Engraftment and Lineage Potential of Adult Hematopoietic Stem and Progenitor Cells Is Compromised Following Short-Term Culture in the Presence of an Aryl Hydrocarbon Receptor Antagonist

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Abstract

Hematopoietic stem cell gene therapy for HIV/AIDS is a promising alternative to lifelong antiretroviral therapy. One of the limitations of this approach is the number and quality of stem cells available for transplant following *in vitro* manipulations associated with stem cell isolation and genetic modification. The development of methods to increase the number of autologous, gene-modified stem cells available for transplantation would overcome this barrier. Hematopoietic stem and progenitor cells (HSPC) from adult growth factor-mobilized peripheral blood were cultured in the presence of an aryl hydrocarbon receptor antagonist (AhRA) previously shown to expand HSPC from umbilical cord blood. Qualitative and quantitative assessment of the hematopoietic potential of minimally cultured (MC-HSPC) or expanded HSPC (Exp-HSPC) was performed using an immunodeficient mouse model of transplantation. Our results demonstrate robust, multilineage engraftment of both MC-HSPC and Exp-HSPC although estimates of expansion based on stem cell phenotype were not supported by a corresponding increase in *in vivo* engrafting units. Bone marrow of animals transplanted with either MC-HSPC or Exp-HSPC contained secondary engrafting cells verifying the presence of primitive stem cells in both populations. However, the frequency of *in vivo* engrafting units among the more primitive CD34+/CD90+ HSPC population was significantly lower in Exp-HSPC compared with MC-HSPC. Exp-HSPC also produced fewer lymphoid progeny and more myeloid progeny than MC-HSPC. These results reveal that *in vitro* culture of adult HSPC in AhRA maintains but does not increase the number of *in vivo* engrafting cells and that HSPC expanded *in vitro* contain defects in lymphopoiesis as assessed in this model system. Further investigation is required before implementation of this approach in the clinical setting.

Introduction

H^{EMATOPOIETIC STEM CELL GENE THERAPY} is a promising strategy for treating neoplastic, monogenic, and infectious disease. Clinical success in treating several monogenic diseases with autologous, gene-modified hematopoietic stem and progenitor cells (HSPC) supports the feasibility of using this approach for other disease indications (reviewed in Naldini, 2011). We previously reported on a pilot clinical trial to assess the safety and feasibility of stem cell-based gene therapy for HIV (DiGiusto *et al.*, 2010). Stem cells were collected from HIV+ patients by apheresis, and the CD34+

HSPC were transduced with a lentiviral vector containing a transgene that encoded three anti-HIV RNA molecules as previously described (Li *et al.*, 2005b). Patients were infused with both gene-modified HSPC (GM-HSPC) and unmodified HSPC (as a safety measure) following myeloablative conditioning with *bis*-chloronitrosourea (BCNU), Etoposide (VP16), and cyclophosphamide (Cytosan). The number of CD34+ HSPC in the unmodified graft exceeded the number of infused GM-HSPC by several hundred-fold and undoubtedly contributed to the low level of gene marking observed *in vivo* (0.1–0.34%). Nonetheless, we demonstrated persistent genetic modification and expression of transgenic

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RNA (≥ 8 months) in blood and bone marrow of all four patients. In one patient, UPN0306, we also demonstrated genetic marking of T- and B-lymphoid and multiple myeloid lineages. Long-term follow-up of UPN0306 revealed that gene marking and transgenic RNA expression persisted for at least 3 years in both the blood and bone marrow and that a transient viremia during a structured treatment interruption led to a transient increase in the level of gene marking in the peripheral blood (DiGiusto *et al.*, 2013).

Having established that GM-HSPC engraft, differentiate, and express antiviral RNA for long periods, we wished to improve the level of HIV-resistant cells in the peripheral blood of patients treated with GM-HSPC. We hypothesized that this could be achieved (in part) through expanding the number of GM-HSPC available for transplant. Numerous studies on the *in vitro* expansion of mouse, nonhuman primate, and human umbilical cord blood HSPC have demonstrated significant increases in the number of *in vivo* engrafting units obtained from short-term cultures under a variety of conditions (reviewed in Watts *et al.*, 2011). Several groups have demonstrated effective expansion of adult HSPC cultured in a combination of hematopoietic cytokines (FMS-like tyrosine kinase-3 ligand [Flt-3L], thrombopoietin [TPO], stem cell factor [SCF], and interleukin-6 [IL-6]) (Herrera *et al.*, 2001; Gammaitoni *et al.*, 2003; Li *et al.*, 2005a), while others have demonstrated loss of *in vivo* engrafting potential and a shift in hematopoietic differentiation toward myelopoiesis under similar conditions (Hollyoake *et al.*, 1997; Tisdale *et al.*, 1998). This change in hematopoietic potential can be explained in part by the observed deregulation of multiple signaling networks, including those involved in stem cell maintenance (Notch and Wnt) and differentiation (Jun, LEF, KLF, and PGE2) (Peters *et al.*, 1996; McNiece *et al.*, 2002; Von Drygalski *et al.*, 2004; Holmes *et al.*, 2012) under expansion conditions.

