Molecular Therapy
Methods & Clinical Development
Original Article

Low-Dose Busulfan Reduces Human CD34$^+$ Cell Doses Required for Engraftment in c-kit Mutant Immunodeficient Mice

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Humanized animal models are central to efforts aimed at improving hematopoietic stem cell (HSC) transplantation with or without genetic modification. Human cell engraftment is feasible in immunodeficient mice; however, high HSC doses and conditioning limit broad use of xenograft models. We assessed human CD45$^+$ chimerism after transplanting varying doses of human CD34$^+$ HSCs (2×10^5 to 2×10^6 cells/mouse) with or without busulfan (BU) pretransplant conditioning in c-kit mutant mice that do not require conditioning (non-obese diabetic [NOD]/B6/severe combined immunodeficiency [SCID]/ interleukin-2 receptor gamma chain null (IL-2r$\gamma^{-/-}$) Kit$^{W41/W41}$ [NBSGW]). We then tested a range of BU (5–37.5 mg/kg) using 2×10^5 human CD34$^+$ cells. Glycophorin-A erythrocyte chimerism was assessed after murine macrophage depletion using clodronate liposomes. We demonstrated successful long-term engraftment of human CD34$^+$ cells at all cell doses in this model, and equivalent engraftment using 10-fold less CD34$^+$ cells with the addition of BU conditioning. Low-dose BU (10 mg/kg) was sufficient to allow human engraftment using 2×10^5 CD34$^+$ cells, whereas higher doses ($\geq$37.5 mg/kg) were toxic. NBSGW mice support human erythropoiesis in the bone marrow; however, murine macrophage depletion provided only minimal and transient increases in peripheral blood human erythrocytes. Our xenograft model is therefore useful in HSC gene therapy and genome-editing studies, especially for modeling in disorders, such as sickle cell disease, where access to HSCs is limited.

INTRODUCTION

Humanized animal models are useful to study long-term hematopoiesis and validate gene therapy and genome-editing methods in hematologic disorders, including sickle cell disease (SCD). The non-obese diabetic (NOD) severe combined immunodeficiency (SCID) interleukin-2 receptor gamma chain null (IL2r$\gamma^{-/-}$) (NSG) mouse strain supports human hematopoietic stem cell (HSC) engraftment and is often used in HSC transplantation and gene therapy models; however, high cell doses and toxic conditioning are required to achieve high-level human chimerism.[1,2] Although total body irradiation (TBI) conditioning ensures engraftment of HSCs, irradiation has toxic effects on many organs including HSCs in NSG mice, and less toxic nonmyeloablative doses of TBI result in feasibility but low-level engraftment.[3–6] We previously established the use of nonmyeloablative parenteral busulfan (BU) as an alternative to TBI, demonstrating sufficient levels of engraftment for analysis at doses >20 mg/kg[7]; however, as many as 2×10^6 peripheral blood (PB) CD34$^+$ selected HSCs or 2×10^7 bone marrow (BM) nucleated cells are required to sustain engraftment beyond 4–6 months.[7–10] Sufficient quantities of CD34$^+$ cells from patients with SCD are limited and not readily available; therefore, a mouse model that utilizes reduced numbers of CD34$^+$ cells is needed.

NOD/B6/SCID/IL-2r$\gamma^{-/-}$ Kit$^{W41/W41}$ (NBSGW) mice are an alternative NSG mouse model that harbors the spontaneous Kit^{W41J} allele, conferring loss of endogenous Kit functions. This loss of function allele impairs endogenous mouse HSCs and allows 9-fold higher human HSC chimerism without the need for any conditioning as compared with engrafted NSG mice.[2,11] Additionally, similar mice (NSGW41) support improved human erythropoiesis up to the normoblast stage and platelet formation as compared with irradiated NSG recipients.[12] In general, mouse models do not support full human erythropoiesis and the survival of human red blood cells (RBCs) into the PB and are, therefore, not ideal in the study of hemoglobinopathies. Several factors, including a lack of human cytokines and a high susceptibility of human RBC rejection by recipient mouse macrophages, do not allow the survival of human RBCs *in vivo*; however, erythropoiesis can be supported after *in vitro* culture from xenograft BM cell,[10] and complete maturation and enucleation, as well as developmentally appropriate globin gene expression, have been demonstrated from the BM of NBSGW mice.[13] c-kit mutant NSG mice demonstrate 5- to 12-fold higher rates of human erythropoiesis in the BM compared with irradiated NSG mice,[1,9,11,14,15] with

Received 6 August 2019; accepted 30 October 2019;
https://doi.org/10.1016/j.omtm.2019.10.017.

