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Authors

Masiuk, Katelyn E
Brown, Devin
Laborada, Jennifer
et al.

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Improving Gene Therapy Efficiency through the Enrichment of Human Hematopoietic Stem Cells

Katelyn E. Masiuk,[1] Devin Brown,[1] Jennifer Laborada,[1] Roger P. Hollis,[1] Fabrizia Urbinati,[1] and Donald B. Kohn[1,2,3]

[1]Department of Microbiology, Immunology & Molecular Genetics, University of California, Los Angeles, Los Angeles, CA 90095, USA; [2]Department of Pediatrics, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA; [3]Department of Molecular & Medical Pharmacology, University of California, Los Angeles, Los Angeles, CA 90095, USA

Lentiviral vector (LV)-based hematopoietic stem cell (HSC) gene therapy is becoming a promising clinical strategy for the treatment of genetic blood diseases. However, the current approach of modifying 1×10^8 to 1×10^9 $CD34^+$ cells per patient requires large amounts of LV, which is expensive and technically challenging to produce at clinical scale. Modification of bulk $CD34^+$ cells uses LV inefficiently, because the majority of $CD34^+$ cells are short-term progenitors with a limited post-transplant lifespan. Here, we utilized a clinically relevant, immunomagnetic bead (IB)-based method to purify $CD34^+$ $CD38^-$ cells from human bone marrow (BM) and mobilized peripheral blood (mPB). IB purification of $CD34^+CD38^-$ cells enriched severe combined immune deficiency (SCID) repopulating cell (SRC) frequency an additional 12-fold beyond standard $CD34^+$ purification and did not affect gene marking of long-term HSCs. Transplant of purified $CD34^+CD38^-$ cells led to delayed myeloid reconstitution, which could be rescued by the addition of non-transduced $CD38^+$ cells. Importantly, LV modification and transplantation of IB-purified $CD34^+$ $CD38^-$ cells/non-modified $CD38^+$ cells into immune-deficient mice achieved long-term gene-marked engraftment comparable with modification of bulk $CD34^+$ cells, while utilizing ~7-fold less LV. Thus, we demonstrate a translatable method to improve the clinical and commercial viability of gene therapy for genetic blood cell diseases.

INTRODUCTION

Lentiviral vector (LV)-based gene modification of autologous hematopoietic stem cells (HSCs) has demonstrated remarkable success for the treatment of genetic blood diseases.[1–6] In this approach, bone marrow (BM) or mobilized peripheral blood (mPB) is collected from a patient, enriched for HSCs, transduced with LV encoding the correct genetic sequence, and transplanted back into the patient. After transplant, HSCs engraft in the BM and produce mature, gene-corrected hematopoietic cells ideally throughout the patient's lifetime. New clinical trials using anti-sickling β-globin vectors show promising early results in the treatment of sickle cell disease (SCD), the most common genetic blood disorder affecting millions of people.[7] However, broad use of gene therapy will require an unprecedented amount of clinical-grade vector production, a process that is currently limited by high cost and commercial manufacturing capacity. Currently, production and clinical validation of β-globin vector for a single patient may require 20–40 L of viral supernatant and cost in the range of $250,000–$500,000. Therefore, new methods that reduce the amount of LV required per patient are needed to make gene therapy clinically and commercially feasible.

One strategy to reduce LV requirements is to improve enrichment of HSCs before transduction. Current clinical HSC enrichment protocols use immunomagnetic beads (IBs) to select for cells expressing the surface marker CD34 prior to LV transduction. This method is inefficient in a gene therapy setting, because the majority of $CD34^+$ cells are short-term progenitor cells with a limited post-transplant lifespan. Further isolation of HSCs from short-term progenitors could improve LV economy by only transducing cells that can endure for the lifetime of the patient. We postulated that purifying $CD34^+$ $CD38^-$ cells would further enrich for HSCs, reduce the absolute number of cells to be transduced with LV, and reduce the total LV dose required per patient. Importantly, these benefits could be achieved while still retaining the target HSCs required for long-term clinical benefit after transplantation.

To date, little is known about clinical transplantation of highly purified HSCs. A small number of clinical trials have shown successful transplantation of $CD34^+CD90^+$ cells,[8–10] which represented ~50% of total $CD34^+$ cells. In contrast, $CD34^+CD38^-$ cells are highly enriched for HSCs and represent ~5%–10% of $CD34^+$ cells. While a large body of work has characterized the repopulation capacity of $CD34^+CD38^-$ cells, the majority of this work has been performed using fluorescence-activated cell sorting (FACS)-purified cord blood

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Correspondence: Donald B. Kohn, Department of Microbiology, Immunology & Molecular Genetics, University of California, Los Angeles, 610 Charles E. Young Drive, 3163 Terasaki Life Sciences Building, Los Angeles, CA 90095, USA.
E-mail: dkohn1@mednet.ucla.edu

(CB). In contrast with FACS purification, IB-based cell sorting is currently available in Good Manufacturing Practice (GMP)-grade platforms with the capacity to purify large cell numbers quickly with high recovery. Here, we investigate an IB-based method to purify $CD34^+CD38^-$ cells from human BM and mPB samples with focus on practical implementation of our findings to current clinical gene therapy trials. We demonstrate the capacity of this method to reduce LV dose by ~5- to 10-fold while maintaining early post-transplant recovery and long-term engraftment of gene-modified cells. These findings have the potential to reduce the total LV required per patient and improve the clinical and commercial viability of gene therapy for genetic blood cell diseases.

RESULTS

More Than 90% of $CD34^+$ Long-Term Repopulating Activity Is Contained within the Lowest 6% of CD38 Expression

Translating $CD34^+CD38^-$ purification to the clinic will require efficient HSC recovery to sustain engraftment of gene-corrected cells after transplant. In order to identify a target cell population to purify for gene therapy, we first sought to determine which subsets of BM $CD34^+$ cells are capable of long-term engraftment after ex vivo culture required for LV transduction. We performed a competitive transplant assay in immune-deficient non-obese diabetic (NOD)/severe combined immune deficiency (SCID)/gamma (NSG) mice utilizing $CD34^+$ cells with varying levels of CD38 expression (Figure 1A). IB-purified $CD34^+$ BM cells were sorted by FACS to obtain three different cell populations with increasing, constant percentiles of CD38 expression (e.g., 6% intervals: 0%–6%, 6%–12%, and 12%–18% of CD38 expression). Each fraction of sorted cells was pre-stimulated with cytokines (interleukin-3 [IL-3], stem cell factor [SCF], fms-related tyrosine kinase 3 [Flt3]-ligand, and thrombopoietin) for 24 hr and then transduced for an additional 24 hr with one of three fluorescent LVs: CCL-ubiquitin C (UBC)-mCitrine, CCL-UBC-mStrawberry, or CCL-UBC-mCerulean. Following transduction, 15,000 cells from each of three differentially labeled CD38 intervals were competitively co-transplanted into sub-lethally irradiated NSG mice. Fluorescent reporter gene color choice for each sorted fraction was rotated to account for potential differences in transduction efficiency among vectors (Figure S1).

At 16–18 weeks post-transplantation, engrafted $hCD45^+$ cells in NSG BM were analyzed by flow cytometry for mCitrine, mStrawberry, and mCerulean expression to determine the long-term engraftment potential of each initially sorted population. Two separate transplants were performed using different intervals of CD38 expression (Figure 1B). In transplant 1, >90% of $hCD45^+$ cells present in the BM were derived from $CD34^+CD38^{0\%–6\%}$ cells, whereas <10% of $hCD45^+$ cells were derived from $CD34^+CD38^{6\%–12\%}$ and $CD34^+CD38^{12\%–18\%}$ cells. In transplant 2, 54% of $hCD45^+$ cells were derived from $CD34^+CD38^{0\%–3\%}$ cells, with an additional 40% of $hCD45^+$ cells derived from the $CD34^+CD38^{3\%–6\%}$ fraction. For each transplant, the observed patterns were consistent in myeloid ($CD33^+$), lymphoid ($CD19^+$), and hematopoietic stem and progenitor cells (HSPCs) ($CD34^+$) lineages. Collectively, these results suggest that after ex vivo culture and LV transduction, the majority of long-term repopulating activity in $CD34^+$ adult BM cells is contained within the lowest 6% of CD38 expression. Furthermore, while the $CD34^+CD38^{0\%–3\%}$ fraction is highly enriched for long-term HSCs, the $CD34^+CD38^{3\%–6\%}$ also contains a substantial proportion of long-term HSCs; thus, purification beyond the lowest 6% of CD38 expression may lead to HSC loss. Based on these results, we defined "$CD34^+CD38^-$ cells" as the lowest 6% of CD38 expression for subsequent experiments tracking phenotypic HSC recovery.

