

Multiple allogeneic progenitors in combination function as a unit to support early transient hematopoiesis in transplantation

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Cord blood (CB) is a valuable donor source in hematopoietic cell transplantation. However, the initial time to engraftment in CB transplantation (CBT) is often delayed because of low graft cell numbers. This limits the use of CB. To overcome this cell dose barrier, we modeled an insufficient dose CBT setting in lethally irradiated mice and then added hematopoietic stem/progenitor cells (HSCs/HPCs; HSPCs) derived from four mouse allogeneic strains. The mixture of HSPCs rescued recipients and significantly accelerated hematopoietic recovery. Including T cells from one strain favored single-donor chimerism through graft versus graft reactions, with early hematopoietic recovery unaffected. Furthermore, using clinically relevant procedures, we successfully isolated a mixture of CD34⁺ cells from multiple frozen CB units at one time regardless of HLA-type disparities. These CD34⁺ cells in combination proved transplantable into immunodeficient mice. This work provides proof of concept that when circumstances require support of hematopoiesis, combined multiple units of allogeneic HSPCs are capable of early hematopoietic reconstitution while allowing single-donor hematopoiesis by a principal graft.

INTRODUCTION

In the 25 years since initial success in sibling cord blood (CB) transplantation (CBT; Gluckman et al., 1989), CBT has been performed >30,000 times worldwide. Clinical experience has proven that CBT is a therapeutic option alongside BM transplantation (BMT) and peripheral blood stem cell transplantation (Barker et al., 2001; Rocha et al., 2001; Frasson et al., 2003; Takahashi et al., 2004). CBT merits attention for its unique characteristics: easy access to source; no risk to donors; immediate “off-the-shelf” availability; reduced HLA match requirements; and low risk of graft versus host disease (GvHD; Barker et al., 2003; Ballen et al., 2013). Many patients who lack an HLA-matched family or nonfamily donor require alternatives, including umbilical cord blood (UCB) or HLA-haploidentical donors. The recent approach taken to improve transplantation using T cell replete grafts from HLA-haploidentical donors and, thereafter, cyclophosphamide to control GvHD, has been shown to be successful and is rapidly spreading worldwide (Luznik et al., 2002, 2008, 2012; Luznik and Fuchs, 2010).

CBT has the major drawback of delayed engraftment resulting from low graft cell numbers, which often limits its use in adult recipients (Laughlin et al., 2001; Wagner et al., 2002; Rodrigues et al., 2009). Current recommendations (Gluckman and Rocha, 2009) suggest 2.5×10^7 nucleated cells (NCs)/kg in graft UCB. In a 60-kg patient, $>1.5 \times 10^9$ NCs would be necessary. However, available single-banked UCB units often contain fewer NCs. Most UCB units in Japan therefore remain unused clinically because of their insufficient graft cell doses (unpublished data). These problems prompted us to seek a new strategy to improve CBT by using multiple units (more than three).

To overcome the cell dose barrier, double-unit CBT has been trialed clinically. It failed to demonstrate significant early engraftment advantages over single-unit CBT (Sanz and Sanz, 2002; Kindwall-Keller et al., 2012; Ruggeri et al., 2014; Wagner et al., 2014). CBT with up to 5 units to provide higher numbers of NC also was not associated with improved kinetics of reconstitution in donor-derived hematopoiesis (Fanning et al., 2008). Multiple unmanipulated whole-UCB units were used in this trial, permitting the inference that un-

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Abbreviations used: BMT, BM transplantation; CB, cord blood; CBT, CB transplantation; GvHD, graft versus host disease; HSC, hematopoietic stem cell; HSPC, hematopoietic stem/progenitor cell; KSL, c-Kit⁺, Sca-1⁺, lineage⁻; KuO, Kusabira Orange; MNC, mononuclear cell; NC, nucleated cell; UCB, umbilical cord blood; WBM, whole BM.

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favorable interactions among mature cells from the individual units, such as B cells, T cells, and dendritic cells, may have disturbed transplantation outcomes, with multidirectional competition between units. We hypothesized that multiple-unit CBT using isolated hematopoietic stem/progenitor cells (HSPCs) from each unit might deploy only profitable effects and result in better transplantation outcomes. We sought to determine if to combine allogeneic multiple-donor-derived HSPCs, irrespective of disparities in donor MHC types, could accelerate early hematopoietic recovery.

We here provide proof of feasibility of such an approach using mouse and xenotransplantation models by appropriately manipulating multiple allogeneic grafts. To our knowledge, this is the first report formally providing experimental evidence of benefits from multiple-donor transplantation.

RESULTS

Allogeneic progenitors in combination can contribute to donor hematopoiesis

To demonstrate that combined allogeneic multiple-donor HSPCs could accelerate early hematopoietic recovery after transplantation regardless of MHC matching, we used mouse BM c-Kit⁺, Sca-1⁺, lineage-marker-negative (KSL) cells as a model donor cell source (Osawa et al., 1996). KSL cells contain HSPCs, but not mature immune cells. They may thus be considered a counterpart of human CD34⁺ cells. To mimic a clinical setting of single-unit CBT, where the cell dose is insufficient for a patient, we first titrated KSL cells in a C57BL/6 (B6) congenic transplantation model by monitoring radioprotective effects in lethally irradiated recipients. As shown in Fig. 1 A, titration studies revealed that 500 B6-Ly5.1 KSL cells were insufficiently radioprotective, whereas transplantation of 2,000 cells rescued all irradiated mice (100%). Similar titration studies confirmed that 500 KSL cells from other allogeneic strains were also insufficient to radioprotect recipient mice (Fig. 1 B). We selected 4 mouse strains as allogeneic donor cell sources: BDF1 (DBA2 × B6 F1, H2b/d), B6D1F1 (DBA1 × B6 F1, H2b/q), B6C3F1 (C3H × B6 F1, H2b/k), and CBF1 (BALB/c × B6 F1, H2b/d). We used F1 strains to avoid inducing GvHD, which might compromise estimation of donor cell engraftment kinetics. Outcomes were compared between mice (B6-Ly5.2) given mixed allogeneic KSL cells (500 cells from each strain, 2,000 cells in total) and mice given congenic B6-Ly5.1 KSL cells (2,000 cells). Mice in both cohorts also received otherwise insufficient doses (500 cells) of B6-Ly5.1 KSL cells (Fig. 1 C). As shown, multiple allogeneic HSPCs, combined, rescued lethally irradiated recipients as effectively as did the same dose of congenic-HSPCs (Fig. 1 D). More importantly, the recipient mice protected by combined allogeneic HSPCs showed prompt peripheral hematopoietic recovery within 2–3 wk, with kinetics undistinguishable from those observed for the recipients of congenic HSPCs alone (Fig. 1 E). These results suggest robust contribution to early hematopoietic reconstitution by a mixture of allogeneic HSPCs. Details of donor cell contribution were determined

by flow cytometry analysis of peripheral blood (PB), using a combination of mAbs detecting distinct MHC and CD45 (Ly5) subtypes (Fig. S1, A and B). As shown, all allogeneic grafts contributed to donor chimerism 2–8 wk after transplantation (Fig. 1 F, right). Interestingly, however, mixed chimerism was sustained in this setting even long-term (up to 12 wk) in PB (from another independent experiment; Fig. S2, bottom); multiple-donor engraftment was also confirmed in BM at 24 (Fig. 1 F) and 30 wk (Fig. S2, bottom). These results demonstrated that combined allogeneic HSPCs were capable of beneficial contribution to donor hematopoietic reconstitution, but that transplantation of HSPCs alone led to long-term existence of multidonor hematopoiesis. Although whether mixed chimerism is favorable or unfavorable to patient outcome remains unclear, likely depending on many variables in each clinical setting, we next sought to find a way to achieve single-donor chimerism even after infusion of multiple combined allogeneic HSPCs.

Modified multiple-donor transplantation enables timely enhancement of early hematopoiesis, followed by single-donor chimerism

Timely enhancement of early hematopoiesis should benefit CBT patients. Stable single-donor chimerism after multiple-donor transplantation can also be desirable clinically. To test whether modeling such transplantation was feasible, and whether single-donor chimerism could be achieved in this setting, we replaced B6-Ly5.1 KSL grafts with B6-Ly5.1 whole BM (WBM) grafts. In this scenario, WBM cells were expected to serve as a principal unit responsible for long-term hematopoietic reconstitution. The mixed units of allogeneic HSPCs, in contrast, were anticipated to work transiently for early hematopoiesis only. We performed a titration experiment using B6 WBM cells to determine an insufficient dose to model single-dose CBT, as we did with KSL cells (Fig. 1 A). As shown in Fig. 2 A, 50,000 WBM cells proved insufficient to radioprotect irradiated recipients, whereas infusion of four times as many cells (2×10^5 cells) allowed survival of most recipients (80%). We thus decided to mimic transplantation of a single-dose unmanipulated UCB unit by infusing 50,000 B6-Ly5.1 WBM cells into B6-Ly5.2-recipient mice. Effects of additional infusion were compared between combined multiple allogeneic HSPCs and congenic HSPCs (Fig. 2 B). As shown, infusion of mixed allogeneic KSL cells in this model completely protected recipients (100%) as effectively as the same dose of congenic KSL cells (Fig. 2 C). In sharp contrast with the previous KSL cells alone model, mixed-donor chimerism in recipient PB was observed for only 2–4 wk; dominant B6-Ly5.1 chimerism was eventually established and maintained long-term (20 wk; Fig. 2 D). Another independent experiment also confirmed single-donor chimerism, with B6-Ly5.1 hematopoiesis in BM long-term (30 wk) after transplantation (Fig. S2, middle). Although mixed allogeneic grafts only transiently contributed to donor cell chimerism in this model, they were capable of enhancing early hematopoi-

