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ORIGINAL ARTICLE

Frequency of $CD4^+FOXP3^+$ regulatory T-cells at early stages after HLA-mismatched allogeneic hematopoietic SCT predicts the incidence of acute GVHD

T Fujioka, H Tamaki, K Ikegame, S Yoshihara, K Taniguchi, K Kaida, R Kato, T Inoue, J Nakata, S Ishii, T Soma, M Okada and H Ogawa

Acute GVHD (aGVHD) is a major obstacle to allogeneic hematopoietic SCT (alloHSCT). Although it is thought that aGVHD is initiated in secondary lymphoid organs at a very early stage of alloHSCT, whether $CD4^+FOXP3^+$ regulatory T-cells (Tregs) have an impact on aGVHD development during this period remains unclear. Here, we measured Tregs in peripheral blood as early as possible after HLA-mismatched alloHSCT, and assessed the incidence of aGVHD. Flow cytometric analyses revealed that at the second week after HSCT, patients with aGVHD had significantly ($P = 0.018$) lower Treg:$CD4^+$T-cell ratios than those without aGVHD. As these differences were seen before the development of aGVHD, these ratios can predict the incidence of aGVHD. The cumulative incidence of aGVHD in patients with ratios of $<9\%$ was significantly higher than that in patients with ratios of $\geqslant 9\%$ ($P = 0.0082$, log-rank test). Additionally, the specific ratio of Tregs:$CD4^+$T-cells was the most significant value among all other possible lymphocyte-associated ratios and absolute cell counts. These findings suggest that the ratio of Tregs:$CD4^+$T-cells at the second week post HLA-mismatched alloHSCT might be a potent predictor of aGVHD in these patients. The practical efficacy of this finding should be verified in further interventional studies.

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Keywords: regulatory T-cells; GVHD; allogeneic SCT; HLA mismatch

INTRODUCTION

Although allogeneic hematopoietic SCT (alloHSCT) has the potential to cure many hematological disorders, GVHD continues to be a major obstacle associated with morbidity and mortality. Naturally occurring regulatory T-cells (Tregs) initially found in $CD4^+CD25^{high}$T-cell fractions[1,2] suppress autoreactive[1] and alloreactive[3–5] immunoreactions. Other researchers have investigated the relationship between the frequency of $CD3^+CD4^+CD25^{high}$Tregs in peripheral blood and the incidence of GVHD after alloHSCT, but results have been inconsistent, possibly due to differences in the definition of $CD25^{high}$.[6–8] The intracellular protein derived from the *FOXP3* gene has since been detected using flow cytometry, and is recognized as both a master regulatory gene and a unique marker for these Tregs.[9,10] This procedure enables the specific measurement of Tregs, and distinguishes them from activated conventional $CD4^+CD25^+$ T-cells. Subsequent studies have applied this procedure and suggested the role of Tregs in attenuating GVHD, mostly after HLA-matched alloHSCT.[11–14]

Here we examined Treg frequencies in the peripheral blood of patients who received alloHSCT from an HLA-mismatched related donor without T-cell depletion. As donor T-cells rapidly recover under our HSCT clinical protocol,[15,16] we analyzed the frequencies of Tregs and other lymphocyte populations as early as possible following HSCT, and examined the relationship between Treg frequency and the subsequent incidence of acute GVHD (aGVHD).

PATIENTS AND METHODS

Patients and samples

Forty-seven patients who underwent alloHSCT from partially HLA-mismatched related donors without T-cell depletion were evaluated. All patients received treatment at the Hyogo College of Medicine Hospital (Nishinomiya City, Japan) between July 2007 and August 2010 in accordance with the protocols approved by the institutional review board. Of these 47 patients, 45 received HLA-haploidentical HSCT. Patient characteristics are summarized in Table 1. After the provision of written informed consent, peripheral blood samples were obtained weekly on a fixed day of the week from the first to the eighth week after transplantation. Data acquired between day 1 and 7 were accordingly defined as data of the first week, those between day 8 and 14 as data of the second week, and so on.

Transplant procedure

Thirty and seventeen patients were preconditioned with a nonmyeloablative and myeloablative regimen, respectively, as reported previously.[15,16] In brief, the nonmyeloablative preparative regimen consisted of fludarabine (30 mg/m^2/day, for 6 days), BU (3.2 mg/kg/day, for 2 days, i.v.), and either anti-T-lymphocyte globulin (Fresenius Biotech GmbH, Munich, Germany) or anti-thymocyte globulin (Genzyme, Cambridge, MA, USA) (8 mg/kg or 2–4 mg/kg of the total dose, respectively). The myeloablative preparative regimen consisted of fludarabine (30 mg/m^2/day, 4 days), cytosine arabinoside (2 g/m^2/day, 4 times over 2 days), CY (60 mg/kg/day, for 2 days) and TBI (8 Gy delivered in 4 fractions). The GVHD prophylaxis regimen for nonmyeloablative HSCT consisted of tacrolimus (0.02 mg/kg/day) and methylprednisolone (1 mg/kg/day), and that for myeloablative HSCT consisted of tacrolimus (0.03 mg/kg/day), MTX

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Table 1. Patient characteristics

	No GVHD	*GVHD*	*P-value*
Number	25	22	
Median age	37	34	0.49
Sex			*0.33*
Male	9	11	
Female	16	11	
Diagnosis			*0.91*
ALL	6	8	
AML	6	6	
Non-Hodgkin lymphoma	7	4	
Myelodysplastic syndrome	3	2	
Hodgkin lymphoma	1	1	
CLL	1	1	
CML	1	0	
Conditioning intensity			*0.98*
Nonmyeloablative	16	14	
Myeloablative	9	8	
Source of stem cells			*0.16*
PBSC	12	15	
BM	13	7	
GVHD grade			*NA*
I		11	
II		7	
III		4	
IV		0	

Abbreviation: NA = not applicable.

(10 mg/m^2 on day 1 and 7 mg/m^2 on day 3), methylprednisolone (2 mg/kg/day) and mycophenolate mofetil (15 mg/kg). After transplantation, degrees of donor – recipient chimerism in T-cell and myeloid lineages of the peripheral blood were assessed by quantitative PCR for STR markers, as previously reported.[17] Assessment of aGVHD was based on clinical symptoms in accordance with commonly accepted criteria.[18,19] Unless patient condition precluded them, skin, liver and gastrointestinal tract biopsies were performed to support the diagnoses. Gastric biopsy was essentially required for the diagnosis of gut GVHD without manifest diarrhea (stage 1).

Flow cytometric analysis of Treg

Peripheral blood samples were collected using EDTA anticoagulant, and PBMCs were isolated by density-gradient centrifugation for analysis without cryopreservation. Flow cytometric analysis was performed using a Coulter cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) with CXP software (Beckman Coulter), using the following Abs: FITC-conjugated anti-CD3, phycoerythrin-Texas Red energy-coupled dye-conjugated anti-CD25, and phycoerythrin-Cy5-conjugated anti-CD4 (Beckman Coulter). For FOXP3 intracellular staining, the phycoerythrin-conjugated anti-FOXP3 Staining Set (eBioscience, San Diego, CA, USA) was used according to the manufacturer's instructions. FOXP3 staining was performed independently after staining with other Abs.

Statistical analysis

Differences in characteristics between patient groups were assessed by the Mann – Whitney *U*-test for continuous variables and the χ^2 test for categorical values. Median Treg frequencies were compared using the Mann – Whitney *U*-test. Treg frequencies were adjusted for differences between patients with and without aGVHD by multiple regression with logistic analysis. The sensitivity and specificity of Tregs in predicting aGVHD were assessed by receiver operating characteristic curve analysis. Cumulative incidences of aGVHD were plotted according to the Kaplan – Meier method and compared using the log-rank test.

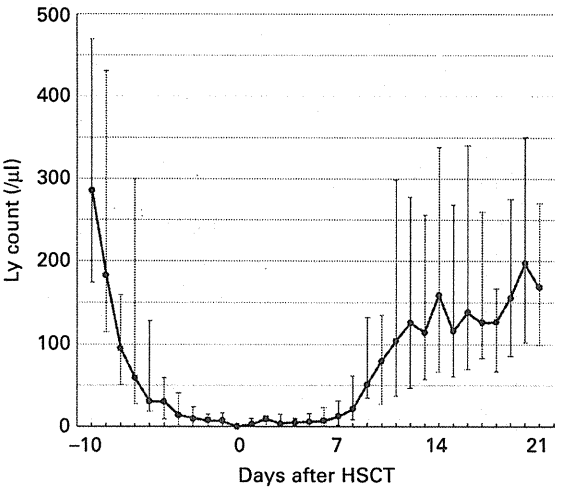

Figure 1. Recovery of lymphocyte (Ly) counts after HLA-mismatched HSCT. Median lymphocyte counts in peripheral blood after HLA-mismatched HSCT are shown. Upper and lower error bars indicate upper and lower quartile ranges, respectively.

RESULTS

Patients

Of the 47 patients, 22 presented with aGVHD *vs* 25 who did not (Table 1). The onset of aGVHD occurred at a median of 38 days after transplantation (range: 14–102). None of the characteristics examined had any significant impact on aGVHD incidence. As described previously, the degree of donor – recipient chimerism in T-cell and myeloid lineages of the peripheral blood achieves the complete donor type within 2 weeks after HLA-mismatched HSCT in our hospital.[15–17] Here, assessment once per week confirmed that on average complete donor-type chimerism in T-cells was achieved on day 10 (median, range: 5–23). Lymphocytes recovered during the second week (Figure 1) were therefore considered to consist almost entirely of donor-originated lymphocytes.

Flow cytometric analysis of Tregs

Representative results of a patient 4 weeks after HSCT are shown in Figures 2a–d. FOXP3$^+$ Tregs were analyzed using a flow cytometric plot gated by CD3$^+$CD4$^+$ fractions (Figure 2a). Although CD25 staining alone showed a large overlap between CD25$^+$ and CD25$^-$ cells (Figure 2c), FOXP3 staining was able to separate FOXP3$^+$ cells as an isolated population (Figure 2d). As demonstrated previously, CD25 staining alone is frequently incapable of revealing an unequivocal boundary that discriminates Tregs from CD4$^+$CD25$^+$-activated conventional T-cells in almost all cases.[20] As FOXP3 staining has apparent objectivity and, moreover, FOXP3 is the key molecule for this type of Treg,[9,10] we defined Tregs simply as CD4$^+$FOXP3$^+$T-cells, regardless of CD25 expression.