Titration of CD38 Magnetic Labeling Optimizes Recovery and Purity of $CD34^+CD38^-$ Cells

We next sought to optimize a clinically relevant, IB-based method to purify $CD34^+CD38^-$ cells. We chose to first deplete $CD38^+$ cells and subsequently select $CD34^+$ cells. CD38 magnetic labeling was performed using a primary anti-CD38-phycoerythrin (PE)-conjugated antibody followed by incubation with anti-PE magnetic beads. Unlabeled $CD38^-$ cells can be relabeled with CD34 beads, thereby preventing the necessity of a bead removal step. $CD34^+$ cells exhibit a gradient pattern of CD38 expression rather than discrete positive and negative populations. We found that the proportion of cells separated into $CD38^-$ and $CD38^+$ fractions could be adjusted by varying the intensity of CD38 magnetic labeling with different concentrations of magnetic beads (Figure 2). Mononuclear cells (MNCs) from CB, BM, and mPB were separated into $CD38^-$ and $CD38^+$ fractions using three different concentrations of anti-PE beads. Strong magnetic labeling (anti-PE beads 1:5) greatly enriched for $CD34^+CD38^-$ cells (13-, 16-, and 15-fold for CB, BM, and mPB, respectively), but resulted in low recovery of $CD34^+CD38^-$ cells (50%, 19%, and 59% for CB, BM, and mPB). Weak magnetic labeling (anti-PE beads 1:25) resulted in high recovery of $CD34^+CD38^-$ cells (93%, 67%, and 96% for CB, BM, and mPB, respectively) but reduced enrichment of $CD34^+CD38^-$ cells (4.6-, 10.3-, and 4.7-fold for CB, BM, and mPB). Thus, there is a tradeoff between recovery and fold-enrichment of $CD34^+CD38^-$ cells that can be optimized by altering the strength of CD38 magnetic labeling. Here, we observe that anti-PE bead concentrations of 1:10 for mPB and >1:25 for BM (1:40 was used in all subsequent experiments) can optimize both enrichment and recovery of $CD34^+CD38^-$ cells.

Co-depletion of $CD15^+$ and $CD38^+$ Cells Increases the Purity of Isolated $CD34^+CD38^-$ Cells

In BM and CB, we observed that CD38 depletion followed by CD34 selection led to a final cell product with <50% $CD34^+$ cells because of contamination of $CD15^+$ granulocytes (Figure 3A, top). Similar absolute numbers of granulocytes were isolated by $CD34^+$ or $CD34^+CD38^-$ purification (Figure 3B), suggesting that the CD38 depletion step does not increase non-specific granulocyte selection. To obtain more highly purified $CD34^+CD38^-$ cells, we included a CD15 magnetic bead during the CD38 depletion step, allowing us to simultaneously co-deplete $CD38^+$ cells and $CD15^+$ granulocytes prior to CD34 selection (Figure 3C). In BM, addition of a CD15 bead increased purity of $CD34^+CD38^-$ cells from 24.5% of total cells to

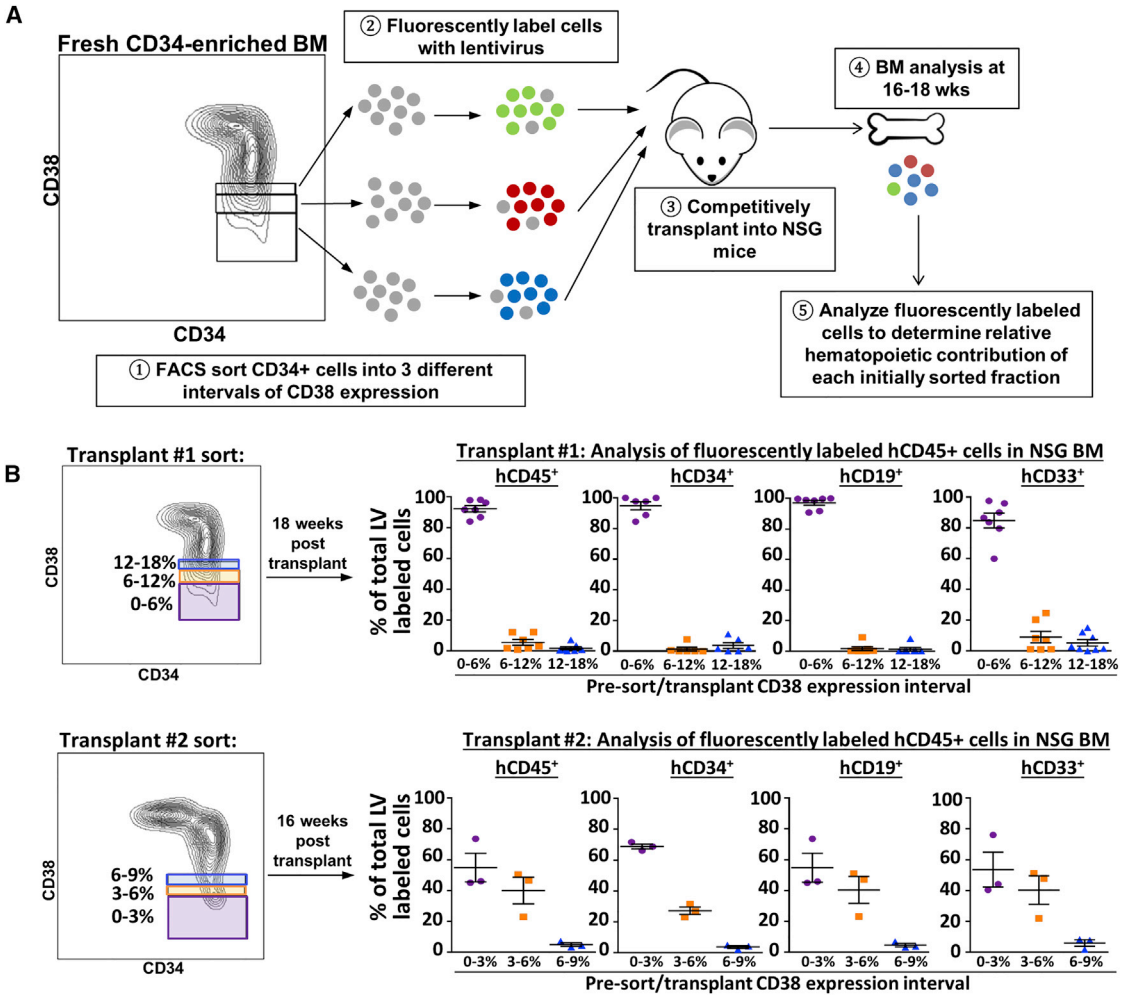


Figure 1. Long-Term Repopulating Activity of CD34$^+$CD38$^-$ Subsets

(A) Experimental setup. (1) FACS sort: BM cells were magnetically enriched for CD34 and sorted by FACS into distinct populations of increasing CD38 expression. Each gate contains an equivalent number of cells. (2) LV transduction: Cells from each interval were transduced with one of three LVs expressing the fluorescent proteins mCitrine, mStrawberry, or mCerulean. (3) Transplant: A combination of cells from each sorted interval (each differentially labeled) were combined and competitively transplanted into sub-lethally irradiated NSG mice. Each mouse received an equal number of cells from each sorted fraction. (4) BM analysis: NSG BM was analyzed at 16–18 weeks for the presence of fluorescently labeled hCD45$^+$ cells. (5) Fluorescently labeled hCD45$^+$ cells engrafted in the NSG BM were analyzed for their relative contribution to human hematopoiesis. (B) Relative abundance of labeled cells in NSG BM. Two separate transplants were performed using different intervals of CD38 expression (top and bottom). Initial sort for CD38 expression is shown in the left panels. At 16–18 weeks post-transplant, fluorescent, LV-labeled hCD45$^+$, hCD34$^+$, hCD19$^+$, and hCD33$^+$ cells were analyzed. y axis represents the relative frequency of each sorted and/or labeled CD38 fraction as a percent of all fluorescently labeled cells. Bars represent mean ± SEM. n = 7 mice for transplant 1; n = 3 mice for transplant 2.

70.5% of total cells, thus further reducing total number of cells isolated prior to transduction. Based on these findings, all subsequent CD34$^+$CD38$^-$ purifications were performed using CD38/CD15 co-depletion.

