**Table 5.** Relationship between first (GVHD-induced) and second (rescue) donors in graft-versus-GVHD treatment

| No. | First donor | Second donor | Relationship of 2 donors (second to first donors) | HLA disparity in the direction of 1st to 2nd donors | GVH-target HLA antigens in 2nd donor* | Engraftment of rescue grafts |
|---|---|---|---|---|---|---|
| 14 | first son | second son | HLA-matched sibling | 0 | – | + |
| 15 | younger sister | youngest sister | HLA-matched sibling | 0 | – | + |
| 17 | brother | sister | HLA-matched sibling | 0 | – | + |
| 1 | mother | sister | Daughter and mother | 2 | – | + |
| 9 | sister | mother | Mother and daughter | 3 | – | + |
| 10 | brother | mother | Mother and son | 3 | – | + |
| 16 | UCB | son | Unrelated | 2 | – | + |
| 5 | first son | second son | HLA-haploidentical sibling | 3 | A24B48DR14 | + |
| 6 | mother | brother | Son and mother | 2 | A26B59 | + |
| 7 | mother | sister | Daughter and mother | 2 | B7DR1 | + |
| 11 | brother | mother | Mother and son | 2 | B52DR15 | +/–† |
| 12 | son | brother | Uncle and nephew | 2 | B54DR4 | – |
| 8 | father | mother | Spouse | 5 | A11B35DR4 | – |
| 13 | father | mother | Spouse | 6 | A33B44DR13 | – |

*HLA determinants of 2nd donors that could be major targets for GVH reaction in first (GVHD-induced) transplantation.
†The patient rejected the first rescue transplantation, but achieved engraftment of the second rescue transplantation.

usually tolerate an intensified conditioning treatment. However, as shown in our murine BMT model, recipients with severe GVHD were in a profoundly immunosuppressive state as a result of GVHD-related activation-induced cell death [27,28] and, therefore, with the help of unmanipulated (T-cell replete) grafts, could easily accept second allografts, even under minimal conditioning treatment, which was advantageous for recipients with serious organ damage.

Regarding GVHD induced by second allogeneic grafts, we demonstrated that second GVHD could be suppressed by conventional GVHD prophylaxis consisting of FK506 and a small dose of mPSL. This was fully expected because, in the unmanipulated HLA-haploidentical reduced-intensity SCT that we recently developed, using a conditioning treatment consisting of fludarabine + busulfan + ATG, and GVHD prophylaxis consisting of FK506 + mPSL 1 mg/kg, the actual incidence of GVHD was only 10% [13]. We consider that, in addition to in vivo T-cell purging by ATG, reduced-intensity conditioning, and a small dose of mPSL effectively suppressed inflammatory cytokine production in the transplantation period, which was shown to be closely involved in the pathophysiology of GVHD [29]. The molecular and cellular mechanisms of the high resistance to GVHD development have not been fully determined: however, in our murine studies, significantly reduced interferon-$\gamma$ levels and a significantly increased percentage of $CD3^+CD4^+foxp3^+$ cells [30,31] were observed in day 7 spleens of second rescue BMT recipients compared with recipients of first BMT with severe GVHD. In addition, antigen-presenting cells (APCs) in the recipient spleen were found to have already been replaced by those of first-donor origin at the time of the second BMT. When APCs are replaced with first donor-derived cells from host cells, the first donor APCs need to cross-present host antigens to second donor T cells to induce GVHD; however, it was reported using major histocompatibility complex–matched, minor antigen-mismatched, murine BMT systems that this cross-presentation was insufficient to induce GVHD [32]. Although the present study includes mostly HLA-mismatched donor/recipient combinations, the limited ability of first donor-derived APCs to cross-present host antigens is considered to reduce the magnitude of the GVH reaction, at least compared with the first transplantation, in which host-type APCs directly present host antigens.

For successful graft-versus-GVHD treatment, engraftment of the rescue donor graft was mandatory in our murine model in which immunosuppressive agents were not used. In the present clinical study, even in patients who rejected the rescue graft, some GVHD symptoms improved within a week after the second transplantation because of conditioning treatment, including immunosuppressive agents and possibly because of the alloreactive response of second donor grafts to dampen first donor lymphocytes. Although these effects may have potential to completely control GVHD coupled with GVHD prophylaxis after second transplantation, as observed in patient no. 11, basically as long as the alloresponse from first donor-derived lymphocytes is maintained, GVHD symptoms continue or are aggravated, as shown in most patients who rejected second grafts. In fact, 8 of the 11 patients achieving rescue donor engraftment had a complete response, and 6 of the 8 patients survived without GVHD symptoms, with a median follow-up of 2128 days. These results strongly suggest, also in humans, that the engraftment of second donor grafts contributes to enduring control of GVHD and longer survival of patients with severe, refractory GVHD. Regarding HLA disparity between the first and second (rescue) donors, when rescue donors did not have HLA determinants that could be major targets for the GVH reaction in transplantation inducing GVHD, no rejection occurred (Table 5). As the extent

of HLA disparity in the direction of the first to second donor became greater, rejection tended to occur more frequently. When the 2 donors were HLA-matched siblings, 100% rescue donor chimerism was gradually achieved over 2 to 3 months.

Furthermore, as suggested in our murine model, the timing of rescue transplantation was another key factor for obtaining a positive graft-versus-GVHD effect. In particular, graft-versus-GVHD treatment in the late stage of GVHD is not effective. When organ damage due to GVHD proceeds fully, although the cell components involved in GVHD are all eliminated, recovery from severe organ damage is difficult, as shown by the lack of long-term survivors among recipients with a low PS score $\leq$20%. Thus, graft-versus-GVHD treatment may be started as one of the treatments for steroid-refractory GVHD before patients are heavily treated.

As patients did not show relapse of the original disease after successful graft-versus-GVHD treatment, and the majority of GVHD patients treated by autologous SCT had a relapse of the original disease [8–10], this strongly suggests GVL effects of second rescue allografts. In autologous transplant settings for GVHD, autografts can reintroduce malignant cells into the recipients in addition to the absence of GVL effects. Furthermore, in rescue transplantation of GVHD by allogeneic grafts, there is a possibility that malignant cells may have been eliminated by allogeneic NK cells as ATG was integrated into the conditioning treatment [33]. Thus, graft-versus-GVHD treatment has a unique feature in that it exerts GVL effects together with treating GVHD, which indicates the achievement of separating GVL from GVHD, a goal in allogeneic SCT.

We have proposed here the novel concept of graft-versus-GVHD and clinically showed that, using reduced-intensity conditioning and T-cell–replete grafts mostly from an HLA-mismatched donor, the second allogeneic SCT succeeded in eliminating harmful lymphocytes responsible for GVHD without the new development of GVHD. Thus, this graft-versus-GVHD strategy may be a promising treatment for refractory GVHD, although our results will have to be confirmed in a large-scale study.

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## Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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# Intrabone Marrow Transplantation of Unwashed Cord Blood Using Reduced-Intensity Conditioning Treatment: A Phase I Study

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The outcome of cord blood transplantation following reduced-intensity conditioning is suboptimal because of fatal infection triggered by prolonged neutropenia and graft-versus-host disease (GVHD) in addition to graft rejection. Intrabone marrow injection (IBMI) may improve the outcome by providing better hematopoietic engraftment and less GVHD. We therefore evaluated IBMI safety in reduced-intensity stem cell transplantation. Furthermore, we used unwashed cord blood to avoid stem cell loss. Ten patients (median age = 61 years old) were enrolled. Cord blood cells were thawed at the bedside and injected into 4 iliac bone sites (2 at each hemipelvis). The procedure was well tolerated with no injection-related complications. Nine patients achieved donor engraftment. The median time to neutrophil recovery ($>0.5 \times 10^9$/L) was 17 days, and platelet recovery was achieved in 8 patients. Early full donor chimerism was achieved (median of 15 and 20 days in T cells and myeloid cells, respectively). Three of 9 evaluable patients developed grade II to III GVHD, and 5 of 10 patients died of treatment-related toxicities. The probability of survival at 1 year was 46.7%. IBMI of unwashed cord blood following reduced-intensity conditioning is safe, well tolerated, and may lead to an increased donor engraftment rate.

