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# Recovery from established graft-vs-host disease achieved by bone marrow transplantation from a third-party allogeneic donor

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***Objective.*** **We investigated whether established graft-vs-host disease (GVHD) could be successfully treated by a second allogeneic bone marrow transplantation (BMT) through elimination of first donor–derived lymphocytes responsible for GVHD.**

***Materials and Methods.*** **In a murine GVHD model of BDF1 (H-$2^{b/d}$)→B6C3F1(H-$2^{b/k}$), GVHD mice underwent a second BMT using a graft ($1 \times 10^7$ bone marrow and $3 \times 10^7$ spleen cells) from a major histocompatibility complex (MHC) antigen haploidentically mismatched (to host and also to first donor) mouse strain, B6B10F1(H-$2^{b/s}$), following low-dose total body irradiation (TBI) 2 to 3 weeks after the first BMT.**

***Results.*** **Results demonstrated that severe GVHD could be successfully and stably treated by a second allogeneic BMT. For successful treatment of GVHD, rapid achievement of full second–donor T-cell chimerism was required. Furthermore, we showed that mice with GVHD could easily accept MHC haploidentically mismatched second-donor hematopoietic cells even after minimal conditioning (2–4 Gy TBI) because they were in a profoundly immunosuppressed state, and that the mice were relatively resistant to new development of GVHD by second-donor grafts. Furthermore, the timing of the second BMT, the intensity of conditioning treatment (GVHD mice are very sensitive), and donor selection were also found to be important for obtaining successful outcomes. Increased regulatory T cells and reduction of interferon-γ levels may be involved in tolerance induction.**

***Conclusions.*** **We demonstrated that established GVHD in a murine GVHD model could be successfully treated by a second BMT from a third-party allogeneic donor.** © **2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Graft-vs-host-disease (GVHD) is a major obstacle to successful allogeneic bone marrow transplantation (BMT), and greatly limits the application and efficacy of allogeneic BMT. GVHD is initiated by donor T cells that recognize differences in major or minor histocompatibility antigens between the recipient and donor. Alloactivated donor T cells attack recipient tissues, especially the skin, liver, and gastrointestinal tract, directly or indirectly, through cellular- and cytokine-mediated effectors, ultimately resulting in the death of recipients [1].

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Autoimmune diseases are caused by self-reactive lymphocytes, which attack various organs, including the muscles, joints, or skin. In murine models, allogeneic BMT was reported to be effective for the prevention [2–4] and treatment [4–7] of autoimmune diseases. In this situation, donor lymphocytes are considered to have the capacity to eliminate all residual self-reactive host lymphocytes through a process known as graft-vs-autoimmunity effects, with analogy to graft-vs-leukemia in leukemia [8]. GVHD resembles autoimmune diseases in that host-reactive lymphocytes induce life-threatening disorders involving various organs, although there is a difference in lymphocyte origin, namely, allogeneic or autologous; therefore, allogeneic (second) BMT may improve established, life-threatening GVHD by eliminating the harmful lymphocytes responsible for GVHD.

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For the successful treatment of established GVHD by allogeneic BMT, the engraftment of second-donor grafts, and the absence of new development of GVHD by second-donor lymphocytes are required; however, mice with ongoing GVHD may have several special features. For example, they might not be able to endure a high dose of total body irradiation (TBI) because of profound organ damage due to GVHD. To overcome this problem, we adopted low-dose TBI as the conditioning treatment for the second BMT. For autoimmune diseases, major histocompatibility complex (MHC)–mismatched murine BMT using a nonmyeloablative regimen has been reported to induce mixed chimerism between the donor and recipient, and to succeed in reversing autoimmunity [9,10]. However, unlike autoimmune diseases, the organ damage caused by GVHD generally progresses rapidly because of the vigorous alloreactive response. Because the GVH reaction must be stopped before organ damage becomes irreversible, the mixed chimerism strategy between first and second donors may be insufficient; therefore, to rapidly eradicate the first-donor lymphocytes responsible for GVHD under nonmyeloablative conditioning, we used a T-cell–replete graft, namely, unmanipulated bone marrow (BM) and spleen cells from the second donor.

In the present study, to investigate whether severe GVHD could be successfully treated by a second BMT, GVHD in MHC haploidentically mismatched BMT of BDF1(H-$2^{b/d}$)→B6C3F1(H-$2^{b/k}$) was treated by a second BMT under various conditions, in which donor selection, TBI dose, and BMT timing varied. The results demonstrated that severe GVHD could be successfully treated by performing a second BMT using a T-cell–replete graft from another MHC haploidentically mismatched donor. Furthermore, we clarified several conditions for successfully treating severe GVHD by allogeneic BMT.

## Materials and methods

*Mice*

Female B6C3F1(B6 × C3H/He; H-$2^{b/k}$), BDF1(B6 × DBA/2; H-$2^{b/d}$), B6B10F1(B6 × B10.s; H-$2^{b/s}$), DBA/1J(H$2^{q/q}$), DBA/2(H-$2^{d/d}$), and C3DF1 (DBA/2 × C3H/He; H-$2^{d/k}$) mice were purchased from Japan SLC (Shizuoka, Japan). Mice used for experiments were between 6 and 11 weeks of age, were housed in sterile microisolator cages in a specific pathogen-free facility, and received autoclaved food and water ad libitum.

*BMT*

BM cells were harvested from the tibia and femur of donor mice by flushing with RPMI-1640 medium. Spleen cells were isolated from donor mice using the nylon-wool purification method as a source of lymphocytes. T-cell purification rate after the nylon-wool purification was 28% to 31%. Because the purification rate was very constant, T-cell normalization was not performed in the following experiments. All BMTs were performed by transfusion of a fixed number of donor cells ($1 \times 10^7$ BM cells and $3 \times 10^7$ spleen cells) after TBI on the previous day. TBI (137Cs source) was given to recipients in a single dose: 8.5 Gy to induce GVHD, and 0 to 5 Gy to treat GVHD. Cells from donors were resuspended in 0.5 mL RPMI-1640 medium and transplanted by tail-vein infusion into recipients.

Survival was monitored daily and GVHD clinical score was assessed weekly using a scoring system incorporating five clinical parameters: body weight, posture (hunching), mobility, fur texture, and skin integrity, as described by Cook et al. [11]. All animal protocols were approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine.

*Flow cytometric analysis*

Fluorescein isothiocyanate-, phycoerythrin-, peridinin chlorophyll protein-, and allophycocyanin-conjugated monoclonal antibodies against mouse antigens (H-$2^d$, H-$2^b$, H-$2^k$, H-$2^s$, CD3) were purchased from BD Pharmingen (San Diego, CA, USA). Cells were first incubated with monoclonal antibody 2.4G2 (specific for Fcγ receptor) for 10 minutes at 4°C to block nonspecific staining. They were then incubated with the relevant fluorescein isothiocyanate-, phycoerythrin-, peridinin chlorophyll protein-, or allophycocyanin-conjugated monoclonal antibodies for 30 minutes at 4°C, and then washed with RPMI-1640 medium containing 1% fetal calf serum and 1 mM ethylenediamine tetraacetic acid. Three- or four-color cytometric analysis was performed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) using Cell Quest software.

T-cell chimerism was analyzed in peripheral blood obtained by eye bleeding. Chimerism analysis of $CD3^+$ cells was performed by three-color flow cytometry. Based on expression of MHC determinants, second-donor cells were discriminated from recipient or first-donor cells.

Regulatory T ($CD4^+foxp3^+$) cells in the spleen were quantitated by flow cytometry as reported previously [12]. Th1 and Th2 cells in spleens were quantitated based on the intracellular expression of cytokines, as reported previously [13]. Cells were treated with phorbol myristate acetate and ionomycin, and were examined for their intracellular interferon (IFN)-γ (Th1) or interleukin-4 (Th2) expression status by flow cytometry.

*Mixed lymphocyte culture*

Spleen cells were isolated and suspended in RPMI-1640 medium supplemented with 10% fetal calf serum. One million responder cells were incubated with $1 \times 10^6$ irradiated (30 Gy) spleen cells (stimulator) in triplicate using 96-well plates at 37°C in 5% $CO_2$ in air for 3 days. Cells were pulsed on the third day with 1 μCi of [$^3$H]-thymidine, and harvested on the fourth day, and thymidine incorporation was measured using a liquid scintillation counter.

*Measurement of cytokine gene expression levels*

Tumor necrosis factor-α (TNF-α) and IFN-γ gene expression levels in spleen cells were measured using quantitative reverse transcription polymerase chain reaction as reported previously.[14] TNF-α and IFN-γ polymerase chain reaction products were normalized in relation to the β-actin internal control.

*Histology*

Formalin-preserved liver, skin, and small and large intestines were embedded in paraffin, cut into 5-μm–thick sections, and stained with hematoxylin and eosin for histologic examination. Slides were coded without reference to prior treatment and evaluated

in a blinded fashion by one individual (Y. Hoshida). A semiquantitative scoring system for GVHD was used as described previously,[15,16] with some modifications: small intestine: villous blunting, crypt regeneration, loss of enterocyte brush border, luminal sloughing of cellular debris, crypt cell apoptosis, outright crypt destruction and lamina propria lymphocytic infiltrate; large intestine: crypt regeneration, colonocyte vacuolization, surface colonocyte attenuation, crypt cell apoptosis, outright crypt destruction, lamina propria lymphocytic infiltrate, and mucosal ulceration; liver: portal tract expansion, mononuclear cell infiltration of the portal tracts, nuclear pleomorphism of the bile ducts, vascular endothelialitis, and hepatocellular changes, including mitotic figures, confluent necrosis, acidophilic bodies, foamy changes, neutrophil accumulation, and macrophage aggregates; skin: intraepithelial apoptosis, exocytosis, liquefaction, dermal lymphocyte infiltration. The scoring system denoted 0 as normal, 0.5 as focal and rare, 1.0 as focal and mild, 2.0 as diffuse and mild, 3.0 as diffuse and moderate, and 4.0 as diffuse and severe. Scores were added to provide a total score for each specimen: the maximum score for small and large intestines was 28 each, for liver 40, and for skin 16.

*Statistical analysis*

Statistical analysis of GVHD clinical scores and histopathological scores was performed with the nonparametric unpaired Mann-Whitney *U*-test. Data from mixed-lymphocyte culture (MLC) analysis were compared with Tukey's HSD test. Estimates of survival probability were calculated by the Kaplan-Meier method. Survival data were compared based on the results of log-rank tests. Cytokine gene expression levels, regulatory T cells, and Th1/Th2 balance between first and second BMT recipients were compared by Student's *t*-test. Results were considered significant when $p$ was $<0.05$.

