ASBMT American Society for Blood and Marrow Transplantation

BIOLOGY

Double Haploidentical Hematopoietic Stem Cell Transplantation Results in Successful Engraftment of Bone Marrow from Both Donors without Graft-versus-Host or Graft-versus-Graft Effects

Chandra S. Biswas, Christopher T. Sauter, Cavan P. Bailey, Daniel Rittenberg, Xiaoling Luo, Michelle M. Panis, Tulin Budak-Alpdogan, Dolores Grosso, Neal Flomenberg, Onder Alpdogan

We established double-haploidentical (DH) hematopoietic stem cell transplantation (HSCT) murine models to explore competitive engraftment, graft-versus-graft effect and graft-versus-host disease (GVHD). T cell–depleted (TCD) bone marrow (BM) cells from B6SJF1 (donor 1 [D1]) and B6D2F1 (donor 2 [D2]) mice achieved >90% donor engraftment when transplanted into B6CBAF1 mice. B6CBAF1 recipients survived without evidence of GVHD when undergoing HSCT with TCD-BM from 2 haploidentical donors, D1 and D2. DH-HSCT recipients had significantly higher leukocyte and neutrophil counts than single-haploidentical HSCT recipients from either D1 or D2. DH recipients consistently showed successful mixed chimerism in both BM and spleen. Two other DH-HSCT models, B6D2F1 + C3D2F1→B6C3F1 and B6CBAF1 + B6SJLF1→B6D2F1, showed similar engraftment patterns. Low-dose T cell infusion from both D1 and D2 increased the degree of early engraftment of the respective donors in BM and spleen; however, this early engraftment pattern did not determine long-term engraftment dominance. In the long term, minimally engrafted D1 BM recovered and comprised >50% of all donor- derived B, T, and natural killer cells. We conclude that early BM engraftment is determined by donor T cell immunodominance, but long-term engraftment is related to the engraftment potential of stem cells after DH-HSCT.

Biol Blood Marrow Transplant 18: 1808-1818 (2012) © 2012 Published by Elsevier Inc. on behalf of American Society for Blood and Marrow Transplantation

KEY WORDS: Haploidentical donor, Graft-versus-leukemia, Allogeneic Stem Cell transplantation, Animal Models, Immune reconstitution

INTRODUCTION

Initially, allogeneic hematopoietic stem cell transplantation (HSCT) was limited to patients with an HLA-identical related donor. To provide options for patients lacking a matched related donor, registries of unrelated donors and cord blood banks have been established as alternative matched stem cell sources. The use of haploidentical (HI) donors broadens the application of HSCT. Unfortunately, HI-HSCT has traditionally been associated with higher mortality rates compared with transplantation from well-matched donors, which has limited its application. HI-HSCT has produced impressive results in transplant recipients in complete remission in selected centers [1-4]. Rigorously T cell–depleted (TCD) HI grafts avoid severe graft-versus host disease (GVHD) and increase the safety profile of HI-HSCT. T cell depletion techniques have been successful in decreasing GVHD, but have been associated with higher rates of relapse, graft rejection, and opportunistic infection owing to a lack of T cells in the donor inoculum [1,5]. Outstanding clinical results have been achieved with large doses of CD34-selected hematopoietic stem cells [5]. Unfortunately, the success rate of HI-HSCT remains low in patients with resistant leukemia, suggesting that leukemic cells are able to escape the donor immune system's graft-versus-tumor (GVT) effects.

MHC molecules play a critical role in immune recognition and response. A decrease in MHC antigen

From the Department of Medical Oncology, Jefferson Medical College, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania.

Financial disclosure: See Acknowledgments on page 1817.

Correspondence and reprint requests: Onder Alpdogan, MD, 1015 Walnut Street, Curtis Building, Suite 1024, Philadelphia, PA 19107 (e-mail: Onder.Alpdogan@jefferson.edu).

Received July 3, 2012; accepted September 20, 2012

1083-8791/$36.00
http://dx.doi.org/10.1016/j.bbmt.2012.09.012

expression is an important contributor to the escape from immune surveillance.

Recently, a striking example in patients with disease recurrence after HI-HSCT was reported in which the malignant clones lost the expression of disparate HLA haplotype, the presumed dominant target after HI-HSCT. Vago et al. [6] evaluated 43 patients who underwent HI-HSCT for acute leukemia or myelodysplastic syndrome and found that 5 of 17 patients with leukemia relapse had mutant leukemic cells that lost mismatched MHC haplotype expression with acquired uniparental disomy. T cells targeting an unshared haplotype were impaired, which allowed expansion of the resistant clones and eventually caused clinical relapse [6]. A Japanese group also reported similar cases with uniparental disomy and leukemia relapse after HI-HSCT [7].

Immunologic escape occurs through other mechanisms as well, but loss of target MHC antigens is an important mechanism that could provide a target for the development of novel treatment options. The use of 2 HI donors, each ideally targeting the opposite host MHC haplotype, could maximize the number of target antigens that can be seen by donor-derived GVT effector cells. In this scenario, the loss of one MHC haplotype (or other target antigens) would not cripple the GVT response. Colvin et al. [8] found that HI donor lymphocyte infusion after administration of a minimal conditioning regimen could produce remission, with all donor lymphocyte infusion products rejected. This finding demonstrates the potency of these cells in eradicating malignancy by targeting haplomismatched tumor cells in even a short period. However, the use of 2 HI donors might trigger other problems, including a graft-versus-graft (GVG) effect, possibly leading to graft failure or poor graft function. Tetraparental chimeric mouse models, in which TCD bone marrow (BM) from both parental strains is transplanted into the F1 strain, have produced stable long-term chimerism [9].

Although each parent is normally alloreactive toward the haplotype inherited from the opposite parent, stable long-term chimerism and the coexistence of lymphohematopoietic cells from both donors occur through tolerance induction. Other methods can be used to induce tolerance in allogeneic HSCT. Chester et al. [10] administered BM from 2 allogeneic MHC-mismatched donors together with TCD syngeneic BM. When donor marrow was TCD, stable mixed chimerism from both donors was seen; however, when donor T cells remained in the graft, 1 of the 2 donors ultimately dominated [10].