Optimized CD34$^+$CD38$^-$ Dual IB Purification Enriches CD34$^+$CD38$^-$ Cells with High Recovery and Results in Efficient HSC Gene Marking

We next compared the optimized CD34$^+$CD38$^-$ IB purification strategy with standard CD34$^+$ selection in multiple BM and mPB samples from healthy donors. For each sample analyzed, two identical fractions of MNCs were purified by either CD34$^+$ single-step IB purification or CD34$^+$CD38$^-$ dual IB purification. The total cell product obtained after purification by each method was analyzed by flow cytometry and counting beads to determine absolute CD34$^+$CD38$^-$ cell counts (Figures 4A and 4B; Figure S2). In BM, CD34$^+$CD38$^-$ dual IB purification of starting MNCs recovered a median 62.5% (range 57.2%–65.6%) of phenotypically defined CD34$^+$CD38$^-$ cells, whereas standard CD34 selection recovered a median of 93.1% (range 80%–95.6%) of CD34$^+$CD38$^-$ cells. CD34$^+$CD38$^-$ dual IB purification

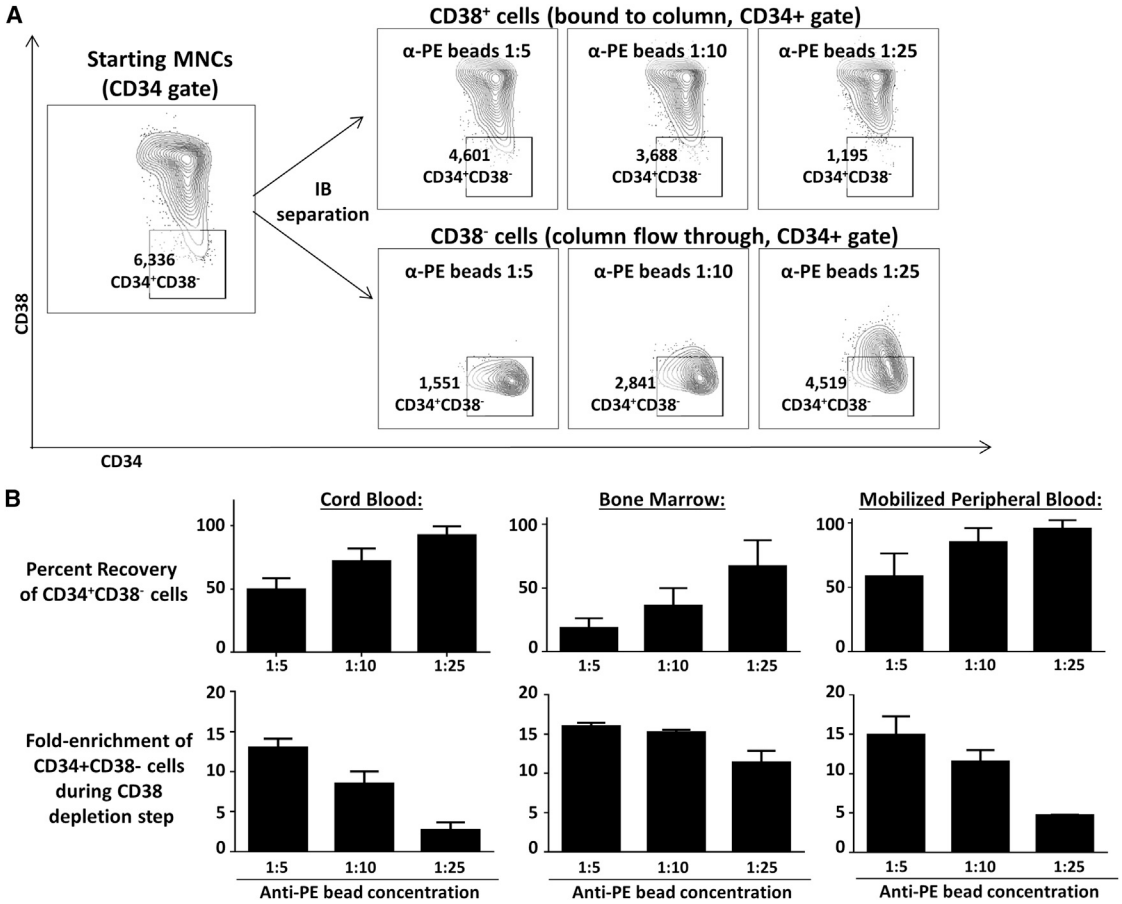


Figure 2. Titration of CD38 Immunomagnetic Labeling Influences Recovery and Enrichment of $CD34^+CD38^-$ Cells

(A) CD38 depletion of BM mononuclear cells (MNCs) using three different concentrations of anti-PE beads (one representative experiment is shown). Plots are gated to show $CD34^+$ cells present in starting MNCs (far left), and after column separation into $CD38^+$ (top) and $CD38^-$ (bottom) fractions. Gates show $CD34^+CD38^-$ cells (defined as the lowest 6% of CD38 expression) and list absolute counts of $CD34^+CD38^-$ cells in each population. (B) Efficiency of CD38 depletion step at different PE bead concentrations. Bar graphs show "percent recovery" and "fold-enrichment" of $CD34^+CD38^-$ cells before and after CD38 depletion using three different anti-PE bead concentrations. Bars represent mean ± SEM. Data represent three independent experiments each for CB, BM, and mPB.

enriched for $CD34^+CD38^-$ cells an additional 8.7-fold (range 4.8–12) beyond standard $CD34^+$ purification. Similar results were achieved in mPB, with a median $CD34^+CD38^-$ recovery of 75.1% (range 58.4%–91.0%) by dual $CD34^+CD38^-$ purification and 87.9% (range 83.7%–93.2%) by standard CD34 selection. $CD34^+CD38^-$ dual IB purification enriched for $CD34^+CD38^-$ cells an additional 12-fold (range 8.7–13.2) beyond standard $CD34^+$ purification.

To further assess recovery of functional HSCs, we transplanted cells from each purified population into NSG mice at limiting doses. Limiting dilution analysis of CCL-UBC-mCitrine LV-transduced cells demonstrated a 12-fold enrichment of SCID repopulating cell (SRC) frequency within $CD34^+CD38^-$ cells (1 in 2,314 cells; 95% confidence interval: 1/1,241–1/4,314) compared with $CD34^+$ cells (1 in 28,248 cells; 95% confidence interval: 1/14,701–1/54,278) (Figure 4C; Figure S3). Analysis of long-term (>16 week) engrafted $hCD45^+$ cells in NSG BM revealed no significant difference in average vector copy number per human cell between groups, suggesting that the dual $CD34^+CD38^-$ IB purification method allows for efficient transduction of HSCs while using less cells and LV (Figure 4D).

$CD34^+CD38^-$ Cells Show Delayed Myeloid Recovery that Can Be Rescued by Increasing Cell Dose or Adding Back Non-transduced $CD38^+$ Cells

Purifying $CD34^+CD38^-$ cells may provide the advantage of utilizing less LV to transduce HSCs. However, transplanting a smaller population of quiescent, stem-enriched cells may cause delayed recovery of neutrophils and leave a patient susceptible to infection early after transplant. Therefore, we compared the early myeloid potential of IB-purified $CD34^+$ and $CD34^+CD38^-$ cells. Here, we transplanted purified cells into the NSG-SGM3 mouse model,[11] an immune-deficient mouse model that supports human myelopoiesis through constitutive expression of human cytokines IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and SCF. In order to