Treg:CD4$^+$T-cell ratios at the second week were significantly lower in patients with aGVHD

As lymphocyte numbers were markedly low during the first week, as shown in Figure 1, flow cytometric analysis was unable to detect any Tregs. By the second week after HSCT, in contrast, lymphocyte numbers increased to levels that made analysis possible in almost all cases (41 of 47 patients). Representative results of patients without and with aGVHD are shown in Figures 2e and f, respectively. Of the remaining six patients with slower lymphocyte recovery, two could be assessed at the third week,

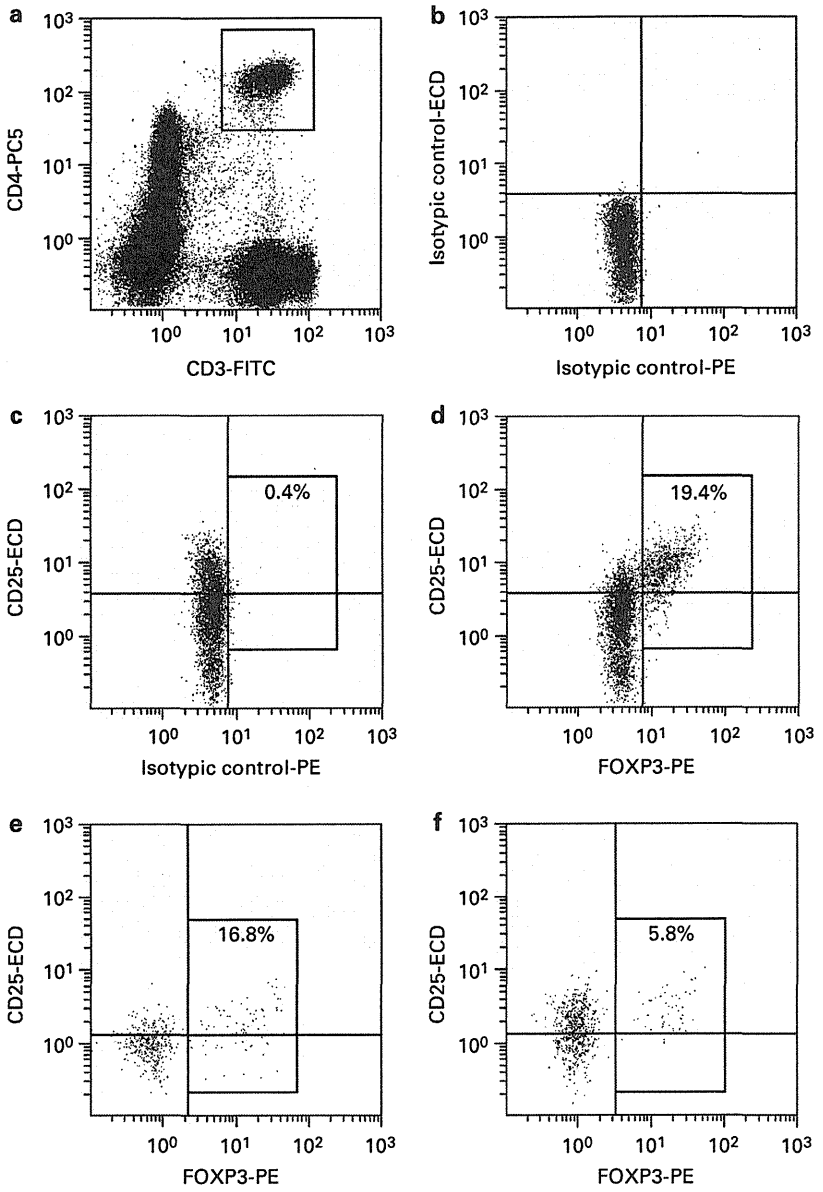

Figure 2. Flow cytometric analysis of Tregs. Representative results of four-color flow cytometric analyses performed for Tregs. Peripheral mononuclear blood cells were stained with CD3-FITC, CD4-PC5, CD25-energy-coupled dye (ECD) and FOXP3-phycoerythrin (PE). All dot plots were gated into lymphocyte populations according to forward- and side-scatter properties, and the gate of $CD3^+CD4^+$ fractions shown on plot **a** was used for the other dot plots with CD25/FOXP3 axes. The percentage of $FOXP3^+$ cells was calculated by subtracting the background percentage of the gate found in plot **c** from the gate shown on plot **d**. Plots **a**–**d** are representative results of a patient 4 weeks after HSCT. Plot **e** is a representative result of a patient without aGVHD in the second week and plot **f** is one of a patient with aGVHD in the second week.

one at the fifth week, one at the sixth week and two at the seventh week.

Figure 3 shows Treg:$CD4^+$T-cell ratios after HSCT ($n=41$), which are the most meaningful values as described in the following paragraph. On average, Tregs were collected on day 12 (median, range: 8–14) during the second week. Patients with aGVHD had significantly lower ratios in the second week after HSCT than those without aGVHD (median (range), 5.23 (0.32–44.8) *vs* 15.5 (0.00–37.1); $P=0.018$). Similar tendencies were seen during the following weeks, but the differences were not statistically significant. Multivariate analysis using logistic regression, which incorporated patient characteristics and transplantation settings, showed that Treg:$CD4^+$T-cell ratio was a unique independent and significant factor related to the incidence of aGVHD (Table 2).

Treg:$CD4^+$T-cell ratio is the most significant value among all other ratios and absolute counts

We also examined the significance of all other ratios between two major lymphocyte populations during the second week. As summarized in Table 3A, although $CD4^+$T-cell:whole T-cell ratio (median (range), 0.32 (0.09–0.78) in the aGVHD (+) group *vs* 0.16 (0.02–0.79) in the aGVHD (−) group; $P=0.026$) and

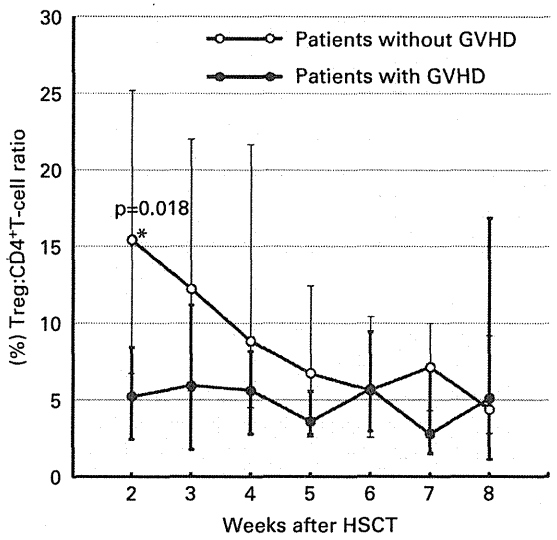

Figure 3. Frequency of Tregs in peripheral blood after HLA-mismatched HSCT. The frequencies of Tregs were assessed by flow cytometry weekly until the eighth week after HSCT. The median Treg:CD4$^+$T-cell ratios of patients with or without aGVHD are shown. Upper and lower error bars indicate upper and lower quartile ranges, respectively. Patients with aGVHD had significantly ($P=0.018$) lower median ratios at the second week after HSCT than those without aGVHD.

Table 2. Multivariate analysis

Parameter	*P-value*
Age	0.643
Sex	0.295
Diagnosis	0.774
Conditioning intensity	0.732
Source of stem cells	0.397
Treg:CD4$^+$T-cell ratio	0.032*

Abbreviation: Tregs = regulatory T-cells. *Indicates statistical significance ($P<0.05$).

CD8$^+$T-cell:CD4$^+$T-cell ratio (median (range), 1.98 (0.43–9.79) in the aGVHD (+) group *vs* 4.13 (0.38–32.3) in the aGVHD (−) group; $P=0.043$) were significant, the Treg:CD4$^+$T-cell ratio ($P=0.018$) had statistically the most significant value. Additionally, neither absolute numbers of whole lymphocytes nor the respective lymphocyte fraction (including Tregs) significantly correlated with the incidence of aGVHD (Table 3B).

Treg:CD4$^+$T-cell ratio at the second week predicts the incidence of aGVHD

As aGVHD occurred at a median of 38 days after HSCT (range, 14–102), while the significant decreases in Treg:CD4$^+$T-cell ratio were observed during the second week, this ratio can serve to predict the incidence of aGVHD. A receiver operating characteristic curve was generated by plotting the true positive rate of aGVHD against the false-positive rate for different cutoff-ratio values (Figure 4). The area under the curve was 0.73, indicating that the Treg:CD4$^+$T-cell ratio at the second week is a good predictor of aGVHD. Further analysis revealed that a cutoff-ratio value of 9% yielded the most accurate predictions of future aGVHD incidence (Figures 4, 69.6% sensitivity and 77.8% specificity). Treg:CD4$^+$T-cell ratios of $<9\%$ predicted a significantly higher incidence of aGVHD than ratios of $\geqslant 9\%$ (Figure 5, $P=0.0082$, log-rank test).

Table 3. *P*-values of each ratio (A) and absolute number (B)

A

numerator / denominator	Ly	T	CD4$^+$T	CD8$^+$T	B	Treg
Ly		0.636	0.093	0.674	0.203	0.478
T			0.026*	0.237	0.318	0.478
CD4$^+$T				0.043*	0.774	0.018*
CD8$^+$T					0.213	0.713
B						0.139
Treg						

B

Ly	T	CD4$^+$T	CD8$^+$T	B	Treg
0.674	0.636	0.083	0.979	0.213	0.875

*Indicates statistical significance ($P<0.05$).