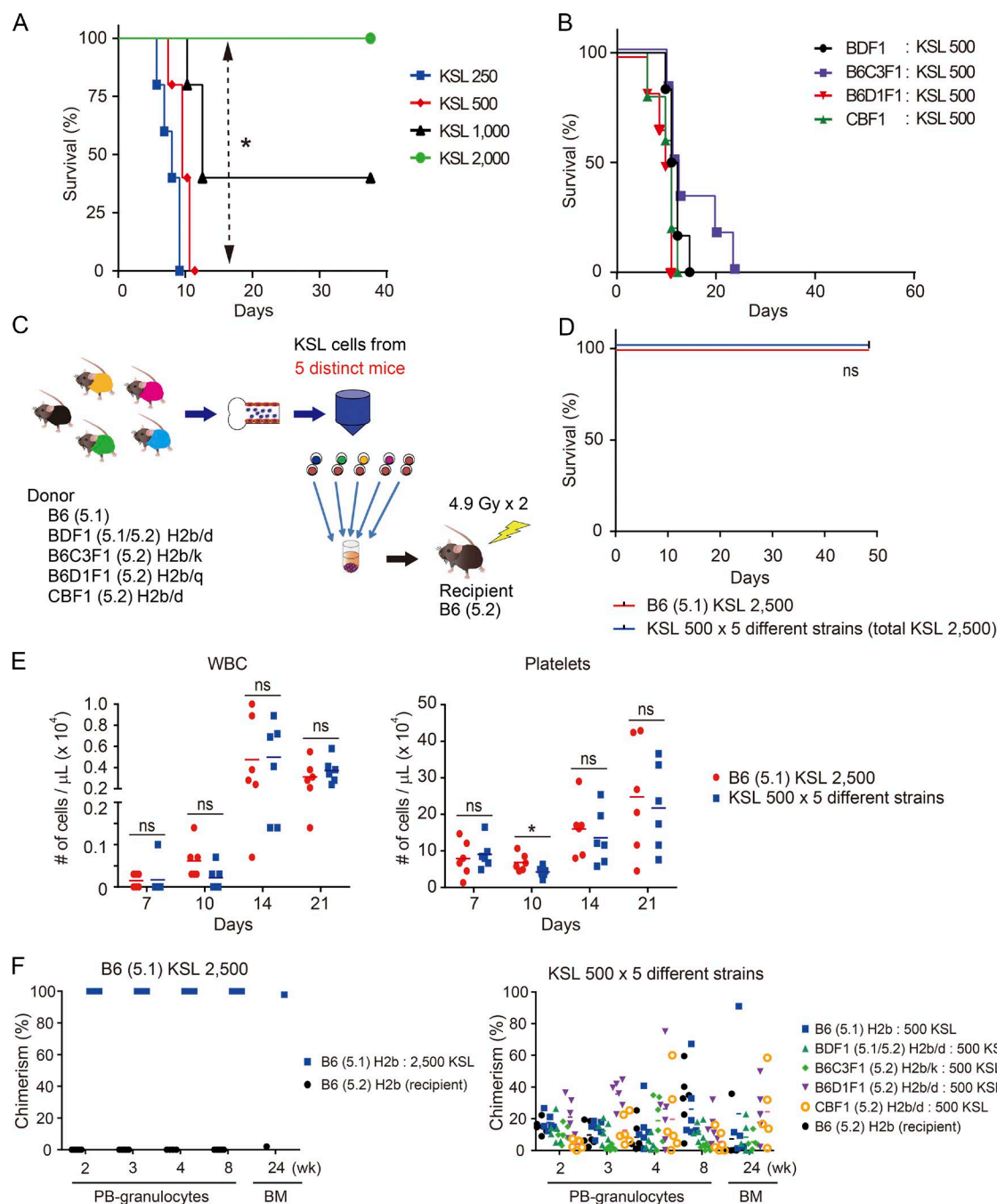


Figure 1. Modeling transplantation of multiple allogeneic progenitors. (A) Cell dose titration by survival assay in congenic mouse transplantation. C57BL/6 (B6-Ly5.2, H2b) mice received cell grafts; congenic B6-Ly5.1 mice (H2b) were principal donors. Recipient mice were transplanted with the indicated dose of KSL cells after lethal irradiation (4.9 Gy \times 2). A Kaplan-Meier plot shows recipient survival rates. $n = 5$ in each group. Data were analyzed by log-rank testing. $P = 0.0026$. $P < 0.008$, Bonferroni-corrected threshold, was considered statistically significant. *, $P <$ indicated Bonferroni-corrected threshold. (B) Kaplan-Meier plot showing survival rates of recipient B6 mice transplanted with the indicated grafts. $n = 6$ in each group. (C) A model of transplantation using multiple-donor-derived KSL cells. Recipient mice are B6-Ly5.2; principal donor mice are B6-Ly5.1. The four allogeneic mouse strains shown are used as additional graft donors. (D) Kaplan-Meier plot showing survival among mice transplanted with congenic KSL 2,500 cells (red) and with mixed allogeneic KSL 2,500 cells (blue). $P < 0.05$ was considered statistically significant. ns, not significant. $n = 6$ in each group. (E) Results of complete blood counts in PB from recipients transplanted with congenic KSL 2,500 cells (red) and with mixed allogeneic KSL 2,500 cells (blue) at indicated times. Mann-Whitney testing was used for statistical analysis. Mean values are indicated as bars. $P < 0.05$ was considered statistically significant. $n = 6$ in each group. (F) Percentages of peripheral donor granulocyte and BM chimerism in individual recipients after transplantation. $n = 6$ in each group (some death observed at 24 wk). Mean values are indicated as bars. Representative data are shown from at least two independent experiments for each panel.

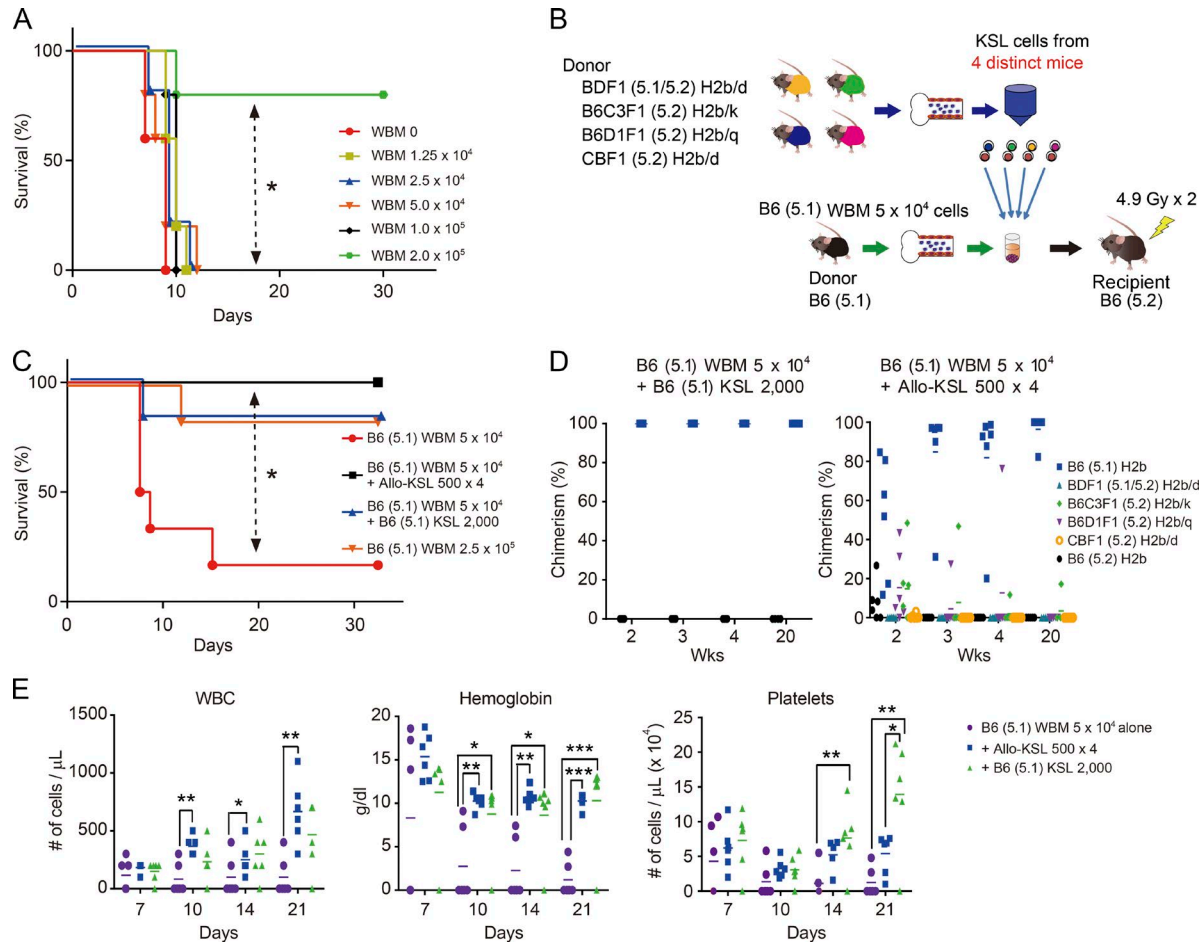


Figure 2. A modified multiple-donor transplantation model using combined allografts yields single-donor chimerism with beneficial radioprotective and bridging effects. (A) Cell dose titration by survival assay in congenic mouse transplantation conducted as shown in Fig. 1 A. Whole BM (WBM) cells were used instead of KSL cells as test cells. $n = 5$ in each group. $P = 0.0031$; $P < 0.01$, Bonferroni-corrected threshold, was considered statistically significant. *, $P < 0.05$, Bonferroni-corrected threshold. (B) The experimental protocol for testing radioprotective and bridging effects in combined allogeneic KSL cells in a modified model. In brief, B6-Ly5.1 KSL cells shown in Fig. 1 C are replaced by B6-Ly5.1 WBM cells. 500 KSL cells from allogeneic strains and 5×10^4 congenic WBM cells are used. (C) Kaplan-Meier plot showing survival among mice receiving indicated grafts. Allogeneic-KSL, Allo-KSL. $n = 6$ in each group. Log-rank testing was used to analyze survival data. $P = 0.0039$. $P < 0.016$, Bonferroni-corrected threshold, was considered statistically significant. *, $P < 0.05$, Bonferroni-corrected threshold. (D) Percentages of peripheral donor-granulocyte chimerism in individual recipients transplanted with congenic grafts (left) and combined allogeneic grafts (right) as a supportive unit. $n = 6$ in each group. Mean values are indicated as bars. (E) Results of complete blood counts in PB from recipients transplanted with congenic grafts alone (purple and green) or mixed allogeneic grafts in addition to congenic WBM (blue). Values in dead mice were regarded as 0 for the analysis. One-way ANOVA with Tukey's multiple comparison test was used for statistical analysis. $n = 6$ in each group. Mean values are indicated as bars. $P < 0.05$ was considered statistically significant. *, $P < 0.05$; **, $P < 0.01$. Representative data are shown from at least two independent experiments for each panel.

etic recovery (Fig. 2 E). Those beneficial effects (henceforth called bridging effects) were clear for leukocyte (WBC) and erythrocyte (hemoglobin) lineages. They were not evident for thrombocyte recovery (platelets).