## Results

*Treatment of GVHD by second BMT from a syngeneic donor*

In a murine GVHD model (BDF1(H-$2^{b/d}$)→B6C3F1(H-$2^{b/k}$)), we first tested whether established GVHD can be alleviated by a second BMT from a mouse syngeneic to the recipient. In this GVHD model, in which recipients received donor BM ($1 \times 10^7$) and spleen ($3 \times 10^7$) cells after receiving a lethal TBI dose (8.5 Gy), recipients began to show clinical features of GVHD 1 week after BMT. Mice began to die from GVHD progression 3 weeks after BMT (Fig. 1A), and only 20% of recipients survived until day 70.

The timing for the second BMT was fixed at day 14 after the first BMT. After receiving TBI of 3 Gy, 4 Gy, or 5 Gy, mice with GVHD underwent a second BMT using BM ($1 \times 10^7$) and spleen ($3 \times 10^7$) cells from B6C3F1 mice (syngeneic to the recipient). The survival of mice that underwent the second BMT using TBI of 3 Gy was not significantly improved compared with control groups that did not undergo a second BMT (Fig. 1A). On the other hand, survival of mice undergoing the second BMT using TBI of 4 Gy was significantly improved compared with control mice; however, all mice that underwent a second BMT using TBI of 5 Gy died by 9 days after the second BMT due to TBI toxicity. The clinical score for GVHD of mice that underwent the second BMT is shown in Fig. 1B. The extent of GVHD in mice that underwent the second BMT using TBI of 3 Gy or 4 Gy was not significantly different from that of the

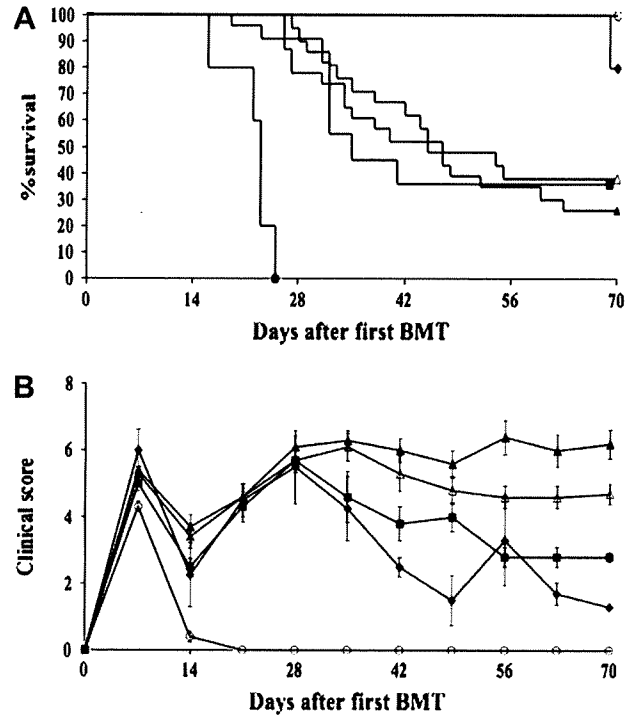


**Figure 1.** Treatment of graft-vs-host disease (GVHD) by second bone marrow transplantation (BMT) using grafts from mice syngeneic to host. B6C3F1 (H-$2^{b/k}$) received bone marrow (BM) ($1 \times 10^7$) and spleen ($3 \times 10^7$) cells from an major histocompatibility complex (MHC) haploidentically mismatched donor, BDF1 (H-$2^{b/d}$) after lethal total body irradiation (TBI) (8.5 Gy). On day 14, recipients underwent a second BMT using BM ($1 \times 10^7$) and spleen ($3 \times 10^7$) cells from mice syngeneic to the host (B6C3F1) after receiving low-dose TBI (3, 4, or 5 Gy). Open circles, B6C3F1→(B6C3F1→B6C3F1) (n = 9); closed triangles, BDF1→B6C3F1 (n = 15); open triangles, BDF1→B6C3F1, in which mice received TBI 3 Gy on day 13 without undergoing a second BMT (n = 21); closed squares, B6C3F1→(BDF1→ B6C3F1) using TBI of 3 Gy (n = 15); closed diamonds, B6C3F1→(BDF1→B6C3F1) using TBI of 4 Gy (n = 8); closed circles, B6C3F1→(BDF1→B6C3F1) using TBI of 5 Gy (n = 10). (**A**) Survival. Data were calculated by the Kaplan-Meier method. The survival of mice that underwent the second BMT using TBI of 3 Gy was not significantly improved compared with GVHD control groups that did not undergo a second BMT with ($p = 0.591$) or without ($p = 0.224$) receiving TBI of 3 Gy. In contrast, the survival of mice undergoing a second BMT using TBI of 4 Gy was significantly better than that of control groups with ($p = 0.048$) or without ($p = 0.004$) receiving TBI. (**B**) GVHD clinical score. Animals were scored for clinical GVHD, as described in Materials and Methods. GVHD scores are shown as the mean ± standard error of the mean. The extent of GVHD in mice that underwent a second BMT using TBI of 3 Gy or 4 Gy was not significantly different from that of the control group by 2 weeks after the second BMT, but thereafter, mice undergoing the second BMT showed GVHD improvement. In particular, remarkable improvement of GVHD was observed in mice undergoing a second BMT using TBI of 4 Gy.

control groups at 2 weeks after the second BMT, but thereafter, mice that underwent the second BMT showed improved GVHD. In particular, remarkable improvement of GVHD was observed in mice undergoing the second BMT using TBI of 4 Gy.

Next, T-cell chimerism in the peripheral blood (PB) of mice that underwent a second BMT was analyzed. In this GVHD model, $CD3^+$ cells in the PB of recipients became composed entirely of BDF1-derived cells by day 14 (of the first BMT) (data not shown). Mice that underwent a second BMT using TBI of 3 Gy showed mixed chimerism between BDF1 and B6C3F1 on day 7 (of the second BMT), and thereafter converted to almost complete chimerism of B6C3F1 on day 14 (experiment 1 in Table 1). On the other hand, mice that underwent the second BMT using 4 Gy achieved almost complete chimerism of B6C3F1 by 7 days after the second BMT (experiment 2 in Table 1). This result suggests that rapid achievement of complete chimerism of B6C3F1 is required to improve the survival of GVHD mice, and that the range of the TBI dose useful for conditioning before the second BMT is narrow, as judged by the improved survival in this system.

*Treatment of GVHD by second BMT from a third-party allogeneic donor*

Next, in this GVHD model (BDF1 ($H\text{-}2^{b/d}$) → B6C3F1 ($H\text{-}2^{b/k}$)), we examined whether GVHD could be treated by a second BMT using a graft from a third mouse strain, B6B10F1 ($H\text{-}2^{b/s}$). In this BMT sequence, the three mouse strains (recipient, first donor, and second donor) shared one MHC haplotype, while the other MHC haplotype was different. Mice with GVHD (BDF1 → B6C3F1) received a graft ($1 \times 10^7$ BM cells and $3 \times 10^7$ spleen) from B6B10F1 on day 14 (of the first BMT) without TBI or following various doses of TBI (2, 3, 4, or 5 Gy). Mice that underwent a second BMT without TBI did not show a significant improvement of the GVHD score by day 28 after the second BMT compared with the control groups that did not undergo a second BMT (Fig. 2B), and the survival rate of these mice was comparable to that of the control groups (Fig. 2A). In contrast, all mice that underwent a second BMT after receiving TBI of 2, 3, or 4 Gy showed an improvement of GVHD symptoms by 7 days after the second BMT, and survived until at least day 70. The survival of these mice was significantly better than that of control mice (Fig. 2A). Some of the mice were observed for a long period (> 150 days), and all survived with or without mild GVHD. All mice receiving TBI of 5 Gy died of severe organ damage (data not shown).

The T-cell chimeric status in the PB was analyzed. Mice undergoing a second BMT without TBI showed mixed chimerism with a low percentage (< 30%) of persistent second-donor components (experiment 4 in Table 1). In contrast, mice undergoing a second BMT using TBI 2 to 4 Gy showed almost complete T-cell chimerism of the second-donor origin by 7 days after the second BMT (experiment 3 in Table 1). These findings indicate that, in the GVHD model of BDF1 → B6C3F1, the second BMT from B6B10F1 ameliorated GVHD and significantly improved the survival of mice by effectively eliminating the first-donor–derived cell components responsible for GVHD, and that the range of the TBI dose (for the second BMT) useful for improving survival was clearly wider in the second BMT using a third-party allogeneic donor than when using a donor syngeneic to recipients. We named the effect by which allografts counteract GVHD "graft-vs-GVHD." To clarify the relationship between the T-cell chimeric status of the first- and second-donors posttransplantation and the graft-vs-GVHD effect, GVHD mice that had received BDF1 → B6C3F1 BMT underwent a second BMT under different conditions, as shown in Table 1 (experiments 5, 6, and 7). The results revealed that the improvement of GVHD occurred only when recipients achieved almost complete T-cell chimerism of the second-donor type, indicating that mixed chimerism was insufficient to improve GVHD symptoms.

**Table 1.** T-cell chimerism between first and second donors after second bone marrow transplantation

| Experiment | BMT sequence | TBI dose for second BMT (Gy) | Second donor components (%)[a] Day 7 | Second donor components (%)[a] Day 14 | Improvement in GVHD score |
|---|---|---|---|---|---|
| 1 | B6C3F1 → (BDF1 → B6C3F1) | 3 | 47.1 ± 20 | 95.7 ± 3.4 | + |
| 2 | | 4 | 99.4 ± 0.8 | 99.1 ± 1.1 | + |
| 3 | B6B10F1 → (BDF1 → B6C3F1) | 3 | 99.3 ± 0.9 | 99.0 ± 1.1 | + |
| 4 | | 0 | 25.9 ± 10 | 18.2 ± 18.5 | − |
| 5 | C3DF1 → (BDF1 → B6C3F1) | 3 | 2.6 ± 2.5 | 1.8 ± 3.6 | − |
| 6 | DBA/2 → (BDF1 → B6C3F1) | 0 | 40.9 ± 7.1 | 25.1 ± 3.1 | − |
| 7 | | 3 | 78.9 ± 8.6 | 96.0 ± 2.5 | + |

TBI = total body irradiation.

BDF1($H\text{-}2^{b/d}$), B6C3F1($H\text{-}2^{b/k}$), B6B10F1($H\text{-}2^{b/s}$), C3DF1($H\text{-}2^{d/k}$), and DBA/2($H\text{-}2^{d/d}$) were used in these experiments.

Flow cytometric analysis of T-cell lineage of peripheral blood (PB) samples was performed on days 7 and 14 of the second bone marrow transplantation (BMT).

[a]PB samples from seven recipients that underwent a second BMT were analyzed. Data are shown as the mean ± standard deviation.