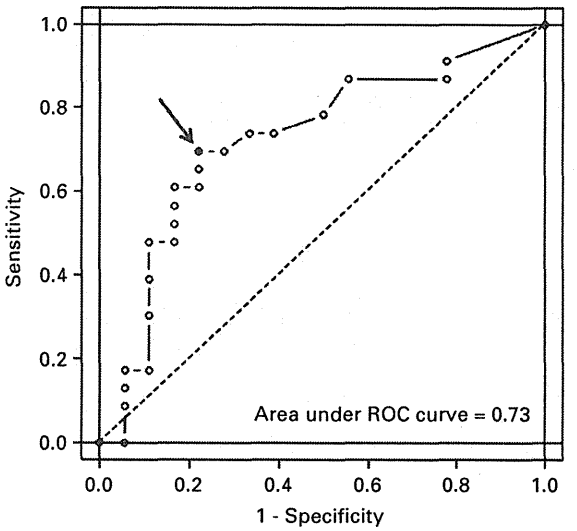

Figure 4. Receiver operating characteristic (ROC) analysis. ROC curve for the ratios of Tregs:CD4$^+$T-cells in identifying patients with aGVHD. The dashed diagonal line represents non-discrimination. Arrow, cutoff ratio at which the sensitivity and specificity resulted in a maximal Youden's index (cutoff ratio, 9%; sensitivity, 69.6%; specificity, 77.8%).

DISCUSSION

In this study, we found that ratios of Treg:CD4$^+$T-cells during the second week after HLA-mismatched HSCT without T-cell depletion accurately predicted the incidence of future aGVHD. Other investigators have used flow cytometry with intracellular staining of FOXP3 to demonstrate a relationship between aGVHD and Treg frequency in peripheral blood. Rezvani *et al.*[11] demonstrated a significant decrease in Treg frequencies at days 30 and 45 when comparing patients with and without aGVHD at the time of Treg sampling, as did Ratajczak *et al.*[14] at a mean of 3 months. Furthermore, Magenau *et al.*[13] assessed Tregs at the onset of aGVHD and demonstrated significant decreases in Treg frequencies in aGVHD patients, with comparison done using samples at GVHD onset and from patients without GVHD, such that the two groups were balanced for the time of acquisition. While their study sampled Tregs more than 4 weeks post transplantation, our study reports significant differences at less than half this time. Although further investigation is needed to determine whether early-stage Treg measurements are possible in other alloHSCT settings, our method under the condition of HLA-mismatched HSCT with rapid hematopoietic reconstitution[15–17]

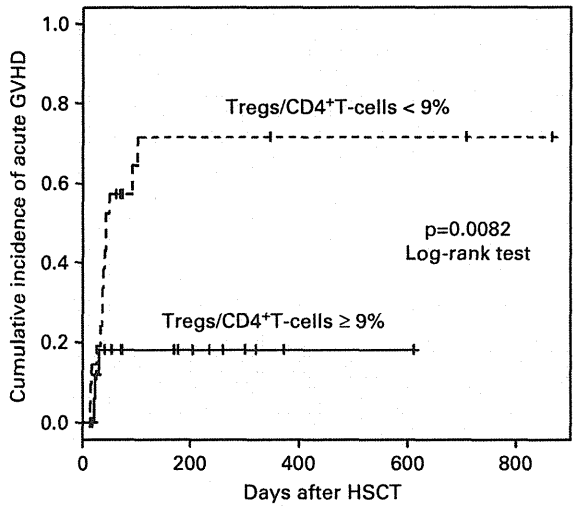

Figure 5. Cumulative incidence of aGVHD. Patients with Treg:CD4$^+$ T-cell ratios $<9\%$ had a significant higher incidence of aGVHD than those with ratios $\geqslant 9\%$ ($P=0.0082$, log-rank test). Measurements were taken in the second week after HSCT.

produced the earliest reported differences in Treg frequency, a finding with practical applications that allows for the prediction of future incidence of aGVHD.

It is a commonly accepted theory that GVHD is initiated in the priming phase, in which donor T-cells activate and proliferate in response to host APCs in secondary lymphoid organs,[21] where Tregs presumably function effectively by suppressing APC function.[22] TNFα is also well known as a central cytokine that peaks immediately after HSCT and stimulates APCs to prime T-cells during this phase.[21,23] Choi *et al.*[24] and Willems *et al.*[25] have both demonstrated that levels of TNFα receptor 1 (a surrogate marker of TNFα) at day 7 correlate with subsequent development of GVHD after myeloablative and nonmyeloablative alloHSCT, respectively. Although a number of studies using animal models have contributed to theories underlying GVHD pathogenesis,[26–30] their finding that this priming phase is limited to a very short duration immediately after HSCT in humans is particularly valuable. The most important point in our study is that conducting investigations at the earliest possible time point after HSCT enabled us to obtain our findings from a very narrow time window. Although it remains unclear whether lower Treg frequencies in peripheral blood reflect lower frequencies in secondary lymphoid organs, integrating our findings and previous studies in which the decline in Treg frequency in peripheral blood was seen during the initial phase of GVHD,[31] it is reasonable to assume that this is indeed the case, and thus that it causes the development of GVHD.

We also found that while absolute numbers of each lymphocyte population did not predict the occurrence of aGVHD, the Treg:CD4$^+$T-cell ratio was the most significant predictor among all other ratios (Table 3). As Tregs can work in cooperation with other cells, including APCs and other T-cells,[22] it is considered rational that ratio rather than absolute number is the relevant factor for predicting aGVHD. However, the reason why the specific ratio of Tregs:CD4$^+$T-cells is the most significant predictor remains uncertain. Although CD4$^+$T-cells recognize MHC class II molecules and have been shown to induce GVHD in a class II-mismatched (class I-matched) murine HSCT model,[32] the importance of CD4$^+$T-cells in GVHD pathogenesis has been demonstrated in fully MHC-mismatched (both class I and class II) murine models[33–35] and even in an HLA class I-mismatched HSCT.[36] Beilhack *et al.*[33] visualized initial proliferation of CD4$^+$T-cells followed by CD8$^+$T-cells in secondary lymphoid organs, and Ewing *et al.*[34] and Yu *et al.*[35] have demonstrated that the activity of CD4$^+$T-cells in the early phase contributes to subsequent development of aGVHD by CD8$^+$T-cells. Accordingly, CD4$^+$T-cells would likely have a leading role during the priming phase of aGVHD, and only then would activation and proliferation of CD8$^+$T-cells proceed. Our observation that both higher CD4$^+$ T-cell:whole T-cell and lower CD8$^+$T-cell:CD4$^+$T-cell ratios in the second week exhibit a significant relationship with aGVHD development does not conflict with these findings, as they both indicate a greater abundance of CD4$^+$T-cells than CD8$^+$T-cells. Furthermore, CD4$^+$T-cells have a particularly direct relationship with Tregs, with CD4$^+$T-cells being the principal targets that Tregs suppress in APC-dependent[37] and -independent[38] manners. Considering this, the high significance of the Treg:CD4$^+$T-cell ratio is reasonable.

Whereas 22 patients developed aGVHD in this study, half of those had grade 1 aGVHD. As previously described,[15] once aGVHD appears in these HLA-mismatched HSCT cases, it inevitably and rapidly progresses to more severe disease, resulting in fatal outcome. All the 11 patients with grade I aGVHD had stage 1 or 2 skin disease at onset. We were therefore obliged to treat them at the earliest time possible, usually within 24 h, with a combination of topical treatment and dose escalation of internal corticosteroid as initial treatment. Additionally, in cases where a skin biopsy was performed, we were unable to delay treatment while waiting for the results, although they would have been helpful for subsequent validation of treatment. Consequently, the disease remained at grade 1 in half of the patients, whereas progression could not be prevented in the other half. It is notable that Treg:CD4$^+$T-cell ratios are able to predict even mild cases of aGVHD, as even grade 1 aGVHD poses a high risk of causing more serious conditions, and should be avoided if possible. In contrast to previous studies,[13,14] we did not observe a significant inverse relationship between aGVHD grade and Treg:CD4$^+$T-cell ratio (data not shown). We attribute this to the early intervention and/or unequal distribution of patients for each grade.

We have demonstrated that patients who developed aGVHD had significantly lower Treg:CD4$^+$T-cell ratios at the second week after HLA-mismatched HSCT, well in advance of clinical aGVHD symptoms. The measurement of Tregs during the second week therefore provides a means to predict the development of aGVHD. Our results suggest that Tregs have a vital role in regulating aGVHD progression, and support the efficacy of early infusions of donor Tregs to prevent GVHD in HLA-haploidentical HSCT.[39] Further studies are needed to confirm whether interventions lead to improved outcomes for patients who show a high risk of aGVHD during the second week post HSCT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Salvage haploidentical transplantation for graft failure using reduced-intensity conditioning

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Graft failure is a major concern after cord blood transplantation (CBT) or HLA-haploidentical transplantation (haplo-SCT). As patients who undergo CBT or haplo-SCT almost always lack both matched-related and -unrelated donors, salvage transplantation would also be limited to either CBT or haplo-SCT. In this study, we assessed eight patients who received haplo-SCT as salvage therapy for graft failure. Five and three patients had received haplo-SCT and CBT, respectively, which resulted in graft failure. The median interval from the failed transplantation to salvage transplantation in six patients with primary graft failure was 33.5 days. The reduced-intensity conditioning regimen consisted of fludarabine, thiotepa, rabbit antithymocyte globulin and low-dose TBI. All eight patients achieved neutrophil engraftment, and seven patients achieved platelet recovery. The median times to neutrophil recovery and platelet recovery were 10 and 20 days, respectively. Three patients died from treatment-related causes: two from GVHD and one from rupture of carotid artery aneurysm. Five patients are alive, at a median follow-up of 946 days. The probability of overall survival at 5 years was 75%. These findings may serve as a rationale for giving precedence to haplo-SCT over CBT in salvage SCT after graft failure.
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Introduction

Graft failure is a life-threatening complication following allo-SCT. Immune rejection mediated by residual cellular immunity[1,2] or humoral immunity,[3,4] defects of the host BM microenvironment[5] and viral infections[6] are the main factors presumed to be involved in the occurrence of this complication. As immune rejection occurs as a result of the balance between residual host immunity and graft-derived immunity, the use of non-myeloablative or reduced-intensity conditioning (RIC),[7] T-cell depletion from the graft,[8] low numbers of infused progenitor cells[9,10] and immunological disparity (that is, HLA mismatch)[11] between the host and donor are known to increase the risk of graft failure. Although the overall frequency of graft failure is less than 5%, it has been reported to reach 12% for HLA-haploidentical SCT (haplo-SCT)[11] and is as high as 20% after cord blood transplantation (CBT).[12,13]

As both CBT and haplo-SCT are being increasingly performed as an alternative to HLA-matched-related or -unrelated transplantations, concerns regarding graft failure are also growing. The treatment options for graft failure are very limited. The survival rate for patients who do not receive salvage transplants are dismal (8%).[14] Salvage transplantation is generally attempted; however, the overall survival varies from 11 to 37%, with major obstacles being infections arising from prolonged neutropenia and damaged organ function as a result of previous transplantation.[14–17] Particularly, patients who undergo CBT or haplo-SCT almost always lack both matched-related and -unrelated donors during the clinically relevant period. Therefore, salvage transplantation is also limited to either CBT or haplo-SCT. We hypothesized that haplo-SCT is superior to CBT as a salvage therapy for graft failure because of the advantage of rapid neutrophil recovery, with respect to the high risk of infection in this particular setting. Therefore, we performed haplo-SCT using RIC for graft failure following CBT or haplo-SCT. Here, we describe the results for eight patients.