More recent studies suggest that hematopoietic stem cells from neonatal and adult sources can be expanded *in vitro* in the presence of aryl hydrocarbon receptor antagonists (AhRA) (Boitano *et al.*, 2010; Carlin *et al.*, 2013) without loss of hematopoietic potential. The aryl hydrocarbon receptor (AhR) is a transcription factor that mediates responses to environmental toxins such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin) and other polychlorinated biphenyls and has been demonstrated to play an important role in regulating stem cell proliferation and differentiation (Thurmond *et al.*, 1999; Singh *et al.*, 2009a,b; Esser, 2012). Screening of a library of over 100,000 heterocyclic compounds has independently identified an AhRA StemRegenin 1 (SR-1) that supported the *in vitro* expansion of both cord blood and adult HSPC (Boitano *et al.*, 2010). Five-week cultures of cord blood HSPC in a combination of growth-promoting cytokines plus SR-1 resulted in a 171-fold increase in CD34⁺ HSPC, while 3-week cultures of adult peripheral blood HSPC resulted in a 73-fold increase in CD34⁺ HSPC. A 17-fold increase in the number of *in vivo* repopulating units was calculated for the cord blood HSPC, but no engraftment data for expanded adult HSPC were reported. Similarly, cord blood and adult peripheral blood HSPC cultured in the presence of cytokines plus two other AhRA (CH-223191 or dimethoxyflavone) showed similar expansion although the level of expansion of the cord blood CD34⁺ HSPC was significantly higher than that of adult

CD34⁺ HSPC (138-fold vs. 6-fold, respectively) and adult HSPC showed impaired T-cell potential when cultured on OP9-Delta cells (Carlin *et al.*, 2013). Evaluation of stem cell potential in AhR^{-/-} knockout mice also demonstrated a loss of HSPC function despite higher rates of division and expansion of HSPC numbers by phenotype (Singh *et al.*, 2014). These data support a role for AhR in regulating HSPC proliferation and renewal but also suggest that antagonizing this function may result in the overall loss of engrafting and balanced differentiation potential of the HSPC from adult sources. The disparity between the level of *in vitro* expansion of the HSPC (as assessed by phenotype) and *in vivo* reconstitution and lineage potential indicates that careful (quantitative) evaluation of *in vivo* engraftment and lineage potential must be performed to assess the effects of *in vitro* culture of HSPC.

In preparation for subsequent clinical trials, we wished to assess the effects of short-term culture in the presence of an AhRA on the *in vivo* engraftment and lineage potential of adult growth factor-mobilized peripheral blood HSPC. Immunodeficient mouse models of transplantation have proven useful for studying hematopoiesis, infectious disease, autoimmunity, and cancer (reviewed in Shultz *et al.*, 2007) and in general support the requirements outlined above. When immunodeficient NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ (NSG) mice are transplanted with cord blood or fetal liver HSPC, the production of T-cells, B-cells, and multiple lineages of myeloid cells has been well documented. However, fetal and neonatal HSPC differ significantly in their hematopoietic potential from adult HSPC (Lansdorp *et al.*, 1993; Tanavde *et al.*, 2002; Lepus *et al.*, 2009) and are not relevant to most gene therapy studies where autologous adult growth factor-mobilized peripheral blood is the source of HSPC. Therefore, we wished to establish our ability to expand the more clinically relevant population. We report here on the systematic evaluation of *in vitro* expansion of HSPC from adult growth factor-mobilized peripheral blood HSPC using engraftment of and lineage differentiation in immunodeficient mice as a readout. Our results demonstrate that the total number of *in vivo* engrafting cells is maintained but does not increase during *in vitro* culture for 7 days with SR-1 and that there is a pronounced loss of lymphopoietic potential. Further investigation and development of this technology is likely to be required before implementation in our clinical gene therapy program.