Here we report for the first time that double HI (DH) HSCT results in successful engraftment of BM from both donors without GVH or GVG effects in TCD models.

MATERIALS AND METHODS

BM Transplantation

Female C57BL/6 (B6, H-2K^{b}), B6CBAF1 (H-2K$^{b/k}$), B6SJL (H-2K$^{b/s}$), C3D2F1 (H2K$^{k/d}$), and B6D2F1 (H2K$^{b/d}$) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice used in the BM transplantation (BMT) experiments were between 10 and 12 weeks of age. The BMT protocols were approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University.

BM cells were removed aseptically from femurs and tibias and rendered TCD by incubation with anti-Thy 1.2 antibody for 30 minutes at 4°C, followed by incubation with Low-TOX-M rabbit complement (Cedarlane Laboratories, Hornby, ON, Canada) for 40 minutes at 37°C or, alternatively, by anti-CD5 magnetic bead depletion (Miltenyi Biotech, Auburn, CA). Typical levels of contaminating T cells after complement depletion ranged from 0.2% to 0.5% of all BM leukocytes.

Splenic T cells were obtained by positive selection with anti-CD5 antibodies conjugated to magnetic beads (Miltenyi Biotech). Cells (5×10^6 BM cells with or without splenic T cells) were resuspended in DMEM and transplanted by tail vein infusion (0.25 mL total volume) into lethally irradiated recipients on day 0. In some experiments, mice were challenged with P815 murine mastocytoma cells by tail vein infusion at the time of transplantation. On day 0 pretransplantation, recipients received 11-13 Gy of total body irradiation (strain-dependent) from a ^{137}Cs source in 2 split doses, with a 3-hour interval between doses to reduce gastrointestinal toxicity. Mice were housed in sterilized microisolator cages and received normal chow and autoclaved hyperchlorinated drinking water (pH 3.0).

Reagents and Antibodies

Antimurine CD16/CD32 FcR block (2.4G2) and all of the following fluorochrome- labeled antibodies against murine antigens were obtained from BD Biosciences (San Jose, CA): anti-H2Kd (SF1-1.1), anti-H2Kb (AF6-88.5), anti-H2Kk (36-7-5), anti-H2Ks (KH49), anti-CD45.1 (A20), anti-CD45R/B220 (RA3-6B2), anti-NK1.1 (PK136), anti-CD3 (500A2), anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-Gr1 (RB6-8C5), anti-CD11b (M1/70), anti-TNF-α (MP6-XT22), anti-IFN-γ (XMG1.2), allophycocyanin-streptavidin, and isotype controls. Rat IgG2a-κ (R35-95), hamster IgG1-κ (Ha4/8), phorbol 12-myristate 13-acetate (PMA), ionomycin, brefeldin A, and concanavalin A were obtained from Sigma-Aldrich (Allentown, PA).

Flow Cytometry Analysis

Cells were washed in FACS buffer (PBS with 0.5% BSA and 0.1% sodium azide), and 10^6 cells/mL

were incubated for 15 minutes at 4°C with CD16/CD32 FcR block. Cells were then incubated for another 30 minutes at 4°C with antibodies and washed twice with FACS buffer. The stained cells were resuspended in FACS buffer and acquired on a FACSCalibur or LSR II flow cytometer with CellQuest or FACSdiva software, respectively (BD Diagnostic Systems, Sparks, MD), and analyzed with Flowjo software (TreeStar, San Carlos, CA).

Intracellular Staining

Cytokine secretion was stimulated in isolated splenocytes by incubation in PMA (10 ng/mL) and ionomycin (2 μM) for 5 hours. After a 2-hour stimulation with PMA and ionomycin, 10 μg/mL brefeldin A was added, After incubation for another 3 hours, cells were collected and counted for intracellular staining. Splenocytes were washed and stained with primary (surface) fluorochrome-conjugated antibodies, fixed and permeabilized with the BD Cytofix/Cytoperm Kit (BD Biosciences), and then stained with intracellular antibodies (ie, TNF-α and IFN-γ).

Cytotoxicity with Cromium Release Assay

Target cells were labeled with 100 μCi ^{51}Cr for 1 hour, and after 3 washes were plated in U-bottomed Costar plates (Corning, Corning, NY) at a concentration of 10^4 cells/well. Splenic T cells were added at various effector-to-target ratios (ie, 40:1, 20:1, and 10:1) in a final volume of 200 μL, followed by incubation for 4-6 hours at 37°C and 5% CO_2. Subsequently, 35 μL of supernatant was removed from each well and counted in a gamma counter (Walec 1450 Microbeta-plus; PerkinElmer, Waltham, MA) to determine experimental release. Spontaneous release was obtained from wells that contained only target cells, and total release was obtained from wells treated with 5% Triton X-100. The spontaneous release was <15% of the total release. Percent cytotoxicity was calculated using the following formula: percent cytotoxicity = 100 × (experimental release − spontaneous release) / (total release − spontaneous release).

Assessment of GVHD

The severity of GVHD was assessed with a clinical GVHD scoring system as described by Cooke et al. [11]. In brief, each week, ear-tagged animals in coded cages were scored on a scale of 0-2 for 5 clinical parameters: weight loss, posture, activity, fur, and skin. A clinical GVHD index was generated by summing the 5 scores (total score, 0-10). Survival was monitored daily. Animals with a score >5 were considered moribund and were euthanized.

Statistics

All values are expressed as mean ± SEM. Statistical comparison of experimental data was performed using the nonparametric unpaired Mann-Whitney *U* test. A *P* value <.05 was considered statistically significant. Log-rank analysis was used in the survival analysis.

RESULTS

SH BM Engrafted Well and Resulted in Better Antitumor Activity

We generated our HI transplant models using hybrid mice with a B6 background that shared the same haplotype ($H2K^b$). Lethally irradiated B6CBAF1 ($H2K^{b/k}$) recipients were transplanted with TCD-BM cells from B6D2F1 ($H2K^{b/d}$) donors. Recipients were euthanized at days 28, 42, and 84, and showed >90% donor cell engraftment, including donor-derived lymphopoiesis and myelopoiesis in the spleen and BM (Figure 1A-D and data not shown), with no evidence of GVHD. Interestingly, all natural killer (NK) cells and B cells were from donor BM even in the early period of HSCT. Conversely, residual host T cells constituted half of the T cell population at day 28, but donor T cell engraftment eventually reached 90% by day 84 posttransplantation.