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**KEY WORDS:** Cord blood transplantation, Reduced-intensity conditioning, Intrabone marrow injection, Unwashed cord blood

## INTRODUCTION

In recent years, umbilical cord blood transplantation (CBT) has been increasingly used for adult patients with hematologic malignancies. The use of reduced-intensity conditioning (RIC) markedly broadened the indication of CBT to elderly patients lacking an HLA-matched sibling donor; however, although the outcome of CBT for younger patients who have received myeloablative conditioning is reportedly similar to that of patients who have undergone unrelated bone marrow transplantation [1-5], the outcome of RIC CBT (RI-CBT) for elderly patients remains suboptimal [6-9]. In particular, the major drawbacks of CBT are the high incidence of graft failure and delayed hematopoietic recovery, both of which are mainly attributable to the small number of progenitors infused.

Studies using animal models have suggested that intrabone marrow injection (IBMI) of hematopoietic stem cells promotes engraftment [10-12] and reduces the risk of graft-versus-host disease (GVHD) [11]. The improvement of engraftment conferred by this technique is likely attributable to the advantage of the seeding efficiency of progenitor cells, which is superior to that of intervenous (i.v.) infusion, where only a small proportion of progenitor cells engraft bone marrow (BM) [12-14].

Thus, we planned to perform a clinical study of IBMI of cord blood after RIC treatment. Moreover, most institutes in Japan, including our transplant team, routinely use unwashed cord blood for i.v.

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CBT in an attempt to avoid the loss of progenitor cells and to reduce the risks associated with the cell-washing procedure, such as bacterial contamination, and have not experienced particular problems. Based on this previous experience, we planned to use unwashed cord blood for IBMI. Because the safety of IBMI of unwashed cord blood containing dimethyl sulfoxide (DMSO) as a cryoprotectant has not been established to date, we conducted a phase I study to evaluate the safety of this procedure.

## METHODS

### Patients

The major objective in this study was to evaluate the safety of reduced-intensity intrabone marrow CBT using unwashed cord blood units. It was planned to enroll 10 patients in this phase I study. The subjects were adult patients age 55 to 70 years, or younger patients who were considered ineligible for myeloablative preparative regimens because of comorbidities. Patients were eligible to enroll in this study if they required allogeneic stem cell transplantation and could not be paired with human leukocyte antigen (HLA)-matched or 1 antigen-mismatched related donors, or unrelated donors (genotypically matching or 1 locus-mismatching in HLA-A, B, and DRB1 loci) within a clinically useful period of time. If even 1 patient had life-threatening adverse effects after the infusion of unwashed cord blood, the study would be discontinued. This protocol was approved by the institutional review board of Hyogo College of Medicine and the ethics committee of the Japan Cord Blood Bank Network. All patients signed an approved consent form before participating in the study.

### Cord Blood Grafts

A cord blood search was performed through the Japan Cord Blood Bank Network. All cord blood units were processed (ie, red blood cells and plasma removed) and cryopreserved at each of the 11 cord blood banks in Japan according to the New York Blood Center method. The final volume of each unit was uniformly 25 mL. HLA-A, -B, and -DR antigens were identified by low-molecular and then high-resolution molecular typing using polymerase chain reaction (PCR) sequence-specific primers. A cord blood unit was chosen for patients after considering the HLA disparity between the donor and recipient, and the cell dose. Cord blood units had $\geq$4 of 6 matches regarding HLA-A, -B, and -DR antigens, and had a cell dose of $\geq 2.0 \times 10^7$ nucleated cells per kilogram of the recipient's body weight before freezing.

### IBMI Procedure

Each cord blood unit was thawed in a 37°C water bath at the bedside in a clean room, and was collected into a 50-mL syringe with a Luer lock just before injection. Patients were premedicated with hydrocortisone 100 mg and hydroxyzine 30 minutes before starting the IBMI procedure. After local anesthesia with 1% lidocaine, standard BM aspiration needles (16-gauge) were inserted into the superior–posterior iliac crest of the patient in the prone position. Two needles were inserted into each hemipelvis at a distance of 4 to 5 cm, except for 1 patient (No. 8) who received cord blood at 2 sites on 1 side of the pelvis. The stylet was removed from the needle, a small volume of BM fluid was aspirated to confirm that the needle was inserted into the BM space, and approximately 6 mL of the collected cord blood aliquot corresponding to one-fourth of the total volume was slowly injected into the BM space. Saline (0.5-1 mL) was subsequently injected to push the small number of cord blood cells left in the needle into the BM space. The stylet was reattached to the needle in order to avoid the backflow of cord blood into the needle. This procedure was repeated for the remaining aliquots. The patient who received cord blood on 1 side of the pelvis received a 12-mL aliquot at 1 site. The entire IBMI procedure took 20 to 30 minutes from the start of local anesthesia to the end of injection.

### Preparative Conditioning and GVHD Prophylaxis

The conditioning regimen consisted of fludarabine (40 mg/m$^2$) for 5 days (day –6 to day –2), cyclophosphamide (50 mg/kg) on day –6, and total body irradiation 3 Gy on day –1 [8]. GVHD prophylaxis consisted of cyclosporine A (CsA) and mycophenolate mofetil (MMF): A continuous infusion of CsA was started at a dose of 3 mg/kg on day –3 with target serum concentration of 250 to 450 ng/mL, and MMF was started at a dose of 15 mg/kg/day (divided into 2 or 3 doses) on day –3 for the first 3 patients and at a dose of 30 mg/kg/day for the next 7 patients because 2 of the first 3 patients developed severe GVHD. When patients did not have acute GVHD (aGVHD) by day 30, tapering of CsA and MMF was started: CsA was tapered over months (approximately 5%-10% per week) and MMF was tapered over 2 weeks.

### Supportive Care

Supportive care was performed as previously described [15]. In brief, the enrolled patients were hospitalized in a single room ventilated with a high-efficiency particulate air filtration system. Each patient received fluoroquinolones and azoles (fluconazole, itraconazole, or voriconazole) from the beginning of the preparative regimen until the discontinuation of immunosuppressants. In addition, acyclovir was administered at a dose of 1000 mg/day for 5 weeks after transplantation, and was subsequently continued at

a dose of 200 mg/day until discontinuation of the immunosuppressants. Patients seropositive for cytomegalovirus received prophylactic ganciclovir (5 mg/kg) for 5 days during the preparative treatment. Cytomegalovirus pp65 antigenemia was monitored weekly. In addition, the reactivation of human herpesvirus-6 was monitored biweekly by PCR analysis of the virus DNA, and was also checked when patients developed a preengraftment immune reaction. Granulocyte colony-stimulating factor (300 $\mu g/m^2$) was started from day 1 and was continued until the neutrophil count was $>2.5 \times 10^9$/L for 2 consecutive tests.

### Chimerism Analysis

The chimerism status of the donor/recipient was monitored using the T cell– or granulocyte-enriched cell fraction of the peripheral blood. The methodology used for cell separation and chimerism analysis has been detailed elsewhere [15,16]. 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 short tandem repeats in the recipient and donor.

### Definitions and Statistical Analysis

Engraftment was defined as the first of 3 consecutive days of absolute neutrophil count $>0.5 \times 10^9$/L. Mixed chimerism was defined as between 5% and 95% donor cells, and full donor chimerism was defined as the presence of >95% donor cells [17]. Primary graft failure was defined as the lack of even partial donor engraftment (mixed chimeric status). Secondary graft failure was defined as a decreased percentage of donor cells to <5% after achievement of at least mixed chimerism. Diagnosis of aGVHD and chronic GVHD (cGVHD) was based on standard clinical criteria [18], and was pathologically confirmed if possible. Cumulative incidence was used to estimate the rates of neutrophil engraftment and platelet recovery, treating nonevent deaths as a competing risk. Overall survival (OS) was calculated using the Kaplan-Meier method.

The data were "locked" for analysis on December 31, 2010.

## RESULTS

### Patient and Graft Characteristics

Ten patients were enrolled in this clinical study between June 2008 and June 2010. The patient and graft characteristics are summarized in Table 1. The median age of the patients was 61 years (range: 51-66 years), and their median weight was 57.1 kg (range: 43.0-86.3 kg). The diagnoses included myelodysplastic syndrome or acute myeloid leukemia evolved from myelodysplastic syndrome (n = 4), acute myeloid leukemia (n = 2), acute lymphoblastic leukemia (n = 2), and non-Hodgkin lymphoma (n = 2). At the time of transplantation, 5 patients were in a nonremission status, and the remaining 5 were hematologically in complete remission but 4 had minimal residual disease detected by real-time PCR analysis using the *WT1* gene [19] or chimeric genes. Patients received cord blood cells containing a median of $2.72 \times 10^7$ (range: $2.04\text{-}3.50 \times 10^7$/kg) nucleated cells/kg and a median of $0.60 \times 10^5$ (range: $0.30\text{-}0.99 \times 10^5$/kg) $CD34^+$ cells/kg.