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Table 1. Complete Blood Count Data for NBSGW Mouse Strain

Complete blood count	Value
WBC (10^3/μL)	1.2
RBC (10^6/μL)	4.0
Hemoglobin (g/dL)	7.3
Hematocrit %	24.7
Mean corpuscular volume (fL)	61.4
Platelets (10^3/μL)	1,181.5
Polys %	60.8
Lymphocytes %	27.0
Monocytes %	7.2
Eosinophils %	3.3
Basophils %	0.9
Polys absolute (10^3/μL)	0.7
Lymphocytes absolute (10^3/μL)	0.3
Monocytes absolute (10^3/μL)	0.1
Eosinophils absolute (10^3/μL)	0.0
Basophils absolute (10^3/μL)	0.0

morphology, composition, enucleate capacity, and maturity (as measured by αβ subunits) comparable with those in the human BM,[12,15] yet human RBC output in the PB is still not seen. Depletion of murine macrophages with clodronate liposomes in this mouse model has demonstrated the appearance of human RBCs in the PB, whereas overexpression of human erythropoietin does not.[12]

An ideal humanized mouse model investigating gene therapy and genome-editing methods in SCD would utilize low $CD34^+$ cell doses, support human erythropoiesis, and demonstrate high-level, sustained engraftment. The NBSGW mouse strain demonstrates high engraftment with 1.25×10^6 $CD34^+$ cells without conditioning[11]; therefore, we hypothesized that engraftment with fewer $CD34^+$ cells could be achieved in this mouse model by applying a known methodology in a novel manner through adding low-dose BU conditioning.[7] We demonstrate successful long-term engraftment of human $CD34^+$ cells at all cell doses in this model and equivalent engraftment using 10-fold less $CD34^+$ cells with the addition of BU conditioning. Low-dose BU (10 mg/kg) is sufficient to allow human engraftment using 2×10^5 $CD34^+$ cells, whereas higher doses ($\geq$37.5 mg/kg) are toxic. We confirmed that NBSGW mice support human erythropoiesis in the BM; however, murine macrophage depletion did not result in sufficient human erythropoiesis in the PB to allow further justification for studying gene therapy methods in the PB over the BM.

RESULTS

Low-Dose BU Conditioning Improves Human $CD34^+$ Cell Engraftment in NBSGW Mice

NBSGW mice demonstrated macrocytic anemia, thrombocytosis, and lymphopenia consistent with their SCID background (Table 1).

In our initial experiment (experiment A) investigating $CD34^+$ cell dose with or without BU conditioning, five cohorts received varying cell doses (2×10^5/mouse with 25 mg/kg BU, n = 4; 2×10^5/mouse without BU, n = 4; 5×10^5/mouse without BU, n = 4; 1×10^6/mouse without BU, n = 4; or 2×10^6/mouse without BU, n = 2) (Figure 1). Only one cohort (2×10^5 cells/mouse) received intraperitoneal (i.p.) BU conditioning 48 h prior to transplantation. Two mice were used as control. We confirmed efficient lentiviral transduction in *in vitro* $CD34^+$ cell culture with 18.3% ± 0.7% of percent GFP-positive (% GFP) and 0.51 ± 0.03 vector copy number per cell (VCN).

All mice transplanted with human $CD34^+$ cells demonstrated $CD45^+$ engraftment (Figure 2A; Figure S1A) and GFP positivity (Figure 2B; Figure S1B). Maximal engraftment occurred around 12 weeks post-transplantation and was sustained beyond 24 weeks. Mice that received 2×10^5 cells/mouse with BU conditioning initially had the highest $CD45^+$ chimerism over the first 12 weeks post-transplantation, which was significantly higher than the equivalent cell dose (2×10^5 cells/mouse) without BU ($p < 0.05$). The average percentage of human $CD45^+$ chimerism sustained from weeks 12 to 24 was equivalent for mice that received 2×10^5 cells/mouse with BU conditioning (47.3% ± 8%) compared with the highest cell doses of 1×10^6 cells/mouse without BU (49.1% ± 3%, not significant [n.s.]) and 2×10^6 cells/mouse without BU (60.3% ± 7%, n.s.). %CD45 chimerism from weeks 12 to 24 was higher for the mice that received 2×10^5 cells/mouse with BU conditioning than the mice that received 2×10^5 cells/mouse without BU (16.5% ± 4.6%; $p < 0.01$) or 5×10^5 cells/mouse without BU (35.1% ± 12%, n.s.). At maximal engraftment at 12 weeks, %GFP in both human and mouse cells was highest in the mice that received 2×10^5 cells/mouse with BU conditioning at 12 weeks (13.5% ± 4.3%) compared with those that received 2×10^5 cells/mouse without BU (4.7% ± 1.1%; $p < 0.05$) reflective of $CD45^+$ engraftment (Figure 2B). Percent GFP-positive human cells was detected among all xenograft mice over 24 weeks post-transplant and were stable over time (Figure S2A).