All B6D2F1 recipients were challenged with P815 murine mastocytoma ($H2^d$) cells to compare the graft-versus-leukemia effect of SH-HSCT and the parent-FI HSCT model. In this model, low-dose T cells from the donors were infused as well. The SH TCD-BM and add-on T cells model demonstrated better antileukemic activity than the parent-FI HSCT model (Figure 2A). Ten days after transplantation, splenocytes of the recipients of either B6- or B6CBAF1-derived TCD-BM and low-dose T cells were harvested and used in a ^{51}Cr release assay to determine eytotoxicity against P815 cells. Compared with B6 splenocytes, B6CBAF1 splenocytes exhibited statistically higher cytotoxic activity against P815 tumor cells. None of the donor cells showed cytotoxicity against C1498 tumor cells, nonspecific control, expressing H2Kb (Figure 2B) (data not shown).

Recipients of TCD-BM transplantation with low-dose T cell infusion but without tumor challenge were euthanized 28 days after transplantation. T cell numbers were slightly increased in recipients of SH HSCT compared with recipients of B6 splenocytes, whereas percentages of T cells in the spleen were not statistically different in the 2 recipients (Figure 2C). $CD8^+$ T cell levels were significantly higher in recipients of SH HSCT, whereas intracellular IFN-γ levels of T cells were similar in the 2 groups (Figure 2D). Interestingly,

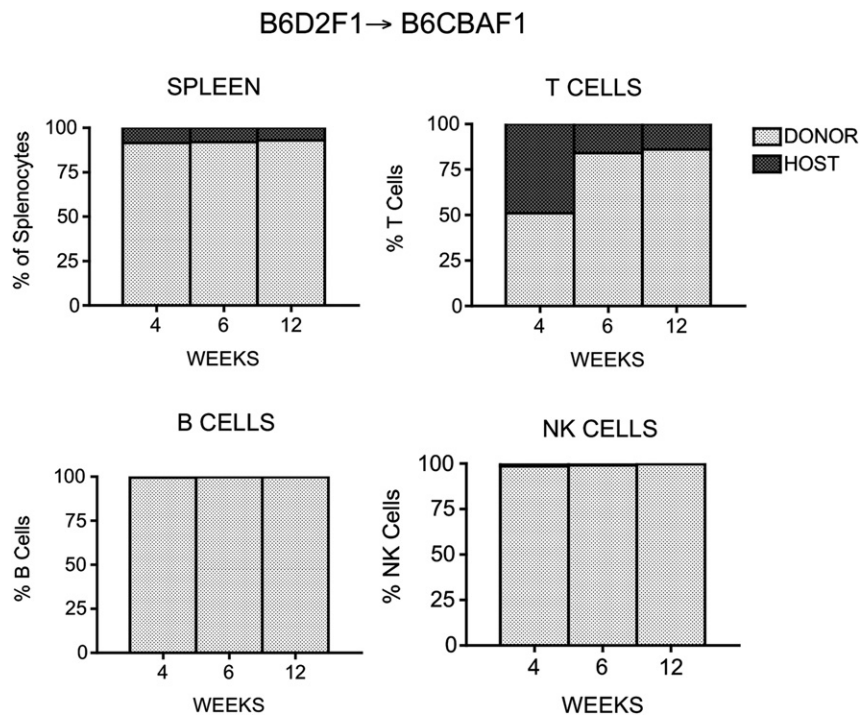

Figure 1. Haploidentical BM engrafted significantly with no signs of GVHD. Lethally irradiated (13 Gy) B6CBAF1 recipients underwent transplantation with 5×10^6 TCD-BM cells from B6D2F1 mice. Mice were euthanized at 4, 6, and 12 weeks after transplantation, and spleens were harvested. Single-cell suspensions were obtained, and splenocytes were stained with anti-H2Kd, anti-CD3, anti-NK1.1, and anti-B220 antibodies. Cells were analyzed by flow cytometry. Donor cells were determined by positive H2Kd expression on the cell surface. Each harvest contained 5 mice.

TNF-α secretion of $CD8^+$ T cells was significantly higher in recipients of B6CBAF1 splenocytes compared with B6 recipients. We concluded that HI T cells provide better antitumor activity in the B6CBAF1→B6D2F1 model compared with the parent→F1 model.

DH HSCT Results in Successful Engraftment of BM from Both Donors without GVG or GVHD

We compared 2 different SH transplant models with 5×10^6 BM cells from either B6SJF1 mice ($H2K^{b/s}$; SH1) or B6CBAF1 mice ($H2K^{b/k}$; SH2) and a DH transplant model with an equal dose of 2.5×10^6 BM cells from both donors (Figure 3). There was no early transplantation-related mortality or graft failure, which might suggest GVG or host-versus-graft (HVG) activity, in the recipients of DH-HSCT. There were no significant differences between the groups in terms of weight loss and GVHD scores (Figure 3A and data not shown). All mice gained weight by week 3. At day 90 posttransplantation, peripheral blood samples revealed significantly higher WBC and neutrophil counts in DH-HSCT recipients compared with SH HSCT recipients (Figure 3B and C). Platelet counts and Hb levels were similar among the groups (Figure 3D and E). DH-HSCT recipients consistently showed successful mixed chimeric engraftment in both BM and spleen. More than 99% of BM cells and 98% splenic cells were of donor origin (Figure 3F, G, and J). Interestingly, there was no difference in donor chimerism between DH and SH transplant recipients. Almost all B cells, myeloid cells, and NK cells in the spleen and BM were of donor origin (Figure 3I, K, and M and data not shown), and DH-HSCT recipients had mixed chimerism. T cell reconstitution patterns were similar in DH-HSCT and SH-HSCT recipients were similar, and even at 90 days posttransplantation, approximately 10% residual host-derived T cells were detected in both spleen and BM (Figure 3H and L).