### Safety and Tolerability of IBMI Procedure

No particular adverse events were observed in the IBMI of cord blood. Injection of cord blood into the BM space could be performed without resistance. During injection, the patients did not experience pain except for some patients who experienced mild pain during the injection of saline to push cord blood cells in the needle into the BM space. Moreover, no abnormalities were observed in general clinical examinations, including blood pressure, heart rate, and oxygen saturation.

For the first 7 patients who received 6-mL injections of cord blood at each IBMI site, a distinct odor derived from DMSO was imperceptible by the staff in the individual clean rooms during and after the injection of cord blood. In contrast, for the eighth patient who received 12-mL injections of cord blood at each IBMI site, the DMSO-derived odor was clearly perceptible in the room. These observations suggest that a considerable proportion of cord blood cells injected into the BM came out into the circulation soon after the 12-mL injection at each BM site; therefore, we used 6-mL injections for the remaining 2 patients, and noted that the DMSO-derived odor was again imperceptible in their rooms.

### Engraftment and Chimerism

One patient (No. 9) showed no signs of neutrophil recovery, with 100% recipient chimerism on day 24, and was diagnosed with primary graft failure. The remaining 9 patients achieved engraftment: the median time to reach a neutrophil count of $\geq 0.5 \times 10^9$/L was day 17 (range: 7-33 days), as shown in Figure 1A. Platelet recovery was achieved in 8 patients, and the median time to reach a nontransfused platelet count of $\geq 20 \times 10^9$/L and $\geq 50 \times 10^9$/L was 41 days (range: 12-55 days) and 43 days (range: 36-103 days), respectively (Table 1, Figure 1A). In the majority of patients achieving donor engraftment, donor T cell recovery preceded donor neutrophil recovery (Figure 1B). Neutrophils increasing in the early period of transplantation were mostly of recipient origin, which

**Table 1. Patient Characteristics and Transplant Outcomes**

| | | | | | HLA Disparity | | Cord Blood Unit | | Time to recovery (days) | | | Acute GVHD | | | | Chronic | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient No. | Age/ Sex | Weight (kg) | Diagnosis | Disease Status | GVH Vector | HVG Vector | NCC ($\times 10^7$/kg) | CD34 ($\times 10^5$/kg) | Neutrophil $\geq 0.5 \times 10^9$/L | Platelet $\geq 20 \times 10^9$/L | Platelet $\geq 50 \times 10^9$/L | Skin | Gut | Liver | Grade | GVHD | Relapse | Current Status (Day) | Cause of Death |
| 1 | 64/M | 48.4 | MDS-AML | Untreated | 4/6 | 4/6 | 3.05 | 0.88 | 7 | 12 | 39 | 2 | 1 | 0 | II | Ex | — | dead (214)† | MRSA sepsis |
| 2 | 58/F | 43.0 | MDS-AML | CR5, MRD+ | 4/6 | 4/6 | 3.50 | 0.69 | 9 | — | — | 3 | 3 | 0 | III | NA | — | dead (81) | TMA |
| 3 | 60/M | 56.5 | ALL, Ph+ | CR1, MRD+ | 5/6 | 6/6 | 3.27 | 0.99 | 9 | 42 | 45 | 2 | 0 | 0 | I | — | — | alive (>809) | — |
| 4 | 66/F | 47.1 | AML, M4 | Refractory | 5/6 | 5/6 | 2.05 | 0.45 | 24 | 55 | 103 | 2 | 0 | 0 | I | — | — | alive (>505) | — |
| 5 | 51/M | 62.4 | MDS | Refractory | 5/6 | 5/6 | 2.58 | 0.45 | 17 | 35 | 40 | 3 | 1 | 0 | II | — | — | dead (111) | CMV pneumonia |
| 6 | 62/M | 66.0 | ALL | CR1 | 5/6 | 5/6 | 2.71 | 0.30 | 33 | 49 | 55 | 2 | 0 | 0 | I | — | — | dead (291) | *Aspergillus* pneumonia |
| 7 | 57/M | 86.3 | NHL (FL) | RR-Auto | 4/6 | 4/6 | 2.04 | 0.63 | 17 | 40 | 40 | 2 | 0 | 0 | I | — | — | alive (>330) | — |
| 8 | 63/M | 70.0 | AML, M4 | CR2, MRD+ | 4/6 | 4/6 | 2.24 | 0.56 | 21 | 36 | 36 | 1 | 0 | 0 | I | — | + (166)* | alive (>239) | — |
| 9 | 61/M | 57.3 | MDS-AML | CR1, MRD+ | 4/6 | 4/6 | 3.00 | 0.70 | — | — | — | NA | NA | NA | NA | — | — | dead (143) | GVHD after second SCT |
| 10 | 57/M | 56.8 | NHL (PTCL) | Refractory | 5/6 | 5/6 | 2.72 | 0.57 | 17 | 47 | 47 | 3 | 0 | 0 | II | — | — | alive (>141) | — |

M indicates male; F, female; AML, acute myeloid leukemia; MDS-AML, AML evolved from myelodysplastic syndrome; ALL, acute lymphoid leukemia; Ph, Philadelphia chromosome; M4, myelomonocytic leukemia; NHL, non-Hodgkin lymphoma; FL, follicular lymphoma; PTCL, peripheral T cell lymphoma; MRD, minimal residual disease; CR, complete remission; RR-Auto, refractory relapse after autologous stem cell transplantation; GVH, graft-versus-host; HVG, host-versus-graft; NCC, nucleated cell dose; NA, not applicable; Ex, extensive type; MRSA, methicillin-resistant *Staphylococus aureus*; TMA, thrombotic microangiopathy.
Figures in the columns of skin, gut, and liver of acute GVHD indicate the stage of GVHD in each organ.
As GVHD prophylaxis, patient Nos. 1, 2, and 3 received CsA and MMF 15 mg/kg, and the next 7 patients received CsA and MMF 30 mg/kg.
*The number in parentheses indicates the day when the patient had a relapse.
†The number in parentheses indicates survival days after transplantation.

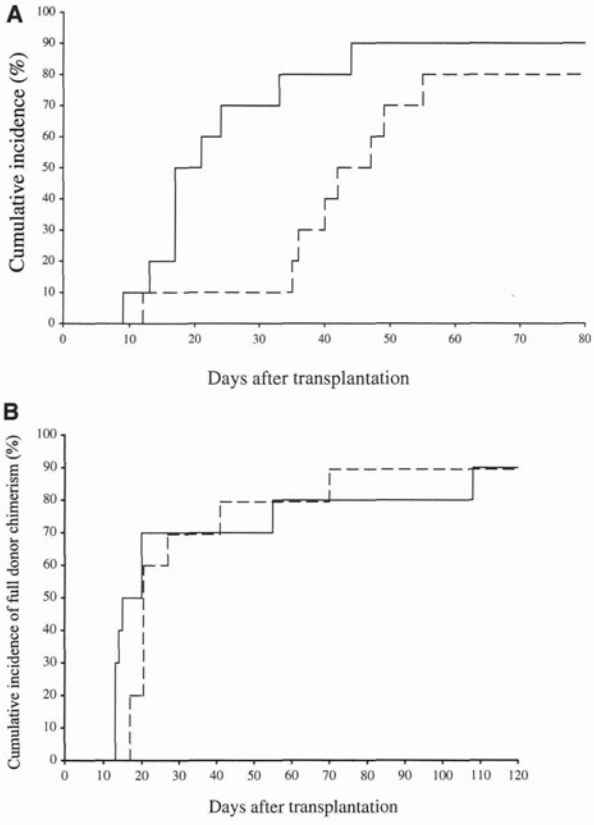


**Figure 1.** (A) Hematopietic recovery. Solid and broken lines indicate cumulative incidence of neutrophil recovery $\geq 0.5 \times 10^9$/L and unsupported platelet recovery $\geq 20 \times 10^9$/L, respectively. (B) Cumulative incidence of achieving full donor chimerism in T (solid line) and myeloid (broken line) cells of the peripheral blood, respectively.

may have contributed to the prevention of infection to some extent: recipient neutrophil counts peaked 0.15 to $1.30 \times 10^9$/L between days 7 and 11, then decreased, and recipient neutrophils were rapidly displaced with donor neutrophils. It took a median of 15 days (range: 13-108 days) to achieve full donor T cell chimerism and a median of 20 days (range: 17-70 days) to achieve full donor myeloid chimerism.