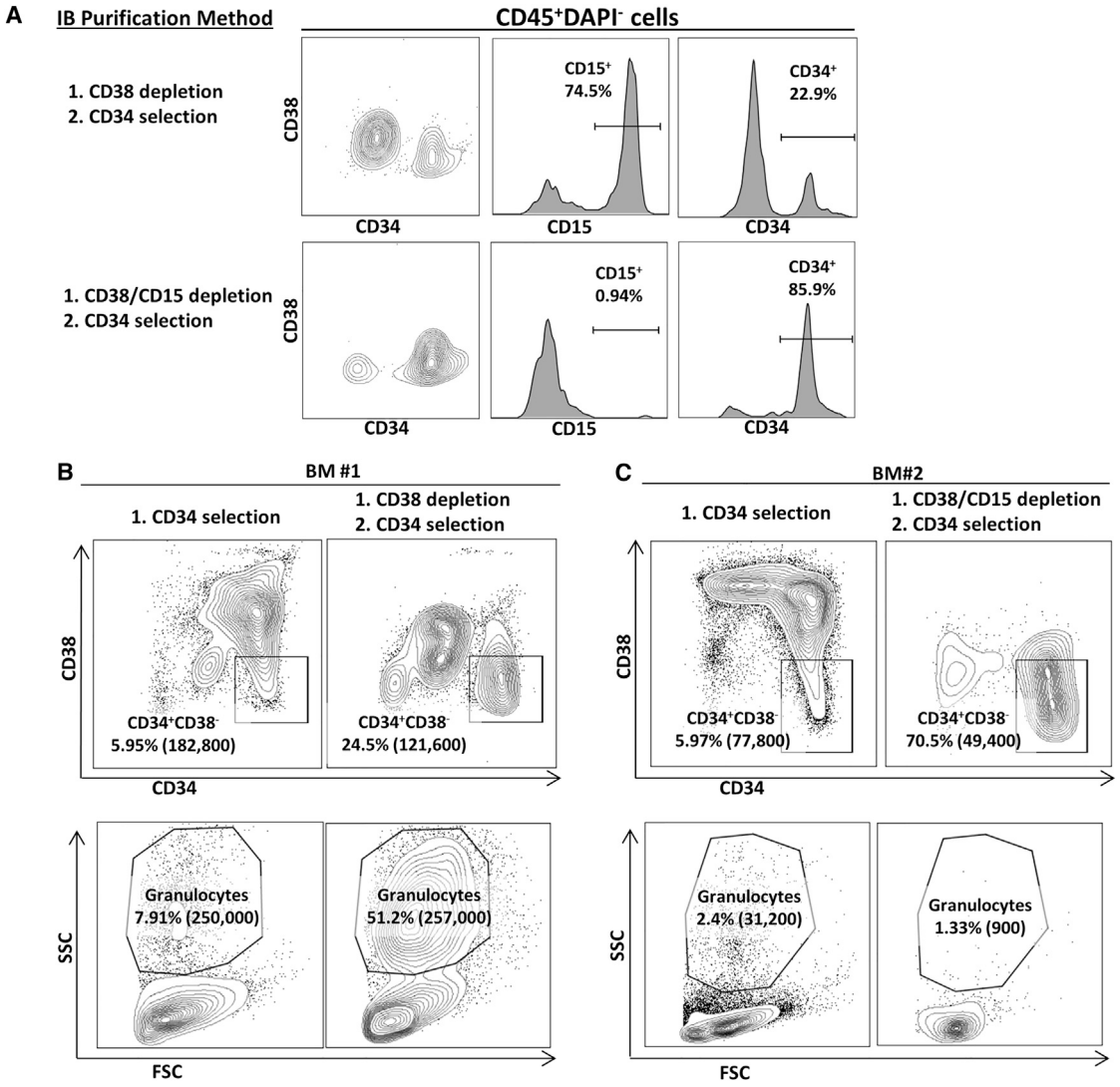
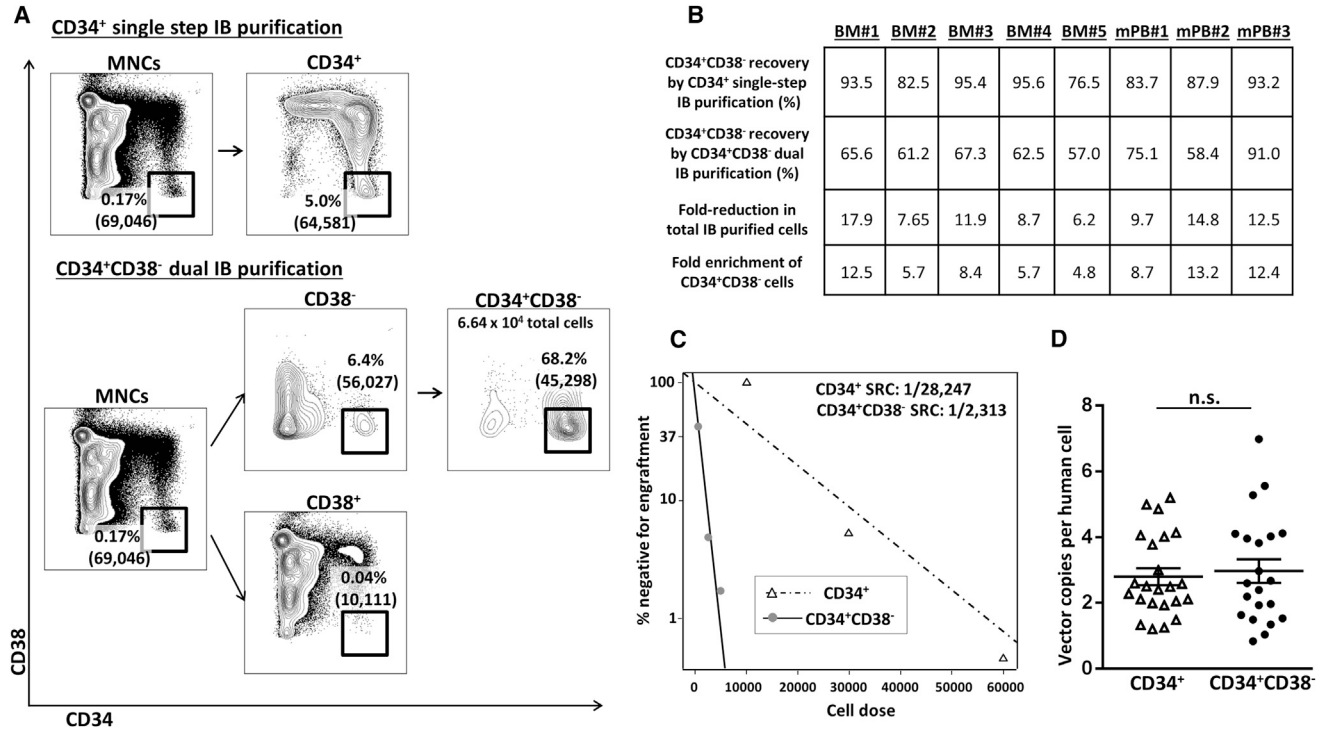


Figure 3. Co-depletion of CD15⁺ and CD38⁺ Cells Increases CD34⁺CD38⁻ Purity

(A) $CD34^+CD38^-$ cells were purified from CB MNCs using one of two methods: CD38 depletion followed by CD34 selection (top) or CD38/CD15 co-depletion followed by CD34 selection (bottom). Leftmost FACS plot shows CD34 and CD38 staining of all viable leukocytes ($CD45^+DAPI^-$) isolated by either method. Histograms indicate the percentage of $CD15^+$ and $CD34^+$ cells within total viable leukocytes isolated by each method. (B) IB purification of BM 1 (no CD15 co-depletion). Left panel shows CD34 purification, and right panel shows parallel $CD34^+CD38^-$ purification (using CD38 depletion only). FACS plots show viable leukocytes ($CD45^+DAPI^-$) isolated by each method. Gates show $CD34^+CD38^-$ cells (top) and granulocytes (defined by high side scatter [SSC], bottom) purified by each method. Absolute cell counts are listed in parentheses. (C) IB purification of BM 2 (with CD15 co-depletion). Left panel shows CD34 purification, and right panel shows parallel $CD34^+CD38^-$ purification (using CD38/CD15 co-depletion). FACS plots show viable leukocytes ($CD45^+DAPI^-$) isolated by each method. Gates show $CD34^+CD38^-$ cells (top) and granulocytes (defined by high SSC, bottom) purified by each method. Absolute cell counts are listed in parentheses.

reflect a clinically relevant scenario, transplanted cell doses for each group represented the total cells obtained from IB purification of a standard volume of marrow (marrow equivalent [ME]). 1 marrow equivalent of $CD34^+CD38^-$ cells showed decreased early production of circulating human myeloid cells as compared with 1 marrow equivalent of $CD34^+$ cells (Figure 5A). 2.5 marrow equivalent of $CD34^+CD38^-$ cells achieved myeloid reconstitution comparable with 1 marrow equivalent of $CD34^+$ cells, whereas 6 marrow equivalent of $CD34^+CD38^-$ cells resulted in myeloid reconstitution ~3-fold higher than 1 marrow equivalent of $CD34^+$ cells. These results demonstrate that dual IB-purified $CD34^+CD38^-$ cells are capable of early myeloid reconstitution, but contain less early myeloid potential than $CD34^+$ cells purified from an equivalent volume of BM.

We next investigated whether co-transplanting non-transduced $CD38^+$ cells (obtained during IB depletion of MNCs) alongside



	BM#1	BM#2	BM#3	BM#4	BM#5	mPB#1	mPB#2	mPB#3
CD34+CD38- recovery by CD34+ single-step IB purification (%)	93.5	82.5	95.4	95.6	76.5	83.7	87.9	93.2
CD34+CD38- recovery by CD34+CD38- dual IB purification (%)	65.6	61.2	67.3	62.5	57.0	75.1	58.4	91.0
Fold-reduction in total IB purified cells	17.9	7.65	11.9	8.7	6.2	9.7	14.8	12.5
Fold enrichment of CD34+CD38- cells	12.5	5.7	8.4	5.7	4.8	8.7	13.2	12.4

Figure 4. Comparison of CD34⁺ Single-Step IB Purification versus CD34⁺CD38⁻ Dual IB Purification

(A) Comparison of a single representative BM sample purified in parallel by either CD34⁺ single-step IB purification (top) or CD34⁺CD38⁻ dual IB purification (bottom). Gates show CD34⁺CD38⁻ cells (defined as the lowest 6% of CD38 expression) with absolute cell counts listed in parentheses. (B) Recovery and enrichment of CD34⁺CD38⁻ cells obtained from IB purification of BM and mPB MNCs. 5 BM samples and 3 mPB sample from independent donors were purified in parallel by either CD34⁺ single-step IB purification or CD34⁺CD38⁻ dual IB purification. For each sample, CD34⁺CD38⁻ cells were defined as those with the lowest 6% of CD38 expression within CD34⁺ cells. Recovery indicates the percentage of CD34⁺CD38⁻ cells recovered after purification from starting MNCs, and it is calculated for both CD34⁺ single-step (row 1) and CD34⁺CD38⁻ dual IB purification (row 2). Fold-reduction (row 3) indicates the reduction in the total number of cells purified by CD34⁺CD38⁻ dual IB selection as compared with standard CD34 selection. This represents the clinically relevant reduction in the number of cells to be transduced and concordant vector dose reduction. Fold-enrichment (row 4) represents the enrichment of CD34⁺CD38⁻ cells as a percentage of total cells obtained by CD34⁺CD38⁻ dual IB purification compared with standard CD34⁺ IB purification. (Figure S2 shows absolute cell counts for each IB-purified fraction.) (C) Poisson statistical analysis of limiting dilution NSG xenotransplant assay of IB-purified BM. Plots demonstrate SRC frequency of CCL-UBC-mCitrine transduced CD34⁺ or CD34⁺CD38⁻ IB-purified cells (n = 5–7 mice transplanted at each cell dose per condition; see Figure S3 for individual values). y axis shows the percentage of recipient NSG mice containing <0.015% hCD45⁺ cells (non-engrafted) in the BM at 16 weeks post-transplantation versus the number of cells injected per mouse (x axis). (D) In vivo VCN analysis. NSG mice were transplanted with IB-purified CD34⁺ or CD34⁺CD38⁻ cells transduced with CCL-UBC-mCitrine LV. 16 weeks post-transplant, engrafted human cells in NSG BM were analyzed for VCN by digital droplet polymerase chain reaction (ddPCR). Data represent three independent experiments performed using BM from independent donors; n = 21–23 mice/group. n.s., not significant. Bars represent mean ± SEM.