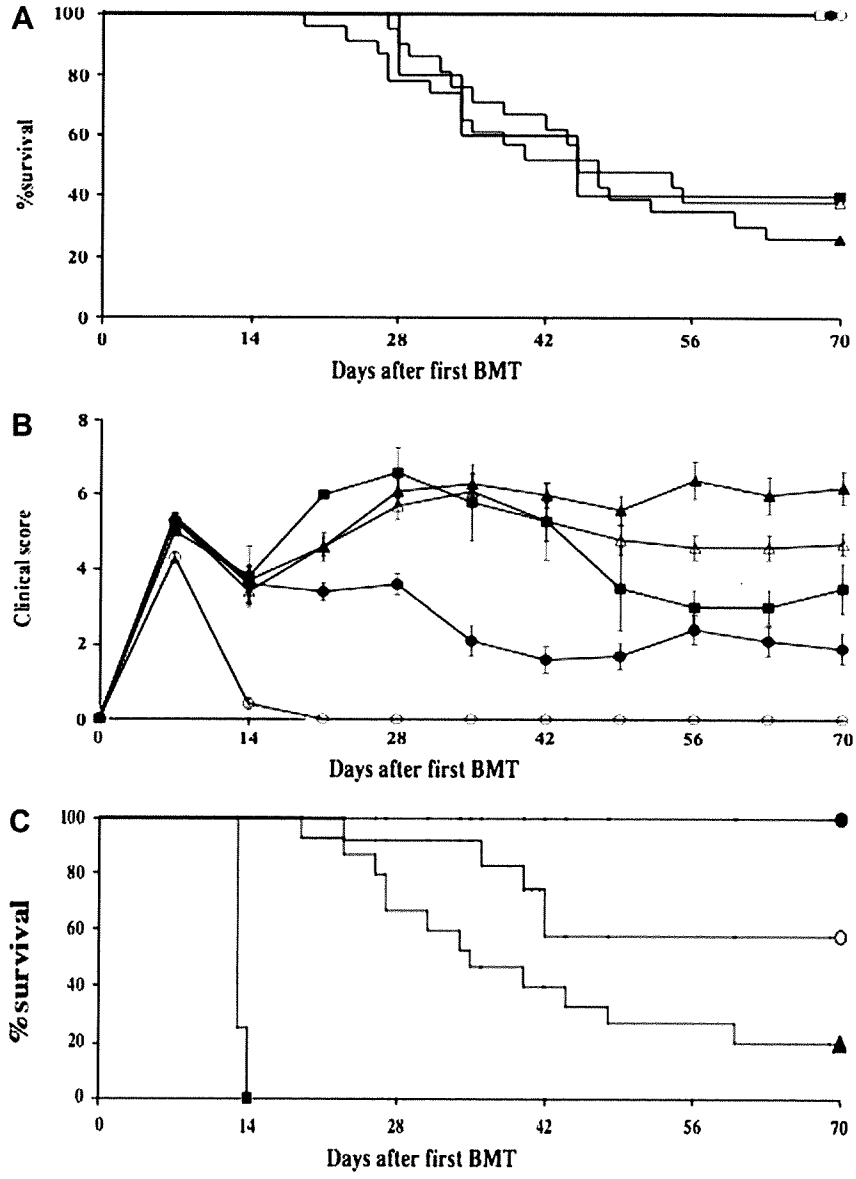


**Figure 2.** Treatment of graft-vs-host disease (GVHD) by second bone marrow transplantation (BMT) using grafts from mice that were major histocompatibility complex (MHC) haploidentically mismatched to the host and first donor. (**A**) Survival of mice receiving a second BMT. Mice that developed GVHD after BMT of BDF1(H-$2^{b/d}$)→B6C3F1 (H-$2^{b/k}$) underwent a second BMT using grafts ($1 \times 10^7$ BM cells and $3 \times 10^7$ spleen cells) from B6B10F1 (H-$2^{b/s}$) without total body irradiation (TBI) or after receiving 2, 3, or 4 Gy of TBI on day 14 after the first BMT. As a control, the survival of mice that did not undergo a second BMT with (open triangles, n = 20) or without (closed triangles, n = 20) receiving TBI of 3 Gy on day 13 of the first BMT was assessed. The survival of mice that underwent a second BMT is shown by the following symbols (closed squares, no TBI [n = 8]; open squares, 2 Gy TBI [n = 8]; closed circles, 3 Gy TBI [n = 8]; open circles, 4 Gy TBI [n = 8]). Mice with GVHD that underwent a second BMT after receiving TBI of 2 Gy, TBI of 3 Gy, or TBI of 4 Gy showed significantly improved survival compared with GVHD control groups with or without TBI ($p < 0.01$, log-rank test). The survival rate of mice that underwent a second BMT without TBI was not significantly different from that of the GVHD control groups with ($p = 0.963$) or without ($p = 0.388$) TBI. (**B**) GVHD clinical score of mice that did or did not undergo a second BMT from B6B10F1 in the GVHD model of BDF1→B6C3F1. Mice not undergoing a second BMT with (open triangles) or without (closed triangles) TBI of 3 Gy; closed squares, second BMT mice without TBI; closed circles, second BMT mice following TBI of 3 Gy; open circles, mice undergoing repeated syngeneic BMT (B6C3F1→[B6C3F1 →B6C3F1]) (n = 9). Scores are shown as the mean ± standard error of mean. Mice that underwent a second BMT with TBI of 3 Gy showed significantly improved GVHD symptoms 7 days and later after the second BMT compared with control mice that did not undergo a second BMT. (**C**) Timing of the second BMT. Mice with GVHD after receiving BDF1→B6C3F1 BMT underwent a second BMT using grafts ($1 \times 10^7$ BM cells and $3 \times 10^7$ spleen cells) from B6B10F1 after receiving a fixed TBI dose (3 Gy) on day 7, 14, or 21 after the first BMT. Closed triangles, no second BMT (n = 15); closed squares, second BMT on day 7 (n = 8); closed circles, second BMT on day 14 (n = 15); open circles, second BMT on day 21 (n = 15). The survival of mice that underwent a second BMT on day 14 ($p = 0.0004$) and on day 21 ($p = 0.031$) was significantly better than that of control mice that did not undergo a second BMT.

We next investigated the influence of the timing of the second BMT on the graft-vs-GVHD effect. To this end, GVHD mice that had received BDF1 → B6C3F1 BMT underwent a second BMT using a graft ($1 \times 10^7$ BM cells and $3 \times 10^7$ spleen) from B6B10F1 after receiving a fixed TBI dose (3 Gy) at various times after the first BMT (Fig. 2C). All mice that underwent a second BMT on day 7 died within 1 week after the second BMT because of severe organ toxicity. On the other hand, all mice that underwent a second BMT on day 14 and 58.3% on day 21 survived at least until day 70 (after the first BMT), with the survivors showing improved GVHD symptoms. The survival of these mice was significantly better than that of control mice that did not undergo a second BMT. Mice undergoing a second BMT on day 21 tended to have a lower survival rate than those undergoing BMT on day 14 ($p = 0.0629$).

Next, to verify that the graft-vs-GVHD effect was observed regardless of donor–recipient pairings, we exchanged the first-donor strain with the second-donor strain. In the GVHD model with BMT of B6B10F1 (H-$2^{b/s}$) → B6C3F1 (H-$2^{b/k}$), in which recipients received $1 \times 10^7$ BM cells and $3 \times 10^7$ spleen cells from the donor after a lethal TBI dose (8.5 Gy), all the mice died of GVHD progression by day 35. Fifty percent of mice that underwent a second BMT on day 14 (after the first BMT) using a graft ($1 \times 10^7$ BM and $3 \times 10^7$ spleen cells) from BDF1 (H-$2^{b/d}$) after receiving 3 Gy TBI survived until day 70, with the survivors showing improvement of GVHD symptoms. Although the extent of GVHD occurring in B6B10F1 → B6C3F1 BMT was found to be slightly greater than that in BDF1 → B6C3F1, a second BMT from BDF1 ameliorated the extent of GVHD, and significantly improved the survival rate compared with that of the control mice that did not undergo a second BMT ($p = 0.002$, log-rank test, Fig. 3A).

*Pathological demonstration of graft-vs-GVHD effects*

The graft-vs-GVHD effect was confirmed histologically. Representative histologic images for the graft-vs-GVHD model of B6B10F1 → (BDF1 → B6C3F1) are shown in Fig. 4A to 4E. The GVHD lesions observed on day 14 in the first BMT (BDF1 → B6C3F1) were almost completely healed on day 50 (36 days after the second BMT) (Fig. 4B and C). In contrast, GVHD-related pathological changes continued in surviving mice in control groups on day 50 (Fig. 4D and E). We evaluated the pathology of GVHD-target organs using a semiquantitative index in which a number of histological parameters were scored. As shown in Fig. 4F, the second BMT group had significantly lower pathological GVHD scores in the skin and liver than control groups. For the large intestine, the second BMT group had a significantly lower score than the control group not receiving a second BMT ($p = 0.007$), and tended to have a lower score than the control group receiving only TBI on day 13 ($p = 0.098$). There was no significant difference in the small intestine among the three groups.

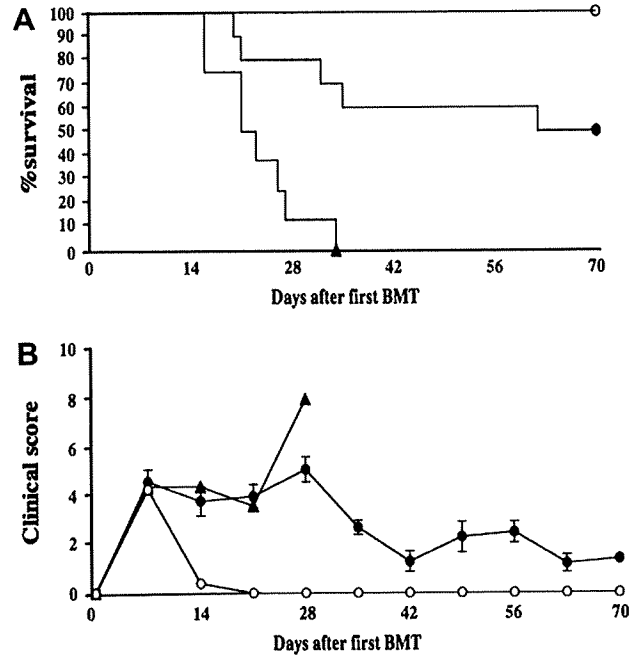


**Figure 3.** Treatment of graft-vs-host disease (GVHD) occurring in bone marrow transplantation (BMT) of B6B10F1 → B6C3F1 by second BMT from BDF1. B6C3F1(H-$2^{b/k}$) received BM ($1 \times 10^7$) and spleen ($3 \times 10^7$) cells from B6B10F1(H-$2^{b/s}$) after lethal total body irradiation (TBI) (8.5 Gy). All recipients (n = 8, closed triangles) died of GVHD by day 35. In this GVHD model, mice with GVHD underwent a second BMT using a graft ($1 \times 10^7$ BM cells and $3 \times 10^7$ spleen cells) from BDF1 (H-$2^{b/d}$) after receiving 3 Gy TBI. GVHD clinical score is shown as the mean ± standard error of mean. Closed circles, BDF1 → (B6B10F1 → B6C3F1) (n = 10); open circles, B6C3F1 → (B6C3F1 → B6C3F1) (n = 10). (**A**) Survival. The survival of mice undergoing a second BMT was significantly better than that of control mice that did not undergo a second BMT ($p = 0.002$). (**B**) GVHD clinical score.