### Outcomes

Patient outcomes are also summarized in Table 1. The majority of patients achieving donor engraftment developed preengraftment immune reactions; however, no particular treatments were needed. Two (Nos. 1 and 2) of the 3 patients who received CsA and MMF 15 mg/kg as GVHD prophylaxis developed grade II and III GVHD, respectively, ultimately resulting in fatal complications of methicillin-resistant *Staphylococcus aureus* sepsis or thrombotic microangiopathy. Among the remaining 6 evaluable patients who received CsA and MMF 30 mg/kg, 4 had grade I GVHD and 2 had grade II GVHD. All patients achieving donor engraftment developed skin GVHD, 4 gut GVHD, and none hepatic GVHD. Of note, all of these patients responded to a small dose of prednisolone. No patients developed chronic GVHD except for 1 patient (No. 1).

Overall, 5 of the 10 patients died from treatment-related toxicities: 3 from infection, 1 from thrombotic microangiopathy, and 1 from GVHD after a second transplantation. Although 1 patient (No. 8) relapsed 166 days after transplantation, he achieved complete remission again by receiving salvage chemotherapy. Five of the 9 patients who achieved donor engraftment are alive at a median follow-up of 392 days (range: 203-871 days). The probability of OS at 1 year after transplantation was 46.7%.

## DISCUSSION

In the present study, we demonstrated that IBMI using unwashed cord blood was safe and well tolerated. In addition, the fact that 9 of the 10 recipients achieved donor-type engraftment suggested that this IBMI procedure coupled with a RIC treatment had sufficient ability to obtain donor engraftment. Recently, Frassoni et al. [20,21] reported the outcome of 32 patients treated by IBM-CBT, the majority of whom received a myeloablative conditioning treatment (including 2 patients receiving RIC). Their study also showed that the IBMI procedure was safe, with the observation of a high engraftment rate, early and robust platelet recovery, and a low incidence of aGVHD. More recently, Brunstein et al. [22,23] reported the outcome of 10 patients who underwent double-unit CBT after myeloablative conditioning, in which 1 unit was injected into the BM and the other into the vein. Although Brunstein et al. [22,23] also showed the safety of the IBMI procedure, they could not observe any superiority of IBMI over i.v. infusion.

In the present study, first, we demonstrated that unwashed cord blood was safely used in the IBMI procedure. In many transplant centers, cord blood units are washed before transplantation, according to a method previously reported by Rubinstein et al. [24]. The 2 aforementioned IBMI studies of cord blood also used washed cord blood. Removal of DMSO may reduce the incidence of adverse reactions associated with the infusion of DMSO [25,26]; however, the clinical implications of cell washing are arguable because a much greater amount of DMSO is routinely infused with autologous peripheral blood stem cell products and, more important, cell washing may lead to the loss of cells to be transplanted. Of note, Barker et al. [27] reported a high engraftment rate in CBT using a simplified method of diluting thawed cord blood with albumin-dextran. Our procedure of IBMI using unwashed cord blood is a particularly simple method that could be conveniently completed at the bedside, although the method might

not be applied to cord blood units of much larger volume, such as cord blood units without plasma reduction.

Even though the aim of the present study was not to evaluate the efficacy of IBMI, better donor engraftment may be expected with this transplant procedure because 9 of the 10 patients achieved donor engraftment with early achievement of full donor chimerism; however, because of the small number of patients treated, we have not yet determined whether this IBMI procedure using unwashed cord blood produces early hematopoietic recovery or a high rate of donor engraftment compared with the usual i.v. infusion methods. Several RI-CBT studies using i.v. infusion methods showed that the platelet recovery rate (platelet count $\geq 20 \times 10^9$/L or $\geq 50 \times 10^9$/L) varied from 42% to 80% [6,8,9,28]. In the present study, platelet recovery occurred in 80% of the patients. This favorable platelet recovery after IBMI is consistent with the results reported by Frassoni et al. [20], but not by Brunstein et al. [22,23], in which only 5 of 10 patients achieved platelet recovery. Regarding the volume of cord blood injected at each BM site, we and Frassoni et al. [20] injected a small aliquot (4-6 mL) of cord blood at each site, whereas Brunstein et al. [22,23] injected a large volume of 20 mL into each site. In order to assess the efficacy of IBMI with cord blood accurately, as many cells as possible should be retained in the BM after injection; however, there is no accurate method at present to assess how many cells are retained. Interestingly, as described by Brunstein et al. [22] who used washed cord blood, we also noticed the distinct odor of DMSO in 1 patient who received a 12-mL injection at each site, which strongly suggested that a substantial number of cord blood cells immediately entered the bloodstream with DMSO and that DMSO was noticed in expiratory air from the lungs; however, with other patients who received a 6-mL injection at 1 injection site, the DMSO-derived odor was imperceptible in their rooms. This observation suggests that the volume of cord blood injected at each BM site may be important in IBM-CBT, which may explain the difference in hematopoietic recovery between the studies by Frassoni et al. [20] and Brunstein et al. [22], although a difference in the strength of preparative regimens can affect engraftment, as shown by Barker et al. [6]; however, delivering hematopietic stem cells to the BM site might not be sufficient to explain any observed advantages of intrabone transplantation if the BM and circulatory system are closely connected. In this regard, Feng at al. [29] reported that intrabone injection facilitated BM homing and redistribution to noninjection-side BM cavities through enhanced expression of cell adhesion molecules on donor cells.

Regarding the occurrence of GVHD, 2 of the first 3 patients in our study who received MMF 15 mg/kg/day as GVHD prophylaxis developed severe aGVHD that ultimately led to transplant-related deaths. The dose of MMF 15 mg/kg may have been too low to obtain a clinically effective blood concentration of the agent. The subsequent 6 evaluable patients who received MMF at an increased dose of 30 mg/kg daily, which was the same dosage as used by Frassoni et al. [20,21], still developed preengraftment immune reactions, and 2 had grade II GVHD; however, it is of note that GVHD occurring in these patients was easily controlled with a small dose of steroid, and none developed cGVHD.

In conclusion, the present study demonstrated the safety of IBMI using unwashed cord blood in a transplant setting of RIC. A larger-scale clinical study is required to determine whether IBMI has advantages in hematopoietic recovery or the risk of GVHD. We are planning to proceed to a multicenter, phase II study to evaluate the efficacy of IBM-CBT.

## ACKNOWLEDGMENTS

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CASE REPORT

# CD20-negative Epstein–Barr virus-associated post-transplant lymphoproliferative disease refractory to rituximab in a patient with severe aplastic anemia

**Hideki Muramatsu · Yoshiyuki Takahashi · Yoshie Shimoyama · Sayoko Doisaki · Nobuhiro Nishio · Yoshinori Ito · Asahito Hama · Akira Shimada · Hiroshi Yagasaki · Masafumi Ito · Seiji Kojima**

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**Abstract** Epstein–Barr virus-associated post-transplant lymphoproliferative disease (EBV-PTLD) is a life-threatening complication following allogeneic hematopoietic stem cell transplantation (HSCT). Monitoring of EBV DNA in high-risk patients with subsequent pre-emptive rituximab treatment is highly effective, and can prevent EBV-associated disease following HSCT. Here, we report a 10-year-old girl with aplastic anemia who developed CD20 negative EBV-PTLD after unrelated bone marrow transplantation that was refractory to rituximab treatment. Similar to other types of lymphoma, the absence of CD20 antigen is likely to be characteristic of rituximab-refractory EBV-PTLD.

**Keywords** Epstein–Barr virus · Post-transplant lymphoproliferative disease · CD20 · Rituximab · Aplastic anemia · Unrelated bone marrow transplantation

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## 1 Introduction

Epstein–Barr virus-associated post-transplant lymphoproliferative disease (EBV-PTLD) is a life-threatening complication following allogeneic hematopoietic stem cell transplantation (HSCT) [1]. Monitoring of EBV DNA in high-risk patients with subsequent pre-emptive rituximab treatment is very effective and can prevent EBV reactivation following HSCT. Absent or diminished expression of CD20 is the most important mechanism responsible for the ineffectiveness of rituximab in treating malignant lymphomas [2]. We provided direct evidence from an immunohistochemical analysis that the clone of rituximab-refractory EBV-PTLD in this patient did not express CD20 antigen but express PAX5 as B cell marker. Similar to other types of lymphoma [3], the absence of CD20 antigen is likely to be characteristic of rituximab-refractory EBV-PTLD.