transduced CD34⁺CD38⁻ cells could produce early hematopoietic recovery comparable with CD34⁺ cells. In order to avoid xenogeneic graft-versus-host disease (GVHD) in recipient mice receiving CD38⁺ cells, we purified CD34⁺, CD34⁺CD38⁻, and CD38⁺ populations from CD3-depleted BM MNCs (Figure 5B). IB-purified CD34⁺ or CD34⁺CD38⁻ cells were transduced with CCL-UBC-mCitrine LV, whereas CD38⁺ cells were cultured in parallel transduction conditions without LV. NSG-SGM3 mice received 1 marrow equivalent of the grafts depicted in Figure 5B: (1) CD34⁺ cells (+mCitrine LV), (2) CD34⁺CD38⁻ cells (+mCitrine LV), (3) CD34⁺CD38⁻ cells (+mCitrine LV) + CD38⁺ cells (non-transduced), or (4) CD38⁺ cells (non-transduced). Irradiated CD34⁻ cells were added as needed so that each mouse received the same number of total cells (Table S1).

Two transplants were performed using BM from independent donors. Transplant 1 used highly purified CD34⁺CD38⁻ cells (~1% of total CD34⁺ cells), and transplant 2 used more moderately purified CD34⁺ CD38⁻ cells (~12% of total CD34⁺ cells) (Figure 5C). In transplant 1, mice receiving CD34⁺CD38⁻ cells showed significantly reduced levels of circulating human neutrophils at 3 weeks compared with mice transplanted with CD34⁺ cells. The addition of non-transduced CD38⁺ cells to the graft significantly increased circulating neutrophil levels. The same pattern was observed in transplant 2, but it did not reach significance when using more moderately purified CD34⁺ CD38⁻ cells. Thus, when compared with CD34⁺ cells, moderately purified CD34⁺CD38⁻ cells (~12% of total CD34⁺ cells) do not show as great of an early myeloid deficit when compared with stringently purified CD34⁺CD38⁻ cells (~1% of total CD34⁺ cells). These results

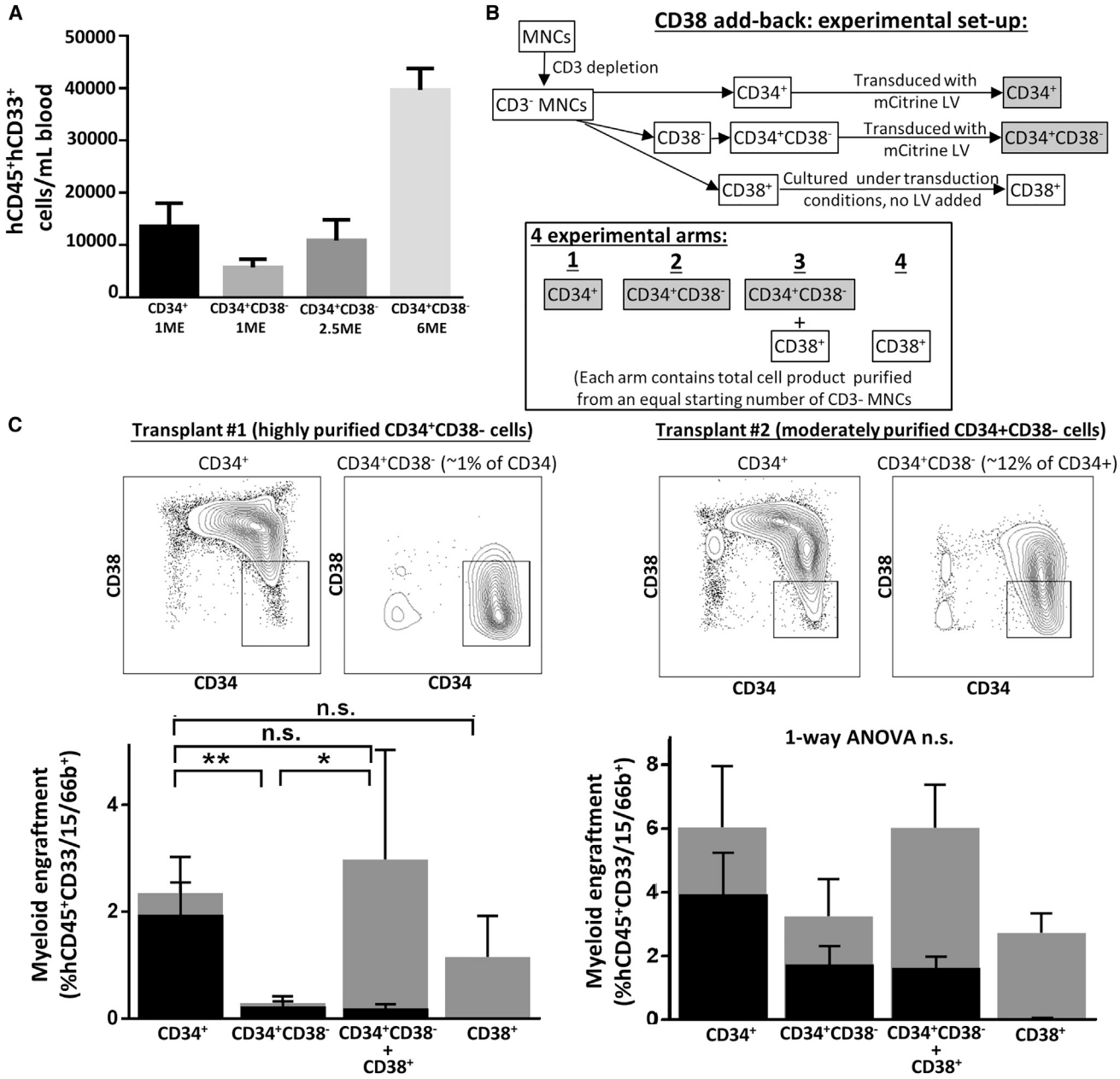


Figure 5. Early Myeloid Potential of IB-Purified CD34$^+$ and CD34$^+$CD38$^-$ Cells

(A) Early myeloid engraftment of CD34$^+$ and CD34$^+$CD38$^-$ IB-purified cells. Peripheral blood hCD45$^+$hCD33$^+$ cells per milliliter was quantified at 4 weeks from NSG-SGM3 mice transplanted with CD34$^+$ cells or increasing doses of CD34$^+$CD38$^-$ cells IB-purified from BM. Cell doses for each group reflect the total cells obtained from IB purification from a standard volume of marrow (marrow equivalent). n = 3 mice/group. Bars represent mean ± SEM. (B) CD38 add-back experimental setup. BM MNCs were IB-depleted of CD3$^+$ T cells to avoid xenogeneic graft-versus-host disease. CD3$^-$ cells were further purified to obtain CD34$^+$, CD34$^+$CD38$^-$, or CD38$^+$ populations. CD34$^+$ and CD34$^+$CD38$^-$ cells were transduced with a CCL-UBC-mCitrine LV, while CD38$^+$ cells were cultured in parallel without the addition of LV. 4 experimental arms were transplanted, with each mouse receiving 1 marrow equivalent of the pictured graft. Gray boxes represent UBC-mCitrine transduced populations; white boxes represent non-transduced populations. (C) Early myeloid engraftment of IB-purified cells in NSG-SGM3 mice. Two transplants were performed using the four arms described in (B). Flow plots show CD34$^+$ or CD34$^+$CD38$^-$ IB-purified cells prior to transplant (left: highly purified CD34$^+$CD38$^-$ cells; right: moderately purified CD34$^+$CD38$^-$ cells). Bar graphs represent mean ± SEM of myeloid engraftment in peripheral blood. Gray bars represent total myeloid engraftment, and overlay of black bars represents gene-marked (mCitrine$^+$) myeloid engraftment. Myeloid engraftment is expressed as the percent of hCD45$^+$CD33$^+$/CD66b$^+$/CD15$^+$ cells out of total CD45$^+$ (human and murine) cells. Each graph represents one experiment performed using BM from independent donors with n = 4–5 mice/arm. *$p < 0.05$, **$p < 0.01$. n.s., not significant.

are consistent with the retention of $CD38^{low}$ myeloid progenitors within the $CD34^+CD38^-$ fraction when using a more moderate purification strategy (~12% of total $CD34^+$ cells). In both transplants, the majority of human neutrophils present at 3 weeks in arm 3 were $mCitrine^-$, confirming that these cells derive from non-transduced $CD38^+$ cells. In the NSG model, we observed a similar defect in early myelopoiesis from transplanted $CD34^+CD38^-$ cells and rescue by the add-back of $CD38^+$ cells (Figure S4), suggesting that these results are not unique to the NSG-SGM3 model. Collectively, these data show that addition of cultured, non-transduced $CD38^+$ cells can restore early myeloid engraftment when added to transduced $CD34^+CD38^-$ cells.

