Supplementary Information

Material and Methods

Vector Preparation

The lentiviral vector pHR'-SEW expressing eGFP under control of the ubiquitously expressing SFFV-promoter was used.[1] The eGFP cassette was exchanged with the gene encoding for TurboBlue fluorescence protein (tuBFP) via insertion into EcoRI and SbfI cutting sites to create the pHR'-SBW vector. VSV- and CD133-targeting envelope pseudotyped lentiviral vectors were produced by PEI mediated transfection of HEK 293T/17 cells as previously described.[2,3] In brief, 2.5 µg of each plasmid encoding H(CD133)-MV and F-MV envelope proteins were mixed with 15 µg 8.91 (gag/pol/rev/tat) packaging plasmid, 15 µg of lentiviral vector and 115 µl of 1 mg/ml branched PEI in 2.5 ml plain DMEM, incubated for 15-30 min and added to a 15 cm cell culture dish. After 10-16 hours media exchange was performed and the supernatants were collected ~48 h after transfection and concentrated via ultracentrifugation at 50,000g for 2 hours. Lentiviral vectors were titered on 10,000 freshly isolated CD34+ cells in a 96-well plate at 2.5 x 10^5 cells/ml in SpemSpan SFEM supplemented with hSCF (50 ng/ml), hTPO (10 ng/ml), hIGFBP2 (100 ng/ml) and hFLT3L (50 ng/ml).[4] Fluorescence was analyzed by flow cytometry three days after transduction.

Transplantation

For transplantation transduced cells were harvested 24 h after isolation or transduction and washed in PBS. Competitor cell populations were mixed and resuspended in 150 µl IMDM without supplements per mouse. Four to eight weeks old NSG mice (NOD/LtSz-scid Il2rg-/-) were sublethally irradiated using a Cesium source (2.4 Gy) and transplanted with 0.5 to 1x 10^6 cells. A small split of the cells were kept in culture and analyzed by flow cytometry three days after transduction to determine percentages of eGFP and tuBFP positive cells.

Flow cytometry

Flow cytometry analysis was performed on BD FACS-Canto II or FACS-Aria machines equipped with violet, blue and red lasers. Doublets were excluded via FSC and SSC properties, dead cells were excluded using eFluor780 viability dye (eBiosciences) and unspecific binding was reduced by preincubation with mouse and human FC-block (Miltenyi). Antibodies used for staining of cell surface antigens were CD34-APC (4H11, eBiosciences), CD34-Vioblue (AC136, Miltenyi), CD133/1-PE or APC (AC133, Miltenyi), hCD45-PE, APC or PerCPCy5.5 (2D1, eBiosciences), CD38-PeCy7 (HIT2, eBiosciences), CD33-PE or APC (P67.7, BD-Bioscience), mCD45-APCCy7 (30-F11, Biolegend), CD19-PECy7 (HIB19, Biolegend), mCD45-eFluor450 (30-F11, eBiosciences).

Competitive transplantation data analysis and representation

For competitive repopulation assays the ratio of VSV- vs. CD133-LV transduced cells was determined three day post transduction using a sample of the transplanted cell mixture. This initial ratio was set to one, and the ratios measured in samples from transplanted mice were expressed relative to this initial ratio. For statistical analysis logarithmic transformation was performed to obtain normally distributed data sets. If data was normally distributed a student's t-test was performed, if not Wilcoxon signed rank test was performed (both Graphpad Prism). For limiting dilution analysis ELDA-software was used http://bioinf.wehi.edu.au/software/elda/. [5]

Barcoded library preparation

The barcoded lentiviral plasmid library containing 485 different equimolar barcodes was kindly provided by L. Bystrykh (Univ. Groningen, Netherlands).[6] The library vector preparation and transduction was performed as described above. The total number of different barcodes transplanted per mouse was calculated from a sample which was analyzed by flow cytometry three days after transduction.