Low-Dose BU 10 mg/kg Is Sufficient to Allow Engraftment Using a 10-Fold Lower Human $CD34^+$ Cell Dose, whereas Doses as High as 37.5 mg/kg Are Toxic

The second experiment (experiment B) investigated the optimal dose of BU conditioning through transplanting a fixed cell dose of 2×10^5 cells/mouse after conditioning with a single dose of i.p. BU (5 mg/kg, n = 4; 10 mg/kg, n = 4; 25 mg/kg, n = 4; or 37.5 mg/kg, n = 4) 48 h prior to transplantation. Two mice were used as control (Figure 1). Efficient lentiviral transduction was observed in *in vitro* $CD34^+$ cell culture with 20.2% ± 0.3% of %GFP and 0.92 ± 0.12 VCN.

All mice transplanted with 2×10^5 cells/mouse with BU conditioning $\leq$25 mg/kg demonstrated $CD45^+$ engraftment (Figure 2C). All mice (n = 4) that received 37.5 mg/kg BU conditioning died within 2–3 weeks after BU conditioning and transplantation of human $CD34^+$ cells ($p < 0.01$, compared with all other groups); therefore, engraftment data 4 weeks post-transplantation were not available for this cohort (Figure 3). Similar to experiment A, maximal

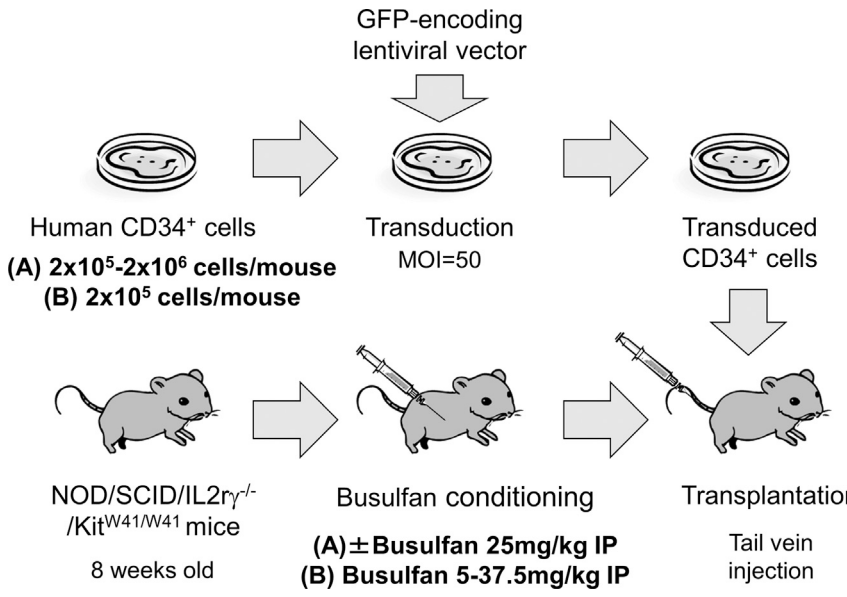

Figure 1. Transplantation of NBSGW Mice

Human CD34+ cells were transduced with a GFP-encoding lentiviral vector at an MOI of 50 and infused via i.v. tail vein injection after (experiment A) either no conditioning or conditioning with BU (25 mg/kg) or (experiment B) conditioning with a single dose of BU (5, 10, 25, or 37.5 mg/kg). Mice in experiment A received varying cell doses (2×10^5, 5×10^5, 1×10^6, 2×10^6, 2×10^5, 2×10^5 cells/mouse), whereas all mice in experiment B received 2×10^5 cells/mouse.

engraftment occurred around 12 weeks post-transplantation. The average percentage of human CD45+ chimerism obtained at 12 weeks in experiment B after 25 mg/kg BU conditioning with a cell dose of 2×10^5 cells/mouse (71.9% ± 14%) was similar to experiment A (58.1% ± 22%, n.s.). Two mice in the 25 mg/kg cohort that had the highest engraftment at 12 weeks (49% and 73.8%, respectively) died between 16 and 18 weeks after transplantation (Figure 3). PB chimerism at the time of death was 46% and 30.5%, respectively. The remaining mice that received BU 5 (n = 4) and 25 mg/kg (n = 2) demonstrated falling chimerism after 12 weeks, whereas mice that received BU 10 mg/kg (n = 4) demonstrated stable chimerism past 24 weeks. The average percentage of human CD45+ chimerism from weeks 12 to 24 for mice that received BU 10 mg/kg was similar (44.1% ± 14%) to that observed for mice that