To confirm that these finding were not strain-specific, we examined the engraftment of DH-HSCT in a different mouse model. In this model, both donors were HI to one another with different haplotypes than the host cells, which is clinically relevant to any parent–child combination with HI-matched sibling donors. Lethally irradiated B6C3F1 recipients underwent transplantation with TCD-BM cells from B6D2F1 and C3D2F1 donor mice and were harvested at day 35 after transplantation. There were no differences in thymic, splenic and BM cellularity posttransplantation (data not shown). Spleen and BM showed >90% donor engraftment from both donors (Figure 4A). Donor 1 (D1) was moderately dominant in the spleen and BM. B cell and T cell reconstitution revealed

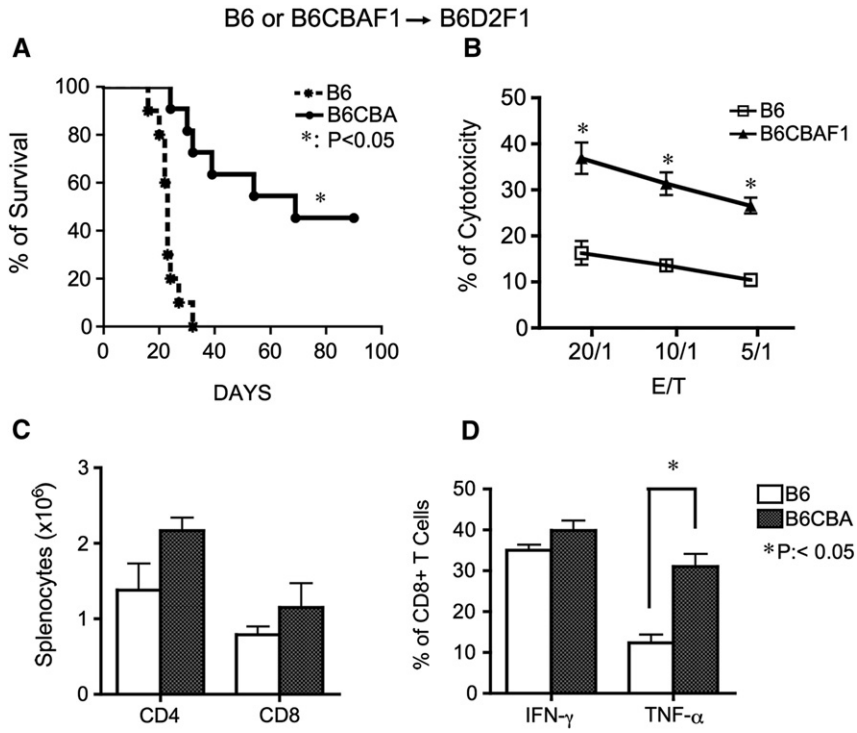

Figure 2. Recipients of HI SCT shows better antitumor effect than recipients of parent-F1 SCT. Lethally irradiated (13 Gy) B6D2F1 recipients underwent transplantation with 5×10^6 TCD BM cells and 1×10^5 T cells from either B6 or B6CBAF1 mice. (A) All recipients also received 1×10^4 P815 murine mastocytoma cells on the day of transplantation. Mice were analyzed for survival at 100 days posttransplantation. Each group contained 10 mice. Kaplan-Mayer curves are shown. Log-rank analysis was used for statistical analysis. (B) Lethally irradiated (13 Gy) B6D2F1 recipients underwent transplantation with 5×10^6 TCD-BM cells from either a B6 donor or a B6CBAF1. Single-cell suspensions were prepared from the spleens harvested from the recipients at day 10 posttransplantation. With the ^{51}Cr release assay, the cytotoxicity effect of the splenocytes (effector, E) on P815 murine mastocytoma cells (target, T) was measured at different E/T ratios, as indicated. Each group contained 5 mice. *$P < .05$. (C and D) B6D2F1 mice underwent transplantation as described in (A). All mice were euthanized on day 28 posttransplantation. Spleens were harvested, and single-cell suspensions were prepared. Splenocytes were stained with anti-H2Kd, anti-CD4, and anti-CD8, followed by intracellular cytokine staining done according to the manufacturer's protocol (BD Pharmingen, San Diego, CA). The cells were analyzed by multicolor flow cytometry. The total numbers of $CD4^+$ and $CD8^+$ cells are shown in (C). The percentage of donor-derived IFN-γ–and TNF-α–secreting $CD8^+$ T cells are shown in (D). The data are representative of 2 independent experiments. Each group contained 5 mice. The Mann-Whitney *U* test was used for statistical analysis.

similar patterns with the transplantation model shown in Figure 3 (Figure 4B-D). None of the mice showed signs of GVHD or died in the early posttransplantation period (data not shown). We again concluded that TCD BM cells from two HI donors could engraft well without GVG and GVHD in HI recipients.

Low-Dose T Cell Infusion from Either D1 or D2 Increased the Degree of Dominance of That Donor's Cells in the Spleen and BM

Low-dose add-on T cell infusions from donors were explored for safety and their effects on engraftment patterns. In these experiments, all recipients received the same amount of DH TCD parent-FI HSCT model BM from B6SJLF1 ($H2K^{b/s}$) and B6D2F1 ($H2K^{b/d}$) mice and were also infused with total 1×10^5 T cells from one or both donor strains. The control group did not receive any T cells. Mice were harvested at day 30 after HSCT. Interestingly, a low-dose T cell infusion did not result in any change in thymopoiesis or BM cellularity but produced a mild decrease in splenic cellularity (data not shown). Donor T cell infusion increased early engraftment of the respective source donor in the spleen and BM and decreased residual host T cells (Figure 5A and B). These data show a similar pattern to the findings reported by Chester et al. [10]. We concluded that add-on T cells increase the same type of donor chimerism. This experiment also demonstrated that D2 cells were dominant in the early engraftment period and that infusion of both D1 and D2 T cells resulted in >95% D2 cell engraftment, suggesting that D2 T cells are immunologically more potent than D1 T cells.