Patients and methods

Patients

This study is a retrospective analysis of eight consecutive patients who received a salvage transplant from an

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HLA-haploidentical related donor (2–3 Ag mismatched in the GVH vector) for primary or secondary graft failure following CBT or haplo-SCT between March 2001 and May 2010 at Osaka University Hospital or Hyogo College of Medicine Hospital. Informed consent was obtained from all the patients, and they were treated according to our institutionally approved protocols, including those for CBT and haplo-SCT.

Table 1 details the patient characteristics. Six patients had primary graft failure, whereas two had secondary graft failure. The median age of the patients was 49 years (range, 29–61 years) at the time of salvage transplantation. The stem cell sources of the previous transplantation, which failed to engraft, were cord blood in three patients, including one with double units, and haploidentical PBSC in five patients. Among these, two patients had received SCT one and two times before the failed SCT. Accordingly, they underwent salvage transplantation as their third and fourth SCT. Chimerism analysis showed no signs of donor hematopoiesis in seven patients. The remaining patient with secondary graft failure showed 100% donor chimerism in the T-cell fraction and 0% donor chimerism in the myeloid fraction.

Preparative regimen for salvage transplantation

All patients were treated with preparative regimen consisting of fludarabine 30 mg/m^2 for 3 days (days −4 to −2), thiotepa 5 mg/kg for 2 days (days −3 to −2), rabbit anti-T-lymphocyte globulin or antithymocyte globulin (ATG) and single-dose TBI 2–4 Gy. The doses of ATG and TBI in each patient are detailed in Table 2. The dose of TBI was determined according to the preparative regimen of previous transplants and the performance status of the patients at the time of salvage transplantation.

Salvage transplantation

Three of the five patients who had graft failure after haplo-SCT received salvage transplantation from the same donor. G-CSF-mobilized PBSCs were collected from the donor on days 0 and 1, with the target CD34+ cell dose of 3×10^6/kg of recipient body weight. The median number of infused CD34+ cells was 4.7×10^6/kg (range, $2.7–7.9 \times 10^6$/kg). The median interval from the failed transplantation to salvage transplantation for the six patients with primary graft failure was 33.5 days (range, 25–54 days).

Table 1 Patient characteristics

Patient no.	*Age (years)/sex*	*Diagnosis*	*Disease stage*	*No. of SCT before the failed SCT*	*SCT resulting in graft failure*				
					Stem cell source	*HLA match*		*Preparatory regimen*	*Pattern of GF*
						GVH vector	*HVG vector*		
1	29/M	MDS-AML	Refractory	0	PBSC	3/6	3/6	Flu/BU/ATG	Primary
2	54/F	CMML-AML	Refractory	2	PBSC	4/6	4/6	Flu/BU/ATG	Secondary
3	49/F	MDS-AML	Relapse after allo-SCT	1	PBSC	4/6	4/6	Flu/CA/BU/ATG	Primary
4	42/M	MDS-AML	Refractory	0	PBSC	4/6	3/6	Flu/CA/CY/TBI (8)	Primary
5	35/M	ALL	CR2	0	Double CB	5/6	5/6	CY/TBI (12)	Primary
						5/6	4/6		
6	57/M	MDS	RA	0	CB	4/6	4/6	Flu/CY/TBI (3)	Primary
7	61/M	MDS-AML	First relapse	0	CB	4/6	4/6	Flu/CY/TBI (3)	Primary
8	49/F	AML	Refractory	0	PBSC	4/6	3/6	Flu/CA/Mel/ATG	Secondary

Abbreviations: CA = cytosine arabinoside; CMML = chronic myelomonocytic leukemia; F = female; Flu = fludarabine; GF = graft failure; GVH = graft versus host; HVG = host versus graft; M = male; Mel = melphalan; MDS-AML = AML evolved from myelodysplastic syndrome.

Table 2 Information regarding salvage transplantation

Patient no.	*Interval from the failed SCT to salvage SCT (days)*	*Salvage transplantation*						
		Donor		*HLA match*		*Preparatory conditioning*		*CD34 (× 10^6/kg)*
		Same as the failed SCT	*Relation*	*GVH vector*	*HVG vector*	*TBI dose (Gy)*	*ATG product/total dose (/kg)*	
1	25	Yes	Sibling	3/6	3/6	4	TMG/5	7.1
2	37	No	Daughter	4/6	3/6	2	TMG/2[a]	7.9
3	54	No	Daughter	4/6	3/6	4	ATG-F/10	4.0
4	31	Yes	Sibling	4/6	3/6	4	ATG-F/8	3.1
5	36	No	Mother	4/6	4/6	2	TMG/3	3.5
6	40	No	Daughter	3/6	3/6	3	TMG/3	5.5
7	31	No	Daughter	3/6	3/6	3	TMG/3	2.7
8	100	Yes	Daughter	4/6	3/6	4	TMG/3	5.3

Abbreviations: ATG-F = anti-T-lymphocyte globulin-Fresenius; GVH = graft versus host; HVG = host versus graft; TMG = thymoglobulin.
[a]Only patient no. 2 received ATG after transplantation (on days 10, 14 and 19).

GVHD prophylaxis and treatment
GVHD prophylaxis and treatment followed the institutional haplo-RIC protocol, which has been detailed elsewhere.[18] Briefly, GVHD prophylaxis consisted of continuous i.v. infusion of tacrolimus with target levels of 10–12 ng/mL and methylprednisolone 1 mg per kg per day. After patients achieved neutrophil engraftment and acute GVHD was considered absent, tacrolimus and methylprednisolone were tapered.

Supportive care
Patients were hospitalized in single rooms ventilated with high-efficiency particulate air filtration systems. All patients received broad-spectrum antibiotics and azoles (itraconazole or voriconazole) at the time of salvage transplantation. Following engraftment, patients received trimethoprim-sulfamethoxazole or aerosolized pentamidine for prophylaxis against pneumocystis pneumonia for at least 12 months post transplantation. Acyclovir was continued at 200 mg per day until the discontinuation of immunosuppressant. Patients received i.v. Ig 100 mg/kg weekly for 2 months after transplantation. CMV was monitored weekly by a pp65 antigenemia test. In addition, human herpesvirus-6 was monitored bi-weekly by PCR for virus DNA. Documented CMV or human herpesvirus-6 reactivation was treated with either ganciclovir or foscarnet. G-CSF 300 μg/m^2 was administered from day 1 or day 5 until the neutrophil count was greater than 2500/μL for two consecutive tests.

Chimerism analysis
Donor chimerism was determined serially in the T-cell- or neutrophil-enriched cell fractions of peripheral blood and BM. The methodology used for cell separation and chimerism analysis has been detailed elsewhere.[18,19] Briefly, T cells were enriched by a negative selection system (RosetteSep; StemCell, Vancouver, Canada) to a purity of >95%, and granulocytes were recovered from the Ficoll-red blood cell interface with a purity of >99%. Chimerism analysis involved quantitative PCR of informative STRs in the recipient and donor. DNA was amplified with fluorescent PCR primers for markers that would distinguish the donor and recipient alleles. Fluorescent PCR products were separated with an Applied Biosystems 310 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA), and GeneScan software (Applied Biosystems) was used to correlate allele peak areas with the percentage of donor or recipient DNA.

Definitions and statistical analysis
Neutrophil engraftment was defined by an ANC of at least 500/μL for three consecutive tests, whereas platelet recovery was defined by a platelet count of at least 20 000/μL without transfusion support. Primary graft failure was defined by an absence of neutrophil recovery associated with no appearance or complete loss of donor cells using STR chimerism analysis by day 18 or an absence of neutrophil recovery by day 60. Secondary graft failure was defined as a recurrent neutropenia less than 500/μL after initial recovery. Diagnosis of acute and chronic GVHD was based on standard clinical criteria,[20] with histopathological confirmation where possible. Overall survival and disease-free survival were calculated using the Kaplan–Meier method.

Results

Engraftment and chimerism
All eight patients achieved neutrophil engraftment, and seven patients achieved platelet recovery following salvage haplo-SCT (Table 3). The median times to neutrophil recovery and platelet recovery were 10 days (range, 8–11 days) and 20 days (range, 17–97 days), respectively. Chimerism analysis showed that all patients achieved complete donor chimerism in both the T-cell and myeloid fractions within 4 weeks after transplantation.

GVHD
Four patients had no clinical acute GVHD. During the tapering of immunosuppressants, two patients developed grade II GVHD, whereas two patients developed grade III GVHD. Although both patients with grade II GVHD were successfully treated with increased doses of steroid therapy, the two patients with grade III GVHD (both with stage 2 liver involvement) were resistant to steroid therapy and subsequently died. None of the evaluable six patients developed chronic GVHD clinically.