T lymphocytes within the congenic graft are responsible for single-donor chimerism

We next sought to elucidate how co-infusion of WBM cells other than KSL cells and of mixed allogeneic HSPCs resulted in B6-Ly5.1 single-donor chimerism. We initially simplified our transplantation model by replacing combined allogeneic

neic KSL cells with KSL cells of only a single donor type (BDF1) to assess whether infusion of test cell populations of B6 origin would limit donor cell contribution from co-infused allogeneic BDF1 KSL cells (Fig. 3 A). Rag2 KO mice (Fig. 3 B) and TCR- α chain KO mice (Fig. 3 C) were used to examine how mature immune cells within B6 WBM grafts affected donor chimerism (Shinkai et al., 1992; Yoshimasu et al., 2004). Consistent with the results of multiple-donor transplantation experiments, infusion of WT B6-KSL cells allowed mixed chimerism with BDF1 hematopoiesis derived from co-infused KSL cells (Fig. 3, B–D, G2). In contrast, WT

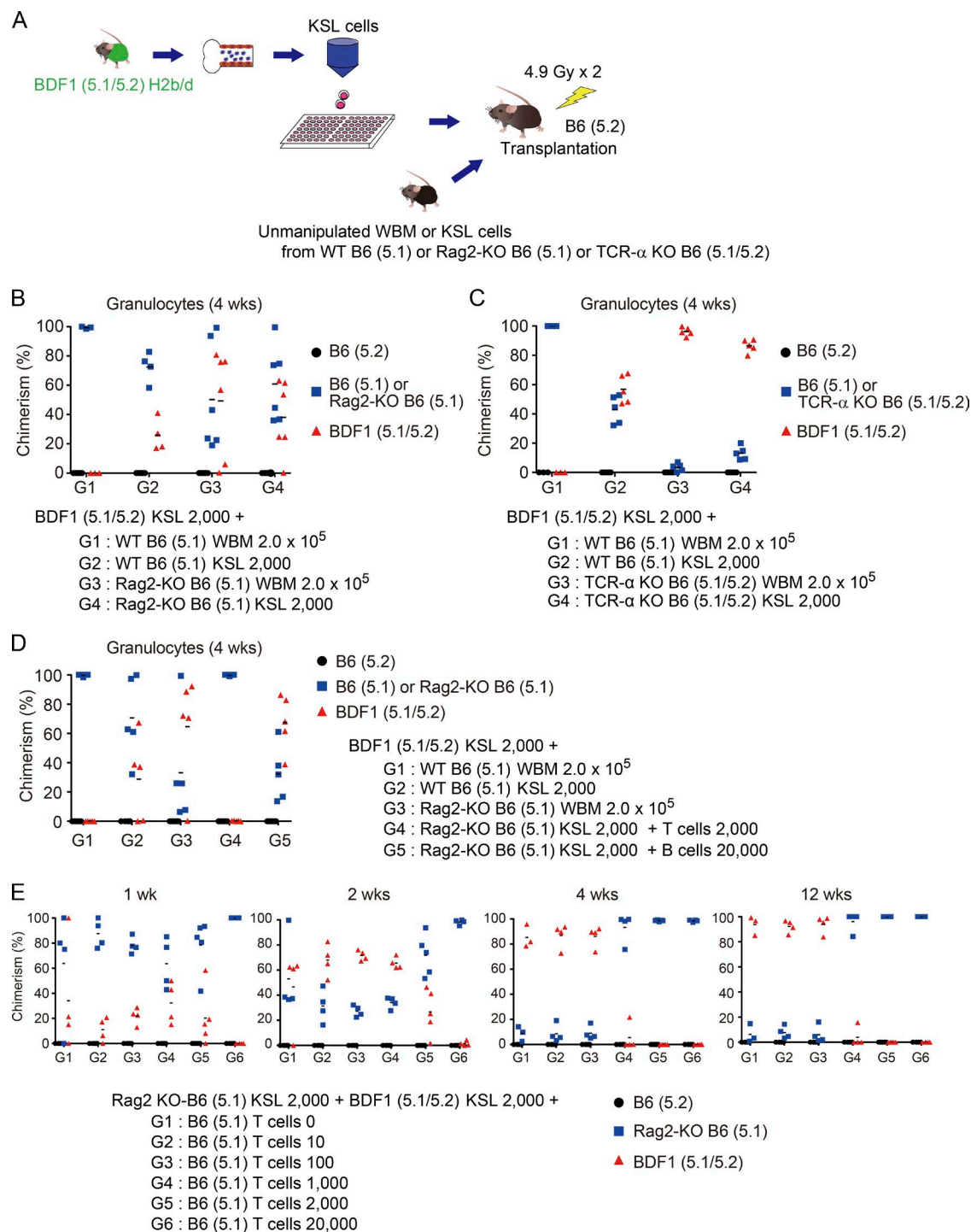


Figure 3. T cells are the critical determinant of single-donor chimerism in transplantation of mixed multiple donor cells. (A) Schematic representation of the experiment. In this simplified model, two donor strains, BDF1 and B6 (Ly5.1), are used. B6 mice are WT, Rag2-KO, or T cell receptor- α chain KO. KSL cells from BDF1 are cotransplanted into recipient B6 (Ly5.2) mice with unmanipulated WBM cells or with KSL cells from B6 strains. (B) Results of cotransplantation of BDF1 and Rag2-KO donor grafts. Percentages of peripheral donor chimerism in granulocytes in individual recipients transplanted with each indicated graft at 4 wk after transplantation. $n = 5$ in each group. Mean values are indicated as bars. (C) Results of cotransplantation of BDF1 and TCR- α KO donor grafts. Percentages of peripheral donor chimerism in granulocytes are shown. $n = 3$ or 5 in each group. Mean values are indicated as bars. (D) Effects on chimerism formation of lymphocytes co-infused with a mixture of Rag2-KO WBM cells and BDF1 KSL cells. Percentages of peripheral donor chimerism in granulocytes at 4 wk in individual recipients. $n = 4$ or 5 in each group. Mean values are indicated as bars. (E) T cell dose titration experiment to convert mixed chimerism into single-donor chimerism. Peripheral blood chimerism was analyzed over time. $n = 3$ –5 in each group. Mean values are indicated as bars. Representative data are shown from at least two independent experiments for each panel.