Early Engraftment Pattern in DH-HSCT Recipients Does Not Predict Long-Term Engraftment Pattern

Evaluation of mixed chimerism at varying time points (days 30, 60, and 90) after DH-HSCT revealed different engraftment dominance patterns. At 90 days posttransplantation, D1 engraftment in the spleen was increased from 17% to 51%, whereas D2

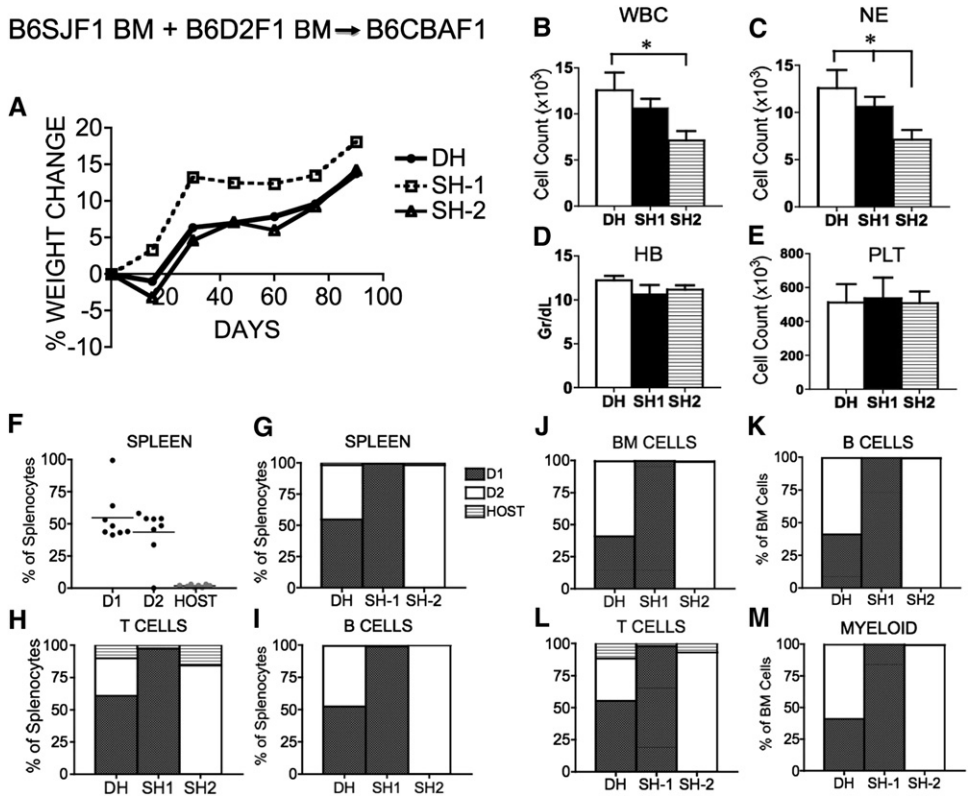

Figure 3. DH-HSCT recipients engrafted well, with no GVHD or graft failure. Lethally irradiated (13 Gy) B6CBAF1 recipients underwent transplantation with 5×10^6 TCD-BM cells from the B6SJLF1 donor (SH1), B6D2F1 donor (SH2), or both donors (DH). (A) Weight curve for the recipients over 90 days posttransplantation. (B-E) Blood was collected via retro orbital bleeding at day 90 from recipients after transplantation and analyzed for WBC (B), neutrophils (NE) (C), hemoglobin (HB) (D), and platelets (PLT) (E). The cells from both HI donors engrafted well in the DH-HSCT recipients. Mice were euthanized at day 90, and spleens and BM cells were harvested. Cells were stained with anti-H2kD, anti-H2kk, anti-H2ks, anti-CD45.1, anti-CD3, anti-B220, Gr-1, and anti-CD11b and analyzed by multicolor flow cytometry. Shown are percent engraftment of total spleen (F and G) and spleen T cells (H), B cells (I), and myeloid cells, and total BM (J) and BM B cells (K), T cells (L), and myeloid cells (M). Each group contained 8 mice, and the experiment was repeated twice. The nonparametric Mann-Whitney *U* test was used for statistical analysis. *$P < .05$.

engraftment was decreased from 70% to 46% (Figure 6A). T cell chimerism exhibited more impressive changes in relative engraftment. D1-derived T cells composed only 7% of splenic T cells at day 30, but expanded to ~60% at day 90 (Figure 6B). Splenic B cells and NK cells followed a similar chimerism pattern, with the exception of residual host cells in the early posttransplantation period (Figure 6C and D). T cell, B cell, and myeloid cell chimerism in BM showed similar patterns after DH-HSCT (data not shown).

Infusion of Low-Dose HI T Cells Results in Enhanced Antitumor Activity after Transplantation

In this experiment, we challenged recipients of DH TCD-BM with P815 tumor cells. B6D2F1 mice underwent transplantation with TCD-BM and add-on low-dose T cells from B6CBAF1, B6SJLF1, or both. Interestingly, recipients of DH TCD-BM exhibited significantly better survival compared with recipients of SH TCD-BM (Figure 7A). Moreover, after a low-dose T cell infusion (1×10^5), survival was significantly better in recipients of B6CBAF1 BM compared with recipients of B6SJLF1 BM. In contrast to the TCD model, survival was similar in recipients of DH TCD-BM with DH T cells and recipients of B6CBAF1 BM with SH T cells (Figure 7B). These data suggest that B6SJLF1 cells have less antitumor activity than B6CBAF1 T cells (Figure 7C). T cells from 2 different donors exhibited different antitumor activity and possibly also antihost activity in DH-HSCT recipients.

This leads to the question of whether or not B6CBAF1 T cells are immunologically more potent than B6SJLF1 cells in terms of engraftment. Lethally irradiated B6D2F1 mice underwent transplantation with TCD B6CBAF1 and B6SJF1 BM cells, and engraftment in the spleen was evaluated. Although most of the early engraftment consisted of B6CBAF1 T cells, comparable chimerism was observed at 100 days after transplantation (Figure 7D and E). When in vitro functions of B6SJLF1 and B6CBAF1 compared with proliferation kinetics, mitogen-induced T cell proliferation of B6SJLF1 T cells were lower than proliferation of B6CBAF1 T cells (data not shown).