Toxicity, relapse and cause of death
In all, three of the eight patients died from treatment-related causes: two from GVHD and one from rupture of

Table 3 Outcomes of salvage transplantation

Patient no.	*Time to engraftment (days)*		*GVHD*		*Relapse*	*Current status*	*Cause of death*
	Neutrophil	*Platelet*	*Acute*	*Chronic*			
1	10	17	0	No	No	Alive, day 3468	
2	8	97	II	No	No	Dead, day 2395	Rupture of carotid artery aneurysm
3	8	35	0	No	No	Alive, day 936	
4	10	17	0	No	Yes (day 718)	Alive, day 916	
5	10	20	II	No	No	Alive, day 459	
6	9	18	0	No	No	Alive, day 246	
7	11	24	III	NE	No	Dead, day 112	GVHD
8	11	NA	III	NE	No	Dead, day 91	GVHD, leukoencephalopathy

Abbreviations: NA = not achieved; NE = not evaluable.

carotid artery aneurysm, possibly related to thrombotic microangiopathy. One patient relapsed 718 days after salvage transplantation and received a third transplantation from a haploidentical related donor.

Survival

Five patients are alive at a median follow-up of 946 days (range, 276–3498 days). The probability of overall survival and disease-free survival at 5 years was 75 and 56%, respectively.

Discussion

We showed that salvage haplo-SCT for graft failure using RIC regimen allowed rapid neutrophil engraftment in all our patients, which translated into no mortality from infectious complications and favorable long-term survival (5-year overall survival = 75%).

Recently, the result of a Japanese nationwide survey of salvage CBT for graft failure was reported by Waki *et al.*[21] Of 80 patients who received salvage CBT, 61 patients who survived for more than 28 days were evaluated for hematopoietic recovery. Among them, 45 patients (74%) achieved neutrophil engraftment at a median of 21 days, and 31 patients (51%) achieved platelet recovery. Thirteen patients developed primary graft failure again. The rate of TRM at day 100 was 45%, with 60% related to infectious complications. The probability of overall survival at 1 year after CBT was 33%. Although the number of patients in this study is too small to draw any conclusions, we found a clear advantage of haplo-RIC over CBT in terms of neutrophil engraftment. Meanwhile, the major drawback of haplo-SCT is the risk of GVHD. Although the rate of severe GVHD was limited, two patients developed fatal GVHD in this study. Optimization of GVHD prophylaxis, such as the use of higher doses of ATG, may further improve the outcome of haplo-SCT for graft failure. To date, reports describing salvage transplantation from haploidentical donors in adult patients are few.[22,23] In the pediatric setting, Lang *et al.*[24] described 11 patients who received haplo-SCT for graft failure, with findings consistent with this report with respect to rapid neutrophil engraftment at a median of day 9, associated with favorable survival (1-year event-free survival = 72%). Although the number of reported cases is limited, double-unit CBT also appears promising.[25]

This study also showed the relative safety and effectiveness of the preparative regimen, consisting of fludarabine, thiotepa, low-dose TBI and ATG. In the majority of recent studies concerning salvage transplantation for graft failure, fludarabine and either ATG or alemtuzumab were included in the preparative regimen.[26–29] These agents are highly immunosuppressive and expected to suppress host immunocompetent cells, including T and NK cells, which are involved in the mechanism of immune-mediated graft rejection. Moreover, the use of ATG or alemtuzumab reduces the risk of GVHD after salvage transplantation. Of note, the aforementioned study by Waki *et al.*[21] showed that the incidence of neutrophil engraftment was higher in patients who received alkylating agents, including melphalan, busulfan and cyclophosphamide, as part of conditioning. Furthermore, the effect of low-dose TBI in promoting donor engraftment in the settings of the first transplantation has been reported by several studies.[14,30] Collectively, the preparative regimen used in this study has a powerful potential in enabling successful donor engraftment with limited toxicity in salvage transplantation for graft failure.

Theoretically, it could be argued that the donor in salvage transplantation should be altered from the previous failed transplantation, as previous studies have shown that cytotoxic T cells targeting mismatched HLA possessed by the donor are aroused at the time of immune rejection.[1] However, in this study, all three patients who received salvage transplantation from the same donor as the previous failed transplantation achieved engraftment. Nevertheless, considering the possible risk to a healthy donor of the administration of high doses of G-CSF twice in a short period of time as well as of poor mobilization, the donor for the salvage transplantation should be chosen cautiously.

This study has several inherent limitations. First, as a retrospective review, our case series is subject to a possible selection bias. In the study period, 12 patients developed graft failure after CBT or haplo-SCT, including eight patients who received salvage haplo-SCT, and thus were analyzed in this study. Of the remaining four patients, two patients received salvage transplantation from HLA one-locus mismatched donors. The other two patients could not receive salvage SCT, as they died as early as on days 20 and 25. Thus, we do not consider this study to be biased. Second, the number of the patients was small and the duration of follow-up for some of them was short. Nevertheless, our case series suggest the usefulness of this approach, indicating the need for further clinical study.

In conclusion, we showed that salvage haplo-SCT for graft failure allowed rapid engraftment in all patients, which translated into favorable overall survival. This study may serve as a rationale for giving precedence to haplo-SCT over CBT in the settings of salvage SCT after graft failure.

Conflict of interest

The authors declare no conflict of interest.

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Allogeneic stem cell transplantation as treatment for heavily treated, refractory acute graft-versus-host disease after HLA-mismatched stem cell transplantation

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Objective. **No effective treatment has been established for patients with steroid-refractory acute graft-versus-host disease (GVHD). Recently, we demonstrated in a murine tandem bone marrow transplantation model that life-threatening GVHD established by the first bone marrow transplantation was successfully treated by engraftment of a second donor graft after reduced-intensity conditioning. We named the effect by which allografts counteract GVHD "graft-versus-GVHD."**

Materials and Methods. **To investigate the efficacy of graft-versus-GVHD treatment clinically, 16 patients who developed, after human leukocyte antigen–mismatched stem cell transplantation, severe GVHD, refractory to three to five lines of GVHD-specific treatments, underwent 17 allogeneic stem cell transplantations using reduced-intensity conditioning regimens with grafts from a second donor.**

Results. **Among the 15 transplantations that could be evaluated, rescue donor grafts were engrafted in 11 cases and rejected in 4 cases. For patients who achieved rescue donor engraftment, the response rate was 90.9% (eight complete response, two partial response, and one stable disease). Six of the eight patients with complete response survived without GVHD symptoms, with a median follow-up of 2128 days. No new development of GVHD by the second graft was observed. No patients had recurrence of the original malignant disease. In contrast, no long-term survivors were observed in patients who rejected rescue donor grafts.**

Conclusions. **We propose here a novel graft-versus-GVHD treatment to treat refractory GVHD, and these results strongly suggest that GVHD can be successfully treated by eliminating the harmful lymphocytes responsible for GVHD by a second allogeneic stem cell transplantation.**

Graft-versus-host-disease (GVHD) is a major obstacle to successful allogeneic bone marrow transplantation (BMT), and greatly limits the applications and efficacy of allogeneic BMT. In particular, for steroid-refractory GVHD, no consensus treatment has been established [1,2], although a number of therapeutic approaches, including mesenchymal stem cells, pentostatin, infliximab, and a variety of monoclonal antibodies, have been reported [3–7].

We and others have attempted to treat patients with severe GVHD by second transplantation using autologous or syngeneic hematopoietic cells to ablate the lymphoid cells responsible for GVHD [8–10]. Although severe GVHD resolved or partially improved after these transplantations, relapse of the original tumor occurred in the majority of patients.

Therefore, we intended to use a second allogeneic donor as a graft source for rescue transplantation against GVHD. We recently demonstrated in a murine tandem BMT model where the three mouse strains shared one major histocompatibility complex haplotype and the other major histocompatibility complex haplotype was different, that

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life-threatening GVHD established by the first BMT using myeloablative conditioning was successfully treated by engraftment of a second donor graft using reduced-intensity conditioning treatment [11]. In allogeneic stem cell transplantation (SCT) for autoimmune diseases, donor lymphocytes are considered to have the capacity to eliminate all residual self-reactive host lymphocytes through a process known as graft-versus-autoimmunity effects [12], with analogy to graft-versus-leukemia (GVL) in leukemia. Thus, we named the effects by which second allografts counteract GVHD through permanent elimination or transient reduction of first donor harmful lymphocytes, "graft-versus-GVHD" [11].

In addition, clinically, we recently developed a novel unmanipulated human leukocyte antigen (HLA)–haploidentical nonmyeloablative SCT using a conditioning treatment consisting of fludarabine + busulfan + anti–T-lymphocyte globulin (ATG), and GVHD prophylaxis consisting of tacrolimus (FK506) + methylprednisolone (mPSL) (1 mg/kg), in which the incidence of acute GVHD was only 20% [13]. As some GVHDs occurred after donor lymphocyte infusion or rapid tapering of immunosuppressive agents for early relapse or severe viral infections, the actual incidence of GVHD was estimated to be 10%; therefore, we applied this HLA-haploidentical nonmyeloablative SCT to rescue transplantation for refractory GVHD.

In the present study, we investigated whether second allogeneic SCT could treat patients with severe, steroid-refractory GVHD.

Materials and methods

Patients

From February 2001 to December 2008, 320 patients underwent allogeneic SCT at Osaka University Hospital or at the Hospital of Hyogo College of Medicine. Among them, 16 consecutive adult patients who developed severe refractory GVHD after HLA-mismatched SCT underwent a second allogeneic SCT to treat GVHD. All of these patients were in remission at the time of rescue transplantation. The major objectives in this study were improved GVHD and survival at 6 months. GVHD was diagnosed from a biopsy of at least one involved organ. Patients with severe GVHD (≥grade II) who did not respond to mPSL (≥2 mg/kg) or who had recurrent GVHD at a dose of steroids ≥1 mg/kg mPSL were eligible for the study; however, patients who were finally enrolled received a median of four (range of two to five) lines of GVHD-specific treatments, including tumor necrosis factor blocker, ATG, and mycophenolate mofetil, by the time of the rescue transplantation (Table 1). In general, GVHD occurring after HLA-mismatched SCT progresses very rapidly, and quickly becomes irreversible; therefore, in the first SCT inducing GVHD, when the manifestations of GVHD worsened during 3 days of treatment, other immunosuppressive agents were added [14], sometimes in combination. Regarding the eligibility criteria for the rescue transplantation, patients who had HLA-identical or HLA 1–3 antigen-mismatched related donors were eligible. Patients were not eligible for rescue transplantation if they had severe renal, heart, or lung disease: serum creatinine level >1.5 times the normal upper limit, ejection fraction <50% on an echocardiogram, or oxygen saturation <93%, respectively. Patients were not eligible for rescue transplantation if they had severe liver disease that was considered to be caused by diseases other than GVHD; total bilirubin level >2.0 mg/dL, and aspartate aminotransferase >2.5 times the normal upper limit.