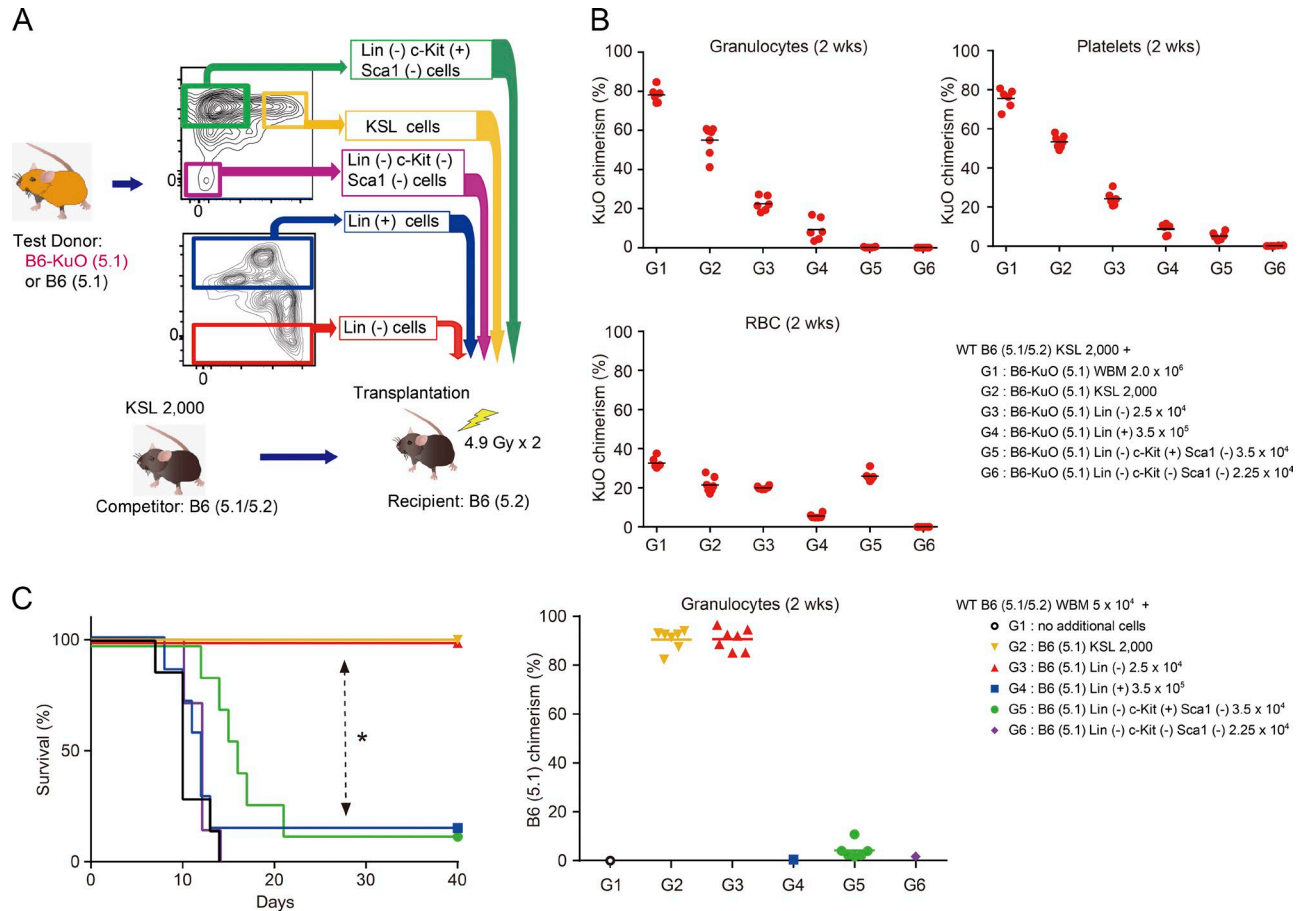


Figure 4. Testing potential in BM subfractions responsible for radioprotection and early hematopoietic reconstitution after transplantation. (A) The experimental protocol to determine which BM subfractions are responsible for early hematopoiesis of granulocytes, erythrocytes, and megakaryocytes after transplantation. Indicated fractions obtained from KuO-Tg- or WT-B6 (Ly5.1) BM are cotransplanted competitively with WT-B6 (Ly5.1/5.2) mice KSL cells into lethally irradiated B6 (Ly5.2) recipients. (B) Percentages of KuO⁺ cell contribution in granulocytes, platelets, and erythrocytes (RBC) in recipient peripheral blood 2 wk after transplantation. Mean values are indicated as bars. $n = 5$ in each group. (C, left) A Kaplan-Meier plot showing survival among mice transplanted with indicated grafts. Log-rank testing was used to analyze survival data. $P < 0.0001$; $P < 0.0125$, Bonferroni-corrected threshold, was considered statistically significant. *, $P < 0.0125$, Bonferroni-corrected threshold. (right) Percentages of B6 (Ly5.1) donor cell contribution in granulocytes in recipient peripheral blood 2 wk after transplantation. Mean values are indicated as bars. $n = 7$ in each group (only one mouse was alive and available at day 14 each for G1, G4, and G6, because of insufficient radioprotection). Representative data are shown from at least two independent experiments for each panel.

WBM cells of B6 origin completely eliminated donor cell contribution by co-infused BDF1 KSL cells, resulting in single-donor chimerism (Fig. 3, B–D, G1). Dominant B6 donor chimerism failed when B6 WBM grafts were genetically depleted of lymphocytes (Rag2 KO) and T cells (TCR- α KO; Fig. 3, B and C, G3). To identify chimerism-enhancing effects of immune cells in grafts more specifically, we tested whether infusion of purified T or B cells of B6 origin could convert mixed chimerism (Fig. 3 D, G3) to single-donor hematopoiesis in mice that received Rag2 KO cells in addition to BDF1 KSL cells. As shown in Fig. 3 D, addition of T cells (G4), but not B cells (G5), conferred ability to establish complete B6 donor chimerism. Dose titration experiments demonstrated a threshold in T cell numbers for full elimination of donor cell

contribution from BDF1 allogeneic grafts (Fig. 3 E). Overall, these results suggested that, in this model, transplantation of a principal unit containing mature T cells achieved long-term single-donor chimerism whereas co-infusion of combined multiple allogeneic HSPCs aided early hematopoiesis via bridging effects during a phase of myelosuppression.

Primitive cell fractions are the most potent enhancer of early hematopoiesis

To maximize the bridging effects achieved by multiple allogeneic units in combination, we examined which subfractions in BM cells were responsible for hematopoiesis in each lineage early after transplantation (Fig. 4 A). Early recovery of platelet counts was particularly important, given the risk of

fatal thrombocytopenia during myelosuppression. To enable assessment of donor cell contribution in erythroid and platelet lineages, we used a transgenic B6 mouse line expressing the fluorescent marker Kusabira Orange (KuO; Hamanaka et al., 2013) as a source of test donor cells. Various subfractions obtained from KuO BM cells were infused into lethally irradiated recipient mice at optimized cell doses, in addition to unlabeled B6 KSL cells. Donor cell contributions in PB were estimated as %KuO⁺ cells 2 wk later. As shown in Fig. 4 B, KuO⁺ KSL cells and WT B6-KSL cells were equal donor cell contributors, with ~50% chimerism observed in both granulocytes and platelets (G2). In respect of donor cell contribution to these two lineages, other tested subfractions that included more committed progenitors, i.e., a Lin[−]/c-Kit⁺/Sca-1[−] population, did not outperform KSL cells (Fig. 4 B, G2 vs. G3–6). Interestingly, this progenitor population showed durable contribution only to RBC chimerism as efficiently as did KSL cells, not to two other lineages, suggesting that it contained active erythroid-committed progenitors (Fig. 4 B, G2 vs. G5). In addition, with a similar fractionation experiment in a radioprotection assay, KSL cells, and Lin[−] cells as well, showed radioprotective ability, whereas Lin[−]/c-Kit⁺/Sca-1[−] cells exhibited only marginal effects (Fig. 4 C, left). Consistent with this result, bridging effects on granulocyte recovery were apparent only for the former two populations, but not for others (Fig. 4 C, right). Collectively, KSL cells, including the most primitive HSCs (defined as CD34^{−/low} KSL cells; Osawa et al., 1996), exerted potent radioprotective and bridging effects, whereas more committed cells did not.

Cultured HSCs efficiently enhance early hematopoiesis, including platelet recovery, during myelosuppression

We then sought to use the long-term HSC fraction experimentally defined in mouse studies (Fig. S3; Osawa et al., 1996; Ema et al., 2006) to exert bridging effects. Because cells in this fraction are scarce, we attempted to amplify early reconstitution ability in HSCs by culturing them in defined serum-free conditions compatible with stem cell amplification (Ema et al., 2000; Lai et al., 2014). To this end, we prepared test cells cultured from 50 B6-Ly5.1 HSCs for varying intervals. As shown in Fig. 5 (A and B), the increase in overall cell numbers peaked on day 7, reaching ~25,000 cells (~500 times); despite massive expansion, HSCs cultured for 7 d (7-day cultured HSCs) retained a lineage-negative (99.3 ± 0.4%) and a lineage[−]/c-Kit⁺ immunophenotype (97.0 ± 0.5%), with a subpopulation showing KSL characteristics (6.8 ± 1.8%). Cells cultured beyond day 8 started showing signs of differentiation (Fig. 5 A) and exhaustion, reflected by a decline in numbers (Fig. 5 B). We then competitively transplanted these 7-d cultured cells into lethally irradiated recipients with 10⁶ B6-Ly5.1/5.2 WBM cells (Fig. 5 C). The early donor cell contribution to myeloid cell lineages from these cells was remarkable (Fig. 5 D). We then sought to determine the cells that mediated bridging effects within these cultured cells. Given that 7-d cultured CD34[−] KSL cells are

almost all Lin[−] c-Kit⁺ (Lai et al., 2014), we divided these cells into three fractions using CD48 and Sca-1 markers (Fig. 5 E, left). We sorted these cells and tested their capacity for hematopoietic contribution in a competitive manner. Interestingly, the CD48[−], Sca-1⁺ fraction (blue) showed stable contribution for up to 4 wk, compatible with its containing long-term reconstituting cells (Noda et al., 2008). In contrast, CD48⁺ Sca-1⁺ c-Kit⁺ Lin[−] (shown in red) and CD48⁺ Sca-1[−] c-Kit⁺ Lin[−] (green) fractions contributed only to early hematopoiesis (1–2 wk), suggesting that these fractions contained cells capable of bridging effects (Fig. 5 E, right). When KuO⁺ HSCs cultured for 7 d were tested for bridging effects (Fig. 5 F), an early contribution to donor hematopoietic reconstitution was clear in both erythroid and platelet lineages. These results suggest that appropriate cultivation methods can render HSCs ideal as a source of supportive cells that will exert bridging effects in transplantation.

Multiple cultured allogeneic HSCs in combination exert ideal radioprotective and bridging effects

We tested whether cultured allogeneic HSCs could support early hematopoiesis without hampering subsequent long-term single-donor chimerism, as such effects would be ideal for many occasions. HSCs purified from four F1 strains allogeneic to parental B6 strains, including one expressing KuO fluorescence, were cultured for 7 d, combined, and cotransplanted into lethally irradiated B6-Ly5.2 mice, in addition to 10⁵ B6-Ly5.1/5.2 WBM cells (Fig. 6 A). As with cultured B6 HSCs (Fig. 5 B), these cultured allogeneic HSCs (initial input, 50 cells) retained lineage[−]/c-Kit⁺ (~95–100%) and KSL immunophenotypes (~10%), despite massive expansion (not depicted). Both radioprotective and bridging effects were evident with combined cultured allogeneic HSCs. They completely rescued lethally irradiated mice receiving a limited number of B6-Ly5.1/5.2 WBM, as did cultured B6-Ly5.1 congenic HSCs (Fig. 6 B). Bridging effects on leukocyte recovery (WBC) and erythropoiesis (hemoglobin) were also apparent (Fig. 6 C). Notably, with thrombocyte recovery (platelets), effects early after transplantation (day 7) were even stronger for multiple allogeneic units than for a control congenic unit (Fig. 6 C). Detailed flow cytometry analysis (Fig. S4 A) demonstrated transient (7–21 d) contribution by allogeneic grafts to donor cell chimerism in granulocytes (Fig. 6 D, right) and in both platelets and erythrocytes (Fig. 6 E and Fig. S4 B). These results indicate that appropriate treatment permits combined allogeneic HSPCs to serve as supportive grafts that exhibit ideal radioprotective and bridging effects.