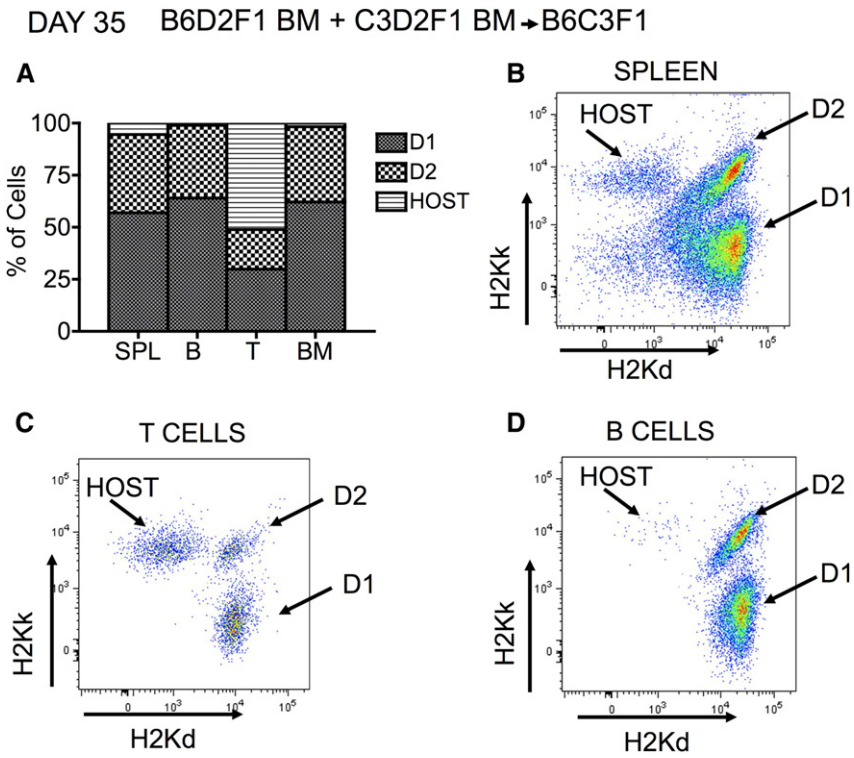

Figure 4. DH-HSCT (B6D2F1 BM + C3D2F1 BM→B6C3F1) engrafted well in recipients of HSCT. Lethally irradiated (13 Gy) B6C3F1 (host) recipients with TCD-BM cells from B6D2F1 (D1) and C3D2F1 (D2) mice, and spleens were harvested on day 35 posttransplantation. (A) In single cell suspensions, cells were stained with anti-H2kd, -H2kk, -H2kb, -CD4, and -CD8 antibodies analyzed by multicolor flow cytometry. (B-D) Chimerism patterns of donor-derived splenocytes (B) and splenic T cells (C), and B cells (D). Each group contained 10 mice.

Interestingly, neither type of cell exhibited a change in intracellular cytokine levels after PMA-ionomycin stimulation. B6D2F1 mice underwent transplantation from B6CBAF1 and B6SJLF1 TCD-BM cells with 1×10^5 T cells (1×10^5) from either B6SJLF1 (D1) or B6CBAF1 (D2) mice. Recipient mice were euthanized and spleens harvested at day 30 posttransplantation. Intracellular IFN-γ levels of donor-derived T cells were measured. There was no difference in intracellular cytokine levels between D1-derived T cells and D2-derived T cells (Table 1). These data suggest that immunodominant T cells are involved in early stem cell engraftment and lymphoid reconstitution as well as in GVT activity, but do not predict long-term engraftment.

DISCUSSION

In this study, we have shown that DH TCD-BM can engraft well without GVG, GVHD, or graft failure in 3 different donor–host combinations. To the best of our knowledge, this is the first study of DH-HSCT in clinically relevant models.

Our SH murine models mimicked possible scenarios that were more clinically relevant than traditional parent→F1 and F1→parent models [12-14]. The lack of host-versus-graft reaction in the parent→F1 model and the presence of only 2 haplotypes in both models urged us to develop new models.

More than 3 decades ago, von Boehmer et al. [9] established that tetraparental BM chimeras were produced by injecting lethally irradiated F1 hybrids with relatively high doses of TCD-BM cells from both allogeneic parental strains. The mice survived in excellent health and showed approximately 50:50 (parent:parent) stable lymphoid cell chimerism persisting for at least several months after irradiation. Moreover, thymus, lymph node, and thoracic duct lymphocytes demonstrated specific unresponsiveness to host mixed leukocyte reaction determinants, suggesting induction of a tolerant T cell population.

An interesting study from the National Cancer Institute demonstrated that 2 allogeneic TCD-BM cells with syngeneic BM cells engrafted well by inducing tolerance to skin grafts from both allogeneic donors [10]. When animals were given non-TCD allogeneic BM from 2 different donors in addition to TCD syngeneic BM, fully allogeneic chimeras were reconstituted in which one or the other allogeneic donor prevailed. The investigators concluded that multiple allogeneic donor BM cells can engraft simultaneously in the mixed marrow model; in addition, multiple allogeneic engraftment confers transplantation tolerance to multiple donors [10].