*Analysis of the allogeneic response of recipient spleen cells by in vitro MLC*

To clarify the immunological state of GVHD mice, the allogeneic response of recipient spleen cells just before the second BMT (on day 14 of the first BMT) was analyzed by in vitro MLC. As shown in Fig. 5A, cells from the recipient spleen, in which T cells of recipient origin (B6C3F1) had been completely replaced by those of first-donor origin (BDF1) by day 14 (data not shown), responded much more weakly to all stimulators, including B6C3F1 (host), B6B10F1 (second donor), and DBA/1J (third party), than control spleen cells obtained from BDF1, although their alloreactive response to the host was still detected.

Furthermore, in the model of graft-vs-GVHD of B6B10F1 → (BDF1 → B6C3F1), to verify the low immunological responsiveness to the host after the second BMT, spleen cells were harvested from recipients 2 months after the second BMT, and were analyzed for their response to

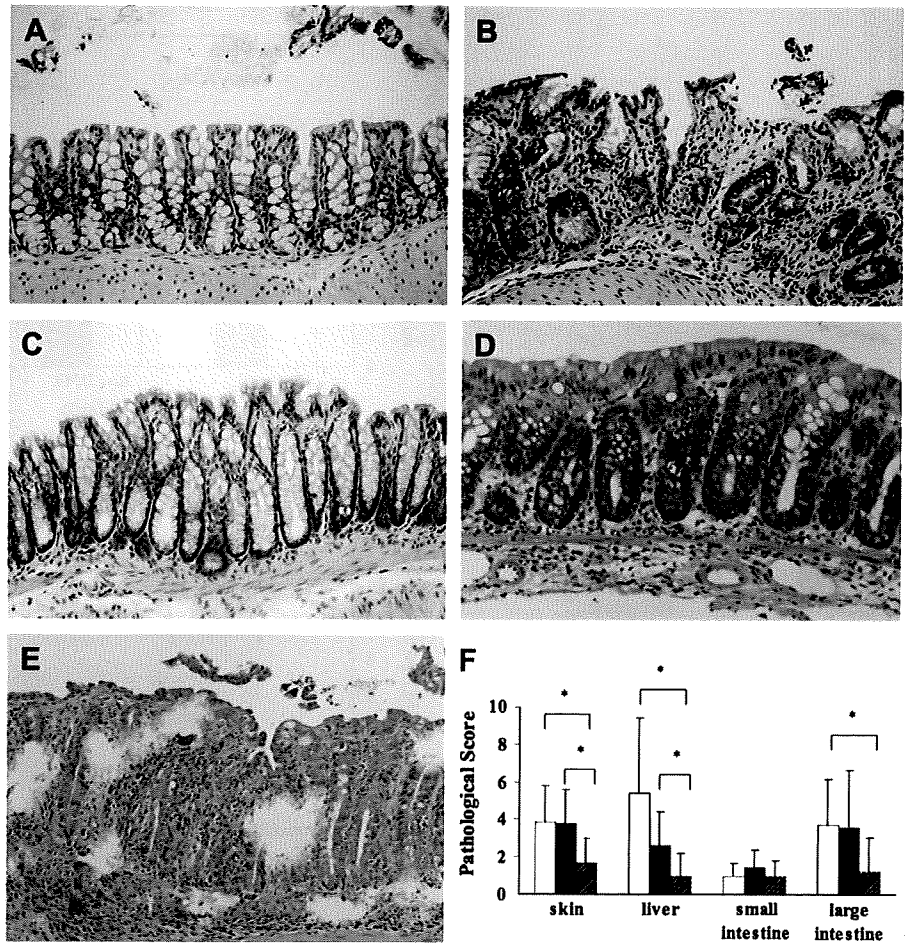


**Figure 4.** Pathological analysis of graft-vs-host disease (GVHD). In the graft-vs-GVHD model of B6B10F1 → (BDF1 → B6C3F1), histological analysis was performed. Representative histologic images of the large intestine are shown. (**A**) On day 14 of syngeneic (B6C3F1 → B6C3F1) bone marrow transplantation (BMT), the image was almost normal. (**B**) On day 14 of allogeneic (BDF1 → B6C3F1) BMT, luminal sloughing, a decrease in the number of glands, apoptosis of the glandular epithelium, and infiltration of small lymphoid cells in the stroma and glandular structures were observed. Arrows indicate apoptosis of the glandular epithelium. (**C**) On day 50 (36 days after the second BMT from B6B10F1), the abnormalities shown in Figure 4B were almost healed. (**D**) GVHD control mouse that did not undergo the second BMT (day 50). (**E**) GVHD control mouse that received total body irradiation (TBI) of 3 Gy only on day 13 but did not undergo the second BMT (day 50). (**F**) GVHD pathological score in GVHD target organs on day 50. The histopathological scoring system used is detailed in Materials and Methods. All data are the mean ± standard deviation. Open bars, GVHD control group (BDF1 → B6C3F1) that did not undergo the second BMT (n = 10); filled bars, GVHD control group (BDF1 → B6C3F1) receiving TBI 3 Gy only on day 13 that did not receive the second BMT (n = 8); hatched bars, second BMT group (B6B10F1 → (BDF1 → B6C3F1) (n = 12). Asterisks indicated statistically significant differences.

BDF1 (first donor) and B6C3F1 (recipient) by in vitro MLC analysis. As shown in Fig. 5B, spleen cells from survivors showed a significantly decreased response to B6C3F1 (host) compared with that to BDF1 (first donor), while the allogeneic response to a third-party antigen (DBA/1J) was relatively well-maintained. These findings are consistent with the conclusion that a second BMT eliminated first-donor lymphocytes without newly inducing GVHD against the host.

*Analysis of cytokine levels, regulatory T cells and Th1/Th2 balance in mice undergoing BMT*

In order to address the mechanisms of tolerance induction after second BMT, we performed relevant experiments using the graft-vs-GVHD system of B6B10F1 → (BDF1 → B6C3F1). TNF-α and IFN-γ gene expression levels in the spleen were compared between first- and second-BMT recipients. As shown in Fig. 6A, IFN-γ levels were significantly repressed in second-BMT recipients compared with first-BMT recipients. There was no significant difference in TNF-α levels between the two groups. Furthermore, to clarify the participation of regulatory T cells in tolerance induction, regulatory T cells were quantitated by analyzing $CD3^+CD4^+foxp3^+$ cells in spleen cells by flow cytometry. As shown in Fig. 6B, a significantly increased percentage of $CD3^+CD4^+foxp3^+$ cells on days 7 and 14 of BMT was observed in second BMT recipients compared with first BMT recipients. On the other hand, there was no significant

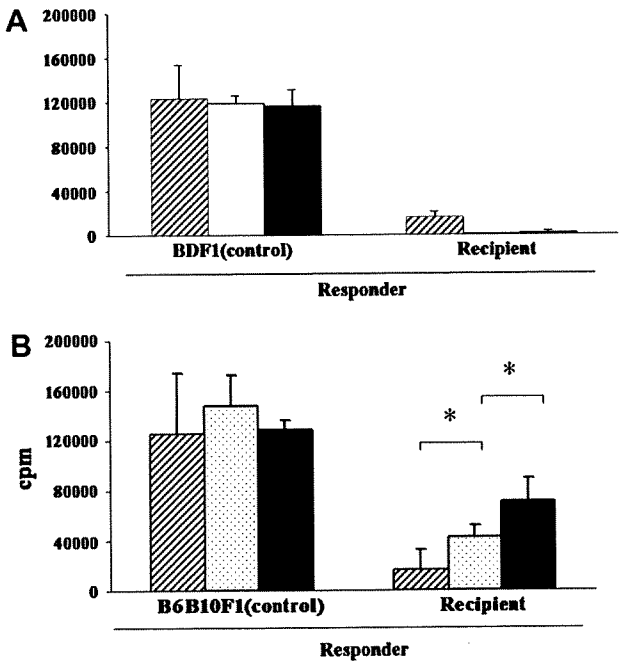


**Figure 5.** Allogeneic response of spleen cells recovered from recipients by in vitro mixed lymphocyte culture (MLC). In the graft-vs-GVHD model of B6B10F1 → (BDF1 → B6C3F1), spleen cells were harvested from hosts immediately before the second bone marrow transplantation (BMT) (on day 14 of the first BMT) (**A**), and 2 months after the second BMT (**B**), and their allogeneic response was analyzed by in vitro mixed-lymphocyte culture (MLC) (see Materials and Methods). Three independent experiments were performed. [$^3$H] Thymidine incorporation in the reaction containing responder cells was subtracted. Values are shown as the mean ± standard deviation. Hatched bars, B6C3F1 (host); stippled bars, BDF1 (first donor); open bars, B6B10F1 (second donor); filled bars, DBA/1J (third party). Asterisks indicate significant differences.