Materials and Methods

Cells

Human mobilized hematopoietic progenitor cells were obtained by Progenitor Cell Therapies (Allendale, NJ) and Key Biologics (Memphis, TN) from healthy donors under informed consent. CD34⁺ HSPC were isolated as previously described (Tran *et al.*, 2012). CD34-enriched HSPC were evaluated by flow cytometry for the coordinated expression of CD34 and CD90 to determine the distribution of progenitors and more primitive stem cells as previously described (Baum *et al.*, 1992; Notta *et al.*, 2011). CD34-enriched HSPC were prestimulated overnight in StemSpan media (Stem Cell Technologies, Vancouver, Canada) containing 100 ng/ml each of SCF and Flt-3L, 10 ng/ml of TPO (Cell Genix GmbH, Freiburg, Germany), and 50 ng/ml of IL-6 (Invitrogen,

Carlsbad, CA)—hereinafter referred to as SFT6. HSPC cultured overnight were considered minimally cultured and are hereinafter referred to as MC-HSPC. HSPC were also cultured for up to 7 days to promote the expansion of HSPC as previously described and are hereinafter referred to as Exp-HSPC (Boitano *et al.*, 2010). Briefly, CD34+ cells were seeded in gas-permeable bags (American Fluoroseal Corporation, Gaithersburg, MD) or T75 flasks (Corning, Corning, New York) at 5×10^4 cells/ml in StemSpan + SFT6 + $0.75 \mu\text{M}$ SR-1 (Cellagen Technology, San Diego, CA) and cultured for 7 days at 37°C and 5% CO_2 . The medium was replaced on days 3 and 5, keeping cell density at $<8 \times 10^5$ cells/ml. Cultures of expanded HSPC (Exp-HSPC) were harvested on day 7, washed in PBS, and formulated in phosphate buffered saline for injection into NSG mice as described below. Analysis of total nucleated cell (TNC) count and coordinated CD34 and CD90 cell surface expression was performed before and after each culture.

Mice

NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ (NSG) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at the City of Hope Animal Resources Center according to protocols approved by the Institutional Animal Care and Use Committee of the City of Hope and the *Guide for the Care and Use of Laboratory Animals* (National Research Council [U.S.] *et al.*, 2011).

Mouse transplantation

Adult (8–10 weeks old) NSG mice were irradiated at 270 cGy with attenuation 24 hr before transplantation. Mice were transplanted with HSPC via intravenous injection of the tail vein with doses ranging from 0 to 2×10^6 CD34+ MC-HSPC per animal or 0 to 4×10^6 CD34+ Exp-HSPC in saline for injection (APP Pharmaceuticals, Lake Zurich, IL). For secondary engraftment experiments, primary animals were transplanted with 1×10^6 MC-HSPC or 4×10^6 Exp-HSPC CD34+ cells and maintained for 16 weeks as above. At 16 weeks posttransplant, bone marrow cells from primary mice that received MC-HSPC were harvested, pooled, and used to transplant eight secondary recipient mice with 3.0×10^7 total bone marrow cells per mouse containing 2.1×10^6 CD34+/CD45+ cells. Similarly, the bone marrow from mice transplanted with Exp-HSPC was pooled and used to transplant 6 secondary recipient mice with 3.7×10^7 total bone marrow cells per mouse containing 7.4×10^5 CD34+/CD45+ cells. Bone marrow from both sets of secondary recipients was analyzed 16 weeks after transplant for human cell engraftment.

Flow cytometric analysis of HSPC cultured *in vitro* in the presence of an AhRA

A representative aliquot of each cultured HSPC population was analyzed for stem cell content before culture and at day 7. Cells were counted by Guava Via Count (Millipore, Billerica, MA), and approximately 2.5×10^5 cells were stained with antibodies to CD34-PC7 (Beckman Coulter, Brea, CA) and CD90-APC (BD Biosciences, San Diego, CA), for 20 min on ice and washed 3 times with PBS containing 0.1% BSA (Sigma-Aldrich, St. Louis, MO). About

$0.5 \mu\text{g/ml}$ DAPI (Invitrogen) was added as a live/dead cell discriminator. For isotype controls, matching Ig isotypes and conjugation from the same vendors were used. Samples were analyzed using Gallios Cytometer (Beckman Coulter) and data analyzed with FCS Express software (De Novo Software, Los Angeles, CA).