The characteristics of the patients and first transplantation inducing severe GVHD are shown in Table 1. Because one patient underwent allogeneic rescue SCT twice, 17 graft-versus-GVHD treatments were performed. Among the 16 patients, 14 had developed acute GVHD after allogeneic SCT, including 3 patients who had developed recurrent acute GVHD >100 days after transplantation and 2 after donor lymphocyte infusion. Institutional review board approval was obtained for the treatment protocol, and written informed consent was obtained from the patients and their families.

Four patients underwent the first transplantation (inducing severe GVHD) using a graft from an HLA 2–3 antigen-mismatched donor, and underwent the second (rescue) transplantation using a graft from an HLA-matched or 1 antigen-mismatched donor (Table 2). The donor in the first transplantation was selected for the following reasons. We recently reported that unmanipulated HLA-haploidentical SCT was useful for treating patients with hematologic malignant diseases in the advanced stage [13,15,16]. Thus, in our HLA-haploidentical SCT protocol, patients with a full-blown relapse can undergo allogeneic SCT using a graft from an HLA-haploidentical donor, even when an HLA-matched (or 1 antigen-mismatched) related donor is available. Such decisions were made at the recommendation of the physicians and with the concurrence of the patient and family members after considering the overall risks of recurrent malignancy, graft rejection, and severe GVHD with the two different types of donors.

Rescue transplantation procedure

Details of the rescue transplantation are shown in Table 2. Median interval between the previous allogeneic SCT and the rescue transplantation was 59 days (range, 32–481 days). All patients received a reduced-intensity conditioning treatment. The conditioning consisted of 30 mg/m^2 fludarabine intravenously for 3 consecutive days on days −6 to −4, ATG (Fresenius) 2 mg/kg/day for 4 days (day −4 to day −1) with or without total body irradiation 3 Gy on day 0. Eight patients could not receive total body irradiation because they had received total body or local irradiation as previous treatments. One patient (no. 10–2) who rejected the first rescue transplantation received thiotepa 10 mg/kg on day −2 and total body irradiation 4 Gy on day −1 in addition to fludarabine and ATG. In all cases, peripheral blood stem cells were used as the stem cell source.

GVHD prophylaxis was performed with FK506 and mPSL (1 mg/kg), as reported previously [13]. In brief, FK506 treatment was initiated the day before transplantation and given at a dose of 0.02 mg/kg/day as a continuous infusion. The target blood concentration of FK506 was set between 8 and 10 ng/mL until day 30, and was thereafter tapered in the absence of acute GVHD. Patients received intravenous FK506 therapy until they could reliably receive oral medications after transplantation. Intravenous administration of mPSL was started at a dose of 1 mg/kg/day from day −4. mPSL tapering was started in the third week and was performed relatively rapidly until day 30 using the serum soluble

Table 1. Patients' characteristics and first transplantation inducing severe GVHD

No	Sex/Age	Disease	Disease status	Conditioning regimen	Donor	HLA disparity	PS	grade	Stage			prior treatment for GVHD
									skin	gut	liver	
1	23/F	ALL	PR	full	Mother	2/2†	50	II	3	1	0	MTX, MMF, mPSL(2), Flu,
2	17/M	LBL	Re3	full	Cousin	2/3	10	III	3	3	1	Flu, ATG, MTX, MMF(inc)
3	33/M	ALL	PR	full	Sibling	3/3	20	III	3	4	0	MTX, MMF(inc), Flu, ATG,
4	37/M	MDS	RAEB	full	Offspring	3/3	20	III	3	4	0	Flu, MMF(inc), infliximab, ATG, pulse mPSL
5	25/M	CML	Re(autoBM)*	full	Sibling	2/2	70	II	3	0	0	PSL(inc). MMF
6	21/F	NHL	CR2(autoPB)	full	Mother	2/0	50	II	3	0	0	MMF, infliximab
7	19/M	HD	RR	full	Father	3/2	50	IV	4	0	0	MTX, ATG, infliximab
8	22/M	ALL	Re2	full	Sibling	3/2	10	III	0	3	3	infliximab, ATG, pulse mPSL, MTX, basiliximab
9	19/F	CML	BC	full	Sibling	2/2	70	II	3	0	0	infliximab, ATG, pulse mPSL, MTX, MMF(inc)
10-1	19/M	SNCL	IF	full	Sibling	2/2	50	III	3	4	0	MTX, infliximab, pulse mPSL, ATG
10-2	19/M	SNCL	IF	RIST	Mother	2/2	30	III	3	2	0	mPSL(inc), infliximab, MMF, ATG
11	41/F	LAHS	IF	full	Offspring	2/3	20	III	3	2	3	ATG, infliximab, MMF, pulse mPSL
12	21/F	AML	Re(alloBM)	RIST	Father	3/3	20	III	2	2	2	infliximab, pulse mPSL, MMF, ATG
13	49/M	CML	CP	RIST	Offspring	2/3	30	IV	4	2	0	ATG, MTX, infliximab, pulse mPSL
14	19/F	ALL	Re2	full	Sibling	3/1	40	III	2	3	1	pulse mPSL, MMF, etanercept, ATG
15	47/F	ALL	Re(alloPB)	RIST	UCB	4/2	40	III	3	3	0	etanercept, MMF, pulse mPSL
16	31/F	ALL	RR	full	Sibling	3/2	60	III	2	2	0	PSL(inc), pulse mPSL, MTX

AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; NHL, non-Hodgkin's lymphoma; HD, Hodgkin's lymphoma; SNCL, small non-cleaved lymphoma; LAHS, lymphoma-associated hemophagocytic syndrome; CR2, second complete remission; PR, partial remission; Re, relapse; Re2 or Re3, second or third relapse; RR, resistant relapse; RAEB, refractory anemia with excess of blasts; CP, chronic phase; BC, blastic crisis; IF, induction failure; full, full regimen; RIST, reduced intensity of conditioning treatment; PS, Karnofsky performance status; MTX, methotrexate; MMF, mycophelate mofetil; mPSL(2), methylprednisolone 2 mg/kg; pulse mPSL, pulse therapy of methylprednisolone; Flu, fludarabine; ATG, anti-T-lymphocyte globulin; inc, increase in dose; autoBM, autologous bone marrow transplantation; autoPB, autologous peripheral blood stem cell transplantation; alloBM, allogeneic bone marrow transplantation; alloPB, allogeneic peripheral blood stem cell transplantation.

*Transplantation in parentheses indicates previous stem cell transplantation.

†Numbers before or after a slash indicate mismatched HLA antigens in GVH or HVG directions, respectively.

Table 2. Details of the rescue transplantation

No	Interval between 2 transplantations (days)	Donor relationship	Donor Sex/ Age	HLA disparity	Conditioning treatment	Cell dose CD34 cells × 10^6/kg	Cell dose CD3 cells × 10^8/kg	Engraftment of rescue graft	days	Hematological recovery Neu>0.5 ×10^9/l (days)	Hematological recovery PLT>20 ×10^9/l (days)	GvGVHD effect	survival (days)	Cause of death
1	94	Sibling	F/25	2/2	chemo	3.90	1.61	+	15	9	101	complete	+3304	-
2	40	Sibling	F/22	0/0	chemo	6.60	5.81	NE		8	-	NA	10	TMA
3	145	Mother	F/58	1/1	chemo	3.76	5.38	NE		-	-	NA	13	Renal failure
4	40	Offspring	M/12	3/3	chemo	16.50	4.19	+	20	10	-	partial	135	GVHD
5	481	Mother	F/55	2/2	chemo+TBI	3.90	2.50	+	34	not decreased	not decreased	complete	+2714	-
6	213	Sibling	F/23	1/0	chemo+TBI	5.20	6.71	+	29	10	36	complete	831	Cardiac failure
7	47	Mother	F/45	2/2	chemo	3.60	3.57	−		26	-	partial	76	Pneumonia
8	98	Mother	F/47	3/3	chemo+TBI	6.20	3.11	+	11	10	-	partial	23	Pneumonia
9	227	Mother	F/48	2/3	chemo+TBI	4.51	2.06	+	17	not decreased	not decreased	complete	+2170	-
10-1	59	Mother	F/51	2/2	chemo+TBI	2.80	2.12	−		not decreased	-	transient	+42	-
10-2	101	Mother	F/51	2/2	chemo+TBI*	2.30	2.27	+	14	9	32	complete	+2086	-
11	63	Sibling	M/37	0/0	chemo	7.10	1.71	−		not decreased	not decreased	partial	33	VOD
12	32	Mother	F/ 51	3/0	chemo+TBI	23.00	3.49	−		not decreased	-	partial	46	GVHD
13	36	Offspring	M/22	2/3	chemo+TBI	7.16	3.22	+	52	8	9	complete	+1637	-
14	59	Sibling	F/12	3/1	chemo+TBI	18.60	8.10	+	14	10	-	transient	72	TTP
15	49	Offspring	M/16	3/3	chemo	17.10	4.30	+	8	8	-	complete	163	Hepatic failure
16	39	Sibling	F/27	3/2	chemo	14.00	2.66	+	107	not decreased	16	complete	+490	-

chemo, chemotherapy consisting of fludarabine 30 mg/m2 and anti-T-lymphocyte globulin; TBI, total body irradiation 3Gy; NE, not evaluable; not decreased, neutrophils or platelet counts did not decrease below 0.5 x 109/l or 20 x 109/l, respectively; GvGVHD effect, graft-versus-GVHD effect; complete, complete response; partial, partial response; TMA, thrombotic microagiopathy; VOD, hepatic veno-occlusive disease; TTP, thrombotic thrombocytopenic purpura.