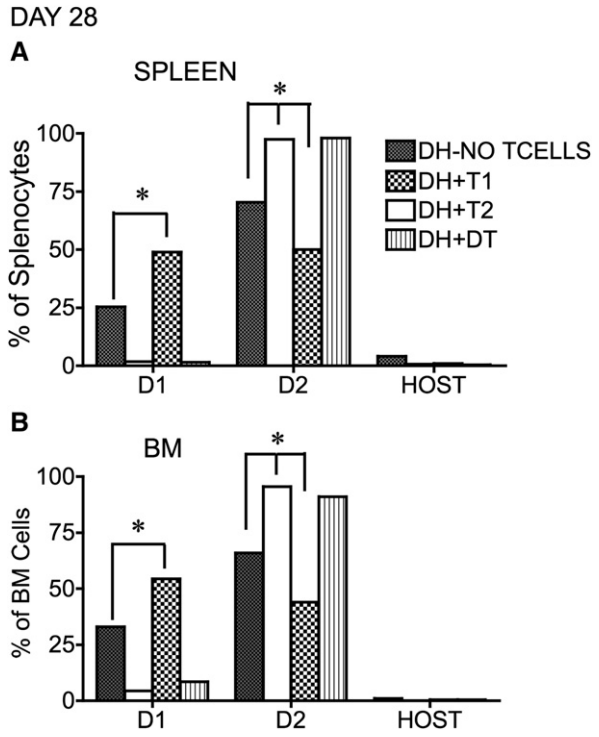

Figure 5. Single or double donor T cells increased the same type of donor BM engraftment in recipients of DH-HSCT. Lethally irradiated (13 Gy) B6CBAF1 recipients underwent transplantation with 5×10^6 TCD-BM cells from B6SJLF1 (D1), B6D2F1 (D2), or both donor mice (DH). Recipients also received 1×10^5 T cells from either B6SJLF1 (T1) or B6D2F1 (T2) or both donor mice (DT) along with TCD-BM. Mice were euthanized on day 28 posttransplantation. Spleens and BM were harvested, and single cell suspensions were prepared from each organ. These cells were then stained with anti-H2Kd, anti-H2kk, anti-H2Ks, and anti-CD45.1. Cells were analyzed by multicolor flow cytometry. The percent engraftment of total spleen (A) and BM (B) cells are shown. Each group contained 8-10 mice. *$P < .05$.

Both of the foregoing studies suggested that TCD is essential for generating mixed chimeras originating from both donors. We used TCD-HI BM in all our HSCT experiments and in some experiments added donor-derived T cells to induce graft-versus-leukemia and GVH effects. Satake et al. [15] reported that recipients of HI transplants had significantly higher antitumor activity and less severe GVHD, correlated with lower CXCR3 expression on donor T cells and higher IFN-γ expression on host NK cells. In contrast, in the present study we did not find increased IFN-γ secretion in host-derived T cells or NK cells after HI-HSCT, although we did detect better antitumor activity both in vivo and in vitro in haplomatched donor T cells compared with parent→F1 mismatched donor cells.

Achievement of tolerance after HI-HSCT is crucial for success and long-term stability of the transplant. Posttransplantation administration of high-dose cyclophosphamide can induce T cell tolerance and decrease the risk of GVHD by eliminating alloreactive T cell clones [16]. The prevention of T cell activation via blocking signal molecules also could induce tolerance after HSCT. BM cells from 2 different donors (BALB/c and CBA mice) engrafted with multilineage double chimerism in MHC-mismatched recipients and induced immunologic donor-specific tolerance to skin allografts in the host after treatment with anti-CD45RB and anti-CD154 monoclonal antibodies [17]. We have found no clinical GVHD in our TCD or T cell–replete models. We suggest that our low T cell dose (approximately 1 log lower than in other transplant models) is sufficient to induce GVT activity without causing lethal GVHD.

To address the tolerance of newly generated T cells in HI-HSCT recipients, we developed a tolerization transplantation model. Donor-derived T cells were separated from HI-HSCT recipients and infused at a higher dose (>1 log) to lethally irradiated mice of an unshared haplotype strain. In our preliminary experiments, we did not observe any signs of GVHD in these mice (data not shown). We suggest that newly generated de novo T cells arise from haplomatched BM and become tolerant to the unshared haplotype of the recipient after HI-HSCT.

Umbilical cord blood (UCB) is an important example of the use of 2 different stem cell sources for HSCT. Transplantation of 2 different UCB units, often UCB units with bidirectional mismatches at 1 or 2 MHC loci, has resulted in successful mixed chimeric engraftment, making this modality an acceptable HSCT option for adult patients. Initial concerns regarding the possibility of a cross-immunologic rejection (ie, GVG response) between 2 mismatched UCB units were allayed with the demonstration of consistent engraftment in practice [18]. In addition, double UCB transplantations have been consistently associated with lower rates of GVHD [19] and relapse [20,21] compared with single UCB transplantations. A recent report demonstrated delayed immune reconstitution, specifically in regard to T cell recovery and increased risk of infection after double UCB transplantation [22]. We did not find delayed immune reconstitution compared with SH-HSCT, and, interestingly, we found better neutrophil recovery in DH-HSCT recipients. In vitro T cell proliferation and cytokine secretion of T cells were comparable in the DH-HSCT recipients and SH-HSCT controls.

Engraftment dominance of UCB units and their varying dominance patterns during the posttransplantation period (ie, early versus late engraftment) have been previously reported after double UCB transplantation [23,24]. The mechanism of dominance is not well understood, but higher $CD34^+$ cell content [23], higher NK cell content, and being first in the order of infusion [23,24] have all been associated with dominance of one UCB unit over the other. Two recent reports noted that early $CD3^+$ peripheral blood chimerism predicts the long-term engraftment outlook [25,26]. Taken together, these findings

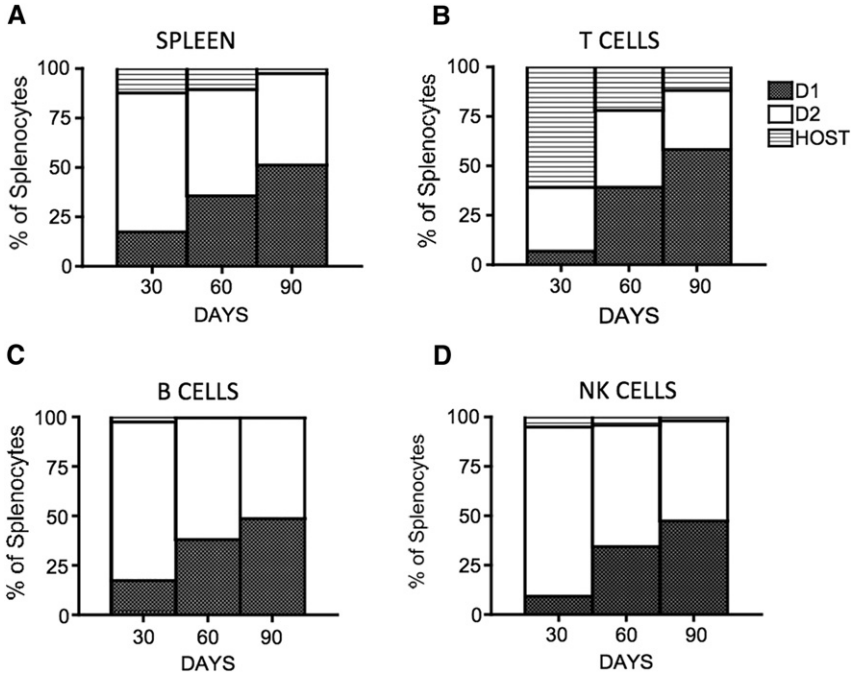

Figure 6. Early engraftment pattern in DH-HSCT recipients does not predict the long-term engraftment pattern. Data from repeated DH-HSCT experiments were compiled and compared for the analysis and prediction of donor (D1 and D2)-derived cellular engraftment pattern in the spleens of host at early and late posttransplantation. Shown are chimerism patterns of spleen cells (A) and splenic T cells (B), B cells (C), and NK cells (D) at days 30, 60, and 90 posttransplantation from DH-HSCT experiments. The total number of mice studied on each day varied between 10 and 20.