Flow cytometric analysis of engraftment

Mice were necropsied at 16 or 25 weeks posttransplantation for analysis of engraftment. Single-cell suspensions of bone marrow (femurs) and spleen were prepared by mechanical dissociation and red cells lysed using ACK lysis buffer (Sigma-Aldrich). All cell suspensions were pretreated with human immunoglobulin (GammaGard; Baxter Healthcare Corp., Deerfield, IL) and mouse immunoglobulin (Invitrogen) for 15 min to block nonspecific antibody staining. Spleen cell suspensions were stained with a human pan-leukocyte antibody to CD45-PC5 (BioLegend, San Diego, CA), and lineage-specific antihuman, CD3-ECD, CD4-APC, CD14-APC-Alexa-750, CD19-PE (Invitrogen), and CD8 β -PC5 (Beckman Coulter), for 20 min and washed 2 times with 1 ml of PBS containing 0.1% BSA (Sigma-Aldrich). BM cells were stained with antibodies to human CD34-PC7, CD45-ECD (Beckman Coulter), CD33-PC5 (BD Biosciences), CD19-PE, and CD14-APC-Alexa750 (Invitrogen) for 20 min and washed 2 times with 1 ml of PBS containing 0.1% BSA. To determine the presence of natural killer (NK) cells, single-cell suspensions of spleen were stained with CD16-FITC (Pharmingen, San Diego, CA) and CD56-PE (Invitrogen) following pretreatment as described above. To establish analytical gates and background staining, bone marrow and spleen samples from 2 to 3 mice that received no human cells were stained with the same antibody panel. Samples were analyzed using a Gallios flow cytometer (Beckman Coulter, Hialeah, FL) and data analyzed with FCS Express software (De Novo Software).

Data analysis

For determination of the relative number of NSG repopulating units (NRU), we performed extreme limiting dilution analysis using ELDA software as previously described (Hu and Smyth, 2009). For all other analyses, either analysis of variance or a two-sample two-tailed Student's *t*-test was employed. The level of significance was set to 0.05.

Results

Durable multilineage engraftment in adult NSG mice transplanted with adult HSPC

We wished to describe the relationship between adult stem cell phenotype and engraftment of NSG mice. In human stem cell transplantation, a dose of $>2 \times 10^6$ CD34+ cells/kg is recommended to ensure rapid hematopoietic recovery (absolute neutrophil count $>500/\text{mm}^3$ for 3 consecutive days) following marrow-ablative conditioning (Bensinger *et al.*, 1995a,b). In order to perform quantitative analysis of engraftment before and after *in vitro* culture, we first established dose-response curves for engraftment of MC-HSPC in NSG mice based on CD34+ cell dose. CD34+ HSPC were isolated from G-CSF-mobilized peripheral blood from

five independent healthy donors by magnetic bead selection and cultured overnight in a combination of hematopoietic cytokines previously shown to support the maintenance of HSPC and *in vivo* engrafting activity (SCF, Flt-3L, TPO, and IL-6—SFT6). We initially transplanted animals with doses of 0.5×10^6 , 1×10^6 , or 2×10^6 MC-HSPC per mouse from a single donor to establish the relationship between cell dose and engraftment. We performed phenotypic analysis of blood, bone marrow, and spleen using antibody to the human pan-leukocyte marker CD45 to identify human cells and calculate extent of engraftment. There was no evidence for human cells in the thymus or lymph nodes of these animals.

In contrast to previous reports in which cord blood progenitors were used to engraft neonatal NSG mice (Andre *et al.*, 2010; McDermott *et al.*, 2010; Choi *et al.*, 2011b; Reimann *et al.*, 2012; Scholbach *et al.*, 2012; Singh *et al.*, 2012), we detected very low levels ($<0.5\%$ CD45+) of human cells in the peripheral blood of most mice at 16 weeks after transplant (Fig. 1A). However, the average human cell engraftment in animals receiving at least 0.5×10^6 CD34+ MC-HSPC was $9.8 \pm 4.8\%$ in bone marrow and $3.5 \pm 1.4\%$ in spleen (Fig. 1B and C). Increasing the dose to 1 or 2×10^6 CD34+ MC-HSPC resulted in modest increases in the level of CD45+ cell engraftment in the bone marrow ($18 \pm 6.4\%$ and $24 \pm 6.8\%$, respectively) and spleen ($12 \pm 5.2\%$ and $19 \pm 5.1\%$, respectively), but these were not significantly different from the 0.5×10^6 dose ($p > 0.05$). Animals from the same cohort were maintained for 25 weeks after transplant and then analyzed for engraftment. All animals were engrafted at levels comparable to or greater than that seen at 16 weeks in this cohort, demonstrating the durability of engraftment with this source of cells (Fig. 1D and E).