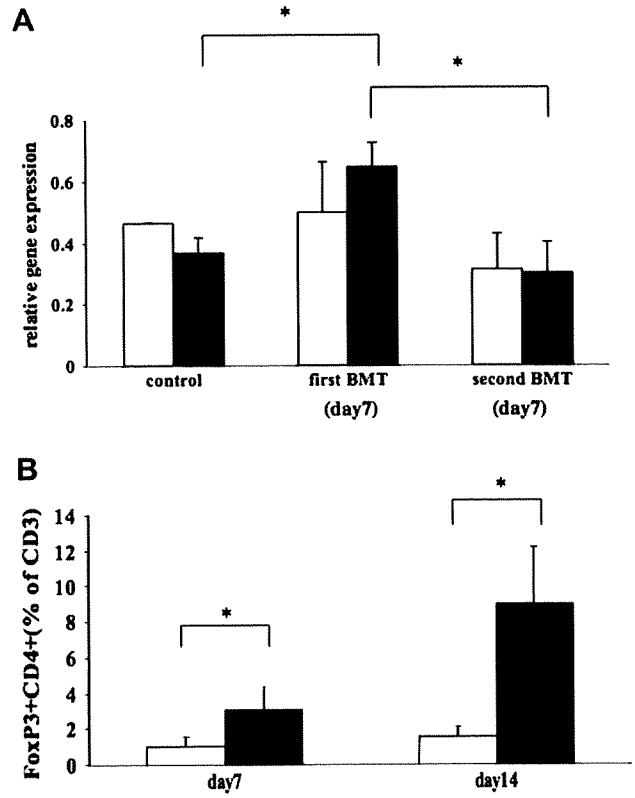


**Figure 6.** Comparison of cytokine gene expression levels or regulatory T-cell counts in the spleen between first and second bone-marrow transplantation recipients. Using the graft-vs-GVHD system of B6B10F1 → (BDF1 → B6C3F1), analyses of cytokine gene expression and regulatory T cell were performed. Values are shown as the mean ± standard deviation. (**A**) Cytokine gene expression levels in spleen cells that were measured by using quantitative reverse transcription polymerase chain reaction. Open bars, tumor necrosis factor-α (TNF-α); closed bars, interferon-γ (IFN-γ). Control, B6C3F1 mice without treatment; cytokine levels were examined 7 days after the first or second BMT. Data present the relative expression of cytokines polymerase chain reaction products to β-actin internal control. (**B**) Regulatory T-cell counts. Regulatory T cells in spleens were measured by flow cytometry on days 7 and 14 of the first or second BMT. Values are expressed as a ratio of $CD4^+Foxp3^+$ cells to CD3 cells. Open bars, first BMT recipients; closed bars, second BMT recipients. Asterisks indicate significant differences.

difference in Th1/Th2 balance between the two groups (data not shown).

## Discussion

In the present study, we addressed the problem of whether a second allogeneic BMT from a third-party donor could eliminate the first-donor–derived harmful lymphocytes responsible for severe GVHD, and whether it could improve survival of recipients by ameliorating GVHD, with analogy to "graft-vs-autoimmunity," namely, the treatment of autoimmune diseases by allogeneic BMT [8]. Results obtained in a mouse BMT model, in which the recipient, first donor, and second donor were each MHC haploidentically mismatched, demonstrated that established GVHD could be successfully treated by a second BMT.

As the graft used to treat GVHD, two grafts, syngeneic and allogeneic (MHC-mismatched) to the host, were first considered. However, syngeneic grafts were found to have problems: the range of TBI dose useful for conditioning before the second BMT was narrow (Fig. 1A) and, in the clinical setting, the majority of patients who underwent autologous stem cell transplantation for life-threatening GVHD were reported to have a relapse of leukemia, although a marked improvement of GVHD symptoms might be observed [17–19]; therefore, we focused on GVHD treatment using allogeneic grafts.

GVHD mice were found to have two unique features that were advantageous for the application of this "graft-vs-GVHD" strategy. First, mice with ongoing severe GVHD were in a profoundly immunodeficient state (Fig. 5A) as a result of GVHD-related activation-induced cell death [20,21]; therefore, GVHD mice easily accepted second donor grafts with minimal conditioning (2–4 Gy TBI), which is an advantage in mice with GVHD, because they are very sensitive to the conditioning treatment. Second, mice with

GVHD were relatively resistant to the new development of GVHD by second-donor grafts even under conditions in which complete T-cell chimerism of second-donor origin was rapidly achieved (Table 1). Consequently, the TBI window in mice with GVHD was clearly widened, as shown in Fig. 2, which should be a key factor for obtaining a stable positive "graft-vs-GVHD" effect. Dendritic cells in the recipient spleen were found to have already been replaced by those of first-donor origin at the time of the second BMT (data not shown). Antigen presentation by first-donor–derived dendritic cells is considered to activate second-donor T cells mainly toward elimination of first-donor components responsible for GVHD rather than host tissues.

In fact, MLC analysis performed 2 months after the second BMT showed establishment of tolerance between the host and second donor, in which the allogeneic response against a third party was, however, maintained (Fig. 5B). Several mechanisms may have been involved in preventing the development of GVHD by second-donor grafts. In addition to GVHD-reducing effects by first-donor lymphocytes that survived after conditioning treatment [22], the allogeneic response of second-donor lymphocytes should have been reduced by a deletion mechanism through activation-induced cell death [20,23]. Conditioning treatment–related cytokine storm is known to enhance the magnitude of the GVH reaction [24]. In the present study, a reduced TBI dose was given to recipients in the second BMT (2–4 Gy) compared with in first BMT (8.5 Gy). In fact, regarding inflammatory cytokine levels after BMT, significantly reduced IFN-$\gamma$ levels were observed in the second BMT compared with the first BMT, although there was no significant difference in TNF-$\alpha$ levels (Fig. 6A). Furthermore, the magnitude of the GVH response may have been further reduced by second-donor–derived regulatory T cells, which can prevent the occurrence of clinical GVHD when alloreactive T cells do not have a high burden [25,26]. In fact, a significantly increased percentage of $CD3^+CD4^+foxp3^+$ cells [27,28] was observed in second BMT recipients, compared with first BMT recipients (Fig. 6B). Thus, decreased cytokine production and increased regulatory T cells may have contributed to the induction of immunological tolerance in this graft-vs-GVHD. On the other hand, there was no significant difference in Th1/Th2 balance between first and second BMT recipients.

For the successful treatment of severe GVHD by second BMT, rapid achievement of full T-cell chimerism by second-donor components was found to be required, in contrast to treatment of autoimmune diseases, in which mixed chimerism may be beneficial as well as safer [8]. This difference is due to the rapidity and vigor of the GVH reaction, and the resultant need to eliminate the causative lymphocytes before they induce irreversible organ changes. In parental to F1 hybrid transplants, rejection by natural killer (NK) cells but not T cells (hybrid resistance) was reported by Bennett et al [29]. Although we could not address this problem, we consider that the rejection occurring in experiments 5 and 6 (Table 1) may have been induced by first-donor–derived T cells as well as NK cells. Particularly in experiment 6, which was a parental to F1 hybrid transplant ($H\text{-}2^d$ to $H\text{-}2^{b/d}$) in the second BMT, first donor–derived NK cells may have partly contributed to the rejection.

Furthermore, at least three elements, namely, BMT timing, the intensity of conditioning treatment, and donor selection were found to be important to achieve successful outcomes. Regarding BMT timing, mice needed to undergo the second BMT after they recovered from organ damage induced by conditioning treatment for the first BMT and before they suffered irreversible organ damage due to GVHD. In our model of BDF1 ($H\text{-}2^{b/d}$) → B6C3F1 ($H\text{-}2^{b/k}$), the optimum timing was 2 to 3 weeks after the first BMT (Fig. 2C). Regarding the intensity of conditioning treatment, mice with GVHD were very sensitive because of severe GVHD-induced organ damage. In our model, they could not tolerate even 5 Gy TBI. Regarding second-donor selection, as indicated by the fact that the alloreactive response of first-donor–derived cells in recipient spleens was detected only in the host cells on day 14 (Fig. 5A), when the second-donor strain had MHC determinants that could be major targets for the GVH reaction in the first BMT, recipients were found to be relatively resistant to engraftment of the second donor, as shown in BMT experiments from syngeneic or allogeneic strains (Fig. 1 and Table 1).

We have presented here the novel concept of "graft-vs-GVHD" and demonstrated that, using appropriately reduced TBI and T-cell–replete grafts from MHC haploidentically mismatched donors, a second allogeneic BMT succeeded in eliminating harmful lymphocytes responsible for GVHD without the new development of GVHD. Mice with GVHD were relatively resistant to the new development of GVHD by second-donor grafts and, therefore, GVHD may be a good target disease for allogeneic BMT. Based on this graft-vs-GVHD concept, we are now testing a protocol for human leukocyte antigen–haploidentical nonmyeloablative stem cell transplantation for the treatment of life-threatening GVHD in a clinical study, and have already accumulated several successful cases (manuscript in preparation).

## Acknowledgments

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# Unrelated Umbilical Cord Blood Transplantation Using a TBI/FLAG Conditioning Regimen for Adults with Hematologic Malignancies

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## ABSTRACT

**A combined chemotherapy regimen comprising fludarabine, cytosine arabinoside, and granulocyte colony-stimulating factor (FLAG) has been used in the treatment of relapsed or refractory leukemias. We here report 38 patients with hematologic malignancies who underwent single-unit cord blood transplantation (CBT) with a conditioning regimen comprising 12-Gy total-body irradiation (TBI) and FLAG therapy (TBI/FLAG). Graft-versus-host disease (GVHD) prophylaxis consisted of tacrolimus or cyclosporin A and/or methotrexate. The median nucleated cell dose was 2.43 × $10^7$/kg (range: 1.96-3.55 × $10^7$/kg). Of 34 evaluable recipients, the cumulative incidence of donor engraftment was 97%. The median time to reach an absolute neutrophil count of 500/μL was 23 days (range: 18-35 days). The median time to an untransfused platelet count of 50,000/μL was 45.5 days (range: 28-208 days). Sixteen patients developed grades II-IV of acute GVHD. Fourteen patients were alive at a median follow-up of 46 months (range: 4-77 months). The estimated event-free survival at 3 years for all patients was 33.5%, with 72.7% in the standard-risk group (n = 11) and 17.7% in the high-risk group (n = 27) ($P$ = .0075). These results showed that this novel regimen was well tolerated by patients and able to establish sustained donor cell engraftment, indicating the feasibility of TBI/FLAG as a conditioning regimen for CBT in adults with hematologic malignancies.**


## KEY WORDS

Cord blood transplantation (CBT) • Adult • Conditioning regimen • FLAG • Total body irradiation (TBI)

## INTRODUCTION

Umbilical cord blood transplantation (CBT) has increasingly been performed as an alternative to human leukocyte antigen (HLA)-matched sibling or unrelated bone marrow transplantation (BMT) [1-3]. The advantages of CBT in comparison to BMT include prompt availability of cryopreserved cells, a less stringent requirement for HLA-type matches between donors and recipients, and a low risk of inducing severe graft-versus-host disease (GVHD). The major drawbacks of CBT are slow hematopoietic recovery and a high incidence of graft failure, mainly because of a small number of progenitors being infused, which is more pronounced in adults with greater body weight [4]. Generally, the overall outcome in adult CBT needs to be improved in comparison to that in adult allogeneic BMT [2]. A standard conditioning regimen with cyclophosphamide and total-body irradiation (TBI) produces favorable results for BMT [5], but a standard conditioning regimen for CBT has not yet been firmly established.

