 1, slightly altered with mild increased collagen density; 2, marked increased collagen density); inflammation (0, none; 1, focal infiltrates; 2, widespread infiltrates); subcutaneous fat (0, normal; 1, reduced number of normal adipocytes; 2, serous fat atrophy); and follicles (0, normal number of hair follicles, ~5 per linear millimeter;1, between 1 and 5 follicles per linear millimeter; 2, <1 follicle per linear millimeter) [15]. Seven parameters were scored for gut (crypt regeneration, crypt epithelial cell apoptosis, crypt loss, surface colonocyte vacuolization, surface colonocyte attenuation, lamina propria inflammatory cell infiltrate, and mucosal ulceration). The scoring system for each parameter denoted 0 as normal; 0.5 as focal and rare; 1 as focal and mild; 2 as diffuse and mild; 3 as diffuse and moderate; and 4 as diffuse and severe, as previously described [16].

To assess the direct effect of inflammatory macrophages on GVHD, 1×10^6 thioglycolate-stimulated peritoneal macrophages from C57BL/6J mice were subcutaneously injected in interscapular region on day 5. All mice were humanely euthanized by overexposure to carbon dioxide on day 7 and GVHD score was pathologically evaluated as described above.

4. Analysis of donor-cell chimerism

Donor-cell chimerism of macrophages in the skin after BMT was analyzed by FACS using anti-MHC haplotype antibodies. An anti-H-2Kb mAb (AF6-88.5) and an anti- H-2Kd (SF1-1.1) recognized cells from C57/BL6 recipient mice and cells from BALB/c donor mice, respectively. Both mAbs were obtained from PharMingen (San Diego, CA).

5. RNA preparation and real-time PCR analysis

Total RNA was extracted from the skin and gut of mice using TRIzol (Invitrogen Carlsbad, CA, USA). The mRNA levels of CCL2 in the skin and gut, and those of TNF-α, IFN-γ and IL-10 in skin macrophages were evaluated using quantitative RT-PCR. Primer pairs (TNF-α: Mm00443258_m1, IFN-γ: Mm01168134_m1, CCL-2: Mm00441242_m1, IL-10: Mm00439614_m1, Arginase-1: Mm00475988_m1, Eukaryotic 18s rRNA: 4333760T) were from Applied Biosystems (Foster City, CA, USA). Obtained data were normalized to internal 18 s rRNA expression and were analyzed using the $2^{-\Delta\Delta}C_T$ Method [17].

6. *In vitro* assay for the effects of DP on macrophages and lymphocytes

The viability of RAW264.7 after DSP or DP treatment was assessed using a colorimetric assay as described elsewhere [18]. Briefly, 10 μl of TetraColor-One (Seikagaku Co., Tokyo, Japan) was added to each well of a 96-well plate, where RAW264.7 cells (10,000 cells/well) were pretreated with various concentrations of DSP or DP (48 hours, 37°C), and the mixture was incubated for

an additional 4 hours. Absorbance at 450 nm was monitored. The viability of splenic T lymphocytes after exposure to DSP or DP was assessed by trypan blue exclusion. Briefly, cells were washed twice with PBS, suspended in culture medium (RPMI containing 10% FBS), plated (1.0×10^5 cells/well in 0.2 mL culture medium) in three independent determinations with DSP or DP (25 nM) onto 96-well plates, and incubated for 48 hours. Viable cells were determined as Trypan blue- negative cells. The percent viability was calculated as follows: (viability in DSP or DP group/viability in control group) ×100 (%).

CCR2 expression on the surface of macrophages after DSP or DP treatment was analyzed by FACS as described previously [19]. Briefly, thioglycolate-recruited peritoneal macrophages were pretreated with 25 μM (as dexamethasone) of DSP or DP for 3 hours, and then, were exposed to LPS (O55:B5, 100 ng/mL, List Biological Laboratories, Inc. Campbell, CA) for 18 hours. After washings with PBS, the cells were incubated for 30 minutes on ice with a rabbit anti-mouse CCR2 polyclonal antibody (pAb) (1: 25 dilution, E68, Novus Biologicals, Littleton, CO) in the presence of an anti-mouse CD16/32 mAb (BioLegend, San Diego, CA) to reduce non-specific binding of a primary antibody to Fc receptor. The cells were washed twice with PBS and were incubated with a fluorescein isothiocyanate -conjugated donkey anti-rabbit IgG pAb (1: 25 dilution, BioLegend, San Diego, CA) as a secondary antibody for 30 minutes. CCR2 expression was assessed from 1.0×10^4 viable cells using a FACSAria flow cytometer (BD Biosciences, San Jose, CA), and the data were analyzed using FlowJo software (TreeStar, San Carlos, CA). Background fluorescence was assessed through staining with the isotype-matched antibody.