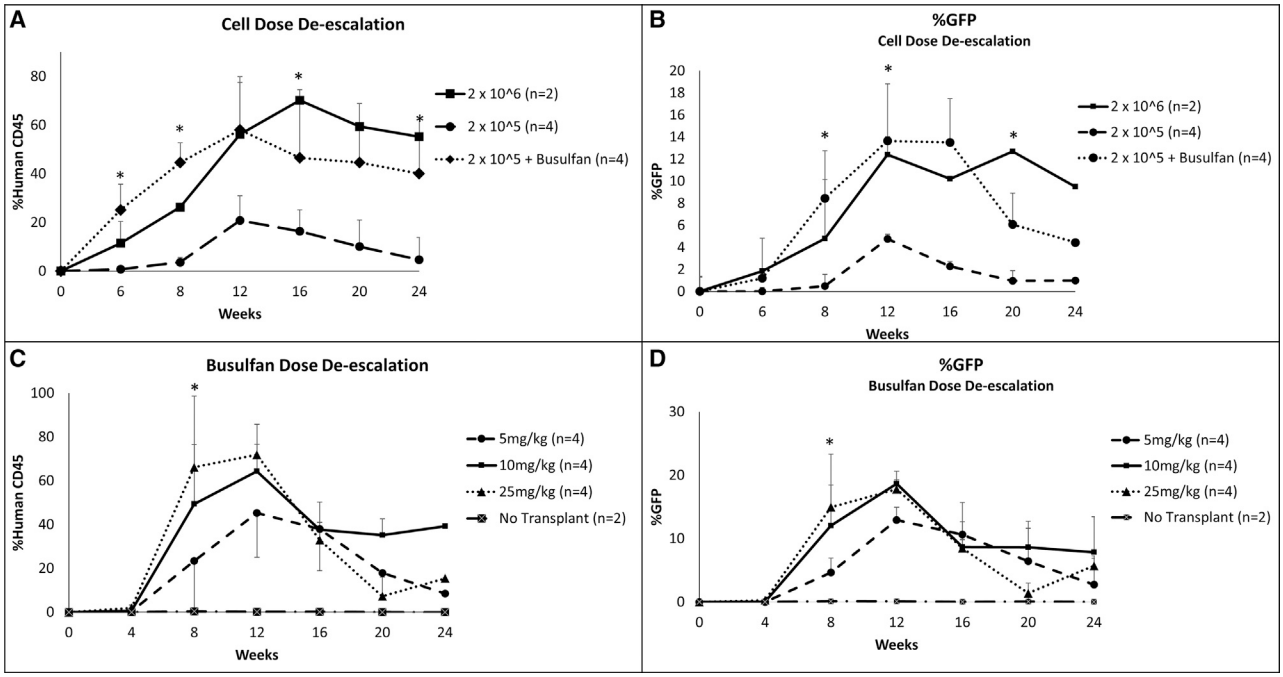

Figure 2. Human CD45 Engraftment

CD45+ chimerism and GFP percentages were measured in NBSGW mice. A: CD45+ chimerism in mice given varying cell doses with or without BU conditioning. B: GFP percentages in mice given varying cell doses with or without BU conditioning. C: CD45+ chimerism in mice given varying doses of BU after a fixed dose of 2×10^5 cells/mouse. All mice that received BU 37 mg/kg died before the first PB draw at 4 weeks. D: GFP percetnages in mice given varying doses of BU after a fixed dose of 2 × 105 cells/mouse. *Statistically significant differences between groups ($p < 0.05$) as determined by one-way ANOVA. Dunnett's post hoc analysis used 2×10^6 as the control (experiment A) and 25 mg/kg (experiment B).