Multiple cultured allogeneic HSCs in combination also can exert radioprotective and bridging effects, despite full mismatch of MHC alleles

These experiments primarily used F1 mouse strains as allogeneic cell sources to exclude the influence of GvHD. We also examined whether fully mismatched MHC allogeneic strains, i.e., DBA/1Jms Slc (DBA1, H2q, and Ly5.2), DBA/2Cr Slc

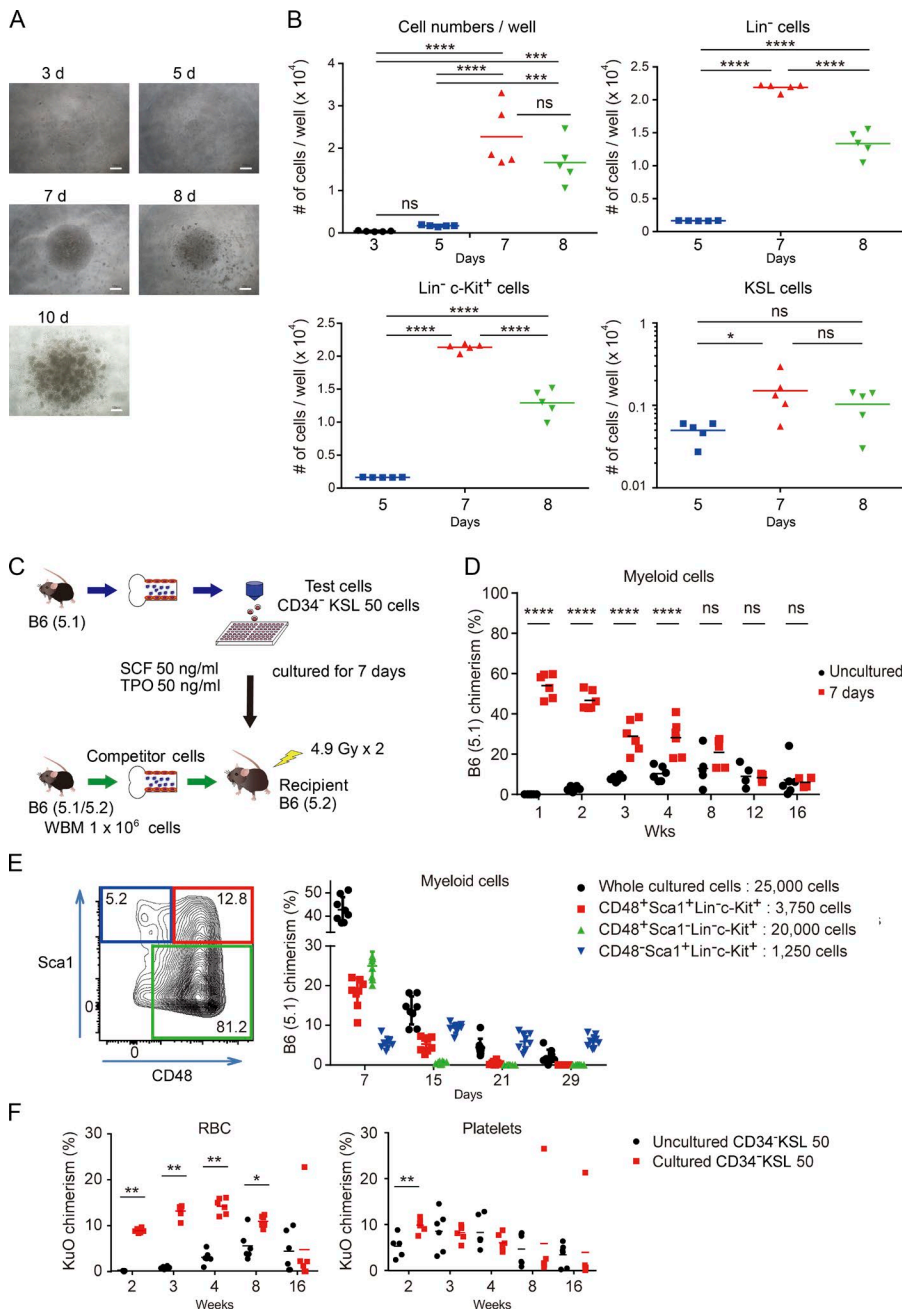


Figure 5. Cultured HSCs can enhance early hematopoietic recovery. (A) Photomicrographs of cultured HSCs at indicated times. Photos were taken and recorded by NIKON digital sight DS-Fi1, L2, and U2 (NIKON) through a microscope (NIKON ECLIPSE TS100, ocular lenses 10X, objective lenses 4X NA 0.13, W.D 16.5). Bars, 300 μ m. (B) Numbers of cultured cells in total or of each indicated population in a well counted by flow cytometry (input = CD34⁺KSL: 50 cells). One-way-ANOVA with Tukey's multiple comparison test was used for statistical analysis. $n = 5$ in each group. Mean values are indicated as bars. $P < 0.05$ was considered statistically significant. *, $P < 0.05$; ***, $P < 0.0001$; ****, $P < 0.00001$. (C) The experimental protocol to test bridging effects in cultured HSCs. Cultured or uncultured HSCs (CD34⁺c-Kit⁺Sca1⁺Lin⁻ cells) derived from B6 (Ly5.1) mice were competitively transplanted with uncultured B6 (Ly5.1/5.2) WBM cells into lethally irradiated B6 (Ly5.2) mice. (D) Donor cell (B6-Ly5.1) contribution to a PB myeloid lineage (granulocytes and monocytes) assessed at indicated times. $n = 6$ in each group. Mann-Whitney test was used for statistical analysis. Mean values are indicated as bars. $P < 0.05$ was considered statistically significant. ****, $P < 0.00001$. (E, left) Phenotypic characteristics of 7-d cultured CD34⁺KSL cells. Note that these cells in total are mostly Lin⁻c-Kit⁺ (~97–98%). Listed subfractions (CD48⁺Sca1⁺Lin⁻c-Kit⁺, 12.8%; CD48⁺Sca1⁻Lin⁻c-Kit⁺, 81.2%; CD48⁺Sca1⁺Lin⁻c-Kit⁺, 5.2%) derived from B6 (Ly5.1) were competitively transplanted with 10⁶ WBM cells from B6 (Ly5.1/5.2) into lethally irradiated B6 (Ly5.2) mice. (right) Donor cell (B6-Ly5.1) contribution to a PB myeloid lineage (granulocytes and monocytes) assessed at indicated times. $n = 8$ in each group. Mann-Whitney testing was used for statistical analysis. Mean values are indicated as bars. $P < 0.05$ was considered statistically significant. *, $P < 0.05$; **, $P < 0.01$. Representative data are shown from at least two independent experiments for each panel.

(DBA2, H2d, and Ly5.2), and C3H HeJ (C3H, H2k, and Ly5.2), could similarly exert favorable radioprotective and bridging effects (Fig. 7 A). HSCs obtained from these strains of mice proliferated well in defined 7-d culture without drastic loss of primitive immunophenotypes (unpublished data). A mixture of these cultured allogeneic HSCs clearly exerted robust radioprotection (Fig. 7 B) and enhanced early hematopoietic recovery (Fig. 7 C). These effects were beneficial: they permitted stable single-donor chimerism in irradiated recipi-

ents (Fig. 7, D and E; and Fig. S5) that, as they received only a limited number (10⁵) of unmanipulated B6-Ly5.1/5.2 WBM cells as a principal graft, would otherwise have died (Fig. 7 B).

Successful preparation of transplantable HSPCs from multiple frozen human UCB units

Finally, as the initial step to translate our findings clinically, we attempted to isolate cells that exert bridging effects using frozen human UCB units in combination. The counterpart of

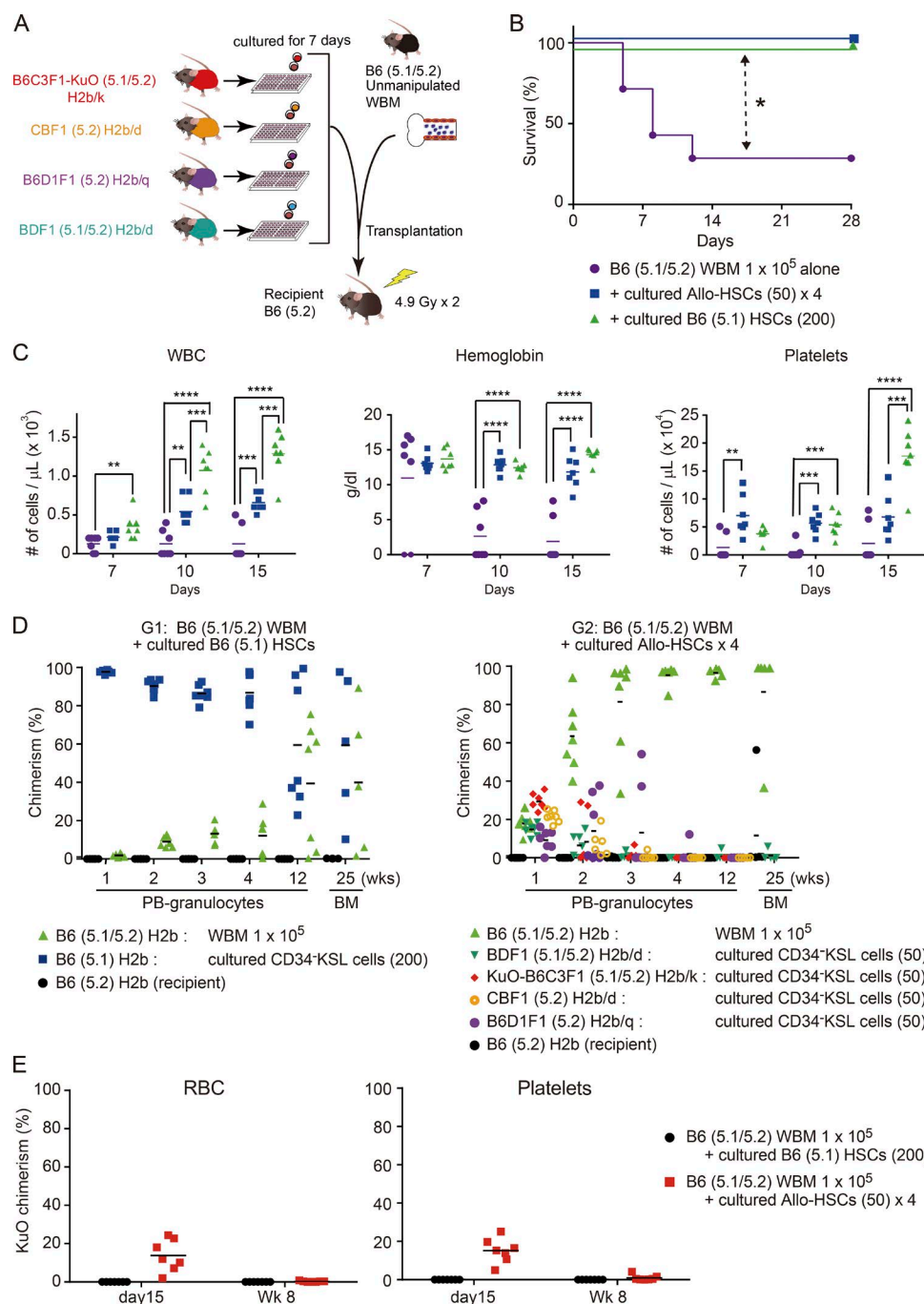


Figure 6. Combined cultured F1 HSCs support early hematopoiesis, while allowing single-donor chimerism from a congenic graft. (A) The experimental protocol to test cultured allogeneic HSCs for their radioprotective and bridging effects in a transplantation model. HSCs (50 cells) harvested from the four allogeneic mouse strains indicated were cultured for 7 d, combined, and transplanted together with uncultured WBM cells derived from B6 (Ly5.1/5.2) into lethally irradiated B6 (Ly5.2) recipients. (B) Kaplan-Meier plot showing survival among mice transplanted with indicated grafts. Allogeneic HSCs, Allo-HSCs. $n = 7$ in each group. Log-rank testing was used to analyze survival data. $P = 0.0066$. $P < 0.025$, Bonferroni-corrected threshold, was considered statistically significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (C) Results of complete blood counts in PB, WBC (left), hemoglobin (middle), and platelets (right), from recipients transplanted with each indicated graft (same experiment as Fig. 6 B). $n = 7$ in each group. One-way ANOVA with Tukey's multiple comparison test was used for statistical analysis. Mean values are indicated as bars. $P < 0.05$ was considered statistically significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (D) Percentages of donor-cell chimerism in granulocytes and BM in individual recipients transplanted with each indicated graft at indicated times after transplantation. $n = 7$ in each group. Mean values are indicated as bars. (E) Percentages of %KuO⁺ cells (B6C3F1 donor) in platelets (left) and red blood cells (right) in individual recipients. Mean values are indicated as bars. $n = 7$ in each group. Representative data are shown from at least two independent experiments for each panel.