## 2 Case report

A 10-year-old girl developed idiopathic severe aplastic anaemia and was received standard immunosuppressive therapy (IST) with horse anti-thymocyte globulin and cyclosporine at a local hospital. She did not respond to the IST after 6 months and was transferred to our institute to undergo bone marrow transplantation from an HLA-matched unrelated donor through the Japan Marrow Donor Program.

The conditioning regimen consisted of alemtuzumab 0.2 mg/kg/day from days −7 to −3, fludarabine 25 mg/m$^2$/day from days −5 to −2, cyclophosphamide 750 mg/m$^2$/day from days −5 to −2, and 3 Gy of total body irradiation on day −1. The prophylaxis for graft-versus-host disease

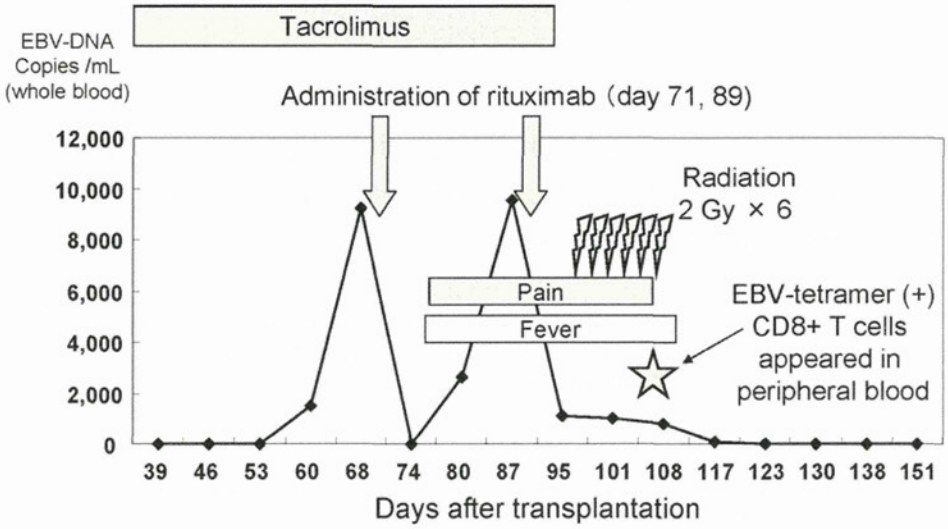


**Fig. 1** Clinical course of EB-PTLD. Copy number of EBV determined by quantitative PCR in peripheral blood (copies/mL of whole blood)

(GVHD) comprised continuous infusion of tacrolimus (0.02 mg/kg/day) from day −1, and short-term methotrexate on day +1 (15 mg/m$^2$/day) as well as on days +3, +6, and +11 (10 mg/m$^2$/day). Granulocyte colony-stimulating factor was administered from day +5. The patient received 20 mg/kg/day of acyclovir and weekly gamma globulin to prevent herpes virus and cytomegalovirus infections, respectively. The unrelated donor was male, and his blood type (A+) was major-minor mismatched with that of the recipient (B+). HLA DNA typing (A, B, Cw, and DRB1) was completely matched between the donor and recipient. On day 0, 2.15 × 10$^8$/kg of nucleated cells (1.48 × 10$^6$/kg of CD34+ cells) were infused. Neutrophil engraftment was achieved on day +15. The patient did not develop acute GVHD.

EBV-DNA copy number was monitored once every week after bone marrow transplantation. Although the patient was relatively asymptomatic, her EBV-DNA copy number was significantly elevated (9,300 copies/mL of whole blood) on day +70 (Fig. 1). She was administered rituximab 375 mg/m$^2$ on day +71, following which the EBV-DNA copy number decreased to the undetectable range by day +74. However, she developed fever along with pain in the right ear on day +78, and her EBV-DNA copy number was again elevated (2,624 copies/mL of whole blood). FDG-positron emission tomography (FDG-PET) (Fig. 2a) and computed tomography (CT) scans identified solid tumors in the right epipharynx and left kidney, which incorporated radioactive FDG highly.

An endoscopy was performed to obtain a biopsy sample from the right epipharynx. Pathological findings after immune staining indicated invasion of abnormal lymphocytes that were CD10−, CD20−, CD5−, CD56−, CD30+, EBER+, and PAX5+ (Fig. 2b). A diagnosis of EBV-PTLD was made on the basis of these results. The EBV-DNA polymerase chain reaction copy number of each lymphocyte subpopulation was checked on day +90.

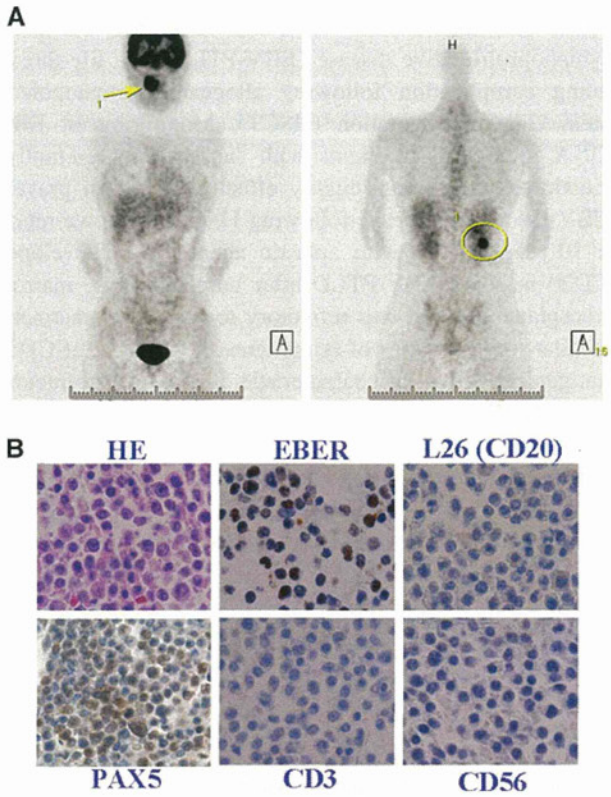


**Fig. 2** FDG-PET scan imaging (**a**) and immune staining (**b**) of EBV-PTLD. **a** The *yellow arrow* and *circle* indicate lesions in the right epipharynx, and left kidney, respectively. **b** HE staining shows infiltration of abnormal lymphocytes. Positive findings on immunostaining of EBER and Pax5 were seen in almost all abnormal lymphocytes. Staining of CD20, CD10, CD3, and CD56 showed negative results

EBV-DNA was detected in CD19 positive lymphocytes in peripheral blood.

The administered dose of tacrolimus was immediately tapered and stopped, and rituximab 375 mg/m$^2$ was administered again on day +89. However, the patient's symptoms did not resolve and her clinical condition deteriorated. She underwent radiation therapy (3 Gy × 4 days) to the left epipharyngeal mass which was causing intractable pain and hence required administration of morphine hydrochloride. Radiation therapy effectively controlled the patient's fever and right-ear pain. EBV-tetramer-positive CD8+ cells were detected in the peripheral blood on day +106. Disappearance of the mass in the epipharynx and right kidney was confirmed on FDG-PET and CT scans on day +152. EBV reactivation has not recurred since then.

## 3 Discussion

Unmanipulated donor lymphocyte infusion (DLI) was first developed as an effective treatment for EBV-PTLD [4]; however, the patients often developed fatal GVHD. As an alternative to DLI, monitoring of EBV-DNA in high-risk patients with subsequent pre-emptive rituximab treatment has been widely used [5–7].

Although various mechanisms are proposed (including an elevated apoptotic threshold, modulation of complement activity, or diminished cellular cytotoxicity), absent or diminished expression of CD20 is the most important one responsible for the ineffectiveness of rituximab in treating malignant lymphomas [2]. Hiraga et al. [3] reported that 5 of 19 relapsed B cell lymphoma patients down-regulated CD20 expression after treatment with rituximab-containing combination chemotherapies. Comoli et al. [8] reported three patients who were diagnosed as having rituximab-refractory EBV-PTLD, coincident with the emergence of CD20-negative/CD19-positive refractory B cells in peripheral blood. We provide direct evidence from an immunohistochemical analysis that the clone of EBV-PTLD rituximab-refractory in this patient did not express CD20 antigen but express Pax5 as a B cell marker. Similar to other types of lymphoma, the absence of CD20 antigen is likely to be characteristic of rituximab-refractory EBV-PTLD.