We also analyzed the bone marrow and spleen of mice at 16 weeks after transplantation to determine the frequency of

lymphoid, myeloid, and hematopoietic progenitor cells among the engrafted human cells. We used multiparameter flow cytometric analysis to establish that the marrow of engrafted mice contained predominantly CD19+ B-cells with evidence of CD4+/CD14+ monocytes as well as CD34+ (multipotent) and CD33+ (myeloid) progenitors (Fig. 2A). The spleens also contained CD4+ and CD8+ T-cells, CD19+ B-cells, and CD4+/CD14+ monocytes (Fig. 2B). The presence of CD16+/CD56+ NK cells was observed in some mice, but we did not routinely assess the presence of this population (Fig. 2C). Similar analysis of engraftment was performed at 25 weeks after transplantation and the grafts were found to be durable, containing multiple lymphoid and myeloid lineages and CD34+ cells (Fig. 2D). The distribution of lineages observed in >200 animals from cohorts transplanted with MC-HSPC from five independent donors was similar to that observed in the initial cohort described above (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hgtb). Taken together, these data demonstrate that transplantation of as few as 500,000 adult CD34+ HSPC leads to long-term engraftment of multiple lymphoid and myeloid lineages in the spleen and bone marrow and that transplant of neonatal mice is not required for the development of T-cells. Having established this baseline activity, we next evaluated the *in vitro* expansion potential of adult HSPC.

In vitro culture of adult HSPC in the presence of an AhRA results in maintenance but not expansion of NSG-engrafting cells

In order to establish the extent to which HSPC from adult growth factor-mobilized peripheral blood could be expanded *in vitro*, we cultured known numbers of CD34+ HSPC from 5 healthy donors overnight in SFT6 (MC-HSPC)

FIG. 1. Engraftment of NSG mice. From mice peripheral blood (A), bone marrow (B), and spleen (C) at 16 weeks posttransplantation. Percentage of human CD45+ cells from bone marrow (D) and spleen (E) at 25 weeks post-transplantation. NSG, NOD.Cg-Prkdc^{scid} IL2rg^{tm1Wjl}/SzJ.

FIG. 2. Phenotypic analysis of engrafted human blood cells. Level of human cell engraftment (CD45/side scatter) including progenitor cells (CD34/CD33) and B or myeloid cells (CD19/CD14) in bone marrow (**A**), or B- and T-lymphocytes (CD19/CD3, CD8 β /CD3, CD4/CD3), monocytes (CD14/CD4), and NK cells (CD16/CD56/CD3) in the spleen (**B** and **C**) 16 weeks after transplantation. Level of progenitor cell engraftment in the bone marrow (CD34/CD33) and T-cell/monocyte engraftment in spleen (CD3/CD4 and CD4/CD14) at 25 weeks after transplantation (**D**).

or for 7 days in SFT6 plus 0.75 μ M SR-1 (Exp-HSPC) to assess the expansion of cells with a CD34⁺HSPC phenotype. However, it is well established that the CD90⁺ (Thy-1⁺) subpopulation of CD34⁺ cells contains more primitive HSPC and accounts for most of the long-term engraftment in animal transplant models (Baum *et al.*, 1992; Craig *et al.*, 1993; Peault *et al.*, 1993; Mayani and Lansdorp, 1994; Donahue *et al.*, 1996). Therefore, all cultures were analyzed for TNC count and the frequency of CD34⁺ and the more primitive CD34⁺/CD90⁺ cells before and after culture. The starting population of MC-HSPC was

>98% CD34⁺, and 24–82% of the CD34⁺ was also CD90⁺ as shown in a representative example in Fig. 3A. The frequency of CD34⁺ and CD34⁺/CD90⁺ cells did not change appreciably after overnight prestimulation in SFT6 (data not shown). However, after 1 week of expansion culture, we observed a marked reduction in the frequency of the more primitive CD34⁺/CD90⁺ Exp-HSPC (range 15–41%) (Fig. 3B). Nonetheless, we observed an overall expansion of TNC (23.0 ± 3.4 -fold), CD34⁺ cells (18.6 ± 3.1 -fold), and CD34⁺/CD90⁺ cells (9.5 ± 0.8 -fold) (Fig. 3C).

FIG. 3. *In vitro* expansion of adult HSPC. Phenotypic analysis of coordinated CD34 and CD90 expression before (**A**) and after (**B**) 7 days of *in vitro* expansion in SFT6+ SR-1. Samples are gated on live cells. (**C**) Fold expansion for TNC, CD34+, CD34+/CD90+ cells on day 7 ($N=5$ donors). HSPC, hematopoietic stem and progenitor cells; SR-1, StemRegenin 1; TNC, total nucleated cell.