To assess the inhibitory effect of DP and DSP on the CCL2-CCR2 axis, transwell migration assays were performed as described elsewhere [18]. Briefly, RAW264.7 cells were pretreated with DSP or DP (25 μM) for 3 hours and then, were serum-starved in DMEM with 1% FBS and 100 ng/ml LPS overnight. After washing, the cells were seeded (2×10^5 cells in DMEM with 1% FBS per well) onto the upper chamber of a cell culture insert with a pore size of 8 μm (BD Biosciences, San Jose, CA). Recombinant murine CCL2 (final concentration, 20 ng/ml; PeproTech, Rocky Hill, NJ) was added to the lower chamber, to which cells were allowed to migrate for 4 hours. The membranes were fixed with 4% paraformaldehyde and were stained with Giemsa. For quantitative analysis, four fields were randomly selected, and migrated cells were counted under a light microscope.

7. Immunohistochemistry

Immunostaining of murine skin and gut specimens was carried out on sections from paraffin-embedded tissues fixed in 10% neutral-buffered formalin solution (Sigma-Aldrich, St. Louis, MO) using streptavidin-biotinylated HRP detection (Beckeman Coulter, Brea, CA) as previously described with slight modification [20]. For antigen retrieval, sections (3.5 μm thickness) on silane-coated slides were heated in a microwave oven for 45 minutes at 98°C in immunosaver (1:200 dilution, Nisshin EM Corp. Tokyo, Japan). After blocking nonspecific binding with normal rabbit serum (1:75 dilution; Dako Inc. Via Real (Carpinteria, CA), sections were incubated with an anti F4/80 mAb (CI:A3-1, Novus, Littleton, CO) (1:100) or an isotype-matched mAb for 15 minutes using intermittent microwave irradiation [21,22]. Sections were then incubated with biotin-labeled rabbit anti-mouse IgG pAb (1:300 dilution; Dako Inc.) and 3,3′-diaminobenzidine (DAB; Vector Laboratories Inc. Burlingame, CA) was used as chromogen. Finally they were counterstained with hematoxylin.

8. Statistical Analysis

Statistical significance of group differences was evaluated using Student's *t*-test between two groups and ANOVA followed by bonferroni test for multiple comparisons using STATA software (StataCorp, Lakeway, TX). Kaplan-Meier product-limit estimates were performed to determine survival, while the different subgroups were compared for significance using the log-rank test.

Results

1. Murine GVHD model mimics human severe GVHD

In a BMT model, mice received TCD-BM (5×10^6) and T lymphocytes (1×10^7) or TCD-BM (5×10^6) alone. Cotransplantation of TCD-BM and T lymphocytes resulted weight loss, poor activity, damaged fur texture and all of the mice died on day 12 even though all of the mice received TCD-BM alone were active and survived. Pathological analysis of the skin showed necrotic keratinocytes, increased collagen density, infiltration of inflammatory cells and serous fat atrophy in mice with TCD-BM and T lymphocytes, but minimal damage in mice with TCD-BM alone (Figure 1A, left panel). The gut was similarly severely damaged in mice with TCD-BM and T lymphocytes (data not shown). A higher number of macrophages infiltrated the skin of mice had received TCD-BM and T lymphocytes compared to the skin of mice had received TCD-BM alone (Figure 1A, middle panel). These results clearly suggest that this fatal mouse GVHD model mimics human severe GVHD with many macrophages [5].

To analyze the mechanism of macrophage infiltration in the skin, we focused on the role of CCL2-CCR2 axis since CCL2 is a potent inducer of macrophage recruitment and activation [23]. Quantitative RT-PCR analysis showed that CCL2 expression in the skin of mice with TCD-BM and T lymphocytes was 10-times higher than that in the skin of mice with TCD-BM alone (Figure 1A, right panel).