EBV-specific cytotoxic T lymphocyte (EBV-CTL) infusion and rituximab reportedly complement each other in preemptive therapy of EBV-PTLD [1]. Comoli et al. [8] reported that all three rituximab-refractory EBV-PTLD patients had their disease controlled by infusion of EBV-CTL. Although we were fortunately able to control EBV reactivation in our patient with radiation and the discontinuation of tacrolimus, the availability of EBV-CTL is important for patients at high risk for EBV reactivation.

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# Reduced-intensity conditioning for alternative donor hematopoietic stem cell transplantation in patients with dyskeratosis congenita

Nishio N, Takahashi Y, Ohashi H, Doisaki S, Muramatsu H, Hama A, Shimada A, Yagasaki H, Kojima S. Reduced-intensity conditioning for alternative donor hematopoietic stem cell transplantation in patients with dyskeratosis congenita.
Pediatr Transplantation 2011: 15: 161–166. © 2010 John Wiley & Sons A/S.

Abstract: DC is an inherited bone marrow failure syndrome mainly characterized by nail dystrophy, abnormal skin pigmentation, and oral leukoplakia. Bone marrow failure is the most common cause of death in patients with DC. Because previous results of HSCT with a myeloablative regimen were disappointing, we used a reduced-intensity conditioning regimen for two patients with classic DC, and one patient with cryptic DC who harbored the *TERT* mutation. Graft sources included two mismatched-related bone marrow (BM) donors and one unrelated BM donor. Successful engraftment was achieved with few regimen-related toxicities in all patients. They were alive 10, 66, and 72 months after transplantation, respectively. Long-term follow-up is crucial to determine the late effects of our conditioning regimen.

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Key words: dyskeratosis congenita – non-myeloablative hematopoietic stem cell transplantation

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DC is an inherited multisystem bone marrow failure syndrome characterized by nail dystrophy, abnormal skin pigmentation, oral leukoplakia, and cancer predisposition. Patients with DC have very short germ-line telomeres compared with normal individuals because of a defect of telomere maintenance. Until now, mutations in six genes (*DKC1*, *TERC*, *TERT*, *NOP10*, *NHP2*, and *TINF2*) involved in telomere maintenance have been identified in patients with DC (1).

Abbreviations: ATG, anti-thymocyte globulin; CMV, cytomegalovirus; DC, dyskeratosis congenita; EBV, Epstein–Barr virus; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; IST, immunosuppressive therapy; RIST, reduced-intensity stem cell transplantation; TBI, total body irradiation.

Bone marrow failure develops in 80–90% of patients with DC and is the most common cause of death, up to 60–70% (2, 3). Although androgen has been used to improve cytopenia since the 1960s, allogeneic HSCT is the only curative treatment for bone marrow failure in patients with DC. However, the outcome in previous reports has been disappointing because of unacceptable transplant-related toxicities such as severe pulmonary/liver complications especially in transplant using a myeloablative conditioning regimen or transplants from an alternative donor (3, 4).

To avoid transplant-related complications, RIST using a non-myeloablative conditioning regimen has been recently used in patients with DC, and encouraging short-term survival has been achieved. Reducing the intensity of

conditioning results in less tissue damage and decreased inflammatory cytokine release compared with myeloablative transplantation (5). However, until now, there have been only a few reports of non-myeloablative transplants, especially from an alternative donor. Here, we report our encouraging results of RIST from an alternative donor using a fludarabine-based conditioning regimen and *in vivo* T-cell depletion by ATG in three patients with DC.

## Patients and methods

### Case 1

Patient 1 was a 21-yr-old man with classical DC with nail dystrophy, abnormal skin pigmentation, oral leukoplakia, and bone marrow failure. He had no family history of physical or hematologic abnormalities. Nail changes began to develop in early childhood. He suffered from cytopenia and was diagnosed with aplastic anemia at the age of 9. At age 18, he was referred to our hospital and was diagnosed as having DC. He had very short telomeres – i.e., less than the first percentile for his age – although mutation analysis did not identify any mutations in *DKC1*, *TERC*, *TERT*, *NOP10*, or *TINF2*. As pancytopenia progressed, we planned HSCT from a sister who was mismatched at HLA DRB1 allele. He did not undergo HSCT before. Conditioning regimen included cyclophosphamide 750 mg/m$^2$/day, fludarabine 25 mg/m$^2$/day, and rabbit-ATG (Thymoglobulin; Genzyme, Cambridge, MA, USA) 2.5 mg/kg/day, all from days −5 to −2, and total lymphoid irradiation 3 Gy (1 fraction) on day −1. GVHD prophylaxis comprised tacrolimus (intravenous infusion of 0.02 mg/kg/day starting on day −1, with dose adjustments to maintain blood levels of 5–15 ng/dL) and short-term methotrexate (15 mg/m$^2$ on day +1 and 10 mg/m$^2$ on days +3, +6, and +11). The administration route of tacrolimus was switched to oral after patients recovered from gastrointestinal toxicity.

### Case 2

Patient 2 was a nine-yr-old girl with aplastic anemia without any physical abnormalities. At first, she was diagnosed with acquired aplastic anemia of unknown cause. However, she was identified as having a heterozygous *TERT* mutation (T726M) and very short telomeres in our retrospective study of mutation screening for telomere-related genes. She was born to healthy non-consanguineous parents and had no family history of physical or hematologic abnormalities. Subsequent screening of her family members revealed that her father had the same heterozygous *TERT* mutation (6). She was diagnosed with very severe aplastic anemia at the age of 8. She received IST with horse-ATG (Lymphoglobulin; Genzyme) 15 mg/kg/day intravenously for five days and cyclosporine because she had no HLA-matched family donor. However, the response to IST was poor, and she was still transfusion-dependent for six months after treatment. At first, she underwent HSCT from an HLA DRB1 one-allele-mismatched unrelated donor. The first conditioning regimen included cyclophosphamide 50 mg/kg/day for four days, TBI 5 Gy (two fractions), and rabbit-ATG (Thymoglobulin; Genzyme) 2.5 mg/kg for four days. Patient failed to engraft and had no autologous recovery of her bone marrow. She underwent a second transplant from an HLA B and DRB1 alleles-mismatched mother, 48 days post-transplant as salvage therapy. Conditioning regimen included fludarabine 30 mg/m$^2$/day and ATG 2.5 mg/kg/day from days −5 to −2, and melphalan 60 mg/m$^2$/day on days −2 and −1. GVHD prophylaxis was the same as for case 1.

### Case 3

Patient 3 was an 18-yr-old man with classical DC with nail dystrophy, abnormal skin pigmentation, oral leukoplakia, and bone marrow failure. He had very short telomeres, and mutation analysis showed *DKC1* mutation. Nail changes began in early childhood, and pancytopenia was noted at age 13. Because pancytopenia progressed, we planned HSCT from an HLA 6/6 alleles-matched unrelated donor. He did not undergo HSCT before. Conditioning regimen included cyclophosphamide 750 mg/m$^2$/day, fludarabine 25 mg/m$^2$/day, and rabbit-ATG 2.5 mg/kg/day, all from days −5 to −2, and TBI 3 Gy (one fraction) on day −1. GVHD prophylaxis was the same as for cases 1 and 2.

Table 1 shows patient and disease characteristics. Pre-transplant cardiac, lung, or liver dysfunction was not observed in any patient except for slight elevation of liver transaminase levels in patient 2. Bone marrow examination

Table 1. Patient and disease characteristics

| Patient no. | Sex | Age at diagnosis of DC | Mutation | Clinical triad | Other synptoms | Pre-transplant hematological data | | | Number of pre-transplant transfusions | | Cytogenetics |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | ANC (×10$^9$/L) | Hb (g/dL) | PLT (×10$^9$/L) | RBC | PLT | |
| 1 | Male | 18 | Not detected | Nail, skin, oral | Cerebellar hypoplasia, growth retardation | 0.9 | 5.7 | 16 | 25 | 2 | 46, XY |
| 2 | Female | 9 | *TERT* | None | None | 0.3 | 6 | 0.9 | 40 | 90 | 46, XX |
| 3 | Male | 15 | *DKC1* | Nail, skin, oral | None | 0.84 | 7.7 | 19 | 0 | 2 | 46, XY |

ANC, absolute neutrophil count; Hb, hemoglobin; PLT, platelet; RBC, red blood cell.