In order to establish the hematopoietic potential of *ex vivo*-expanded HSPC, we transplanted >280 NSG mice with varying doses of MC-HSPC or Exp-HSPC from 5 healthy donors. CD34+ and CD34+/CD90+ cell dose infused were recorded for each animal. We noted a significant increase in the number of Exp-HSPC required for engraftment compared with the MC-HSPC in both phenotypes (Fig. 4A and B). We also noted that the absolute level of engraftment of human cells in the mice (% CD45+) only

reached an average of $13.2 \pm 2.1\%$ at the highest dose of Exp-HSPC tested (4×10^6 CD34+ cells), while MC-HSPC reached $33.6 \pm 3.4\%$ at the highest dose tested (2×10^6 CD34+ cells). Since both multipotent progenitors and stem cells are capable of engrafting primary recipient mice (Notta *et al.*, 2011), we performed secondary transplants of bone marrow from primary mice receiving either MC-HSPC or Exp-HSPC. Femurs from mice receiving HSPC were pooled, and the CD34+ HSPC content and total nucleated

FIG. 4. Engraftment of NSG mouse bone marrow with minimally cultured or expanded HSPC. (**A**) The level of human cell engraftment at 16 weeks after transplantation measured as percentage of CD45+ cells in bone marrow based on CD34+ MC-HSPC or Exp-HSPC dose or (**B**) CD34+/CD90+ MC-HSPC or Exp-HSPC dose. Line represents regression curve for engraftment. $N=174$ mice for MC-HSPC and 110 mice for Exp-HSPC transplanted with cells from five healthy donors. (**C**) Secondary engraftment of NSG mice with bone marrow isolated from MC-HSPC- or Exp-HSPC-engrafted primary mice. *** $p < 0.001$. MC-HSPC, minimally cultured HSPC; Exp-HSPC, expanded HSPC (cultured for 7 days).

cell count were determined. For MC-HSPC, 8 mice received 3.0×10^7 total bone marrow cells per mouse containing 2.1×10^6 CD34+/CD45+ cells. For Exp-HSPC, 6 secondary recipient mice received 3.7×10^7 total bone marrow cells per mouse containing 7.4×10^5 CD34+/CD45+ cells. Each cohort was analyzed for the presence of human cells 16 weeks after secondary transplant (Fig. 4C). Animals receiving secondary transplants of bone marrow from both MC-HSPC ($N=8$)- and Exp-HSPC ($N=6$)-engrafted primary mice were engrafted with human cells at low but significant levels above the background of mice receiving only saline ($p \leq 0.001$). Therefore, primitive hematopoietic stem cells were present in each population used to transplant the primary mice.

In order to estimate the frequency of an engrafting dose in minimally cultured and expanded populations, we performed extreme limiting dilution analysis of 68 mice transplanted with MC-HSPC and 38 mice receiving Exp-HSPC. We used CD34+/CD90+ dose to estimate the frequency of NSG engrafting activity in the two populations. Animals were transplanted with limiting doses of CD34+/CD90+ HSPC ranging from 3250 to 220,000 cells (MC-HSPC) and 81,250 to 349,300 cells (Exp-HSPC), which included engraftment rates from 0% to 100% for each of the groups but did not include doses above the minimum dose that resulted in 100% engraftment. We defined engraftment as $\geq 1\%$ CD45+ cells in bone marrow at 16 weeks. The slope coefficient for CD34+/CD90+ cells (0.94) was not different from 1, indicating that the single-hit Poisson model is ap-

propriate. The estimated frequency of NRU in the CD34+/CD90+ population was 10-fold lower (range 5.11–19.67) in the Exp-HSPC compared with the MC-HSPC (Fig. 5). When considering that the mean expansion of CD34+/CD90+ cells was 9.5 ± 0.8 during 7 days of culture in SFT6+ SR-1, we concluded that there was no expansion of NRU in adult HSPC during culture in SFT6+ SR-1.

Expanded adult HSPC have reduced lymphoid potential

We also performed phenotypic analysis of the bone marrow and spleen of >200 primary animals transplanted with MC-HSPC or Exp-HSPC. Both populations of HSPC engrafted and produced multiple lymphoid and myeloid lineages, but the frequency of CD4+ T-cells and CD19+ B-cells among the human CD45+ cells in the spleen of animals receiving Exp-HSPC was significantly reduced compared with animals transplanted with MC-HSPC ($p < 0.05$), and monocytes were significantly increased at the highest Exp-HSPC dose tested ($p < 0.01$) (Fig. 6). No significant differences were observed in the level of CD3+/CD8+ T-cells in the spleen (data not shown) or CD34+ cells in the bone marrow of animals at 16 weeks after transplantation with MC-HSPC and Exp-HSPC. These results demonstrate that in addition to the reduction in the frequency of *in vivo* engrafting cells among the Exp-HSPC population, there is a deficit in CD4 T-cell and B-cell potential. There was no gross evidence of tumors in any mice, and >90% of mice survived to the endpoint of 16 or 25 weeks in these assays.