2. Characterization of macrophages increased in GVHD

The phenotypes of dermal macrophages isolated on day 7 after BMT (the purity of macrophages >90%, not shown) were evaluated by quantitative RT-PCR analyses. Macrophages from GVHD mice showed that skin macrophages from GVHD mice expressed much higher levels of TNF-α and IFN-γ, and a significantly lower level of IL-10 than those of sham mice (no GVHD) (Figure 1B), suggesting that the macrophages involved in GVHD possess inflammatory properties [1]. To assess the direct effect of inflammatory macrophages on GVHD, 1×10^6 thioglycolate-stimulated peritoneal macrophages from C57BL/6J mice were subcutaneously injected in the interscapular region on day 5 and evaluated on day 7. Skin pathological score of the injected site was significantly higher among mice injected macrophages than PBS-injected control mice (Figure 1C). Donor-cell chimerism analyzed by FACS showed that >90% of dermal macrophages possessed the recipient phenotype (data not shown). These data indicated that recipient monocytes recruited to the skin GVHD site acquired inflammatory phenotypes and deteriorated GVHD subsequently.

3. Effects of DP on macrophage functions in vitro

Based on these results, we hypothesized that GVHD with many macrophages would be ameliorated by inhibiting macrophage functions. We therefore compared DP with conventional DSP on macrophage functions. Both DSP and DP inhibited proliferation of RAW 264.7 cells in a dose dependent manner. However, DP possessed a significantly higher ability than DSP (Figure 2A left panel). DP decreased the viability of RAW 264.7 cells by 75% at a