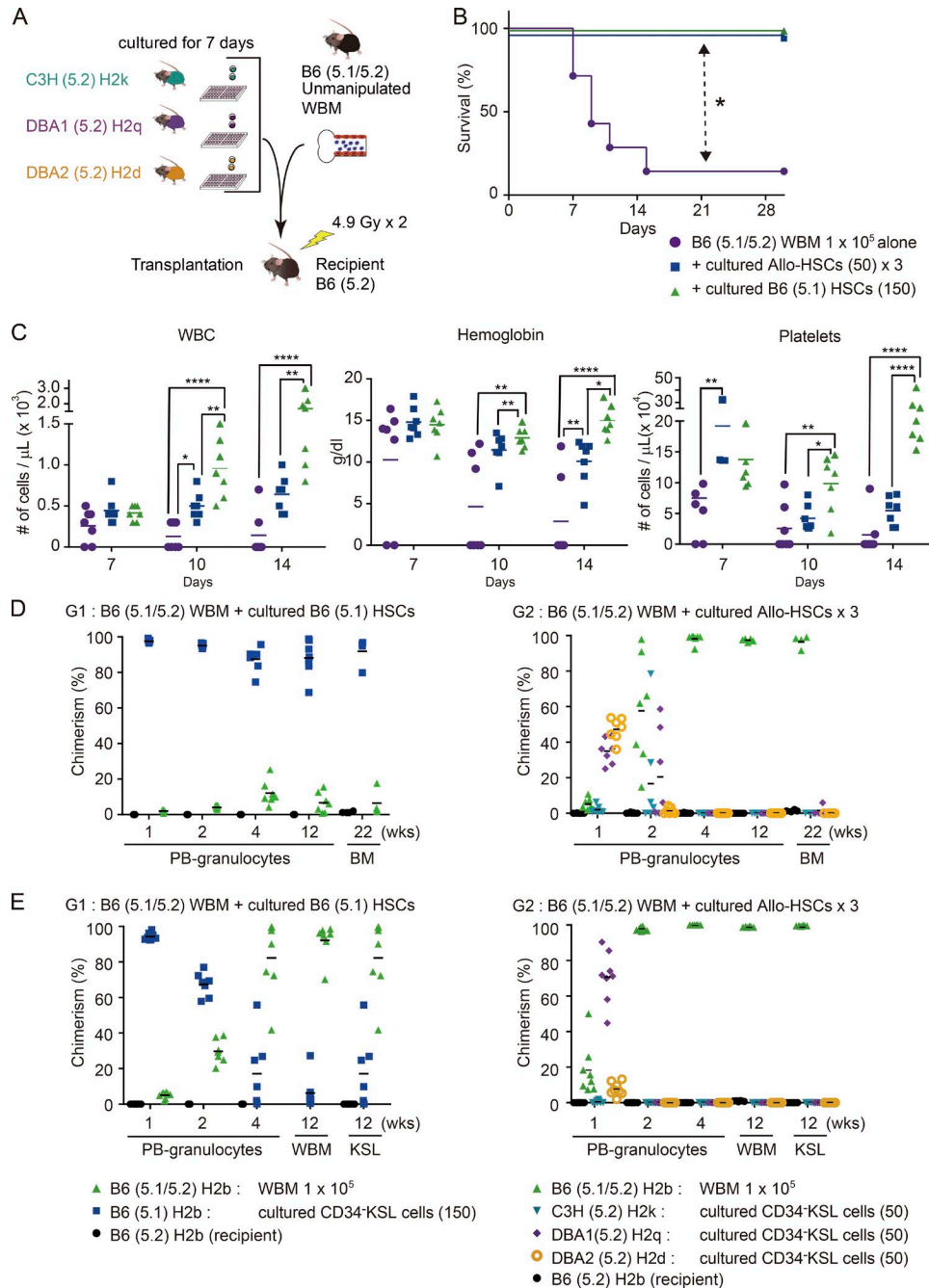


Figure 7. Combined cultured allogeneic HSCs with fully mismatched MHC also exhibit beneficial radioprotective and bridging effects. (A) The experimental protocol used to test radioprotective and bridging effects in cultured allogeneic HSCs of strains fully MHC-mismatched to recipients. Three allogeneic strains, DBA/1JmsSlc (DBA1, H2q, and Ly5.2), DBA/2Cr Slc (DBA2, H2d, and Ly5.2), and C3H/HeJ (C3H, H2k, and Ly5.2), were used. HSCs (50 cells) harvested from these allogeneic mice were cultured for 7 d, combined, and transplanted together with uncultured WBM cells derived from B6 (Ly5.1/5.2) into lethally irradiated B6 (Ly5.2) recipients. (B) Kaplan-Meier plot showing survival among mice transplanted with indicated grafts. Allogeneic HSCs, Allo-HSCs. $n = 7$ in each group. Log-rank testing was used to analyze survival data. $P = 0.0013$; $P < 0.025$, Bonferroni-corrected threshold, was considered statistically significant. *, $P < 0.05$ indicated Bonferroni-corrected threshold. (C) Results of complete blood counts in PB, WBC (left), hemoglobin (middle), and platelets (right), from recipients transplanted with each indicated graft. $n = 7$ in each group. One-way-ANOVA with Tukey's multiple comparison test was used for statistical analysis. Mean values are indicated as bars. $P < 0.05$ was considered statistically significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (D and E) Percentages of donor cell chimerism in granulocytes and BM in individual recipients transplanted with each graft at indicated times after transplantation. $n = 7$ in each group. Mean values are indicated as bars. D and E represent two independent experimental results. To confirm single-donor chimerism originated from a principal graft (B6-5.1/5.2) at the primitive cell levels in group two, percentages of donor cell chimerism were determined in not only whole cells (WBM) but also a KSL fraction in individual recipients' BM at 12 wk in E.