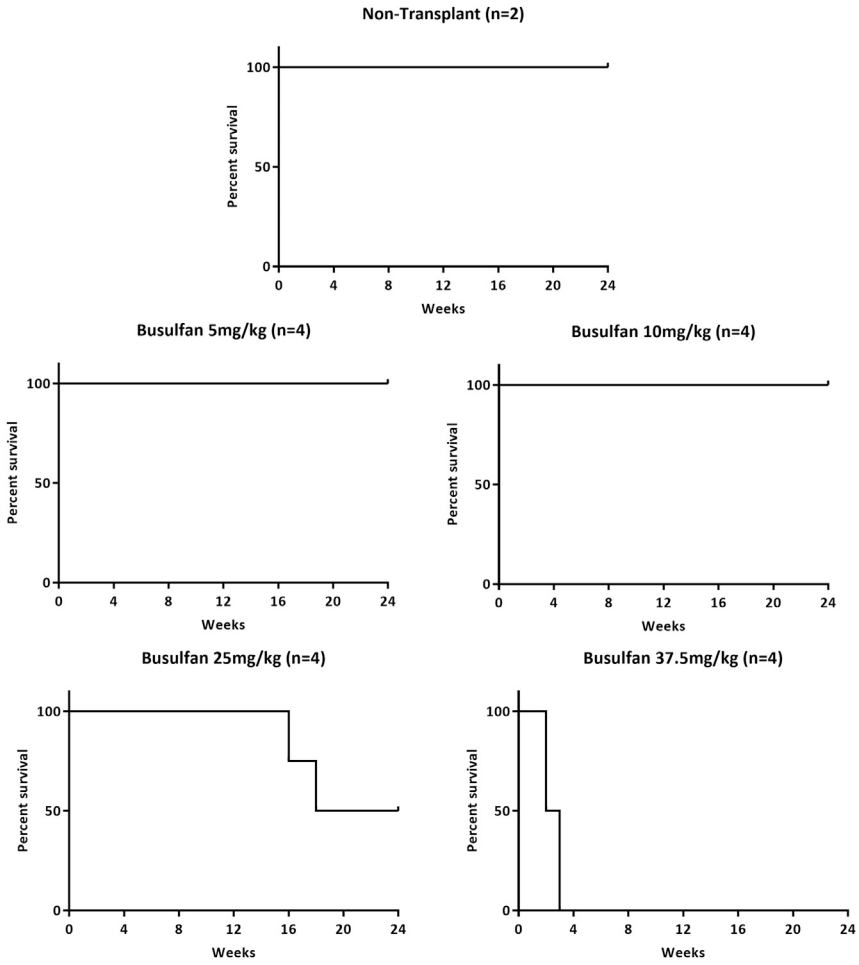

Figure 3. Survival of NBSGW Mice after Low-Dose BU Conditioning

All mice that received BU 37.5 mg/kg (n = 4) died within the first 4 weeks after transplantation, whereas lower doses of BU appear to be well tolerated.

received 25 mg/kg in experiment A (47.3% ± 8%, n.s.). At maximal engraftment at 12 weeks, there was no difference in %GFP in both human and mouse cells based on BU dose (5 mg/kg: 12.9% ± 9.0%; 10 mg/kg: 18.7% ± 5.0%; 25 mg/kg: 17.8% ± 1.5%, n.s.) (Figure 2D). %GFP levels weaned in all cohorts over weeks 12–24 (5 mg/kg: 8.2% ± 9.0%; 10 mg/kg: 10.9% ± 5.0%; 25 mg/kg: 8.3% ± 1.5%, n.s.). Percent GFP-positive human cells were detected among all xenograft mice over 24 weeks post-transplant and were stable over time (Figure S2B).

NBSGW Mice Support Human Erythropoiesis in the BM; However, RBC Output in the PB Is Severely Impaired

NBSGW mice demonstrated erythrocyte engraftment in the BM (54% ± 15%) with all differentiation stages ($CD71^+GPA^-$ [glycophorin-A^-], $CD71^+GPA^+$, $CD71^-GPA^+$) present (Figures 4A and 4B). RBC output into the PB was severely impaired and never reached >1% (Figure 4C). Maximal RBC output occurred 8 weeks post-transplantation and declined steadily until human GPA^+ RBCs were not more detectable than the negative control after 20 weeks.

In an attempt to increase PB erythrocyte detection, murine macrophages were depleted by either i.p. or intravenous (i.v.) injection of 200 μL of clodronate liposomes after 6 months of sustained engraftment. PB RBC output was not significantly higher in mice that received liposomes (0.2% ± 0.15%) compared with mice that did not (0.07% ± 0.02%, n.s.), despite a distinctly visible population seen on flow cytometry (Figures 4D and 4E). GPA positivity in the non-transplant control was felt to be due to non-specific antibody binding. RBC output was not influenced by the method of liposome administration (i.v. versus i.p., n.s.) or the timing of analysis after i.v. injection (day 1 versus day 4, n.s.) (Figure 4F). Average RBC percentage was no higher than the control mice (0.1% ± 0.001%) after i.v. depletion with analysis after 24 h (0.35% ± 0.07%, n.s.), but was higher after i.v. depletion (0.5% ± 0.13%; $p < 0.05$) and i.p. depletion (0.53% ± 0.02%; $p < 0.05$) 4 days after injection.