Table 2. Pre-transplant characteristics of donors and patients

| Patient no. | Age at transplant | Donor | Donor sex | Donor age | ABO incompatibility | Source | HLA match | Mismatch locus |
|---|---|---|---|---|---|---|---|---|
| 1 | 21 | Sister | Female | 24 | Compatible | BM | 5/6 | DR |
| 2 | 9 | Mother | Female | 36 | Major | BM + PBSC | 4/6 | B, DR |
| 3 | 18 | UD | Female | 37 | Compatible | BM | 6/6 | – |

UD, unrelated donor; BM, bone marrow; PBSC, peripheral blood stem cell.

revealed severe hypocellularity and normal karyotypes in all three patients. Table 2 shows pretransplant characteristics of donors and patients.

### Supportive care

All patients received trimethoprim-sulfamethoxazole orally or inhaled pentamidine as prophylaxis against *Pneumocystis jiroveci*. Patients received standard doses of oral amphotericin B and acyclovir as fungal and viral prophylaxis. Patients received pre-emptive therapy with ganciclovir when CMV antigenemia became positive. Weekly viral studies for CMV, EBV and human herpesvirus 6 were obtained until day 90 post-transplant (7). Granulocyte colony-stimulating factor was started from day 5 to neutrophil engraftment. Acute and chronic GVHD was diagnosed and graded according to established criteria (8, 9).

### Results

Transplant outcomes are shown in Table 3. Engraftment day was defined as the first of three consecutive days in which the patient had an absolute neutrophil count greater than $0.5 \times 10^9$/L. Neutrophil engraftment was achieved in all patients, although the days of platelet recovery were delayed. Analysis of short tandem repeats or fluorescent *in situ* hybridization of sex chromosomes revealed that all patients achieved >95% donor chimerism by day 100.

Engraftment syndrome developed in patient 3 and responded well to steroid therapy (10, 11). Acute GVHD did not occur in any patient, while chronic GVHD of the skin occurred in patient 3 and responded to tacrolimus therapy. Patients 1 and 2 discontinued their treatment with immunosuppressive drugs at 18 and 14 months, respectively, following transplant.

Increases in the EBV genome load were observed in patients 1 and 3. The dose of tacrolimus was decreased in patient 1, and one course of rituximab was administered in patient 3. As a result, EBV genome load decreased in both patients. Positive CMV antigenemia was seen only in patient 3. Preemptive therapy with ganciclovir was administered until the test for CMV antigenemia became negative. He did not progress to CMV disease.

To date, all three patients are alive with a follow-up of 10, 66, and 72 months, respectively. No patients have developed pulmonary or liver complications or malignancies.

### Discussion

In our case reports, we report the outcome of two patients with classical DC and one patient with aplastic anemia harboring the *TERT* mutation to assess the feasibility and efficacy of a fludarabine-based non-myeloablative regimen. Our regimens are promising, as all three patients achieved complete chimerism and hematologic recovery without severe transplant-related toxicities.

Previously, results of HSCT using a myeloablative regimen for patients with DC were disappointing mainly because of pulmonary/liver complications and GVHD (12–19). Until recently, there were no survivors who received unrelated sources of stem cells (3). A high transplant-related mortality rate is considered

Table 3. Outcomes of transplantation

| | Cell dose | | Engraftment | | GVHD | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Patient no. | NCC ($\times 10^8$) | CD34 ($\times 10^6$) | ANC (>500 $\mu$L) | PLT (>20 $\times 10^9$/L) | Acute | Chronic | Complication | Follow-up | Outcome |
| 1 | 3.45 | 1.73 | 16 | 31 | No | No | EBV reactivation | 5 yr 6 months | Alive |
| 2 | 9.6 | 2.6 | 23 | 123 | No | No | DM, enteritis | 6 yr | Alive |
| 3 | 0.81 | N.E | 19 | 111 | No | Skin | Sepsis, engraftment syndrome, CMV antigenemia, EBV reactivation | 10 months | Alive |

NCC, nuclear cell count; ANC, absolute neutrophil count; PLT, platelet; N.E, not evaluated.

to be associated with impaired restorative ability of tissue damage because of defective telomere maintenance. To avoid these complications, reduced-intensity regimens have been recently used and have achieved engraftment with fewer complications in both related and unrelated settings (16, 20–24). Most recently, Dietz et al. reported encouraging results of six patients with DC who underwent HSCT using fludarabine-based non-myeloablative regimens (26). Their non-myeloablative regimen consisted of cyclophosphamide 50 mg/kg for one day, fludarabine 40 mg/m$^2$ for five days, and TBI 2 Gy and alemtuzumab 0.2 mg/kg for five days. Engraftment was achieved in five of six patients. Four patients are alive, three of whom were recipients of unrelated grafts. Our regimen is similar to theirs, including cyclophosphamide, fludarabine, low-dose irradiation, and ATG instead of alemtuzumab. The results of HSCT from an alternative donor for DC are shown in Table 4.

It is still unclear whether HSCT can prolong the overall survival of patients with DC. Dietz et al. combined 18 cases who had undergone RIST in the literature with their six cases and calculated an overall survival rate of 65%, which was similar to another historical cohort that included both myeloablative and non-myeloablative transplants reported by Alter et al. (4). However, the follow-up periods in non-myeloablative transplants seem to be shorter than in myeloablative transplants. Although bone marrow failure is the most common cause of death in patients with DC, pulmonary fibrosis is another common cause of death (27). Alter et al. reviewed 65 patients who had received HSCT until 2008 (4). According to the review, nine of 30 deaths after HSCT were because of pulmonary fibrosis, suggesting that the high rate of this lung complication might originate from the natural history of DC. A prospective long-term follow-up study is necessary to clarify whether HSCT procedures, including conditioning agents and allogeneic immune responses to recipient's organ such as the lungs and liver, affect the natural course of DC.

Fludarabine is a potent immunosuppressive and less myeloablative agent, which has been used successfully in RIST for aplastic anemia (28) and other bone marrow failure syndromes

Table 4. Summary of HSCT from an alternative donor for dyskeratosis congenita

| Patient | Age/sex | Donor source | HLA | Conditioning regimen | Outcome | Complication | References |
|---|---|---|---|---|---|---|---|
| 1 | 23/M | MUD BM | 6/6 | CY 120 mg/kg and TBI 12 Gy | Death | Disseminated candidasis | Langston et al. (16) |
| 2 | 20/M | MUD BM | 6/6 | CY 120 mg/kg and TBI 12 Gy | Death | Disseminated candidasis | Langston et al. (16) |
| 3 | 29/M | MUD BM | 6/6 | CY 200 mg/kg and TBI 6 Gy | Death | Rejection<br>Died of respiratory failure after 2nd BMT | Dokal et al. (17) |
| 4 | 3/M | MUD | 6/6 | CY 120 mg/kg, Flu 180 mg/m$^2$ and ATG 160 mg/kg | Alive >15 months | | Dror et al. (24) |
| 5 | 8/F | MUD | 6/6 | CY 120 mg/kg, Flu 180 mg/m$^2$ and ATG 160 mg/kg | Alive >16 months | EBV reactivation | Dror et al. (24) |
| 6 | 15/M | MUD | 6/6 | CY 120 mg/kg, Flu 180 mg/m$^2$ and ATG 160 mg/kg | Death | Cardio-respiratory arrest on day 0<br>Diffuse capillaritis | Brazzola et al. (25) |
| 7 | 24/M | MMUD dUCB | 4/6<br>4/6 | CY 50 mg/kg, Flu 200 mg/m$^2$, TBI 2 Gy and Alem 1 mg/kg | Death | Sepsis outside of hospital | Dietz et al. (26) |
| 8 | 5/F | MUD BM | 6/6 | CY 50 mg/kg, Flu 200 mg/m$^2$, TBI 2 Gy and Alem 1 mg/kg | Alive >40 months | | Dietz et al. (26) |
| 9 | 2/M | MUD BM | 8/8 | CY 50 mg/kg, Flu 200 mg/m$^2$, TBI 2 Gy and Alem 1 mg/kg | Death | Adenoviral sepsis | Dietz et al. (26) |
| 10 | 18/F | MMUD dUCB | 4/6<br>5/6 | CY 50 mg/kg, Flu 200 mg/m$^2$, TBI 2 Gy and Alem 1 mg/kg | Alive >12 months | Acute GVHD grade IV (gut) | Dietz et al. (26) |
| 11 | 25/M | MMUD dUCB | 4/6<br>5/6 | CY 50 mg/kg, Flu 200 mg/m$^2$, TBI 2 Gy and Alem 1 mg/kg | Alive >12 months | | Dietz et al. (26) |
| 12 | 21/M | MMRD BM | 5/6 | CY 3 g/m$^2$, Flu 100 mg/m$^2$, TLI 3 Gy and ATG 10 mg/kg | Alive >5 yr | EBV reactivation | This report |
| 13 | 9/F | MMRD BM+PBSC | 4/6 | MEL 120 mg/m$^2$, Flu 120 mg/m$^2$ and ATG 10 mg/kg | Alive >6 yr | DM, enteritis | This report |
| 14 | 18/M | MUD | 6/6 | CY 3 g/m$^2$, Flu 100 mg/m$^2$, TBI 3 Gy and ATG 10 mg/kg | Alive >10 months | Sepsis, engraftment syndrome,<br>EBV reactivation | This report |