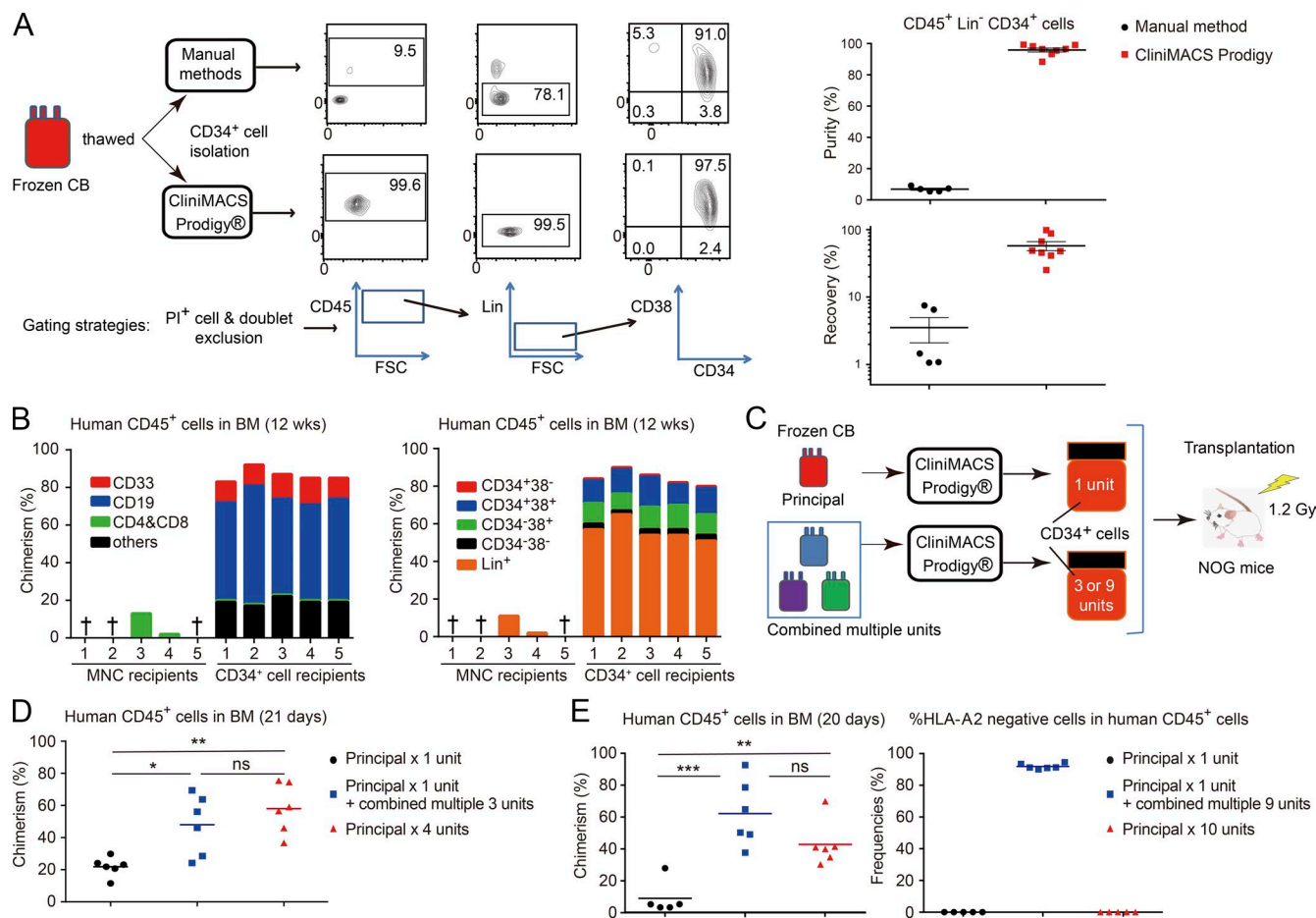


Figure 8. Preparation of HSPCs from frozen human UCB units and their transplantability shown in xenotransplantation. (A) Comparison of procedures and cell-collection efficiencies between a manual method and that using CliniMACS Prodigy. Shown are the purity of CD45⁺CD34⁺ cells (top right) and %recovery (bottom right). $n = 5$ (manual); $n = 8$ (CliniMACS Prodigy). Percent recovery (CD34⁺ cells) was calculated by the ratio between the number of CD34⁺ cells estimated before freezing (provided by Japan Red Cross Society) and the number of CD34⁺ cells isolated. (B) Percentages of human CD45⁺ cells with further characterization by CD33, CD19, CD4, and CD8 (left) and by lineage markers, CD34, and CD38 (right) within BM of individual recipients at 12 wk after transplantation. $n = 5$ in each group. Each bar indicates the chimerism of individual recipients. †, death. (C) The experimental protocol to obtain CD34⁺ cells from multiple UCB units at one time and test their transplantability. One donor UCB was used as a model of principal unit, whereas multiple units were combined before purification of CD34⁺ cells. (D) Results of transplantation that used four unrelated UCB units. A dose of 3.2×10^4 cells was defined as a single unit. Percentages of human CD45⁺ cells in BM of individual recipients at 21 d after transplantation. $n = 6$ in each group. One-way-ANOVA with Tukey's multiple comparison test was used for statistical analysis. Mean values are indicated as bars. $P < 0.05$ was considered statistically significant. *, $P < 0.05$; **, $P < 0.01$. (E) Results of transplantation that used 10 unrelated UCB units. A dose of 1.0×10^4 cells was defined as a single unit. (left) Percentages of human CD45⁺ cells in each indicated recipient BM at day 20. $n = 5$ or 6 in each group. One-way ANOVA with Tukey's multiple comparison test was used for statistical analysis. Mean values are indicated as bars. $P < 0.05$ was considered statistically significant. **, $P < 0.01$; ***, $P < 0.001$. (right) Percentages of HLA-A2⁻ cells (representing combined multiple units) in human CD45⁺ cells among recipients. Mean values are indicated as bars. The setting shown in D was tested twice independently, whereas the 10-unit experiment in E was performed once.

murine BM KSL cells was human UCB CD34⁺ cells, representing a progenitor-rich population containing HSCs, shown in a xenotransplantation model to determine pace of engraftment in a dose-dependent manner (Liu et al., 2010). Repeated attempts initially failed to obtain viable CD34⁺ cells from frozen UCB units; success with frozen samples, enabling use of banked UCB stocks with relatively small cell numbers, was preferable to fresh UCB units. The CliniMACS Prodigy, an all-in-one cell-processing instrument, efficiently harvested viable mononuclear cells (MNCs) after protocol optimization, and vi-

able CD34⁺ cells as well from frozen UCB cells (see Materials and methods). The use of CliniMACS Prodigy improved both purity and yields of CD34⁺ cells, with percentage of recovery being >10 times higher than that achievable with a standard manual purification method for MNCs (% recovery [mean \pm SEM]; manual, $3.5 \pm 1.4\%$ vs. Prodigy, $57.6 \pm 8.7\%$; Fig. 8 A). We then tested transplantability and long-term engraftment ability of these isolated UCB CD34⁺ cells in a xenotransplantation setting. As shown, these CD34⁺ cells successfully engrafted into sublethally irradiated immunodeficient mice

and showed long-term multilineage reconstitution of human hematopoiesis (Fig. 8 B). Recipients of unfractionated MNCs showed frequent death (three out of five), most likely caused by xeno-GvHD (unpublished data), and allowed only marginal human T cell engraftment in survivors (Fig. 8 B). These results prompted us to exclude the use of human grafts containing T cells in xenotransplantation and to attempt modeling multiple CBT only in “HSPCs alone” settings. With these limitations, we first attempted to extend our novel method to isolation of mixed CD34⁺ cells from multiple frozen UCB units at one time using CliniMACS Prodigy (Fig. 8 C). After thawing multiple UCB units, we combined them for Prodigy processing. This successfully yielded viable mixed CD34⁺ cells (% recovery [mean \pm SEM]; 37.2 \pm 5.0%). These cells in combination repopulated recipient mouse BM 3 wk after transplantation as efficiently as did the same dose of single unit-derived CD34⁺ cells. (Fig. 8 D). Up to nine thawed UCB units could be combined in a single bag before MNC and CD34⁺ cell separation. When transplanted together with single-unit CD34⁺ cells (HLA-A2 positive), these mixed CD34⁺ cells in combination extensively contributed short-term to human cell chimerism in mouse BM, as indicated by the dominant (~90%) presence of human cells that were HLA-A2⁺, a marker common to the combined 9 units (Fig. 8 E). This work overall establishes, with proof of concept, that this novel strategy of using allogeneic progenitors in combination may feasibly overcome the cell dose barrier in CBT.

DISCUSSION

Clinical studies of double-unit CBT in graft cell dose augmentation have shown inconsistent results (Laughlin, 2004; Barker et al., 2005; Majhail et al., 2006; Kurtzberg, 2011; Kindwall-Keller et al., 2012; Kai et al., 2013; Scaradavou et al., 2013; Somers et al., 2013; Wagner et al., 2014). The consensus is that rapid BM reconstitution has not been achieved (Wagner et al., 2014). Moreover, multiple-unit CBT did not improve donor cell reconstitution kinetics and often resulted in autologous hematopoietic recovery (Fanning et al., 2008), permitting the inference that unfavorable reactions among infused mature lymphocytes from multiple units might have occurred. Some groups have reported that additional purified CD34⁺ cells are effective in rescue (Fernández et al., 2003; Delaney et al., 2010; de Lima et al., 2012). Recently, co-infusion of CD34⁺ cells from HLA-mismatched donors was shown to yield rapid engraftment of neutrophils and platelets in CBT and to lower treatment-related mortality (Kwon et al., 2014). Several clinical studies have also reported that the addition of a graft expanded *ex vivo* by Notch stimulation or co-culture of an unmanipulated graft with mesenchymal stem cells supported early hematopoiesis after CBT (Delaney et al., 2010; de Lima et al., 2012). These findings prompted us to hypothesize that isolated HSPCs devoid of lymphocytes could be combined and used as supportive grafts capable of bridging effects in multiple-donor transplantation.

Using BM KSL cells as a test population, we formally demonstrated that even HSPCs of allogeneic origins could

contribute to donor hematopoietic reconstitution when used as a combined mixture. This was confirmed initially using KSL cells originating from four F1 strains and, later, by using cultured HSCs obtained from three fully allogeneic strains. To our surprise, however, hematopoiesis reconstituted with a mixture of F1 KSL cells was sustained long-term, although Ly5.2-B6 recipients could reject those grafts due to MHC disparity. Furthermore, cotransplanted KSL cells of congenic Ly5.1-B6 origin did not exhibit any advantage over combined allogeneic grafts in hematopoietic reconstitution. We concluded that combined allogeneic grafts using HSPCs had the potential to reconstitute donor hematopoiesis, but that measures were required to use this beneficial effect only early after transplantation, while allowing subsequent single-donor hematopoiesis.

Single-donor hematopoiesis was achievable by transplanting the congenic graft as WBM cells, even with cotransplantation of a combined multiple allogeneic graft. Of note is that this was feasible with a limited dose of WBM cells, one insufficient to sustain recipients without support from co-infused allogeneic grafts. Consistent with a previous report (Yahata et al., 2004), we inferred that inclusion of a small dose of T cells in the graft underlay single-donor chimerism after transient mixed-donor chimerism, likely through graft-versus-graft reactions. Based on these findings, we proposed a model of transplantation in which a principal graft would be used in a form containing mature T cells with support from multiple allogeneic HSPCs only during a high-risk period of myelosuppression.

We have defined bridging effect as the ability of supportive grafts to transiently provide donor-derived hematopoiesis during myelosuppression. To our knowledge, the specific populations in the graft responsible for bridging effect in each of the granulomonocytic, thrombocytic, and erythropoietic lineages must still be established. Our fractionation studies found that the most primitive stem/progenitor cells, i.e., KSL cells, were better suited as supporting grafts than further-committed progenitors, i.e., c-Kit⁺/Sca-1⁺/Lin[−] cells containing common myeloid progenitors, granulocyte/macrophage progenitors, and megakaryocyte/erythrocyte progenitors; this was particularly true for platelet recovery. This is consistent with work demonstrating that a myeloid bypass pathway brings HSCs directly to a thrombocytic lineage (Yamamoto et al., 2013). We proceeded to test whether highly purified HSCs could also be used as supporting grafts in transplantation that used only a limited dose of principal WBM. A defined culture protocol compatible with HSC amplification (Ema et al., 2000; Lai et al., 2014) yielded a mixture of cultured allogeneic cells, each originating from as few as 50 HSCs, which showed robust bridging effect, especially in platelet recovery, without hampering single-donor chimerism formation from the congenic graft. This suggests that for well-balanced bridging effect, primitive progenitors containing HSCs are likely suitable, either with or without modification.

To establish clinically relevant procedures, we optimized a protocol to obtain viable MNCs from frozen human UCB using the all-in-one CliniMACS cell processor. The protocol allows combining up to nine UCB units for processing without unwanted reactions. To prove the applicability of a transplantation model established in mouse studies, we used a mixture of CD34⁺ cells derived from multiple UCB units. CD34⁺ cells were used because they are well-characterized primitive cells containing HSCs, but lacking mature immune cells, and because they have for decades been a source in clinical transplantation. As xenotransplantation models limit experimentation, we did not prove that CD34⁺ units in combination exert radioprotective or bridging effects with enhanced hematopoietic recovery in peripheral blood, including thrombocytopoiesis. Nevertheless, we believe that our studies show that a beneficial effect inheres in human allogeneic CD34⁺ cells, because analysis confirmed their contribution to the human chimerism observed.