Intensive combination chemotherapy has significantly improved the prognosis of patients with hematologic malignancy [6]. FLAG therapy using fludarabine (Flu), cytosine arabinoside (Ara-C), and

granulocyte colony-stimulating factor (G-CSF) has been shown to be effective against a variety of hematologic malignancies, including high-risk acute myeloid leukemias [7,8] and acute lymphoblastic leukemia [9]. The use of FLAG therapy for the treatment of leukemias is based on the following arguments: (1) infusion of fludarabine before Ara-C increases the accumulation of the active metabolite ara-C triphosphate in leukemic cells [10], (2) G-CSF shortens the duration of neutropenia and reduces infection rates in leukemia patients [11], and (3) G-CSF may sensitize leukemic blasts to S-phase-specific Ara-C by recruiting quiescent cells into the cell cycle and increasing Ara-C phosphorylation [12]. Thus, FLAG therapy was pharmacokinetically designed to increase antileukemic metabolites, and was intended to exert an efficient antileukemic effect in the treatment of relapsed or refractory leukemias.

Fludarabine is highly immunosuppressive and shown to be especially effective in a nonmyeloablative preparative regimen for allogeneic stem cell transplantation (SCT) [13]. Pawson et al. [14] used FLAG with or without idarubicin as a reduced-intensity conditioning (RIC) regimen for second allogeneic peripheral blood SCT in the treatment of relapsed leukemia patients. Thus, FLAG therapy may act not only as an effective antileukemic chemotherapy regimen, but also as an efficient preparative regimen for SCT.

In the present study, we developed a new conditioning regimen consisting of FLAG therapy combined with 12-Gy TBI (TBI/FLAG). We performed CBT using this regimen in 38 adult patients with hematologic malignancies in our single institution. Our results demonstrated the feasibility of this TBI/FLAG as a novel myeloablative preparative regimen for CBT.

## PATIENTS AND METHODS

### Eligibility

Patients were eligible if they were in a condition requiring SCT but had no 6/6 or 5/6 allele HLA-matched related donor or 6/6 HLA-matched unrelated donor available, or needed urgent SCT within 3 months. Patients receiving a transplant during the first or second complete remission of leukemia or non-Hodgkin's lymphoma, or those who had refractory anemia of myelodysplastic syndrome (MDS) were placed in the standard-risk group. Patients in their third or subsequent remission, relapse, or partial remission with refractory leukemia and those with chronic myelogenous leukemia (CML) beyond the first chronic phase at the time of CBT were considered to be in the advanced phase of disease and were placed in the high-risk group. Patients with diseases with high-risk cytogenetics, such as acute lymphoblastic leukemia (ALL) with t(9;22) and acute myelogenous leukemia (AML) with −5, del(5q), −7, del(7) or del(11), were also included in the high-risk group [15]. This study was approved by the institutional review board of Hyogo College of Medicine. All patients provided written informed consent.

### CB grafts

Appropriate cord blood (CB) was identified through the Japan Cord Blood Bank Network (JCBBN), which maintains information on the holdings of 11 local CB banks in Japan [16]. In the first 19 patients, CB grafts were selected on the basis of serologic matching at 4-6 of 6 HLA loci (class I HLA-A and -B, and class II HLA-DR alleles) as determined by a standard complement-dependent microlymphocytotoxicity test [17]. In the subsequent 19 patients, high-resolution DNA typing of class II DRB1 alleles was used for selection of class II alleles according to the availability of the high-resolution class II data. CB grafts selected had a cryopreserved cell dose of at least $2 \times 10^7$ nucleated cells (NC) per kilogram of recipient body weight (NC/kg). Confirmatory high-resolution DNA typing of class I HLA-A and -B and class II DRB1 alleles was also performed [18-20]. All CB used were single units and were not depleted of T lymphocytes.

### Preparative Regimen

The TBI/FLAG regimen comprised TBI (12 Gy), Flu (150 mg/m$^2$), Ara-C (10 g/m$^2$), and G-CSF. TBI was administered daily at 3 Gy for 4 days (day −10 to day −7). Flu, Ara-C, and G-CSF were administered daily for 5 days (day −6 to −2). Flu (30 mg/m$^2$) was administered intravenously over 2 hours. Four hours after the completion of Flu infusion, Ara-C (2 g/m$^2$) was administered intravenously over 2 hours. The TBI/FLAG regimen was performed irrespective of prior Ara-C treatment. G-CSF (300 μg/m$^2$) was administered subcutaneously. In the first 24 consecutive patients, G-CSF was administered to all patients, but in the subsequent 14 patients, the G-CSF administration was omitted in patients with lymphoid leukemias and lymphomas (n = 7), because efficacy of G-CSF on lymphoid malignancy is not firmly established.

### GVHD Prophylaxis and Treatment

GVHD prophylaxis was tacrolimus (n = 11) or cyclosporin A (CsA) (n = 1) alone during the years 2000 to 2002. Tacrolimus plus short-term methotrexate (MTX) (n = 9) or CsA plus short-term MTX (n = 17) was used since August 2002. Administration of tacrolimus (0.02 mg/kg/day) or CsA (3 mg/kg/day) in a continuous infusion was started on day −1 and continued until the patient became tolerate to oral administration. Short-term MTX was administered at 10 mg/m$^2$ on day 1 and 7 mg/m$^2$ on days 3 and 6 [21]. After neutrophil engraftment, and in the absence of acute

GVHD (aGVHD), tacrolimus or CyA was tapered 10% per week starting at approximately day 35. Acute GVHD was clinically diagnosed using the criteria of Glucksberg et al. [22]. Grade II to IV aGVHD was treated with methylprednisolone at 1-2 mg/kg/day. Patients who survived for >100 days were analyzed for chronic GVHD (cGVHD).

### Supportive Care

Each patient was isolated in a laminar air-flow room. Ciprofloxacin at 400 mg/day and fluconazole at 300 mg/day were administered from day −14 until neutrophil recovery. G-CSF at 300 μg/m$^2$ was again administered to all patients from day 5 until neutrophil recovery. Acyclovir was administered at 750 mg/day for 5 weeks after transplantation to prevent herpes simplex virus infection. Ganciclovir 10 mg/kg was administered in 2 divided doses from day −10 to day −3 as prophylaxis for cytomegalovirus (CMV) infection. Detection of CMV antigenemia was performed using an immunoperoxidase-conjugated antibody, HRP-C7, which binds to an immediate-early antigen of CMV, pp65 antigen. After grafting, ganciclovir administration was reinstituted in patients demonstrating positive CMV antigenemia.

### Donor Chimerism Analysis

Donor chimerism was analyzed using marrow and/or blood samples. Chimerism was determined by quantitative PCR analysis of informative short tandem repeat regions in the recipients and donors (STR-PCR) [21,23]. DNA was extracted from marrow or blood cells using a SepaGene isolation kit (Sankyo Pure Chemical, Tokyo, Japan), and amplified with fluorescent PCR primers (AmpFlSTR profiler PCR amplification kit; Applied Biosystems, San Jose, CA). The fluorescent PCR products were separated by capillary electrophoresis using a 310 Genetic Analyzer (Applied Biosystems). GeneScan software and GeneMapper software (Applied Biosystems) were used to calculate the percentage of donor and recipient DNA.

### Engraftment

Engraftment was considered to have occurred when whole blood cell counts of absolute neutrophil counts of >500/μL were obtained for 3 consecutive days after transplantation, accompanied by the detection of donor chimerism. Graft failure was considered to have occurred when peripheral and marrow hypoplasia were noted after transplantation, and donor markers could not be detected by using cytogenetic and/or molecular techniques.

### Regimen-Related Toxicity (RRT) and Transplantation-Related Mortality (TRM)

RRT, the nonhematologic toxicities directly caused by a given preparative regimen by day 28, were analyzed using Bearman's criteria [24]. TRM was defined as death without primary disease progression.

### Statistical Analysis

The probability of event-free survival (EFS) was estimated using the Kaplan-Meier method with Mantel-Cox log rank test. In this analysis, graft failure, relapse, disease progression, and death were defined as events. We used Cox proportional hazards models to determine which independent patient-, disease- and transplant-related variables predict EFS. We first fitted univariable models, then all variables with $P <$ .10 were included in a multivariable model. Hazard ratios were estimated with 95% confidence intervals. Categoric variables were compared using the $\chi^2$ test. Values of $P <$ .05 were considered to be significant. All statistical analyses were carried out with StatView version 5.0 software (SAS Institute, Cary, NC).

## RESULTS

### Patient Characteristics

Thirty-eight patients underwent CBT with a TBI/FLAG conditioning regimen between December 2000 and February 2007 at our institution. The median age of the patients was 38.5 years (range: 16-52 years) and the median weight was 58 kg (range: 39-81 kg). Details of patients' characteristics are listed in Table 1. Eleven patients (29%) who were in first or second remission were placed in the standard-risk group. The remaining 27 patients (71%), who were placed in the high-risk group, include 14 in relapse or partial remission with

**Table 1.** *Patient Characteristics*

| | | | |
|---|---|---|---|
| **Number of patients** | | **38** | |
| **Sex (male/female)** | | **20/18** | |
| **Age (year); median (range)** | | **38.5 (16-52)** | |
| **Disease** | | | |
| | **Standard-Risk CR1/CR2 RA, CP** | **High-Risk > CR3/>AP HRC** | **High-Risk Rel/Ref** |
| **AML** | **5** | **4** | **5** |
| **ALL** | **4** | **5** | **2** |
| **MLL** | **2** | **0** | **0** |
| **NHL** | **0** | **1** | **1** |
| **ATL** | **0** | **0** | **2** |
| **MDS** | **0** | **0** | **2** |
| **CML** | **0** | **3** | **0** |
| **CLL** | **0** | **0** | **2** |
| **Total** | **11** | **13** | **14** |

AML indicates acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; MLL, mixed-lineage leukemia; NHL, non-Hodgkin lymphoma; ATL, adult T cell leukemia; MDS, myelodysplastic syndrome; CML, chronic myelogenous leukemia; CLL, chronic lymphocytic leukemia; CR1, CR2, CR3 first, second, and third complete remission; RA, refractory anemia; CP, chronic phase of CML, Rel, relapse; Ref, refactory disease; AP, accelerated phase of CML; HRC, high-risk cytogenetics.

refractory disease, 13 in their third or subsequent remission or with high-risk cytogenetics (Table 1).

### Graft Characteristics

The median number of nucleated cells infused was $2.43 \times 10^7$/kg body weight (range: $1.96\text{-}3.55 \times 10^7$/kg) and that of $CD34^+$ cells was $0.87 \times 10^5$/kg body weight (range: $0.24\text{-}3.98 \times 10^5$/kg) (Table 2). CB grafts were primarily selected on the basis of serologic matching at HLA-A, -B, and -DR alleles (n = 19) or serologic matching at HLA-A and -B and high-resolution DNA typing of DRB1 alleles (n = 19). Only 1 graft (2%) in primary selection was 3 HLA mismatches. However, confirmatory high-resolution DNA typing of both class I and class II alleles revealed that 12 (31%) of the CB grafts had 3 or 4 mismatched antigens (Table 2).