Addition of $CD38^+$ Cells Enhances Long-Term Engraftment of Gene-Modified HSCs

We next assessed the effect of adding back non-transduced $CD38^+$ cells on long-term gene-marked engraftment. Here, we transplanted IB-purified $CD34^+$, $CD34^+CD38^-$, and $CD38^+$ cells into NSG mice using the same four experimental arms depicted in Figure 5B. To reflect a clinically relevant scenario, each mouse received the total cell product purified from a set volume of BM. At 16 weeks post-transplant, recipients of $CD34^+CD38^-$ cells showed decreased engraftment of total ($hCD45^+$) and gene-marked ($mCitrine^+$ $hCD45^+$) cells as compared with recipients of $CD34^+$ cells (Figure 6A). Addition of non-transduced $CD38^+$ cells to the graft of transduced $CD34^+CD38^-$ cells significantly increased engraftment of gene-marked ($hCD45^+mCitrine^+$) cells to levels achieved by $CD34^+$ cell transplants. Importantly, this was accomplished using 6.2- and 7.8-fold less LV (in two independent experiments) compared with the volumes required to transduce bulk $CD34^+$ cells. All transplanted arms showed similar lineage distribution, with the exception of mice transplanted with $CD38^+$ cells alone, which showed a significant myeloid bias in engrafted cells (Figure 6B). PB analysis showed comparable engraftment of total $hCD45^+$ cells at all time points after transplant of transduced $CD34^+$ cells or transduced $CD34^+CD38^-$ cells co-transplanted with non-transduced $CD38^+$ cells (Figure 6C, left). At early time points (3–12 weeks), transduced $CD34^+$ cells showed higher levels of gene-marked ($mCitrine^+$) engraftment than transduced $CD34^+CD38^-$ cells co-transplanted with non-transduced $CD38^+$ cells (Figure 6C, right). However, after 12 weeks, we observed equivalent levels of gene-marked engraftment in these two groups. These data suggest that non-transduced $CD38^+$ cells drive early hematopoiesis (3–12 weeks), while gene-marked $CD34^+CD38^-$ cells take over hematopoiesis 12 weeks after transplant.

DISCUSSION

We have demonstrated a clinically relevant, IB-based method for purifying $CD34^+CD38^-$ cells from CB, mPB, and BM (Figure 7). We further show that modification of $CD34^+CD38^-$ cells with a reduced LV dose coupled with co-transplantation with non-modified $CD38^+$ cells produced early myeloid reconstitution and long-term engraftment of gene-marked cells comparable with traditional methods of modifying bulk $CD34^+$ cells.

The strategy explored here is broadly applicable to a number of HSC-based gene therapy applications and may be especially useful in gene therapy for hemoglobinopathies, where clinical-scale LV production has proven difficult because of the large size and complexity of the human β-globin gene expression cassette. We estimate that the additional cost to purify 1×10^{10} total BM or mPB cells beyond standard $CD34^+$ IB purification is ~$10,000 per patient (based on the cost of commercially available CD38 antibody, CD15 antibody, and anti-biotin beads). Therefore, the per-patient cost of improving stem cell enrichment is highly cost-efficient compared with current vector production costs (~$500,000 per patient). Furthermore, this strategy may improve efficiency in clinical-scale HSC gene-editing applications such as CRISPR/Cas9.

While a number of in vivo repopulating studies have shown that HSCs are enriched within the $CD34^+CD38^-$ fraction,[12–16] we sought to more thoroughly define a target population to isolate for gene modification. The majority of prior work has evaluated the engraftment capacity of FACS-sorted/uncultured cells, whereas current gene therapy protocols require a period of ex vivo culture that likely alters repopulating capacity. Additionally, prior work has used variable definitions for $CD34^+CD38^-$ cells ranging from the lowest 1% to 30% of CD38 expression. Some work has also suggested that a $CD34^+CD38^{low}$ fraction contains long-term repopulating activity.[14,16] Here, we find that within freshly isolated BM $CD34^+$ cells, only the lowest 6% of CD38 expression is capable of long-term NSG repopulation after LV transduction/ex vivo culture. Thus, purifying $CD34^+$ cells based on CD38 expression alone could lead to a theoretical ~16-fold enrichment of long-term repopulating activity.

High individual donor variability in CD38 expression could be a potential hurdle to clinical translation. Because the lowest 6% of CD38 expression may not encompass >90% of HSCs for every individual, a conservative cutoff for CD38 expression may increase the chance of retaining HSCs. Our results show that the level of CD38 expression retained within the $CD38^+$ and $CD38^-$ populations can be adjusted by titration of magnetic beads, thereby achieving optimal recovery of $CD34^+CD38^-$ cells while also maximizing HSC enrichment. Clinical scale-up to GMP-grade IB sorting systems will likely require additional titration of CD38 magnetic labeling specific to the system parameters (magnetic field strength, column flow rate, etc.).

In the dual $CD34^+CD38^-$ IB protocol, we found that $CD38^+/CD15^+$ co-depletion increased purity of $CD34^+CD38^-$ cells by preventing the non-specific enrichment of $CD15^+$ granulocytes. Due to the relative rarity of $CD34^+CD38^-$ cells (~0.1%–0.3% of MNCs), non-specific enrichment of granulocytes can represent >50% of total cells after $CD34^+CD38^-$ purification. In contrast, $CD34^+$ cells are ~10 times more abundant than $CD34^+CD38^-$ cells (~1%–3% of MNCs); thus, the relative contribution of granulocytes is minor in $CD34^+$ purified cells (<10%). Because clinical vector doses are calculated on a per-cell basis, contaminating granulocytes present in $CD34^+$ $CD38^-$ purified populations could reduce vector MOI and limit the