To translate this into clinical use, several issues must be considered. Although our purification method achieved ~98–99% CD34⁺ cell purity, whether residual small numbers of T cells in each graft would affect outcomes is untested. If such activity is demonstrated, complete removal or chemical inactivation of T cells may be needed for combined allogeneic grafts. Alternatively, as involvement of T cells was shown to be critical in ensuring single-donor chimerism from a principal graft, the effects of immunosuppressive drugs that patients usually receive may be a concern. To address these issues, it may be justifiable to conduct clinical trials after careful estimation of risk–benefit balances. Possible benefits of combined allogeneic CD34⁺ cells as supportive grafts may include decreased frequency of transfusions and infections, and even lower medical costs.

To our knowledge, this is the first report of proof that combination of multiple units of allogeneic HSPCs can exert radioprotective and bridging effects. This method is relatively simple; it likely will be time-saving and cost-effective. Some measures to overcome the cell dose barrier to CBT have been previously described (Lauret et al., 2004; Delaney et al., 2010; Himburg et al., 2010; Pajcini et al., 2011; de Lima et al., 2012; Robinson et al., 2012; Cutler et al., 2013; Dahlberg et al., 2014; Fares et al., 2014). We believe that these do not preclude our strategy, but instead complement it. Its core is the bridging effect that allogeneic UCB CD34⁺ cells provide, which can be accessed by combining several units with manipulation that may or may not include cultivation steps. We believe that use of this new strategy using multiple UCB units will open a door for wider application of CBT.

MATERIALS AND METHODS

Mice

Mice of strains C57BL/6 (B6, H2b, Ly5.2), Slc:CBF1 (CBF1, H2b/d, Ly5.2), DBA/1JmsSlc (DBA1, H2q, Ly5.2), DBA/2Cr Slc (DBA2, H2d, Ly5.2), C3HHeJ (C3H, H2k, Ly5.2), and Slc:B6C3F1 (B6C3F1, H2b/k, Ly5.2) were purchased from Japan SLC. Rag 2 KO-Ly5.1 mice, B6-Ly5.1 mice (H2b), and B6-Ly5.1/Ly5.2 mice (H2b) were

purchased from Sankyo Laboratory Service. DBA/1JNCrlj mice (DBA/1, H2q, Ly5.2) were purchased from Charles River Japan. Kusabira-Orange transgenic mice (KuO mice; Hamanaka et al., 2013) were used after backcrossing more than seven times with C57BL/6-Ly5.1 mice. KuO-B6C3F1, B6D1F1, and BDF1 mice were obtained from mating of, respectively, male KuO mice and female C3H mice, male DBA/1JNCrlj mice and female B6-Ly5.2 mice, and male B6-Ly5.1 mice and female Slc:DBA2 mice. The Departments of Nephrology and Immunology (Kitasato University School of Medicine, Kanagawa, Japan) contributed male TCR- α chain KO B6-Ly5.1/Ly5.2 mice, which were generated on a C57BL/6 background (Yoshimasu et al., 2004). NOD/Shi-SCID, IL2R γ -null (NOG) mice were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan).

Purification of murine HSPCs

CD34⁺KSL cells were purified as previously described (Osawa et al., 1996; Ema et al., 2006; Morita et al., 2010; Lai et al., 2014). With our stringent gating strategy, the resulting cell population is generally ~100% CD48⁺ and ~80% CD150⁺ (Morita et al., 2010), resembling so-called SLAM HSCs (Kiel et al., 2005) with respect to purity (Fig. S3). Murine KSL cells were isolated from femora, tibiae, and pelvic bones of adult mice (8–12 wk) and were incubated with a c-Kit-specific antibody. Cells expressing c-Kit were obtained using a MACS LS column after incubation with anti-c-Kit MicroBeads (Miltenyi Biotec). These cells were stained with a cocktail of biotinylated mAb specific for CD4, CD8, B220, IL-7R, Gr-1, Mac-1, and Ter-119 (eBioscience; Lin markers), and then stained with these dye-coupled mAbs: PE-Sca-1 or PE-Cyanin7-Sca-1, APC-c-Kit, and APC-Cyanin7-Streptavidin (eBioscience). FITC-CD34 mAb (eBioscience) was used to obtain CD34⁺KSL cells. Dead cells were identified by propidium iodide staining. Live cells were sorted into 96-well plates (round-bottomed wells) using FACSARIA equipment (BD).

Cell culture procedures

50 CD34⁺KSL cells were sorted into individual wells of 96-well plates containing 200 μ l of S-clone medium (Eidia) supplemented with 10% BSA (Sigma-Aldrich), 1% penicillin-streptomycin-glutamine (Life Technologies), 50 ng/ml mouse stem cell factor, and 50 ng/ml mouse thrombopoietin (PeproTech). Cultured cells were counted using Flow-Count Fluorospheres (Beckman Coulter) and FACSARIA. Surface phenotypes after cultivation were analyzed by staining cells with a cocktail of biotinylated mAbs against Lin markers, and the dye-conjugated mAbs APC-anti-c-Kit (eBioscience), PE-anti-CD48, and PE-Cyanine7-anti-Sca-1 (BioLegend). The biotinylated antibodies were developed with streptavidin-APC-Cyanine7. Cells were analyzed by FACSARIA.

Isolation of murine B cells and T cells

Murine WBM cells were isolated from femora, tibiae, and pelvic bones of adult mice (8–12 wk). WBM was frac-

tionated by treatment with ACK lysis buffer (NH_4Cl 8,024 mg/l, KHCO_3 1,001 mg/l, $\text{EDTA.Na}_2.2\text{H}_2\text{O}$ 3.722 mg/l), staining with PE-Cyanine7-B220- and APC-Cyanine7-CD3e/4/8a-mAbs, and sorting into 96-well plates by FACSARIA.

Isolation of human CD34⁺ cells

Frozen UCB units were provided by the Japanese Red Cross Kanto-Koshinetsu Cord Blood Bank (Tokyo, Japan). Isolation of human CD34⁺ cells from frozen UCB samples starts with introduction of thawed UCB units into CliniMACS Prodigy equipment (Miltenyi Biotec). In a closed tube-bag system, UCB cells were washed in Dulbecco's Modified Eagle's Medium (Sigma Aldrich) containing DNase I (Roche), followed by centrifugation (420 g). This medium was replaced by CliniMACS Buffer and MNCs were isolated from the entire unit in a gradient formed using Ficoll-Paque Premium buffer (GE Healthcare; $P = 1.084$). MNCs were then washed three times in CliniMACS Buffer and mesh filtered. All these steps were conducted in an automated manner directed by an optimized program (available upon request). Beyond this point, manual positive selection was conducted (CD34 MicroBeads kit; Miltenyi Biotec), generally twice, to increase CD34 purity (possibly ~100%), as analyzed by staining cells with an FITC-conjugated anti-human lineage antibody cocktail, APC-CD34, Pacific Blue-CD45 (BioLegend), and PE/Cyanine7-CD38 (eBioscience).

Transplantation

Recipient mice (8–12 wk) were pretreated orally with acid water (pH, ~2.5) for >1 wk before transplantation, lethally irradiated on day 0 with 1.2 Gy for NOG mice (6–8 wk) or 4.9 Gy twice (total 9.8 Gy) for other mice, and transplanted with test cells intravenously. In some experiments (Figs. 4 B and 5, D–F), recipient mice were treated with water containing enrofloxacin (Baytril 10%; Bayer Animal Health). For studies using mixed KSL cells, KSL cells from different strains were sorted into the same single well of 96-well plates before transplantation.

Chimerism analysis and CBC measurement

Cells from PB samples collected by retroorbital bleeding or BM samples were treated with ACK lysis buffer and stained with these dye-conjugated mAbs: Pacific Orange-Ly-6G/6C (Gr-1; Life Technologies), PE-Cyanin5-CD45R/B220, Alexa Fluor 700-CD4, Alexa Fluor 700-CD8a, PE-Cyanin7-CD45.1, APC-cyanin7-CD45.2, Pacific Blue-H2Kd, FITC-H2Kk, FITC-H2Kb, PE-H2Kb, Alexa Fluor 647-H2Kq, Brilliant Violet 510 Streptavidin (BioLegend), Alexa Fluor 700 Sca-1, and APC c-Kit (eBioscience). Data were acquired by FACSARIA and analyzed using FlowJo software (Tree Star). Complete blood counts were obtained with MEK-6450 Celltac- α (Nihon Kohden). For flow cytometry analysis of human cell engraftment in NOG mice, BM samples were stained with

these dye-conjugated mAbs: PE-HLA-A2, Pacific Blue-human CD45, FITC-conjugated anti-human lineage antibody cocktail, APC-CD34, APC-CD4, APC-CD8, PE-Cyanin7-mouse CD45 (BioLegend), PE-CD33, FITC-CD19 (BD), and PE/Cyanine7-CD38 (eBioscience).

Statistics

One-way ANOVA testing with Tukey's multiple comparison testing and Mann-Whitney testing were used in statistical analysis. A p -value <0.05 was considered statistically significant. We plotted survival curves using Kaplan-Meier estimates. Log-rank testing was used to analyze survival data. In multiple comparisons of survival curves, we used Bonferroni-corrected thresholds rather than $P < 0.05$ to avoid either overestimation or underestimation of results.

Study approval

All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of the Institute of Medical Science (University of Tokyo, Tokyo, Japan). The use of UCB samples for experiments was approved by institutional review boards at both the University of Tokyo and the Japanese Red Cross Society.

Online supplemental material

Fig. S1 shows the gating strategy to distinguish mouse strain cell derivations applicable to Figs. 1 and 2. Fig. S2 shows the results confirming attainability of single congenic-donor chimerism. Fig. S3 shows the stringent CD34⁺ KSL cell gating strategy. Fig. S4 shows the gating strategy to distinguish mouse strain cell derivations applicable to Fig. 6. Fig. S5 shows the gating strategy to distinguish mouse strain cell derivations applicable to Fig. 7. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20151493/DC1>.

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