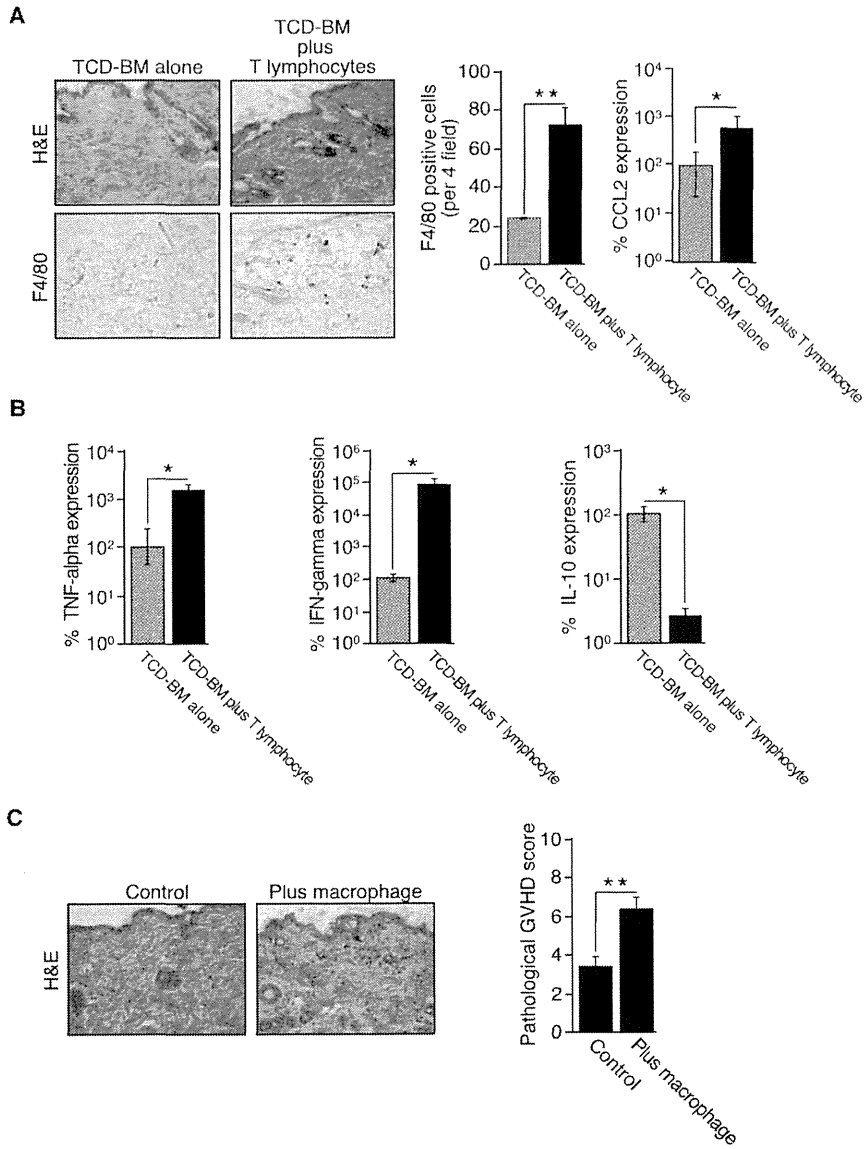

Figure 1. Characterization of macrophage-involvement in GVHD. A: Skin specimens from mice in which T-cell depleted bone marrow (TCD-BM) cells and spleen-derived T lymphocytes were co-transplanted were histopathologically compared with those of mice transplanted with TCD-BM alone. Skin specimens (7 days after transplantation) were stained with H&E (upper left panels) and macrophages were detected with the anti-mouse F4/80 monoclonal antibody (lower left panels). Original magnification, ×400. Representative images of two independent experiments are shown. For quantitative analysis of macrophage numbers, 4 microscopic fields were randomly selected from each of three mice and the total number of $F4/80^+$ cells was counted. The mean number of $F4/80^+$ cells per 4 fields ± SD is shown for each group (middle panel). Original magnification, ×200. The results are representative of two independent experiments. Statistical significance: $^{**}P<0.01$. CCL2 mRNA expression was evaluated using quantitative real-time RT-PCR. Total RNA was extracted from the skin (3 specimens per group) and was subjected to RT-PCR using specific primer pairs. Each reaction was performed in duplicate sets. The obtained data were normalized to internal eukaryotic 18 s rRNA expression, were analyzed using the $2^{-\Delta\Delta}C_T$ Method and are expressed as percent expression, where expression of the control is designated as 100%. The results reflect the mean ± SD of three independent determinations. The results are representative of two independent experiments. Statistical significance: $^{*}P<0.05$. **B:** Macrophages in the skin were characterized by measurement of TNF-α, IFN-γ and IL-10 mRNA expression. Skin dermis was incubated in RPMI containing 2% Liberase for 2 h at 37°C. After digestion, residual tissue was minced and mechanically disaggregated. After separation of dermal macrophages by using the magnetic isolation system, total RNA was extracted and mRNA levels of TNF-α, IFN-γ and IL-10 were quantified by RT-PCR using specific primer pairs (3 specimens per group). Each reaction was performed in duplicate sets. The obtained data were normalized to internal eukaryotic 18 s rRNA expression, were analyzed using the $2^{-\Delta\Delta}C_T$ Method and are expressed as percent expression, where expression of the control is designated as 100%. The results reflect the mean ± SD of three independent determinations. Statistical significance: $^{*}P<0.05$. The results are representative of two independent experiments. **C:** Direct effects of inflammatory macrophages on GVHD were assessed by injecting thioglycolate-stimulated macrophages (1×10^6 cells per mouse) or PBS (three mice per group) subcutaneously into the interscapular region of GVHD mice 5 days after BMT. The animals were killed 7 days after BMT. Skin specimens of the injected sites stained with H&E were photographed (left panel) and skin GVHD was pathologically scored based on five parameters (epidermal damage, alteration of dermis, degree of inflammation, alteration of subcutaneous fat and number of follicles). The results reflect the mean ± SD of three independent determinations. Statistical significance: $^{**}P<0.01$. The results are representative of two independent experiments.

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concentration of 10 μM, which is 25-fold lower than the concentration at which DSP similarly worked (by 71% at 250 μM) (Figure 2A left panel). Interestingly, the toxic effect of DP on splenic T lymphocytes is rather weak toxic than DSP, when tested at 25 μM as dexamethasone (Figure 2A right panel). DP also significantly decreased CCR2 expression on the surface of primary peritoneal macrophages (Figure 2B) and RAW 264.7 cells (data not shown), and subsequently decreased migration of primary macrophages towards CCL2 (Figure 2C) compared to DSP. This decreased number of macrophage migration could not be attributed to decreased number of the input cells, as 3 hour-treatment with DSP or DP at 25 μM minimally affected on macrophage viabilities (data not shown). These results clearly suggest that DP attenuates macrophage functions more efficiently than DSP.