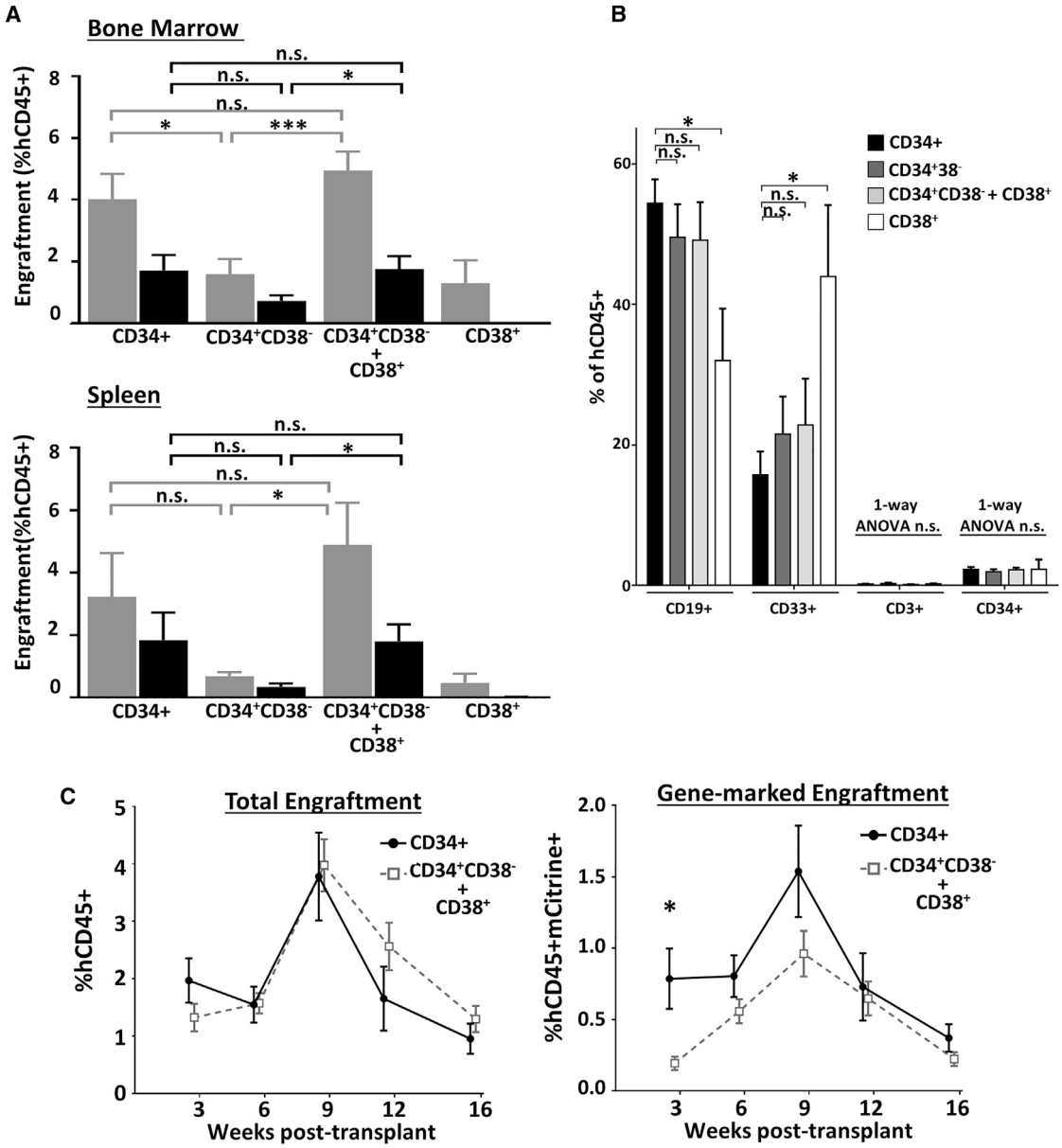


Figure 6. Long-Term Engraftment of IB-Purified Cells

(A) Long-term engraftment of IB-purified cells in NSG BM (top) or spleen (bottom) 16 weeks post-transplant. Gray bars represent mean ± SEM of total human engraftment (% $hCD45^+$); black bars represent gene-marked engraftment (%$hCD45^+mCitrine^+$). Engraftment is expressed as the percent of $hCD45^+$ or $hCD45^+mCitrine^+$ cells out of total $CD45^+$ (human and murine) cells. Data represent two experiments using BM from independent donors with a total of eight to nine mice per arm. (B) Lineage analysis of total $hCD45^+$ in NSG BM 16 weeks post-transplant. Bars show mean ± SEM of each lineage ($CD19^+$ B cells, $CD33^+$ myeloid cells, $CD3^+$ T cells, and $CD34^+$ HSPCs) expressed as percent of total $hCD45^+$ cells. Data represent two experiments using BM from independent donors with a total of eight to nine mice per arm. (C) PB engraftment over time after transplant of CCL-UBC-mCitrine transduced $CD34^+$ cells (filled circles) or CCL-UBC-mCitrine transduced $CD34^+CD38^-$ cells combined with non-transduced $CD38^+$ cells (open squares). Left panel shows total PB engraftment (%$hCD45^+$); right panel shows gene-marked engraftment (%$hCD45^+mCitrine^+$). *$p < 0.05$, ***$p < 0.001$. n.s., not significant.

vector dose reduction achieved by $CD34^+CD38^-$ IB purification. Furthermore, cryopreservation of a granulocyte-rich cell population could potentially lead to poor cell recovery upon thawing because of granulocyte death and/or clumping.

Application of the optimized $CD34^+CD38^-$ dual IB method to samples of healthy BM and mPB achieved ~75% recovery and ~10-fold enrichment of phenotypically defined $CD34^+CD38^-$ cells in mPB, and ~60% recovery and ~5-10-fold enrichment in BM. In BM,

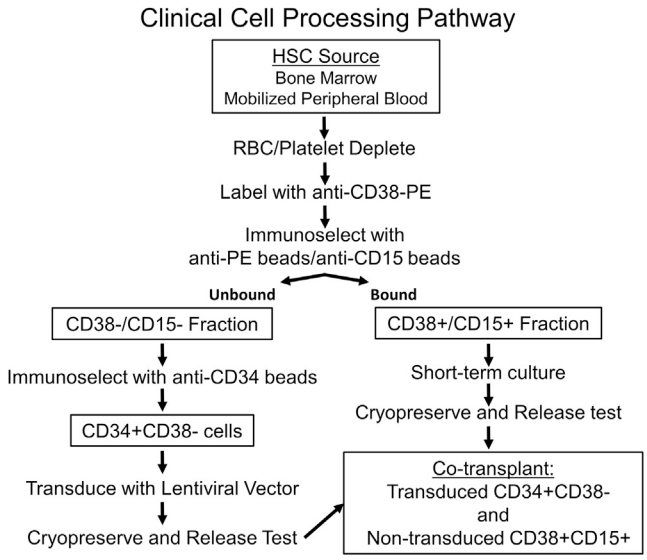


Figure 7. Proposed Clinical Cell Processing Pathway

BM or mPB will be RBC/platelet depleted, followed by labeling with CD38-PE, anti-PE beads, and anti-CD15 beads. The $CD38^+/CD15^+$ fraction (bound to the column) will undergo short-term culture followed by cryopreservation and release testing. The $CD38^-/CD15^-$ fraction (column flow-through) will be labeled with anti-CD34 beads, followed by selection of $CD34^+CD38^-$ cells. $CD34^+CD38^-$ cells will undergo transduction with LV followed by cryopreservation and release testing. After successful release testing, transduced $CD34^+CD38^-$ cells and non-transduced $CD38^+/CD15^+$ cells will be thawed and co-transplanted into a conditioned patient.

$CD34^+CD38^-$ purification results in a 12-fold enrichment of SRC beyond standard $CD34^+$ selection. We observed no difference in transduction efficiency of IB-purified $CD34^+CD38^-$ or $CD34^+$ cells when evaluated by vector copy number (VCN) analysis of long-term NSG-engrafted human cells. This is in contrast with our lab's previous demonstration that highly purified (FACS-sorted) CB $CD34^+CD38^-$ cells demonstrate enhanced transduction in vitro compared with $CD34^+$ cells.[17] This discrepancy could be because of differences in the tissue analyzed (BM versus CB), $CD34^+CD38^-$ purity (moderately purified IB-sorted cells versus highly purified FACS-sorted cells), or post-transduction assay (in vitro culture versus in vivo xenografts). Importantly, our results here suggest that the dual IB $CD34^+CD38^-$ purification does not impede LV transduction of long-term HSCs.

We next explored the early myeloid potential of IB-purified $CD34^+CD38^-$ cells. Prior work has shown that short-term repopulating activity is enriched in the $CD34^+CD38^+$ fraction,[18] suggesting that $CD34^+CD38^+$ progenitors may be necessary for early myeloid recovery. However, murine studies have suggested that high doses of purified stem cells can lead to rapid hematopoietic recovery.[19,20] Clinical trials using FACS-sorted $CD34^+CD90^+$ cells have demonstrated prompt neutrophil and platelet engraftment in patients transplanted with $>0.8 \times 10^6$ $CD34^+CD90^+$ cells/kg.[8–10] However, the purified $CD34^+CD90^+$ populations in these studies represented ~50% of total $CD34^+$ cells, whereas the IB-purified $CD34^+CD38^-$ cells evaluated here represent ~10% of total $CD34^+$ cells. Therefore, it is largely unknown whether high doses of purified $CD34^+CD38^-$ cells could provide rapid myeloid reconstitution in a clinical transplant setting, or if co-transplant of $CD34^+CD38^+$ cells will be required.

Here, we observed that IB-purified $CD34^+CD38^-$ cells were capable of early myeloid reconstitution, but contained less early myeloid potential than an equivalent marrow volume of $CD34^+$ cells. Our xenograft data suggest that approximately two to three times as many IB-purified $CD34^+CD38^-$ cells (when transplanted alone) may be required to achieve early myeloid reconstitution comparable with $CD34^+$ cells. In a clinical scenario, it is possible that the total product of $CD34^+CD38^-$ cells obtained will be above the threshold required for sufficient myeloid reconstitution. However, we favor the safer approach of adding non-transduced $CD38^+$ cells (containing $CD34^+CD38^+$ progenitors) to the graft, because transplant of bulk $CD34^+$ cells in gene therapy clinical trials has historically provided prompt myeloid reconstitution.

Furthermore, add-back of non-transduced $CD38^+$ cells may also play a critical role in achieving optimal long-term engraftment of gene-modified HSCs. In long-term NSG transplant assays, we observed decreased engraftment of both total and gene-marked cells in recipients receiving $CD34^+CD38^-$ cells alone as compared with recipients receiving $CD34^+$ cells. However, when non-transduced $CD38^+$ cells were added to transduced $CD34^+CD38^-$ cells, levels of gene-modified engraftment were equivalent to those achieved by standard $CD34^+$ transduction/transplant. These data suggest that the $CD34^+CD38^-$ dual IB purification method efficiently recovers HSCs but requires co-transplantation of $CD34^+CD38^+$ cells for optimal long-term engraftment. In agreement with our findings, prior work has demonstrated that cycling $CD34^+CD38^+$ cells facilitate HSC engraftment[21–23] through enhancement of stromal cell-derviced factor 1 (SDF-1)-mediated homing and secretion of metalloproteinase (MMP)-9.[23]

An important advantage of using CD38 as an enrichment marker is that it can cleanly separate HSC-containing and HSC-devoid fractions; virtually no repopulating activity is contained within the $CD38^+$ fraction, and almost all repopulating activity is contained within the $CD34^+CD38^{0\%–6\%}$ fraction. Although additional HSC markers have been identified[24,25] (e.g., CD90, CD45RA, and CD49f), in vivo xenograft analysis of human HSC populations suggests that these markers *enrich* for HSCs but do not exclusively *define* HSCs. For example, $CD34^+CD38^-CD45RA^-CD90^+$ cells contain more HSCs per cell than $CD34^+CD38^-CD45RA^-90^-$ cells; however, $CD34^+CD38^-CD45RA^-90^-$ cells *do* contain long-term HSCs capable of multi-lineage reconstitution of NSG mice.[24] Thus, discarding the $CD34^+CD38^-CD45RA^-90^-$ population prior to gene modification or transplant would likely result in a lower total HSC dose and could be disadvantageous in a clinical scenario.