Discussion

The expansion of adult HSPC is of great interest in stem cell transplantation and gene therapy. The provision of increased numbers of HSPC for autologous transplantation will improve rates of engraftment and increase the likelihood of successful replacement of defective or disease-susceptible blood cells with functional and/or disease-resistant versions. In this study, we evaluated the use of an AhRA to expand adult HSPC as this methodology was previously shown to expand both cord blood and adult HSPC (Boitano *et al.*, 2010), but no *in vivo* engraftment data were provided for the expanded adult HSPC population. Other groups using unique AhRA or AhR knockout mice have suggested that there may be defects in engraftment and lineage potential using this approach (Thurmond *et al.*, 1999; Carlin *et al.*, 2013; Singh *et al.*, 2014). Therefore, we conducted extensive analysis of engraftment and lineage potential of *in vitro*-expanded adult HSPC to determine the suitability of this procedure for clinical implementation in our HIV gene therapy program.

We describe here the use of an immunodeficient (NSG) mouse model to quantitatively and qualitatively assess *in vitro* expansion of adult HSPC and their progeny. The use of adult (8–10 weeks old) NSG mice in these studies avoided the difficulties of timing, transplant, and survival of neonates that has been previously observed (Shultz *et al.*, 2005), and by combining adult source of stem cells and adult mice, we were able to create large numbers of animals and perform robust statistical analysis of the effects of *in vitro* expansion of HSPC from a clinically relevant source of cells. Dose–response curves for engraftment were

FIG. 5. Limiting dilution analysis of the frequency of NSG-engrafting cells. The results from engraftment of NSG mice with limiting numbers of CD34+/CD90+ cells from MC-HSPC and Exp-HSPC are shown. Log fraction of nonresponding (nonengrafting) animals is shown on the Y-axis, while CD34+/CD90+ cell dose is shown on the X-axis. MC-HSPC ($N=68$) are shown in red circles, and Exp-HSPC ($N=38$) are shown in black circles. Downward triangles indicate the dose for each population at which there were no nonresponding animals. Dotted lines indicate 95% confidence interval for each population.

FIG. 6. Phenotypic distribution of CD45+ cells in the spleen and bone marrow of humanized NSG mice. Average frequency ($\pm$ SEM) of CD3+/CD4+ and CD3+/CD8+ cell populations in the spleen (A and B) and CD34+, CD19+, CD4+/CD14+, and CD33+ cell populations in the bone marrow (C–F) is shown for different CD34+ MC-HSPC and Exp-HSPC doses (M, million). $N=203$ mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NS, not significant.

established using limiting numbers of cells as a basis for quantitative comparison of MC-HSPC with Exp-HSPC. Phenotypic analysis of populations before and after expansion combined with quantitative analysis of *in vivo* engraftment allowed us to establish the relationship between phenotype and function of HSPC. Secondary engraftment and lineage analysis were used to verify the presence of primitive stem cells in each population and assess any defects in multilineage hematopoietic potential, respectively.

Investigation of lineage development in transplanted animals revealed that engraftment of both MC-HSPC and Exp-HSPC was multilineage (monocytes, T-cells, B-cells, NK cells, and progenitors) and specifically included both CD3+/CD4+ T-cells and CD4+/CD14+ monocytes, which are targets for HIV infection and thus critical for evaluating our HIV gene therapy strategy *in vivo*. Multilineage engraftment was durable, lasting for at least 25 weeks after transplant, and human stem and progenitor cells capable of engraftment of secondary recipients were present in the

bone marrow of the primary recipients for at least 16 weeks, confirming the presence of primitive hematopoietic stem cells in the primary grafts. These results are similar to previous studies in which adult growth factor-mobilized peripheral blood was used to transplant NSG mice and further support the use of this model system for investigating human disease (Herrera *et al.*, 2001; Shultz *et al.*, 2005; Andre *et al.*, 2010; Gille *et al.*, 2012; Hakki *et al.*, 2014). However, unlike transplantation of neonatal mice with cord blood HSPC, no human cell engraftment of lymph nodes or thymus and minimal engraftment of cells in the circulation of these mice were observed. It is not clear whether this is because of a failure of the adult HSPC to home to these organs or the inability of these organs to support development of human blood cells from HSPC in adult mice. Since lymphoid engraftment of spleen and bone marrow was robust, we conclude that the failure to engraft some lymphoid organs (thymus and lymph nodes) was not a property of the HSPC but rather a reflection of changes in