4. The effect of DP on murine fatal GVHD

We next investigated whether DP could affect the fatal murine GVHD with many macrophages. DP or DSP was administered 10 mg/kg as dexamethasone on day 7 and day14 (control: n = 9, DSP: n = 9, DP: n = 10). DP significantly lowered the clinical GVHD score compared to DSP. The difference became apparent 5 days after second administration (Figure 3A, left panel). Subsequently DP could rescue about 20% of these mice, whereas mice treated with no steroid or with DSP had all died by days 12 and 30, respectively (Figure 3A, right panel). The effect of DP was also confirmed by pathological analyses, where tissue damage and pathological GVHD scores in the skin (Figure 3B, left panel) and gut (Figure 3B, right panel) were significantly improved in mice treated with DP. The number of F4/80$^+$ macrophages was also lower in mice treated with DP. Since DP had a weaker effect on lymphocytes than DSP (Figure 2A, right panel), the combined facts suggest that DP can attenuate GVHD with many macrophages by inhibiting inflammatory macrophages.

Discussion

Macrophage infiltration in the skin of patients with GVHD is a maker of poor prognosis [5]. Here, we identify macrophages in the GVHD sites are inflammatory and an exacerbator of GVHD, and provide evidence that such GVHD can be effectively treated by DP, not conventional DSP in a mouse model.

Inflammatory cells such as macrophages and mast cells have been proved to be durable to even high dose chemotherapy and irradiation [24,25]. Accordingly, donor-cell chimerism analysis showed that >90% of dermal macrophages possessed the recipient phenotype (not shown).

Macrophages are divided into two major classifications: classically activated, i.e. inflammatory, and alternatively activated, i.e. anti-inflammatory macrophages [26]. Since persistence of macrophage activation can be harmful to the host, phenotypic switch from inflammatory macrophages to anti-inflammatory macrophages can be occurred via various stimuli [27]. We revealed by RT-PCR that macrophages in the skin of a murine GVHD model possessed inflammatory properties (Figure 1B) although the macrophages in patients with GVHD expressed CD163 [5], a marker of the alternatively activated macrophages [26]. Recently, compelling studies revealed that CD163$^+$ macrophages could be unrestrained proinflammatory macrophage population with an incomplete switch to anti-inflammatory macrophages under certain circumstances such as iron-overloading condition [28]. An elevated level of ferritin, a marker of tissue iron overload, closely correlates with increased risk of acute GVHD, higher mortality and lower overall survival [29]. These