DISCUSSION

NBSGW mice support robust levels of humanization in PB and BM without a requirement for conditioning. Here we demonstrate the ability to use 10-fold less $CD34^+$ cells after conditioning NBSGW mice with low-dose BU. Mice that received the lowest cell dose with low-dose BU demonstrated high, sustainable engraftment with evidence of high erythroid chimerism in the BM. Therefore, in this novel setting, NBSGW mice are a superior mouse model compared with historically used NOD/SCID, C57BL6, or BALB/c mice to study gene therapy and genome-editing methods in SCD, where patient-derived $CD34^+$ cells are scarce.[7–10]

Investigations of gene therapy strategies in SCD historically utilize immunodeficient NSG mice over others because of high human engraftment as a result of polymorphisms in the *signal-regulatory protein alpha* (*Sirpa*) gene that abrogates the phagocytosis of human cells.[14,16] Although NSG mice support high levels of human chimerism, conditioning is required and as many as 20×10^6 PBMCs or $1–3 \times 10^6$ $CD34^+$ selection HSCs are needed to achieve ~50% human $CD45^+$ chimerism.[17] Studies investigating various gene therapy and genome-editing strategies in SCD, including zinc-finger nuclease (ZFN) versus CRISPR/Cas9 editing of SCD $CD34^+$ cells using various homologous donor templates,[18] lentiviral addition of the anti-sickling βAS3 globin gene,[19] CRISPR correction,[20] γ-globin gene correction,[21] BCL11a editing,[22] site-specific correction of the sickle mutation with ZFNs,[23] β-globin gene transfer,[24] and comparisons studies of

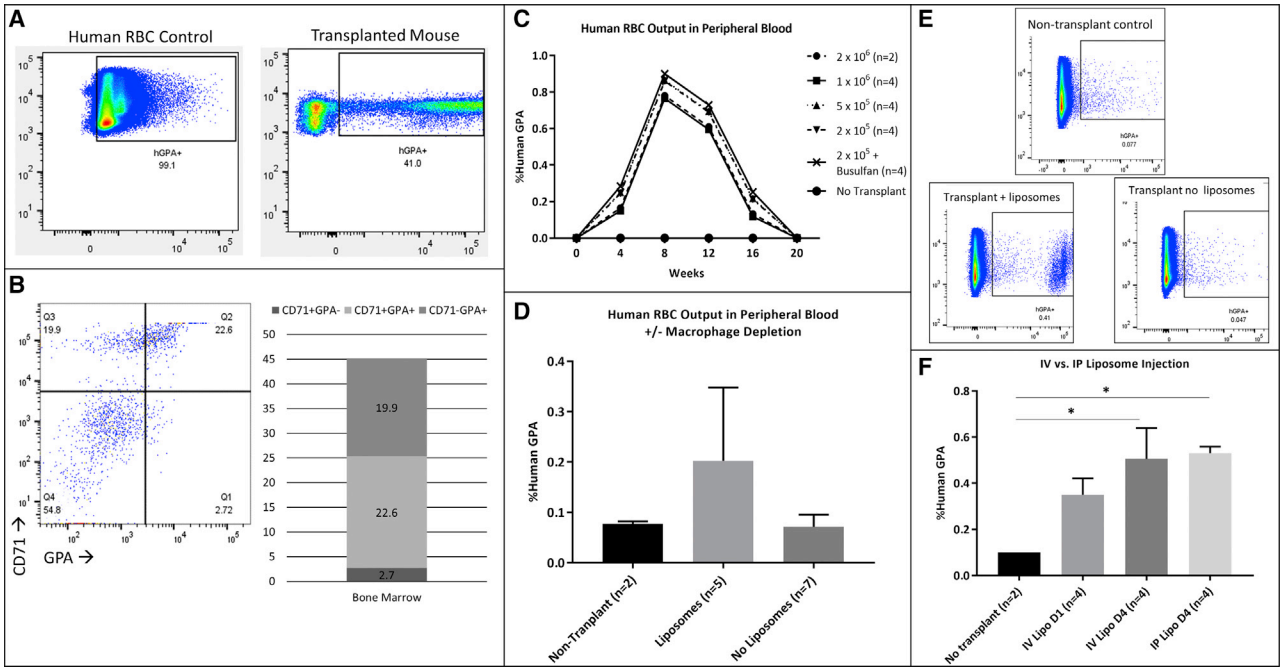

Figure 4. Human Erythropoiesis in NBSGW Mice

Flow cytometry evaluation of GPA$^+$ RBCs (A) and GPA$^+$CD71$^+$ RBCs (B) after bone marrow harvest demonstrate human RBC engraftment. (C) Human RBCs never reached >1% in the PB despite continued human WBC engraftment. Mouse macrophage depletion with clodronate liposomes demonstrated a non-significant rise in measurable GPA$^+$ RBCs (D), which could be seen on flow cytometry (n.s.) (E). (F) Measurement of human RBCs in the PB was equivalent after i.v. versus i.p. liposome injection if measured 4 days (D4) after injection rather than 1 day (D1) after i.v. injection. *$p < 0.05$.