### Recovery of Peripheral Blood Cell Counts and Engraftment

Four of 38 patients were not evaluated for donor engraftment because of early death from sepsis (n = 2) (day 7, day 21) or bleeding (n = 2) (days 17, 22). Of 34 evaluable recipients, the cumulative incidence of primary donor engraftment was 97% (33 patients) as 1 patient experienced graft rejection with autologous marrow recovery. The median time for neutrophil recovery (>500/μL) was 23 days (range: 18-35 days; n = 33) (Figure 1A). All of these patients accompanied by donor chimerism by 86% to 100% using STR-PCR analysis of bone marrow cells at approximately day 21. After neutrophil recovery, 6 patients did not achieve subsequent reticulocyte recovery; 5 patients died between day 25 and day 100, and 1 patient experienced relapse. The median time for reticulocyte recovery (>1%) was 29 days (range: 25-57 days; n = 27) (Figure 1B). Thereafter, 1 patient, who experienced relapse, failed to achieve platelet recovery. The median time for platelet recovery (>50,000/μL) was 45.5 days (range: 28-208 days; n = 26) (Figure 1C).

**Table 2.** *Graft Characteristics and GVHD Prophylaxis*

| | | |
|---|---|---|
| **Cord blood** | | |
| **Total cells ($\times 10^7$/kg)** | **2.43 (1.96-3.55)** | |
| **$CD34^+$ cells ($\times 10^5$/kg)** | **0.87 (0.24-3.98)** | |
| **HLA mismatch** | **Primary*** | **Confirmatory†** |
| **0/6** | **1** | **0** |
| **1/6** | **9** | **6** |
| **2/6** | **27** | **20** |
| **3/6** | **1** | **10** |
| **4/6** | **0** | **2** |
| **GVHD prophylaxis** | | |
| **CsA/CsA + sMTX** | **1/17** | |
| **Tacrolimus/tacrolimus + sMTX** | **11/9** | |

GVHD indicates graft-versus-host disease; CsA, cyclosporin A; sMTX, short-term methotrexate.

*Primary HLA mismatches were detected on the basis of serological HLA-A, -B, and -DR alleles (n = 19) or serologic HLA-A and -B and high-resolution DRB1 alleles (n = 19).

†Confirmatory HLA mismatches were detected on the basis of high-resolution HLA -A, -B, and -DRB1 alleles.

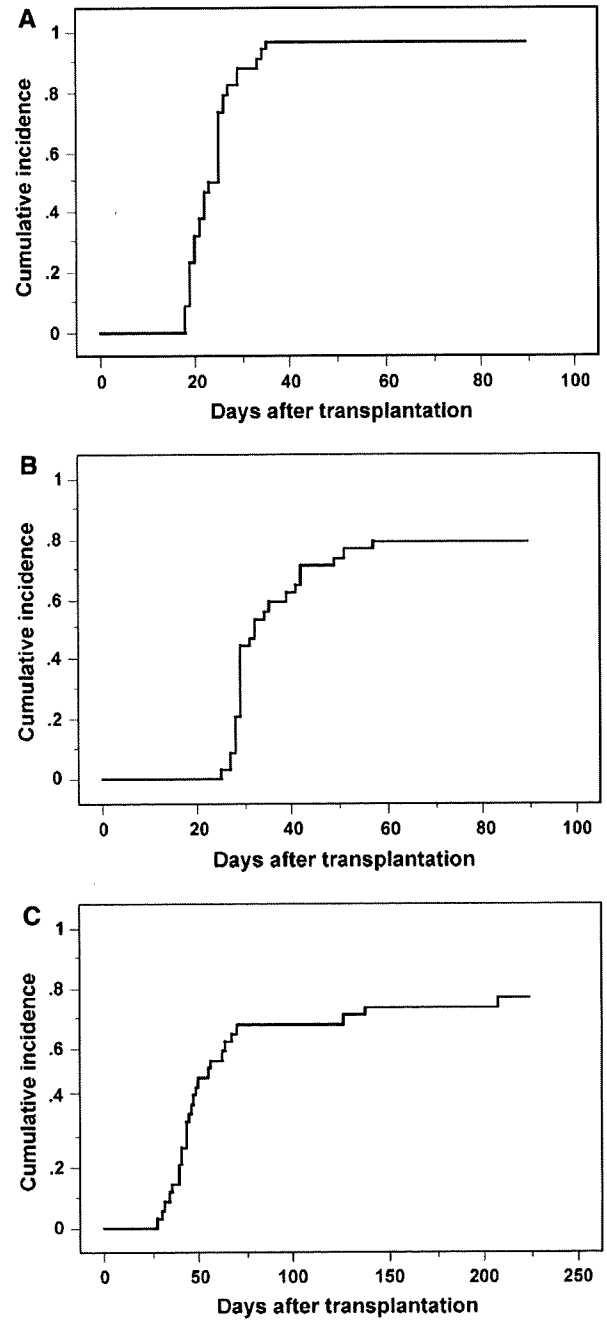


**Figure 1.** Neutrophil, reticulocyte, and platelet engraftment. Kaplan-Meier estimates of the probability of achieving a neutrophil count of >500/μL (A), a reticulocyte count of >1% (B), and an untransfused platelet count of >50,000/μL (C).

### Early Organ Toxicity

Early organ toxicity caused by the TBI/FLAG preparative regimen by day 28 was graded by the regimen-related toxicity (RRT) grading system [24]. Toxicities because of infection, bleeding, GVHD, and drugs administered posttransplant were excluded from this

analysis. Grade I stomatitis was observed in 13 patients, grade I hepatic toxicity in 7 patients, and Grade I gastrointestinal toxicity (diarrhea) in 11 patients. No patient developed cardiac toxicity (electrocardiograph abnormality), pulmonary toxicity (dyspnea), renal toxicity (increase in creatinine), or bladder toxicity (haematuria).

### Infection

Five patients developed sepsis, 6 pneumonia, 1 human herpesvirus-6 (HHV-6) encephalitis and 1 interstitial pneumonitis. Reactivation of cytomegalovirus was documented in 16 patients and gancyclovir was administered. One of them developed fatal interstitial pneumonitis because of CMV. No obvious fungemia and invasive aspergillosis were observed.

### GVHD

Twenty-eight patients who attained engraftment and survived >40 days were evaluated for aGVHD. The cumulative incidence of grade II-IV aGVHD was 57% (16/28), with grades II, III, and IV occurring in 7, 8, and 1 patients, respectively. Appearance of aGVHD varied depending on GVHD prophylaxis used. The incidence of grade II-IV aGVHD was 88% (n = 8) when single agent (tacrolimus or CsA alone) was used in 2000 to 2002, and it was significant reduced (45%, n = 20) when short-term MTX was used in combination with tacrolimus or CsA after August 2002 ($P = .04$ by the $\chi^2$ test). Chronic GVHD developed in 11 (41%) of 27 evaluable patients who survived >100 days. Of the 11 patients, 8 patients developed limited cGVHD and 3 extended cGVHD.

### Relapse

Overall, 11 patients (28.9%) relapsed after CBT. Cumulative incidence of relapse is shown in Figure 2. Of these patients, 10 were in the high-risk group (3 with AML, 1 with MDS, 3 with ALL, 2 with non-Hodgkin lymphoma), and 1 was in the standard risk group (1 with ALL).

### Causes of Death

TRM within 100 days was 23.6% (9 of 38 patients). The main cause of death was bleeding (pulmonary and cerebral) in 2 cases, sepsis in 2, multiple organ failure in 2, herpes simplex virus-6 encephalitis in 1, and pneumonia in 2. One patient died of relapse within 100 days. The cause of death after 100 days was relapse in 10 cases, sepsis in 1, pneumonia in 1, interstitial pneumonitis in 1, and cGVHD in 1. TRM at day 365, which excluded primary disease progression, was 34.2% (13 out of 38 patients).

### Survival and Prognostic Factors

Fourteen out of 38 patients were alive at a median follow-up of 46 months (range: 4-77 months). Three-year EFS was 33.5% (Figure 3). Using Cox proportional hazards models, sex, weight, HLA match (primary and confirmatory), cell dose, GVHD prophylaxis, and the presence of grade II-IV GVHD had no apparent effect on EFS (Table 3). In contrast, age and disease status at transplantation had significant impacts on EFS in both univariable and multivariable analysis (Table 3). Kaplan-Meier estimates indicated that patients who were 42 years old or younger (n = 26) showed significantly better survival (39.8%) than those who were older than 42 years (n = 12) (19.4%) ($P = .0422$) (Figure 4). Regarding the disease status at transplantation, EFS was 72.7% in the standard risk group (n = 11) and 17.7% in the high-risk group (n = 27) ($P = .0075$) (Figure 5). As the number of patients included in this study is small, the results shown above should be interpreted with caution.

## DISCUSSION

Intensified chemotherapy can be effective in the treatment of chemotherapy-sensitive malignant

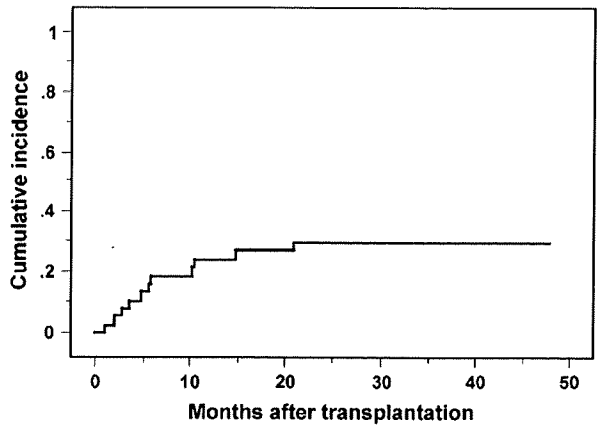


**Figure 2.** Cumulative incidence of relapse estimated by Kaplan-Meier method.

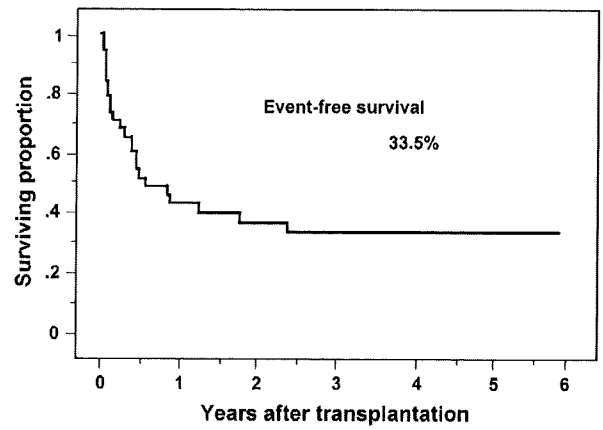


**Figure 3.** Kaplan-Meier estimates of EFS in CBT (n = 38).

**Table 3.** *Risk Factors for Event-Free Survival (EFS) after CBT*

| Factors | Hazard Ratio | 95% Confidential Interval | P-Value |
|---|---|---|---|
| **Univariate analysis** | | | |
| Sex | 0.881 | 0.394-1.966 | .7568 |
| Age >42 | 2.325 | 1.007-5.493 | .0481 |
| Weight | 1.016 | 0.969-1.064 | .5168 |
| Disease status: high risk | 4.604 | 1.356-15.634 | .0143 |
| Cell dose: total cells | 0.696 | 0.239-2.064 | .5140 |
| HLA match (primary)* | 0.885 | 0.458-1.708 | .7152 |
| HLA match (confirmatory)† | 1.022 | 0.666-1.567 | .9218 |
| GVHD prophylaxis | | | |
| CsA vs tacrolimus: | 0.701 | 0.301-1.585 | 0.3939 |
| CsA | | | |
| MTX + vs −: + | 0.738 | 0.322-1.691 | 0.4730 |
| Grade II-IV acuteGVHD | 0.688 | 0.215-2.200 | 0.5282 |
| **Multivariate analysis** | | | |
| Age >42 | 2.828 | 1.140-7.091 | 0.0250 |
| Disease status: high-risk | 5.245 | 1.505-18.281 | 0.0093 |

GVHD indicates graft-versus-host disease; CsA, cyclosporin A; MTX, methotrexate.