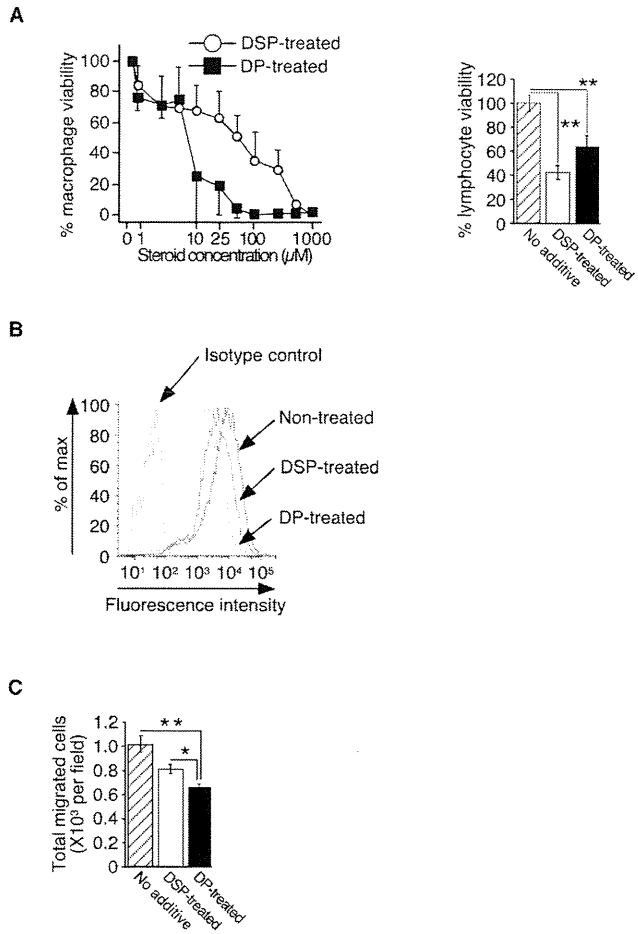

Figure 2. Effect of dexamethasone palmitate on macrophages *in vitro*. **A:** The viability of mouse macrophage-like RAW264.7 cells after dexamethasone sodium phosphate (DSP) or dexamethasone palmitate (DP) treatment (48 hours) was evaluated by using a colorimetric assay (left panel). The percentage viability was calculated as follows: (O.D value in the presence of each concentration of steroid/O.D value without steroid) ×100. The results reflect the mean ± SD of three independent determinations (representative experiment of three performed). The viability of splenic T lymphocytes after exposure to DSP or DP (25 nM each, 48 hours) was assessed by trypan blue exclusion (right panel). Viable cells were determined as Trypan blue-negative cells. The percent viability was calculated as follows: (viability in DSP or DP group/viability in control group) ×100 (%). The results reflect the mean ± SD of three independent determinations (representative experiment of three performed). **B:** CCR2 expression on the surface of mouse primary peritoneal macrophages after DSP or DP treatment was evaluated by FACS. The results are representative of three independent experiments (left panel). **C:** The migration of peritoneal macrophages towards CCL2 after DSP or DP treatment was analyzed using transwell assays. For quantitative analysis, four fields were randomly selected, and migrated cells were counted under a light microscope (×200). The results reflect the mean ± SD of four independent determinations. Representative results of three independent experiments are shown (right panel). Statistical significance: *P< 0.05 and **P<0.01.
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evidences and results suggest that CD163$^+$ macrophages in patients with GVHD can be inflammatory and exacerbate GVHD similarly with the mouse GVHD model.

CCL2-CCR2 signaling is known to play a major role in recruitment of monocytes/macrophages [23]. Inflammatory mediators released from activated macrophages not only induce tissue

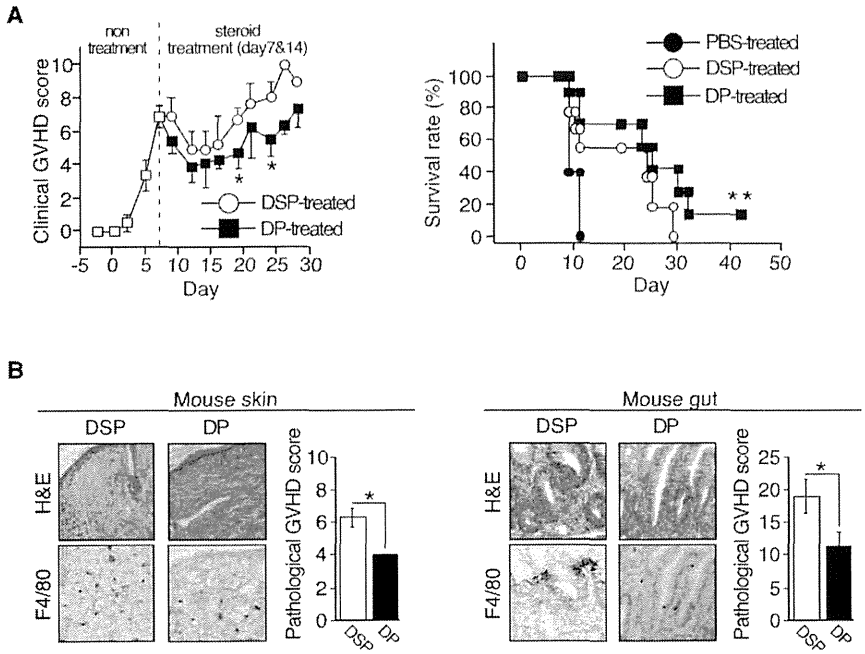

Figure 3. Effect of dexamethasone palmitate on macrophages in mice with fatal GVHD. A: Clinical assessment of GVHD after DP or DSP treatment. Mice were treated with DP or DSP (10 mg/kg/each day) on day 7 and day14 after co-transplantation of TCD-BM and spleen-derived T lymphocytes (control: n = 9, DSP: n = 9, DP: n = 10). Clinical GVHD was assessed 3 times a week, using a scoring system consisting of 5 clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10, left panel). Statistical significance: $*P<0.05$ (days 19 and 24). Mortalities were counted daily for up to 42 days after transplantation (right panel). Statistical significance: $**P<0.01$ (PBS-treated versus DP-treated). **B:** Pathological assessment of GVHD after DP or DSP treatment. Skin (left panels) and gut (right panels) specimens were stained with H&E (top panels) and skin macrophages were detected with the anti-mouse F4/80 monoclonal antibody (bottom panels). Original magnification, ×400. Representative images of the skin of three mice are shown. Skin and gut GVHD were scored based on five parameters (epidermal damage, alteration of dermis, degree of inflammation, alteration of subcutaneous fat and number of follicles) and seven parameters (crypt regeneration, crypt epithelial cell apoptosis, crypt loss, surface colonocyte vacuolization, surface colonocyte attenuation, lamina propria inflammatory cell infiltrate and mucosal ulceration), respectively, and are presented as histograms. Statistical significance: $*P<0.05$.
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damage but also recruit and activate macrophages [1]. DP treatment in a mouse GVHD model decreased the number of macrophages in the skin and gut, and attenuated GVHD without severe complications compared to DSP treatment (Figure 3), suggesting that DP inhibited the positive feedback loop between macrophages and inflammation more efficiently than by DSP.