the thymic and peripheral organ microenvironment in mice by 8 weeks that precludes repopulation of these organs from the donor graft. The ability of T-cells to mature in an extrathymic microenvironment is encouraging considering our intent to treat older patients and patients on long-term combination antiretroviral therapy who also have compromised lymphopoietic niches (reviewed in Gaardbo *et al.*, 2012). This model system may then be useful for studying the development of T-cells outside of the thymus and trafficking via functional comparisons of microenvironment in young versus older mice.

Using a single-hit kinetic model of engraftment, we determined that the frequency of NSG-engrafting cells among the CD34+ and CD34+/CD90+ population was reduced in the Exp-HSPC relative to MC-HSPC. This reduction was balanced by the expansion of the CD34+ and CD34+/CD90+ cells *in vitro* and resulted in the overall maintenance of total NSG-engrafting units. CD34+ cells are known to be comprised of both long-term and short-term engrafting progenitor cells, and it was not unexpected that their expansion did not necessarily reflect the expansion of long-term repopulating cells in this model. However, even the primitive stem cell phenotype (CD34+/CD90+) did not maintain fidelity with biological activity; therefore, this marker combination was still insufficient to accurately predict differences in engraftment among the MC-HSPC and Exp-HSPC. This type of discordance between *in vitro* estimates of expansion by stem cell surface phenotype and *in vivo* estimates of engraftment was also observed with cord blood in previous studies using identical conditions, but longer culture periods (Boitano *et al.*, 2010).

The skewing of engraftment away from the lymphoid lineage and toward myeloid lineage was also noteworthy and similar to results from prior HSPC expansion studies. It is not clear whether this bias is the result of the preferential expansion of HSPC with myelopoietic potential or a shift of the overall fate of HSPC in this direction as has been observed in aged stem cells (Van Zant and Liang, 2012; Snoeck, 2013). We are currently investigating intracellular signaling pathways in expanded HSPC to determine whether disruption of pathways known to maintain stem cells in a primitive state is altered. The maintenance of balanced lymphoid and myeloid potential will likely be required in any *in vitro* expansion protocol intended for use in a clinical setting. This is especially true in the setting of our HIV gene therapy trials in which recovery of CD4+ T-cells is a central goal.

In these studies, we have established the utility of the adult NSG transplant model in quantitative evaluation of the hematopoietic stem cell potential of adult HSPC. The number of transplantable HSPC derived from a single adult apheresis product ($1-4 \times 10^8$ HSPC) allows for the creation of hundreds of mice from a single donor and thus supports statistical evaluation of cohorts treated under different conditions, with gene therapy vectors or other candidate therapeutic agents. Further improvements to this model system may be realized through the use of alternative preconditioning procedures or transgenic expression of human cytokines and/or major histocompatibility complex (MHC) molecules. Several groups have demonstrated that preconditioning animals with busulfan leads to efficient cell engraftment (Kramer *et al.*, 2006; Choi *et al.*, 2011a; Singh

et al., 2012; Chevalere *et al.*, 2013), and this procedure is more likely to reflect the conditioning used to prepare patients for transplantation. Alternatively, newborn NSG mice that constitutively express SCF support enhanced engraftment of HSPC in the absence of irradiation of chemical conditioning, and animals develop functional immune systems that are capable of rejecting allogeneic skin grafts (Brehm *et al.*, 2012). Finally, the introduction of class I MHC molecules into NSG mice has allowed for the development of HLA-restricted human T-cell responses and promotes the evaluation of immune function to viral Epstein-Barr Virus antigens in these animals (Strowig *et al.*, 2009).

In summary, we have developed a robust animal model of human HSPC engraftment that has proven useful for qualitative and quantitative analysis of *ex vivo* manipulations related to enhancing the outcome of HSPC transplantation. We were able to demonstrate the maintenance but not expansion of NSG-engrafting units during short-term culture with a skewing, although not loss, of lineage potential. Understanding differences in the ability to expand fetal, neonatal, and adult sources of stem cells is critical in designing clinical interventions, and much of what can be learned by studying these differences may aid in improvements in the engineering of hematopoietic stem cell grafts for a wide variety of indications. We believe that the data presented here represent a significant step toward this goal.

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Author Disclosure Statement

The authors declare no competing financial interests.

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