*Primary HLA matches were detected on the basis of serological HLA-A, -B, and -DR alleles (n = 19) or serologic HLA-A and -B and high-resolution DRB1 alleles (n = 19).

†Confirmatory HLA matches were detected on the basis of high-resolution HLA -A, -B, and -DRB1 alleles.

diseases [6]. The curative effect of allogeneic SCT is derived partly from the antileukemic effect of myeloablative therapy and partly from a graft-versus-leukemia effect of donor immune cells on the residual leukemia. Transplantation using CB cells as alternative to the bone marrow cells or peripheral blood cells has increasingly been performed for the treatment of hematologic malignancies [1-3]. However, a standard preparative conditioning regimen has not been firmly established. We here report the results of CBT using a new myeloablative regimen, TBI/FLAG.

In the present study, we used 12-Gy TBI (in 4 fractions) and FLAG comprising 10 g/m$^2$ Ara-C, 150 mg/m$^2$ Flu, and G-CSF. High-dose Ara-C has been found to be effective in the treatment of myeloid and lymphoid leukaemia patients with poor prognoses [25,26]. A conditioning regimen using TBI and high cumulative doses of Ara-C (24 or 36 g/m$^2$) achieves a lower relapse rate [27]. However, a significant proportion of allogeneic or autologous BMT patients who received high cumulative doses of Ara-C (36 g/m$^2$) has been reported to die early as a result of toxicity [28]. The incidence of pulmonary complications, including interstitial pneumonia and obvious infection, and the risk of pulmonary toxicity, increases with age. These results suggest that the use of high cumulative doses of Ara-C (36 g/m$^2$) for conditioning should be avoided. Tomonari et al. [29] carried out a preliminary trial in which 5 patients who received CBT were conditioned with 24 g/m$^2$ Ara-C, 90 mg/m$^2$ Flu, and 12-Gy TBI. All patients showed favorable prognosis. Furthermore, Takahashi et al. [30] reported that a conditioning regimen comprising 12-Gy TBI, Ara-C (12 g/m$^2$), cyclophosphamide (120 mg/kg), and G-CSF produced very good outcomes. Thus, appropriate doses of Ara-C may be effective as part of a preparative regimen. In the TBI/FLAG regimen used in this study, the total dose of Ara-C was limited to 10 g/m$^2$, and its activity was pharmacokinetically augmented by concomitant use of fludarabine [10]. This preparative regimen was found to be associated with minimal early RRT within 28 days and without any enhancement of later pulmonary or other life-threatening toxicities.

In CBT, the incidence of grade II-IV aGVHD has been reported to be 40% to 70% [31,32]. It was 58% in this study. When single-agent (tacrolimus or CsA alone) was used in 2000 to 2002, the incidence of grade

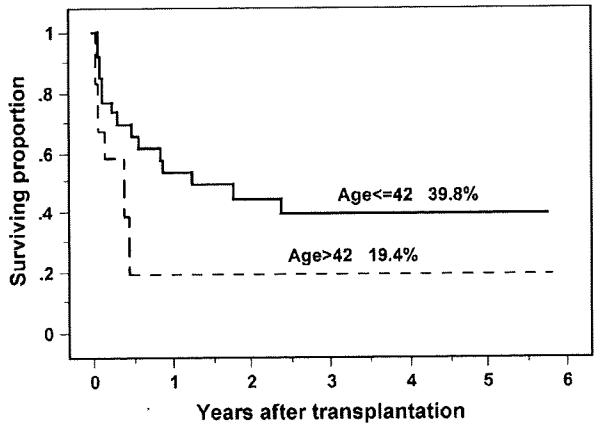


**Figure 4.** EFS in relation to age. Kaplan-Meier estimates of EFS in patients 42 years old or younger (n = 26) and older than 42 (n = 12) ($P$ = .0422).

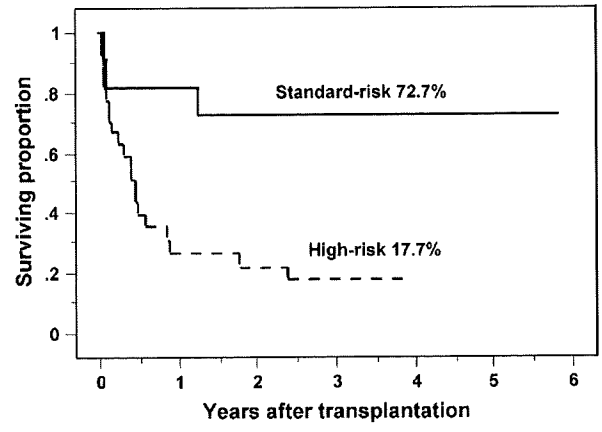


**Figure 5.** EFS in relation to disease status. Kaplan-Meier estimates of EFS in standard-risk patients (n = 11) and high-risk patients (n = 27) ($P$ = .0075).

II-IV aGVHD was high (88%, n = 8), but it was significantly reduced to 45% (n = 20) when short-term MTX was used together with tacrolimus or CsA after August 2002. However, the combined use of MTX for GVHD prophylaxis did not result in the improvement of the survival rate in our analysis (Table 3). Rather, the disease status had the strongest impact on the survival rate (Table 3). Our patients' response to steroid therapy was generally good for those with grade II and III aGVHD (data not shown).

Rocha et al. [33] reported the results of CBT on 98 patients in multicenter analysis, which showed 36% of 2-year survival. Laughlin et al. [2] also reported multicentric analysis of CBT including 150 patients that showed 26% of 3-year survival. In single-institution studies of adult CBT, Long et al. [34] reported 3-year survival of 19% of 57 patients, whereas Takahashi et al. [30] showed 2-year survival of 74% of 113 patients. In our single-institution study using a single-conditioning regimen, 3-year EFS was 33.5% (Figure 3). Cell dose of CB graft is known to be one of the critical factors that affect EFS in CBT [31], but we did not find this is to be the case in our analysis (Table 3). This is probably because we used CB with relatively large number of cells, with the median cell number of $2.43 \times 10^7$ cells/kg. We found that patients older than 42 showed poor EFS (Figure 4), and this FLAG/TBI conditioned CBT is favorable to those who are 42 or younger. Regarding disease status and survival, 3-year EFS was 72.7% in the standard risk group (n = 11) and 17.7% in the high-risk group (n = 27) ($P = .0075$) (Figure 5). The EFS of 17.7% in the high-risk group in our study is comparable to previously reported rates of 15% to 20% [2]. The results of the present study are encouraging because standard-risk patients had 72.7% survival, which is comparable to that seen in standard-risk patients receiving allogeneic BMT or peripheral blood stem cell transplantation from HLA-matched donors [35]. This may indicate that CBT has almost the same efficacy as BMT in standard-risk patients. Although the finding must be confirmed in a larger scale study, our study suggests that CBT following conditioning with the TBI/FLAG regimen may be a reasonable option for adults with hematologic malignancies.

The results presented above show that CBT with a TBI/FLAG preparative regimen was well tolerated without significant RRT, and offered sustained donor cell engraftment. Patients who are 42 years old or younger and in standard risk may obtain a favorable outcome in this TBI/FLAG regimen. Further studies are needed to optimize this procedure to establish an effective treatment modality for hematologic malignancies.

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poorly phrased. There is no standard by which one can compare an odds ratio for CR to a hazard ratio for OS. Given how difficult it has been historically to improve upon the results with MP, and the conflicting results of the various MPT vs MP trials, the clear survival advantage seen with MP-Bortezomib in the VISTA trial is truly remarkable, and by far the most important take-home message. It is not appropriate to directly contrast the results of the E4A03 and VISTA studies (which I agree are both excellent). The contrast I tried to make in the editorial was in the reaction to these trials of stock analysts (whose obtrusive presence at the meeting has recently been noted[16]), who appear to place an excessively high value on improvements in CR. In my opinion this is not always appropriate, and a more balanced approach is warranted, with better surrogates (for example, molecular CR, suppression of cytogenetic abnormalities) for OS needed.

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# Molecular detection of *AML1-MTG8*-positive cells in peripheral blood from a patient with isolated extramedullary relapse of t(8;21) acute myeloid leukemia

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A few studies have reported that *AML1-MTG8* expression levels in bone marrow (BM) are 1- to 3-log higher than those in peripheral blood (PB) when detected by quantitative PCR methods in acute myeloid leukemia (AML) with the t(8;21) translocation.[1–3] However, the relationship between BM and PB is retained at any time during the clinical course is unknown. Here we present a patient with t(8;21) AML who demonstrated isolated ovarian relapse after allogeneic BM transplantation (BMT). *AML1-MTG8* chimeric transcripts could be repeatedly detected in both BM and PB during the clinical course. Moreover, the *AML1-MTG8* expression levels detected by real-time quantitative (RQ)-PCR methods in PB were higher than those in BM before and at the time of the extramedullary relapse (EMR). Thus, we propose that the presence of EMR is responsible for repeated detection of minimal residual disease (MRD) and discuss the clinical significance of different *AML1-MTG8* expression levels between BM and PB for the diagnosis of isolated EMR.

A 22-year-old woman was diagnosed with AML (French-American-British (FAB) subtype M2) with thoracic vertebrae involvement in March 1998. Cytogenetic evaluation revealed the t(8;21)(q22;q22) chromosomal translocation. She achieved complete remission (CR) with induction chemotherapy and