MUD, matched unrelated donor; MMUD, mismatched unrelated donor; MMRD, mismatched related donor; BM, bone marrow; PBSC, peripheral blood stem cell; dUCB, double unrelated cord blood; CY, cyclophosphamide; Flu, fludarabine; Alem, alemtuzumab; MEL, melphalan; BMT, bone marrow transplantation; DM, diabetes mellitus.

such as Fanconi anemia (29), Shwachman-Diamond syndrome (30), and Diamond-Blackfan anemia (31). In this study, fludarabine seemed to be well tolerated in patients with DC who achieved engraftment even after transplant from an alternative donor.

Reduction in the dose of cyclophosphamide may contribute to a decrease in transplant-related toxicity. We administered cyclophosphamide at a total dose of 3000 mg/m$^2$, which was a tolerable dose for our patients. In several reports, the total dose was reduced to 40–50 mg/kg with durable engraftment. However, one patient who received 50 mg/kg cyclophosphamide and an unrelated double-cord graft (one set of 4/6 and 4/6 HLA match) developed primary graft failure (26). The appropriate dose of cyclophosphamide remains undetermined.

The dose of irradiation is another important issue to achieve engraftment without increasing toxicities. Because patients with DC possess chromosomal instability, they are suspected to show increased radiosensitivity. In fact, a full-dose TBI regimen resulted in unacceptable toxicities in previous reports (16). From our experience as well as other reports, inclusion of low-dose TBI may contribute to achieve durable engraftment without undesirable complications.

Dietz et al. tried to provide a natural pulmonary compensation by delivering irradiation side-to-side, instead of anterior-to-posterior, with the patient in a seated position and the arms resting at the side of the thoracic cage (26). In our institute, patients are in a supine position with the arms at the side of the thoracic cage during TBI, which is delivered side-to-side. Our method also can provide for pulmonary compensation. In addition to the dose of irradiation, the method of irradiation may be important to assess the true effects on lungs in patients with DC.

GVHD prophylaxis is another important issue for successful transplant from an alternative donor. *In vivo* T-cell depletion can reduce the risk of GVHD in HSCT for bone marrow failure syndrome (32). Our conditioning regimen included rabbit-ATG for the purpose of *in vivo* T-cell depletion to prevent severe acute GVHD. Acute GVHD did not occur in any patient in our series, even in the patient who received both bone marrow and peripheral blood from an HLA haploidentical donor. Finke et al. reported the outcome of patients with hematologic malignancies who underwent HSCT from unrelated donors using a regimen containing rabbit-ATG (33). The cumulative incidence of grade II-IV acute GVHD and chronic GVHD for HLA-mismatched transplantation was 20% and 44%, respectively, which were equal to 21% and 43% for HLA-matched transplants. The authors concluded that a single-antigen mismatch might not compromise the outcome after HSCT from an unrelated donor when ATG is used in addition to standard GVHD prophylaxis.

In conclusion, our study indicated that RIST can provide successful engraftment with few complications in patients with DC, even in transplants from an alternative donor. Long-term follow-up is crucial to monitor the late effects of conditioning agents and allogeneic immune responses to the recipient's organs, such as the lungs and liver. Given these encouraging results, we believe that RIST should be explored further.

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**702. Experimental Transplantation - Immune Function, GVHD and Graft-versus-Tumor Effects: Posters I**

# Prevention of Idiopathic Pneumonia Syndrome by Intra-Bone Marrow Injection of Donor Cells

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## Abstract 1902

Several clinical and experimental studies have reported that a recently developed intra-bone marrow (IBM) stem cell transplantation (SCT) technique gives high rates of engraftment and is associated with a low incidence of acute graft-versus-host disease (GVHD). Idiopathic pneumonia syndrome (IPS) is a significant cause of mortality and remains a major obstacle after allogeneic SCT. In the present study, the extent of IPS after IBM-SCT was compared with that after conventional intravenous SCT (IV-SCT) using a lethally irradiated B6(H-2b) into F1 (H-2b/d) mouse IPS model.

Compared with IV-SCT, IBM-SCT significantly improved the clinical GVHD score and reduced total and CD3+ T cell numbers in bronchoalveolar lavage fluid (9.6± 3.5 vs. 21.3 ± 0.5 x$10^4$/ml; $p < 0.05$). Histopathological examination of lung tissue at 6 weeks post-SCT showed significantly reduced IPS pathology in mice that underwent IBM-SCT. To explore the mechanisms of the reduction in IPS pathology in mice that underwent IBM-SCT, we monitored the *in vivo* distributions of infused donor cells and compared them between mice that underwent IBM-SCT versus IV-SCT. Recipient mice were imaged at different time points (1, 2, 3, and 6 h, and 1, 2, 3, and 5 days), using a lethally irradiated luciferase-expressing transgenic FVB/N (FVB/N luc+)(H-2q) into BALB/c (H-2d) mouse model. *In vivo* bioluminescence imaging (BLI) analysis revealed that the majority of injected donor cells were trapped in the lung 1 h after IV-SCT. In contrast, almost all donor cells were localized in the injected limbs 1 h after IBM-SCT, and significantly fewer cells had reached the lung (3.1± 0.7 vs. 16.7± 1.1 x$10^5$ photons/sec/animal, IBM-SCT vs. IV-SCT, $p < 0.01$; Figure). After syngeneic (FVB/N luc+ into FVB/N) SCT, the majority of the injected cells were also trapped in the lung 1 h after IV-SCT, and a similar difference was observed in donor cell distribution in the lung after IV-SCT versus IBM-SCT (2.4± 0.6 vs. 11.6± 1.3 x$10^5$ photons/sec/animal; $p < 0.01$). These results suggest that initial cell localization to the lung is dependent on the SCT method. At 2 days post-SCT, we examined the profiles of chemokines produced locally in the lung (CCL2, CCL3, CXCL1, CCL5, and CCL8). The mRNA expression of CC chemokines, especially CCL2, was more strongly induced in the lung after allogeneic IV-SCT than after allogeneic IBM-SCT (0.098 ± 0.020 vs. 0.020 ± 0.003 units/GAPDH mRNA; $p < 0.05$). A similar difference was observed between mice that underwent syngeneic IV-SCT and syngeneic IBM-SCT, suggesting that increases in chemokine levels in the lung early post-SCT are also dependent on the SCT method. At 5 days post-syngeneic SCT, BLI analysis revealed that no difference was observed in donor cell distribution in the lung after IV-SCT versus IBM-SCT (4.8± 1.1 vs. 4.6± 2.5 x$10^7$ photons/sec/animal; $p = 0.94$). On the other hand, the BLI signals dramatically increased in the lungs of mice that had undergone allogeneic IV-SCT after day 2 post-SCT and there was a significant difference in the BLI signals between IV-SCT and IBM-SCT mice at 5 days post-SCT (50.9± 6.6 vs. 16.0± 6.2x$10^7$ photons/sec/animal; $p < 0.05$). These results suggest that increases in chemokine levels in the lung at day 2 post-SCT lead to increases in the allogeneic response in the lung.