Not a few attempts to prevent severe acute GVHD in animal models by inhibiting macrophage function as an antigen-presenting cell or modulating macrophage phenotype have been reported and some were successful [30–35]. However, severe adverse effects such as infections occurred occasionally [31] since inflammatory macrophages play important roles in both innate and acquired immune response. Minimal risk of infection can give DP treatment an advantage over those pretreatment.

Our small preclinical study showed that DP treatment in patients with macrophage-rich GVHD (3 day, 5 mg/day) ameliorated GVHD with efficient reduction of skin macrophages. Severe adverse effects such as infections were not observed during and after DP treatment (not published).

Again, these observations provide further evidence that macrophages directly exacerbate GVHD and that DP treatment against such macrophages improves the outcome of refractory GVHD.

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Author Contributions

Conceived and designed the experiments: SN T. Nakayama MM T. Naoe. Performed the experiments: SN T. Nakayama MM S. Terakura SS TK HM. Analyzed the data: SN T. Nakayama MM S. Terakura SS HM T. Naoe. Contributed reagents/materials/analysis tools: AS YO MI S. Toyokuni KN. Wrote the paper: SN T. Nakayama MM T. Nishida S. Terakura SS KT HM NI KM HK MI RU T. Naoe.

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Letter to the Editor

Successful unrelated cord blood transplantation for adult acquired aplastic anemia using reduced intensity conditioning without ATG

Acquired aplastic anemia (aAA) patients who are transfusion-dependent and who have failed or relapsed after immunosuppressive therapy need further treatment. In cases in which a human leukocyte antigen (HLA)-identical sibling donor is not available, the use of alternative donor including HLA-matched unrelated donor and unrelated cord blood (CB) are commonly considered, but this strategy is associated with worse outcomes [1]. Because of the abundant availability of acceptable CB units, the use of cord blood transplantation (CBT) has been increasing. Although the use of CBT in patients with aAA has recently been evaluated [2], relatively little information is available on how to achieve proper engraftment with a reduced intensity conditioning (RIC) regimen in aAA patients undergoing CBT. We describe here three adult patients with aAA who underwent transplantation with unrelated CB after a RIC regimen without ATG. The patient characteristics are shown in Table 1. All three patients received single-unit CB containing more than 2.2×10^7/kg of total nucleated cell (TNC) with no more than two of six HLA-mismatches. The conditioning regimen consisted of six doses of fludarabine (Flu) 30 mg/m^2, two doses of cyclophosphamide (CY) 60 mg/kg and total body irradiation (TBI) 2 Gy × 2 with no use of anti-thymocyte globulin (ATG). The graft-versus-host disease (GVHD) prophylaxis regimen was a combination of short-term methotrexate (15, 10, and 10 mg/m^2 on days 1, 3, 6, respectively) and tacrolimus. Because patient #3 had a high titer of anti-HLA antibody, a CB unit that was not cross-reactive with this antibody was chosen.

All three patients rapidly exhibited sustained CB engraftment (Table 2). Chimerism analyses of the CD3$^+$ fraction using various numbers of tandem repeats showed initial full-donor conversion from the first point of analysis in all patients. No secondary graft-failure was observed. Acute GVHD was observed in patient #2 (skin only stage 2, Grade I) and resolved spontaneously. Chronic GVHD was observed in patient #3 (skin, oral involvement) and symptoms resolved quickly after the administration of 0.5 mg/kg oral prednisolone. The regimen was generally well tolerated, and no significant organ damage or severe toxicity occurred. The patients remain alive without transfusion dependence at 68, 44 and 9 months, with Karnofsky scores of 70% (due to postherpetic neuralgia), 100% and 100%, respectively.