*Thiotepa 10 mg/kg and TBI 4 Gy were given in addition to fludarabine and ATG.

interleukin-2 receptor level [17,18], as an indicator, and was thereafter continued carefully.

Acute GVHD was graded according to standard criteria [19] and GVHD beyond 100 days after transplantation was diagnosed based on the proposed National Institutes of Health criteria [20]. Patient status before rescue transplantation was assessed by the Karnofsky performance rating. We defined the response to treatment as follows: complete response: loss of all symptoms of acute GVHD; partial response: improvement of at least one GVHD grade; stable disease: no change in GVHD grade; progressive disease: worsening of GVHD. Regarding the assessment of GVHD after the rescue transplantation, if the symptoms of patients were considered to have been caused mainly by a complication other than GVHD, their GVHD stages were downgraded by one stage, according to the recommendation in the 1994 consensus conference on acute GVHD grading [21]. A diagnosis based on autopsy directly reflected the assessment of response.

Each patient was isolated in a laminar air-flow room and standard decontamination procedures were followed. Oral antibiotics (ciprofloxacin, vancomycin, amphotericin B) were administered to sterilize the bowel. Patients with negative cytomegalovirus (CMV) IgG titers received blood products from CMV seronegative donors. Intravenous immunoglobulin was administered at a minimum dose of 100 mg/kg every 2 weeks until day 100. Cotrimoxazole was given for at least 1 year for prophylaxis of *Pneumocystis jirovecii* infections. Acyclovir was administered at a dose of 1000 mg/day for 5 weeks after transplantation to prevent herpes simplex infections.

Ganciclovir 7.5 mg/kg divided in three doses per day was administered from day −10 to day −3 as prophylaxis for CMV infection. Thrombotic microangiopathy was diagnosed according to Zeigler's criteria [22], and based on the recommendations reported by Nishida et al. [23].

Chimerism analysis

Chimerism between the donor and recipient was analyzed as described previously [13]. Chimerism analysis was continued twice a week after transplantation until donor engraftment or rejection. Blood samples were analyzed to determine the degree of donor/recipient chimerism in the T-cell or neutrophil-enriched cell fraction, using polymerase chain reaction amplification of informative microsatellite regions, which identified differences between the donor and recipient (based on polymorphisms found in pretransplantation donor/recipient samples) [24]. To remove monocytes, KAC-2 silica beads (Japan Immunoresearch Laboratories Co., Ltd., Gunma, Japan) were mixed with heparinized peripheral blood and incubated at 37°C for 1 hour. To enrich T cells, a negative selection system (RosetteSep; StemCell Technologies) was used [25]. To obtain a T-cell–enriched cell fraction, a cocktail containing anti-CD16, anti-CD19, anti-CD36, and anti-CD56 antibodies was added to the blood samples after they were treated with Silica beads. After Ficoll-Paque (GE Healthcare, Little Chalfont, Buckinghamshire, UK) density gradient centrifugation, $CD3^+$ cells were recovered from the Ficoll: plasma interface with a purity >95%. Neutrophils were recovered from the Ficoll:RBC interface with a purity >99%.

Statistical analysis

The protocol was designed as a phase II study with sufficient power to detect a response rate of ≥20% with a standard error of 10%. Comparison of patients who did or did not achieve rescue donor engraftment for the response for GVHD was evaluated using the χ^2 test. Survival data from patients achieving rescue donor engraftment or not were compared based on the results of log-rank tests. Results were considered significant at $p < 0.05$.

Data were "locked" for analysis on May 31, 2010.

Results

Engraftment of rescue donor grafts

To treat GVHD, patients received peripheral blood stem cells from a second allogeneic donor with a median of 6.40×10^6 (range, $2.30–23.00 \times 10^6$) $CD34^+$ cells/kg, including a median of 3.22×10^8 (range, $1.61–8.10 \times 10^8$) $CD3^+$ cells/kg, without T-cell depletion. As shown in Table 1, 16 patients received 17 rescue transplantations to treat GVHD. Because of a poor performance status at transplantation, two patients (nos. 2 and 3) died early (days 10 and 13, respectively) and could not be evaluated for the effects of rescue transplantation; therefore, data from 15 transplantations were analyzed.

Among the 15 transplantations that could be evaluated, rescue donor grafts engrafted in 11 cases, but not in 4 cases. T-cell engraftment preceded neutrophil engraftment (data not shown). In chimerism analysis, all patients showed 100% first donor chimerism in both T-cell and myeloid cell components before the rescue transplantation. It was difficult to obtain continuous chimerism data between first and second (rescue) donors within 1 week after transplantation because of lymphocytopenia. Changes of T-cell chimerism of patients, in whom the chimeric status could be consecutively measured, are shown in Figure 1. In the four patients rejecting a rescue graft, although transiently increasing up to 35% on day 4, rescue donor-derived T cells, thereafter decreased and became undetectable up to 2 weeks after transplantation. Regarding patients who achieved engraftment, donor T-cell chimerism rapidly or gradually increased after transplantation, and full T-cell chimerism of the rescue donor was achieved in a median of 15 days (range, 7–106 days).

Regarding neutrophil recovery, in 6 of the 15 patients, absolute neutrophil counts did not decrease to $<0.5 \times 10^9$/L, and in the remaining 9 patients, absolute neutrophil counts increased to $>0.5 \times 10^9$/L at a median of 10 days (range, 8–26 days). The platelet counts did not decrease to $<20 \times 10^9$/L in three patients (nos. 5, 10–2, and 12). Among the remaining 12 patients, platelet recovery occurred in 5 patients at a median of 32 days (range, 9–101 days), but not in the remaining 7 patients because of early death or subsequent transplantation.

Graft-versus-GVHD effects

Clinical effects of rescue transplantation are shown in Table 3. For successful graft-versus-GVHD treatment, engraftment of the rescue donor graft was mandatory in our murine model [11], in which immunosuppressive agents were not used. In the present clinical study, in which immunosuppressive agents

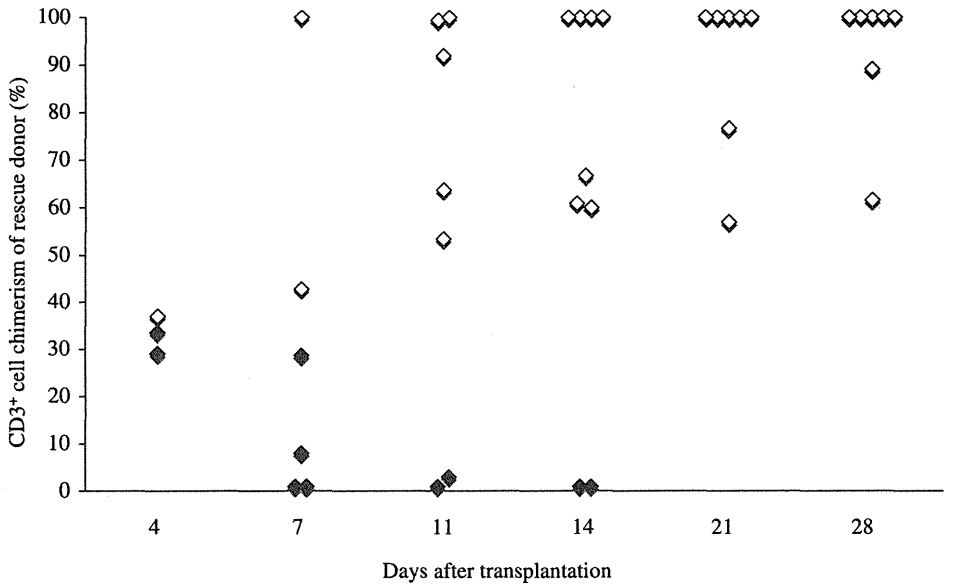

Figure 1. T-cell chimerism between first and second (rescue) donors in patients who did or did not achieve rescue donor engraftment. Open or closed diamonds denote patients who did or did not achieve rescue donor engraftment, respectively.

were naturally used in the transplantation, the response rate for patients achieving rescue donor engraftment or not was 90.9% (eight complete response, two partial response, and one stable disease) and 50% (one complete response, one partial response and one stable disease), respectively. Patients achieving rescue donor engraftment tended to show a higher response than patients not achieving engraftment ($p = 0.080$, χ^2 test). For the response of each organ, patients achieving rescue donor engraftment showed a significantly higher response with cutaneous GVHD than patients not achieving engraftment ($p = 0.016$), but there was no significant difference in response for intestinal and hepatic GVHDs between patients who did and did not achieve rescue donor engraftment. Regardless of achieving engraftment of the rescue donor graft, most GVHD symptoms began to improve during the conditioning treatment, and continued to improve by 1 week after transplantation. Thereafter, in patients who achieved rescue donor engraftment, the majority of GVHD symptoms continued to improve and disappeared within 40 days after transplantation, whereas in patients not achieving engraftment, some GVHD

Table 3. Change of the severity of GVHD

		stage			
No.	engraftment	skin	gut	liver†	grade
1	yes	3 → 0 (19)*	1 → 0 (0)	0 → 0	II → 0 (19)
4	yes	3 → 0 (6)	4 → 2 (12)	0 → 0	III → III
5	yes	3 → 0 (19)	0 → 0	0 → 0	II → 0 (19)
6	yes	3 → 0 (21)	0 → 0	0 → 0	II → 0 (21)
8	yes	0 → 0	3 → 1 (9)	3 → 0 (11)	III → II (11)
9	yes	3 → 0 (4)	0 → 0	0 → 0	II → 0 (4)
10-2	yes	3 → 0 (7)	2 → 0 (15)	0 → 0	III → 0 (15)
13	yes	4 → 0 (38)	2 → 0 (20)	0 → 0	IV → 0 (38)
14	yes	2 → 0 (−5)	3 → 0 (30)	1 → 1	III → II (30)
15	yes	3 → 0 (10)	3 → 0 (30)	0 → 0	III → 0 (30)
16	yes	2 → 0 (−6)	2 → 0 (5)	0 → 0	III → 0 (5)
7	no	4 → 1 (2)	0 → 0	0 → 0	IV → I (2)
10-1	no	3 → 1 (5)	4 → 1 (5) → 2 (19)	0 → 0	III → II (5) → III(19)
11	no	3 → 0 (−5)	2 → 0 (−5)	3 → 0†	III → 0†
12	no	2 → 0 (10)	2 → 0 (15)	2 → 3 (4) → 2(13)	III → III