In our CD38 add-back transplant studies, we used a conservative CD38 depletion strategy (6.2- to 7.8-fold reduction in cell

number/LV) in order to maximize the number of HSCs retained in the $CD38^-$ fraction. It is possible that further reductions in total cell number and LV dose could be achieved using a more stringent CD38 depletion or using additional cell surface markers (e.g., CD90, CD49f). These strategies may be advantageous if highly purified HSCs can be modified and expanded with compounds such as StemRegenin-1 (SR-1),[26,27] prostaglandin E2 (PGE2),[28,29] or UM171.[30]

In summary, we demonstrate a method to improve the efficiency of gene therapy for genetic blood cell diseases through improved HSC enrichment and reduced LV dose.

MATERIALS AND METHODS

MNC Isolation

Healthy adult BM and mPB were obtained from commercial sources (All Cells and Hemacare). Umbilical CB was obtained after vaginal and cesarean deliveries at UCLA Medical Center. All specimens obtained have been deemed as anonymous medical waste exempt from institutional review board review. MNCs were isolated using Ficoll-Paque PLUS (GE Healthcare) density centrifugation within 48 hr of collection. The total number of MNCs used for IB purification varied with each cell product and ranged from 4×10^6 to 4×10^7 (absolute cell numbers are summarized in Figure S2B).

IB Purification of $CD34^+$ and $CD34^+CD38^-$ Cells

All microbeads and magnetic columns used for cell separation were purchased from Miltenyi Biotec. All incubation steps were performed in magnetic-activated cell sorting (MACS) buffer (PBS/0.5% BSA/2 mM EDTA) at 4°C with 10^7 cells/100 μL total volume. Total reagent/buffer volumes were scaled accordingly based on MNC number in order to keep cell density and reagent concentration constant for each processed sample. Concentrations described for each reagent represent the reagent volume:total volume of reagent and MACS buffer.

MNCs were first stained with CD38-PE (1:15, Clone IB6; Miltenyi Biotec), CD34-fluorescein isothiocyanate (FITC) (1:10, Clone 581; BD Biosciences), and CD45-allophycocyanin (APC) (1:10, Clone HI30; BD Biosciences) for 30 min. Stained MNCs were washed once with MACS buffer and divided into equal fractions for further IB processing. Each experiment comparing $CD34^+$ and $CD34^+CD38^-$ IB purification (cell counts and xenograft studies) utilized the total purified cell product isolated from an equal starting volume of MNCs (defined in the text as marrow equivalent).

To purify $CD34^+$ cells, we incubated MNCs with anti-CD34 microbeads (1:5) for 30 min, washed them, and added them to an LS column. The column was washed with 3×3 mL of MACS buffer. $CD34^+CD38^-$ cells were purified by first incubating MNCs with anti-PE microbeads (1:5–1:25 for bead titration studies, 1:10 for mPB purification, 1:40 for BM purification and xenograft studies) and anti-CD15 microbeads (1:5) for 15 min. Cells were washed and applied to an LD column. The column was washed with 2×1 mL fractions of MACS buffer. After washing, the $CD38^+/CD15^+$ fraction was flushed from the column. The $CD38^-/CD15^-$ flow-through fraction was collected, washed, and subsequently selected with anti-CD34 microbeads as described above. To obtain absolute $CD34^+CD38^-$ cell counts in each fraction, we added 3×100 μL aliquots to 300 μL of MACS buffer and 50 μL of counting beads (eBioscience) and DAPI (1:1,000; Life Technologies), and analyzed them on an LSRII or LSR Fortessa flow cytometer (BD Biosciences) (Figure S2). In xenograft experiments where $CD38^+$ cells were added back to the graft, CD3-depleted MNCs were used as a starting material to deplete mature human T cells and avoid xenogeneic GVHD. MNCs were incubated with anti-CD3 microbeads (1:5) at 4°C for 15 min, washed, and separated on an LD column. $CD3^-$ cells were immediately processed by further $CD34^+$ or $CD34^+CD38^-$ IB purification.

Cell Sorting

BM MNCs were enriched for $CD34^+$ cells as described and were stained with CD38-PE (1:15, Clone IB6; Miltenyi) and CD34-APC (1:10, Clone 581; BD Biosciences) in MACS buffer for 30 min at 4°C, followed by washing to remove unbound antibody. DAPI (1:1,000; Life Technologies) was added just before analysis. Cells were gated on viable, single $CD34^+$ cells (Figure S1A) prior to defining CD38 intervals. Cells were sorted according to defined intervals of CD38 expression using a FACSAria II (BD Biosciences).

LV Transduction

Construction, packaging, and titering of CCL-UBC-mCitrine-PRE-FB-2XUSE, CCL-UBC-mStrawberry-PRE-FB-2XUSE, and CCL-UBC-mCerulean-PRE-FB-2XUSE have been described.[17] Cells were plated on retronectin (Takara Shuzo)-coated plates (20 μg/mL) at a density of $0.5–1 \times 10^6$ cells/mL in X-VIVO15 medium (Lonza) containing 1× glutamine/penicillin/streptomycin (Gemini BioProducts), 50 ng/mL SCF, 50 ng/mL fms-related tyrosine kinase 3 ligand (Flt3-L), 50 ng/mL thrombopoietin (TPO), and 20 ng/mL IL-3 (PeproTech). LV was added to a final concentration of 2×10^7 TU/mL and incubated with cells for 24 hr prior to transplant. All LV concentrations (transducing units [TU]/mL), cell concentrations (cells/mL), and cell plating densities (cells/cm^2) were kept constant during transduction of $CD34^+$ and $CD34^+CD38^-$ cells by adjusting the total number of wells for plating/transduction proportional to cell counts ($CD34^+$ cells required ~5–10 times more cell culture wells than $CD34^+CD38^-$ cells).

Xenografts

All work with mice was done under protocols approved by the UCLA Animal Care Committee. 6- to 10-week-old male and female NOD.Cg-Prkdsci-dIl2rgtm1Wjil/SzJ (NSG) and NOD.Cg-PrkdcscidIl2rgtm1WjlTg(CMV-IL-3, CSF2, KITLG)1Eav/MloySzJ (NSG-SGM3) mice (Jackson Laboratory) were used as transplant recipients. Mice were irradiated 4–6 hr prior to transplantation using a ^{137}Cesium source at a total dose of 250 rad (~101 rad/min). Cells were harvested from retronectin-coated dishes by gentle pipetting and PBS washes. Collection of all cells from each well was verified by visual inspection under the microscope. Collected cells were

washed, resuspended in PBS, and administered via intravenous injection into the retro-orbital sinus. $CD34^-$ cells were irradiated (10 Gy) and added to grafts as filler cells (total cell doses per mouse are listed in Table S1).

Engraftment Analysis

Engraftment of human cells in PB, BM, and spleen of xenograft recipient mice was evaluated by flow cytometry using anti-human CD45-APC (HI30), anti-murine CD45-PE (Clone 30-FII), anti-human CD33-V450 (Clone P67-6), anti-human CD66b-V450 (Clone G10F5), anti-human CD15-V450 (Clone HI98), anti-human CD3-PerCP-Cy5.5 (Clone SK7), anti-human CD19-APC-Cy7 (SJ25CI), and anti-human CD34-PE-Cy7 (Clone 581) (all antibodies are from BD Biosciences).

Determination of Vector Copies per Human Cell

The average vector copies (VCs) per human cell was measured in engrafted NSG BM samples as previously described.[17] In brief, LV DNA content was quantified using a digital droplet PCR probe specific to the HIV-1 Psi region and normalized to the autosomal human gene SDC4.

Statistics

Pairwise comparison was performed by unpaired t test within the framework of one-way ANOVA. Two group comparisons by Wilcoxon rank-sum test were performed when the assumption of normality was not met. Hypothesis testing was two-sided, and a significance threshold of $p = 0.05$ was used. Limiting dilution analysis was performed using online software provided by Walter and Eliza Hall Institute (WEHI) bioinformatics.[31]

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ymthe.2017.05.023.

AUTHOR CONTRIBUTIONS

K.E.M. designed, performed, and analyzed experiments and wrote the manuscript. D.B. and J.L. performed portions of the research studies. R.P.H. provided study materials and advised experiments. F.U. advised experiments. D.B.K. participated in conception and design of the studies, provided financial support through grant funding, and co-wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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