Here we report three CBT recipients who received successful single-unit CBT after a RIC regimen. All three patients exhibited sustained full donor-type hematopoiesis without further intervention to increase donor-type chimerism. The conditioning regimen included 180 mg/m^2 Flu and 120 mg/kg CY with 2 Gy × 2 TBI, which may be regarded as a relatively strong regimen in terms of immunosuppressive and cytotoxic ability. Thus, one might think this regimen too potent for the induction of sustained engraftment of CB. However, Liu et al. reported that RIC regimen, consisting of Flu 120 mg/m^2, CY 1200 mg/m^2 (equivalent to 40 mg/kg if the patient's body weight was 50 kg) and rabbit ATG 30 mg/kg, was not sufficiently potent enough to induce engraftment after CBT in patients with aAA. They reported two early deaths and 16 graft-failures among the 18 CBT recipients conditioned with the above regimen [3]. Thus, it is reasonable to use a CY dose >40 mg/kg, and further study to determine the optimal CY dose between 40 and 120 mg/kg is warranted.

To ensure rapid and proper CB engraftment, graft cell contents, such as TNC, CD34$^+$ cell count and CD8$^+$ cell count, are important factors [4]. In Western countries, ATG is commonly used as the conditioning regimen for CBT. Nevertheless, the use of ATG will decrease lymphocytes, including graft-facilitating CD8$^+$ lymphocytes, which may lead to attenuation of total potency for the facilitation of engraftment in exchange for the beneficial effect of reducing the incidence of severe acute GVHD. Indeed, only one of seven CB recipients for aAA who received ATG-containing regimen achieved engraftment in a previous retrospective study in Japan [5]. Thus, we replaced ATG with 4 Gy TBI in our regimen, which may be another reason for successful engraftment.

One of the biggest differences in CBT between Western countries and Japan may be the attitude toward the use of ATG. In the recent protocol of European group, two doses of ATG 2.5 mg/kg and a single agent GVHD prophylaxis are recommended [6]. To reduce the incidence of severe acute GVHD, physicians in Europe and US would be likely to use ATG more frequently, which might result in failure to observe better engraftment. In fact, it is reported that a conditioning regimen without ATG provided a low incidence of graft-failure [7]. Taken together, we believe that ATG should not be included in the conditioning regimen for CBT, not only for a single-unit CBT but also for a double-unit CBT. We also have shown the superiority of two-drug GVHD prophylaxis (including methotrexate) over single-drug prophylaxis in CBT [8]. To compensate prophylactic effect of ATG to control severe GVHD, it would be preferable to develop the GVHD prophylaxis after transplantation without ATG. Further study to determine whether or not ATG should be used in order to achieve prompt engraftment and subsequent higher quality of life and survival after RIC-CBT is warranted.

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Table 1
Patient demographics and CB unit characteristics.

Pt no.	Age/sex	BW (kg)	Disease status at transplant	Interval from diagnosis to CBT (year)	Transfusion dependency	ABO mismatch	HLA serological mismatch	HLA allele mismatch	HLA-antibody	Donor-specific antibody	TNCC (10^7/kg)	$CD34^+$ (10^5/kg)
1	48/M	51	Severe	1.2	RBC	Match	2/6	3/8	–	–	3.67	0.50
2	53/M	65	Severe	22.1	RBC/PC	Major/minor	1/6	4/8	–	–	2.79	0.44
3	37/F	51	Non-severe	26.9	RBC/PC	Major/minor	2/6	3/8	+	–	2.24	0.55

Pt, patient; M, male; F, female; BW, body weight; RBC, red blood cell concentration; PC, platelet concentration; HLA, human leukocyte antigen; TNCC, total nucleated cell count.

Table 2
Engraftment, chimerism and other outcomes.

Pt no.	Days to ANC >500/μl	Days to reticulocyte >1%	Days to plt > 20,000/μl	Days to plt > 50,000/μl	Chimerism after CBT	Acute GVHD	Chronic GVHD	Other complications	Survival, mo	KS (%)
1	19	30	25	191	Day 20, 95% donor	No	No	Postherpetic neuralgia	Alive, 68	70
2	21	28	37	44	Day 19, 100% donor	Grade I (skin 2)	No	Polymyalgia rheumatica	Alive, 44	100
3	22	37	32	43	Day 25, 100% donor	No	Yes (skin, oral)	No	Alive, 9	100

Pt, patient; ANC, absolute neutrophil count;plt, platelet; KS, Karnofsky score.

Conflict of interest

All authors declare that there are no competing financial interests.

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