suggest that cells from the donor that engrafts earlier or in larger numbers may exert effector responses against the cells of the other donor. This is supported by the detection of evolving cord dominance as early as the day of engraftment [27]. Despite the dominance of hematopoietic cells of one donor over the other, the use of 2 donor UCB units has been associated with decreased rates of relapse compared with single UCB unit transplantation [20,21]. Liu et al. [28] reported that the combination of unrelated UCB and

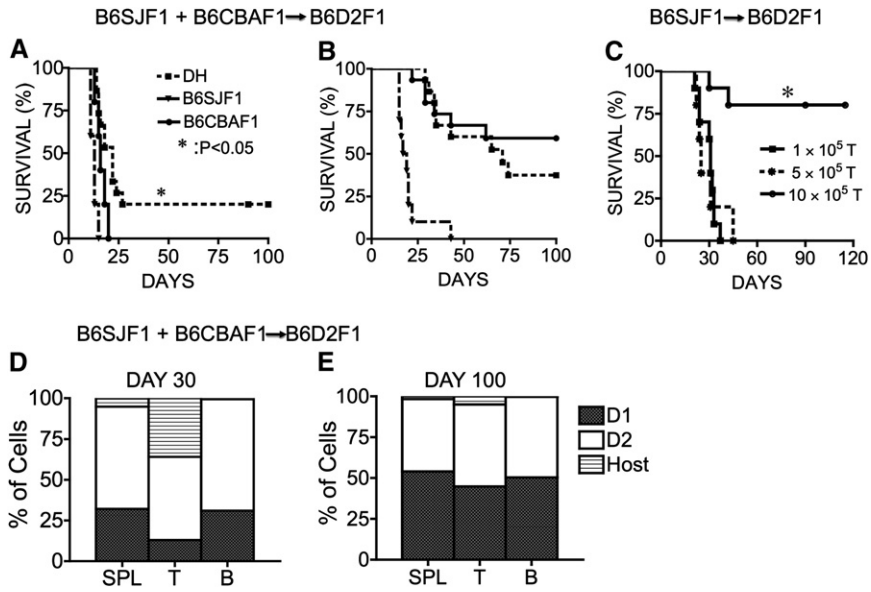

Figure 7. DH-HSCT does not abrogate antitumor activity. (A) Lethally irradiated (13 Gy) B6D2F1 recipients underwent transplantation with 5×10^6 TCD-BM cells from B6SJLF1, B6CBAF1, or both donor mice. (B) Some mice received 1×10^5 T cells from B6SJLF1, B6CBAF1, or both donor mice. (C) B6D2F1 mice received BM from B6SJLF1 with a titrated T cell dose between 1×10^5 and 1×10^6. All recipients also received 1×10^4 P815 murine mastocytoma cells on the day of transplantation. Survival was assessed for 100 days posttransplantation. Each group contained 10-20 mice. Kaplan-Mayer curves are shown. Log-rank analysis was used for statistical analysis. (D and E) Chimerism patterns of splenocytes and splenic T and B cells were analyzed on day 30 and day 100 posttransplantation, respectively, using similar methods as described in Figure 3. In brief, lethally irradiated (13 Gy) B6D2F1 recipients underwent transplantation with 5×10^6 TCD-BM cells from B6SJLF1 (SH1), B6CBAF1 (SH2), or both donor mice (DH).

Table 1. B6CBAF1- and B6SJLF1-Derived T Cells Show Similar Intracellular Cytokine Secretion after PMA-Ionomycin Stimulation in HI-HSCT Recipients

	B6SJLF1	B6CBAF1
CD4, %	8.24 ± 1.84	9.92 ± 1.17
CD8, %	11.3 ± 2.8	13.3 ± 2.7

HI-HSCT resulted in early neutrophil and platelet recovery after transplantation. Interestingly, early HI engraftment shifted to the durable engraftment of UCB at day 180 posttransplantation, suggesting that stem cell function is critical for long-term engraftment. Murine models have demonstrated better proliferative potential of UCB cells compared with adult BM-derived stem cells [29], which might affect engraftment kinetics posttransplantation. In the present study, we have shown that the engraftment potential of stem cells can affect long-term chimerism, and, unlike in double UCB transplantation studies, both BM cells continue to show mixed chimerism even 3 months after transplantation. In addition, cell dose, order of infusion, and compatibility of MHC molecules do not apply in our transplantation models, given the infusion of the same number of cells through the same tail vein injection procedure.

In conclusion, TCD-BM cells engraft well in recipients of DH-HSCT, without the risk of GVG and GVHD. Dominant T cells play a role in early engraftment of stem cells but might not be critical in long-term engraftment, specifically in TCD models. We believe that HI-HSCT has a potential to enhance the GVT effect after transplantation, which warrants further clinical trials in patients at high risk for relapse after HI-HSCT.

ACKNOWLEDGMENTS

Financial disclosure: This study was supported by the Leukemia Research Foundation. O.A. is the recipient of an Amy Strelzer Manasevit Scholar Award from the National Marrow Donor Program and the Marrow Foundation.

Authorship statement: Christopher T. Sauter, Cavan P. Bailey, Daniel Rittenberg, Xiaoling Luo, and Tulin Budak-Alpdogan performed experiments. Chandra S. Biswas and Michelle M. Panis performed experiments and analyzed data. Dolores Grosso and Neal Flomenberg designed experiments. Onder Alpdogan designed experiments, analyzed data, and wrote the manuscript.

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