lentiviral vectors[25] all demonstrate human cell engraftment of NSG mice utilizing between 5×10^5 and 3×10^6 SCD CD34$^+$ cells per mouse. Even higher starting CD34$^+$ cell doses are often required *ex vivo* to account for less than 100% transduction efficiency, viability, loss during electroporation, incomplete CD34$^+$ cell recovery after immunomagnetic bead selection, and the lack of long-term engrafting hematopoietic cells characteristic of BM-derived CD34$^+$ cells from patients with SCD.[26,27] Here we demonstrate $\geq$40% human CD45$^+$ chimerism that is sustained until at least 24 weeks post-transplantation in NBSGW mice utilizing only 2×10^5 CD34$^+$ cells/mouse after low-dose BU conditioning. We utilized mobilized PB stem cells in these experiments; however, based on our previous work utilizing other stem cell sources and BU conditioning in the NOD/LtSz-scid/IL2Rγ^{null} mouse model,[7,10] we hypothesize similar engraftment patterns utilizing BM and cord blood-derived HSCs. Our new method would be helpful for lentiviral gene therapy and gene-editing experiments in patient-derived CD34$^+$ HSCs, especially for hemoglobinopathies where stem cell sources are limited.

NBSGW mice are NSG mice characterized by an additional mutation in *c-Kit*, the tyrosine kinase transmembrane receptor for SCF that is involved in HSC self-renewal and differentiation.[28] Immunodeficient mice with loss-of-function Kit mutations,[29] or those treated with a neutralizing anti-mouse Kit antibody,[30] provide a competitive advantage for donor cells by rendering mouse HSCs uncompetitive in occupying the BM niche. Such mice therefore accept human CD34$^+$ HSCs without the need for conditioning and give human CD34$^+$ cells with a full Kit function a proliferative advantage over mouse HSCs. The addition of low-dose BU at 10 mg/kg to further expand the BM niche for donor cells was of minimal toxicity to NBSGW mice and enabled equivalent and sustained engraftment of 2×10^5 cells/mouse as compared with doses $\geq 1 \times 10^6$ cells/mouse without conditioning. BU doses as high as 37.5 mg/kg were too toxic in this mouse model, immediately impairing host hematopoiesis to a degree that was unsustainable while awaiting engraftment of human CD34$^+$ cells. Maximal human cell engraftment occurred at 12 weeks post-transplantation, coinciding with a 2-fold reduction at 12 weeks in stably engrafting human blood cells described in adult Kit$^{W41/W41}$ mice.[31]

Despite high erythroid chimerism in the BM, NBSGW mice do not support survival of human erythrocytes in the PB. Less than 1% of erythrocytes were found in the PB in this study, and clodronate liposome murine macrophage depletion non-significantly increased erythrocyte output. Although fully matured CD71$^-$CD235a$^+$ enucleated human RBCs can be detected in the mouse PB, their levels in the PB are insufficient, limiting the value of immunodeficient mouse models as an *in vivo* model for the study of human hemoglobinopathies.[32] The exact mechanisms of human erythrocyte depletion by phagocytic cells in these mice remain unknown, although macrophage depletion alone is not sufficient, and targeting other factors such as mouse complement, neutrophils, and endothelial cells may be necessary.[33] At this time, erythrocyte analysis requires a BM

harvest and is supported by high BM erythroid chimerism in the NBSGW mouse model.[13]

Conclusions

The *c-Kit* mutation in NBSGW mice enables robust, uniform, and sustained engraftment of human $CD34^+$ cells without the need for conditioning. By employing our previous methodology in a novel way, we were able to support robust human $CD34^+$ cell engraftment with a 10-fold reduction in $CD34^+$ cells by conditioning NBSGW mice with low-dose BU. Used in this way, this mouse model is superior to existing NSG models to study gene therapy and genome-editing methods in SCD.

MATERIALS AND METHODS

Mice

Male NOD.Cg-Kit^{W-41J} Tyr + $Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/ThomJ (NBSGW, Stock No.: 026622) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA; https://www.jax.org). Mice were 8 weeks old at the time of transplantation. Mice were housed in a pathogen-free facility and handled according to an animal care and use protocol (H-0228) approved by the Animal Care and Use Committee at the NIH.

Conditioning of NBSGW Mice for Transplantation

The initial experiment (experiment A) investigated cell dose with or without BU conditioning. A single cohort (n = 4) received 25 mg/kg BU (Busulfex; PDL BioPharma, Redwood City, CA, USA; https://www.pdl.com). All other mice received no conditioning. BU was diluted with PBS (Biofluids, Rockville, NJ, USA) to a final volume of 200 μL and injected i.p. 48 h prior to the infusion of human cells. In the second experiment (experiment B) investigating BU dosing, all mice received an i.p. dose of BU (5, 10, 25, or 37.5 mg/kg) diluted in 200 μL PBS 48 h prior to the infusion of human cells. Each experiment (A and B) had a group of control mice (n = 2) that received neither BU nor human cells. Experimental design is shown in Figure 1.