*Numbers in parentheses denote the day after rescue transplantation when the stage or grade of GVHD was changed.
†Staging of hepatic GVHD was decided based on the serum bilirubin levels. Patient No.11 had an increased bilirubin level and died on day 33, but the main cause of death of the patient was diagnosed from autopsied samples with hepatic veno-occusive disease without no evidence of GVHD.

symptoms disappeared and others became stable or rebounded. Once a complete response was achieved, no rebound of GVHD occurred. In 8 patients who achieved rescue donor engraftment and who had a complete response, the median time for achieving a complete response was 19 days (range, 4–38 days) after transplantation. Among three patients not achieving a complete response despite rescue donor engraftment, one patient (no. 4) showed a complete response for cutaneous GVHD, but had continued diarrhea. The diarrhea was diagnosed to be mainly caused by thrombotic microangiopathy because of partial improvement of the symptom by tapering the immunosuppressants [23]. In another patient (no. 8), the serum bilirubin level was normalized after rescue transplantation and diarrhea had also improved (stage 3→stage 1) by day 23 when the patient died of aspergillus pneumonia. The remaining patient (no. 14) showed a complete response of cutaneous and gut GVHDs, but serum bilirubin levels continued to increase. The aggravation of jaundice was diagnosed to be caused by thrombotic thrombocytopenic purpura based on the presence of severe hemolysis and renal failure. In four patients who rejected rescue donor grafts, one patient (no. 11) showed a complete response of cutaneous and intestinal GVHDs, but showed a progressive increase in serum bilirubin levels and died on day 33. The patient was diagnosed from autopsied liver samples with hepatic veno-occlusive disease with no evidence of GVHD. Patient no. 7 achieved a partial response (stage 4→stage 1) of cutaneous GVHD but died of pneumonia on day 76. Patient no. 12 showed a complete response for cutaneous and intestinal GVHDs, but showed no response of hepatic GVHD, and died of aggravated GVHD on day 46. The remaining patient (no. 10–1) showed a partial response of cutaneous GVHD and also showed partial improvement of intestinal GVHD by day 5, when diarrhea rebounded and was progressively aggravated; therefore, he underwent a second rescue transplantation, after which he achieved rescue donor engraftment and ultimately had a complete response.

Regarding chronic GVHD, only 1 of the 10 patients who survived for >100 days developed limited-type chronic GVHD (skin lesion).

Adverse effects (Table 4)

CMV antigenemia occurred in 11 of 15 transplants (73.3%). The median peak number of CMV antigen-positive leukocytes was 15.4 per 50,000 white blood cells (15.4/50,000), with a range of 2.8/50,000 to 285.7/50,000. No CMV disease was observed.

Three patients developed bacterial infections: one (no. 7) had fatal pneumonia from *Enterococcus cloacae*, and one (no.16) had *Escherichia coli* sepsis, and one (no. 15) had sinusitis, all were successfully treated with administration of antibiotics. Two patients developed aspergillus pneumonia: one patient (no. 13) was successfully treated by antibiotics and another patient (no. 8) with a pulmonary aspergillus lesion before rescue transplantation died of aggravated pneumonia and brain fungal embolism. One patient (no. 14) developed fatal thrombotic thrombocytopenic purpura and one (no. 11) fatal hepatic veno-occlusive disease. One patient (no. 10–1) developed pancreatitis, which was improved by conventional treatment. Ten patients (62.5%) developed liver dysfunction with an increase to more than three times the normal upper limit of the transaminase level. The majority of cases of liver dysfunction were due to steroid- or drug-induced toxicities, and the transaminase level in these patients was normalized after tapering or discontinuation of the causative drugs. Other adverse events are shown in Table 4.

Relapse, cause of death, and overall survival

No patients had recurrence of the original disease. Two patients died early because of a poor performance status at rescue transplantation. Among them, 1 patient (no. 2) had severe GVHD accompanied by sepsis hyperbilirubinemia (10.2 mg/dL), and died of multiorgan failure on day 10. Another (no. 3) developed renal failure after the start of conditioning treatment. Despite receiving hemodialysis, he died of renal failure on day 13.

Overall survival at 6 months and 3 years was 44.6% (95% confidence interval [CI], 19.8–86.8%), and 37.2% (95% CI, 12.4–62.0%), respectively. Patients who achieved rescue donor engraftment showed a significantly improved survival rate compared with those who rejected grafts (log-rank test, $p = 0.013$) (Fig. 2). Six of the eight patients who achieved a complete response survived without any GVHD symptoms or relapse of the original diseases, with a median follow-up of 2128 days (range, 490–3304 days). Two of these patients needed no immunosuppressive agents and the others a small dose of steroids. Two of the patients who achieved a complete response died of cardiac failure on day 831 (no. 6) and of hepatic failure on day 163 (no. 15). Three patients who achieved rescue donor engraftment and who did not achieve a complete response died of multiorgan failure, including thrombotic microangiopathy on day 135 (no. 4), fungal pneumonia on day 23 (no. 8), and thrombotic thrombocytopenic purpura on day 72 (no. 14), as described previously. On the other hand, no long-term survivors were observed in patients who rejected rescue donor grafts. The causes of death for patients who rejected grafts were as described here. Performance status at rescue transplantation was important because no long-term survivors were observed among patients with ≤20% Karnofsky performance score.

Discussion

In the present study, we clearly showed that severe, steroid-refractory GVHD was successfully treated by allogeneic SCT using grafts from a second allogeneic donor. The response rate was 80.0% (90.9% for patients achieving engraftment and 50.0% for patients rejecting graft).

Table 4. Adverse events (%)

Infection	bacteria	bacteremia	1 (5.9)
		others	2 (11.8)
	fungus		2 (11.8)*
	virus	cytomegalovirus	0 (0)
		herpes zoster	2 (11.8)
	pneumocystis jiroveci		0 (0)
Hypoxemia			1 (5.9)
Hemorrhagic cystitis			2 (11.8)
Thrombotic thrombocytopenic purpura			1 (5.9)
Thrombotic microangiopathy			2 (11.8)
Venoocclusive disease			1 (5.9)
Pancreatitis			1 (5.9)
Liver dysfunction†			10 (58.8)
Hypertension			4 (23.5)
Aseptic necrosis			2 (11.8)
Cataract			2 (11.8)
Hyperglycemia‡			8 (47.1)
Nephrotoxicity§			1 (5.9)
Insufficiency of adrenal gland			1 (5.9)

*One patient had aspergillus pneumonia before transplantation.
†An increase to > 3 times the normal upper limit of transaminase.
‡Insulin dose of >30U/day was needed to control blood sugar.
§Nephrotoxicity that needed hemodialysis.

Although patients who were enrolled in the present study had a severe GVHD after HLA-mismatched SCT, which is known to be very difficult to control [26], the overall survival at 6 months and 3 years was 44.6% and 37.2%, respectively. Furthermore, the GVHDs were not only steroid-resistant, but also heavily treated: these patients were refractory to a median of four lines of GVHD-specific treatments (12 patients received tumor necrosis factor blockade, 12 ATG, 11 mycophenolate mofetil, and 9 a pulse therapy of mPSL). The rationale for graft-versus-GVHD treatment is that allogeneically harmful lymphocytes responsible for GVHD are all eliminated by retransplantation using a second allogeneic graft [11]. In the realization of the graft-versus-GVHD concept, there are two major barriers to be overcome: organ toxicity by conditioning treatment and new development of GVHD by a second allogeneic graft.

Regarding the organ toxicities of conditioning treatment, patients with severe GVHD are in a poor state of health due to GVHD-related organ damage, and therefore cannot

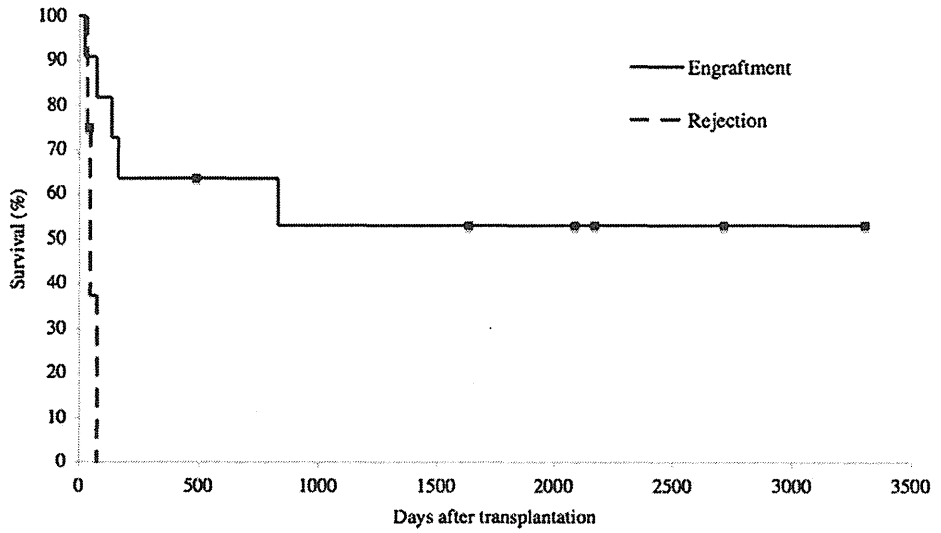

Figure 2. Overall survival of patients with refractory GVHD who did or did not achieve rescue donor engraftment. Patients achieving rescue donor engraftment showed a significantly improved survival rate compared with those rejecting grafts (p = 0.013). The survival rate of patients (n = 11) who achieved rescue donor engraftment was 63.6% (95% CI, 34.6–92.6%) at 6 months and 53.0% (95% CI, 22.0–84.0%) at 3 years, respectively.