Cell Dose

Mice in experiment A (cell dose with or without BU) received varying cell doses (2×10^5/mouse with BU, 2×10^5/mouse without BU, 5×10^5/mouse without BU, 1×10^6/mouse without BU, or 2×10^6/mouse without BU). All mice in experiment B (BU dose) received 2×10^5 cells/mouse.

Cell Preparation and Infusion

Granulocyte colony-stimulating factor-mobilized $CD34^+$ cells from healthy donors were collected under studies 08-H-0156 and 03-H-0015, which were approved by the institutional review board of the National Heart, Lung, and Blood Institute (NHLBI). All individuals gave written informed consent for sample donation. Consent documents were approved by the institutional review board prior to study initiation and are reviewed and updated annually.

Human $CD34^+$ cells (4e6/mL) were cultured in X-VIVO10 media (Lonza, Basel, Switzerland) containing 100 ng/mL each of stem cell factor (SCF), fms-like tyrosine kinase 3 ligand, and thrombopoietin (R&D Systems, Minneapolis, MN, USA).[34] After overnight pre-stimulation, the culture medium was changed to fresh medium containing identical cytokines and a self-inactivating lentiviral vector encoding GFP under the control of a murine stem cell virus promoter with a vesicular stomatitis virus G glycoprotein (VSVG) envelope at MOI 50.[32] GFP is used as a cellular tag and is a valuable method of measuring gene expression and cell tracking. After 1-day transduction, transduced cells were suspended in PBS to a final volume of 200 μL and infused i.v. via the tail vein.[33] After infusion of donor human cells, murine blood was obtained for complete blood count (CBC) using a Celldyne 3500 automated cell counter (Abbott Laboratories, Abbott Park, IL, USA).

Small aliquots of transduced cells were cultured *in vitro*; GFP expression was analyzed by flow cytometry (BD FACSCanto II; Becton Dickinson, East Rutherford, NJ, USA) 3–4 days after transduction, and average VCN was measured by qPCR (QuantStudio 6 Flex real-time PCR system; Thermo Fisher Scientific, Waltham, MA, USA) 6–7 days after transduction.[35]

Flow Cytometric Analysis

Mouse PB was obtained every 4 weeks for human CD45 and GFP analysis by flow cytometry (BD FACSCanto II). PB cells were stained for human RBC chimerism with phycoerythrin (PE)-conjugated anti-human GPA (clone HIR2) and human white blood cell (WBC) chimerism with PE-conjugated anti-human CD45 (clone HI30) after RBC lysis with ammonium chloride potassium lysis buffer (Quality Biological, Gaithersburg, MD, USA). WBC and RBC chimerism were measured from harvested BM upon death using PE-CD45, fluorescein isothiocyanate (FITC)-GPA, and allophycocyanin (APC)-conjugated CD71 (clone L01.1), as previously described.[36] All antibodies were purchased from BD Biosciences.

Macrophage Depletion

Murine macrophages were depleted by either i.p. or i.v. injection of 200 μL of clodronate liposomes (Liposoma, Amsterdam, the Netherlands) after 6 months of sustained engraftment. Depletion of liver and splenic macrophages is complete 24 h after i.v. injection and 3 days after i.p. injection. Therefore, mouse PB was obtained for flow cytometry analysis 1–4 days after i.v. injection or 4 days after i.p. injection per the manufacturer's recommendation.

Statistical Analysis

Pairwise comparison was performed by two-tailed Student's t test or ANOVA for multiple group comparison. Survival data were analyzed by Cox proportional hazards model. A p value <0.05 was deemed statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.10.017.

AUTHOR CONTRIBUTIONS

A.L. designed the research, performed experiments (mouse and flow cytometry), analyzed results, and wrote the paper; M.Y. performed experiments (mouse and flow cytometry); T.N. performed experiments (vector preparation and cell culture); J.G. performed experiments (DNA and PCR); C.M.D. performed experiments (vector preparation and flow cytometry); J.J.H.-M. performed experiments (mouse); S.D. performed experiments (mouse); M.M.H. performed experiments (HSC collection); N.U. designed the research, performed experiments (vector preparation and cell culture), and wrote the paper; J.F.T. designed the research and wrote the paper.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Program of the National Heart, Lung, and Blood Institute (NHLBI) and the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK) at the NIH.

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