Sequence analysis

Bar codes were amplified by PCR, using primers with the IonTorrent adapter, an 8 bp multiplexing code and a vector specific sequence. Product size was confirmed by agarose gel electrophoresis and the product were isolated from the gel and purified using Qiagen Gel extraction columns according to the manufacturer's protocols. The resulting amplicons were submitted to the LUMC sequencing core facility (LGTC) to be sequenced on an IonTorrent 314 or 316 chip according to the manufacturer's instructions (Life Technologies, Bleiswijk, Netherlands). FASTQ files were retrieved from the IonTorrent sequencer and underwent overall quality control using FASTQC (Simon Andrews, Babraham Institute, Cambridge, UK (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), after which the sequences were separated by multiplex barcode using a custom BioPerl script (http://www.bioperl.org) that uses regular expressions to sample multiplex sequences. From the resulting FASTA files, the DNA barcodes were extracted using a custom BioPerl script that determined barcode location based on a regular expression matching the surrounding virus sequences and the invariable doublets within the DNA barcode. These barcodes were clustered using R (R-3.0.0, www.r-project.org)[7] by calculating dissimilarity between all barcodes for all samples of an individual animal. Read numbers for barcodes with dissimilarity of less than 2 nt were clustered (http://igraph.sf.net). This resulted in a set of barcodes that corresponded with the expected size of our DNA barcode library (~485 barcodes, based on single bacterial clone selection). The contribution of each clone was calculated by dividing the reads for each clustered clone by the total number of reads per sample.

Cell cycle analysis

For cell cycle analysis freshly prepared mPB CD34+ cells were stained with CFSE using the CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes) according to the manufacturer’s instructions and subsequently subjected to transduction. Data was analyzed using ModfitLT 4.0 software (Verity Software House).

Apoptosis stain

Freshly prepared mPB CD34+ cells were transduced with the indicated amounts of vector supernatant overnight and resuspended in fresh media 24h later. 36h later cells were stained with the Dead Cell Apoptosis Kit with Annexin V APC (Molecular Probes) and 7AAD according to the manufacturer's instructions.

References

1. Demaison C, Parsley K, Brouns G, et al. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of imunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. *Hum Gene Ther*. 2002;13(7):803-813.
2. Anliker B, Abel T, Kneissl S, et al. Specific gene transfer to neurons, endothelial cells and hematopoietic progenitors with lentiviral vectors. *Nat Methods*. 2010;7(11):929-935.
3. Brendel C, Muller-Kuller U, Schultze-Strasser S, et al. Physiological regulation of transgene expression by a lentiviral vector containing the A2UCOE linked to a myeloid promoter. *Gene Ther*. 2012;19(10):1018-1029.
4. Zhang CC, Kaba M, Iizuka S, Huynh H, Lodish HF. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. *Blood*. 2008;111(7):3415-3423.
5. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *J Immunol Methods*. 2009;347(1-2):70-78.
6. Verovskaya E, Broekhuis MJ, Zwart E, et al. Heterogeneity of young and aged murine hematopoietic stem cells revealed by quantitative clonal analysis using cellular barcoding. *Blood*. 2013;122(4):523-532.
7. Csardi G, Kutalik Z, Bergmann S. Modular analysis of gene expression data with R. *Bioinformatics*. 2010;26(10):1376-1377.

Supplementary Figures

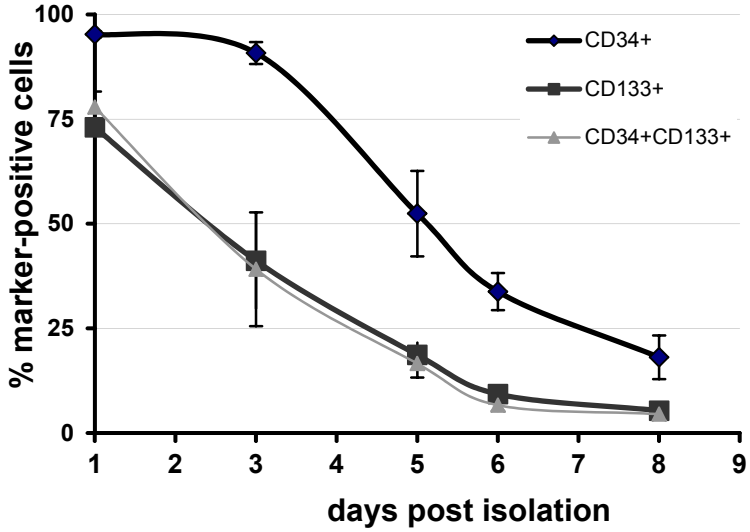

Figure S1. Cell surface marker loss during prolonged culture of CD34+ cells.

CD34+ cells freshly isolated from mobilized peripheral blood were monitored for surface marker expression (CD34+, CD133+, double positive) during prolonged *in vitro* culture by flow cytometry (n=5-21). Error bars indicate SD.

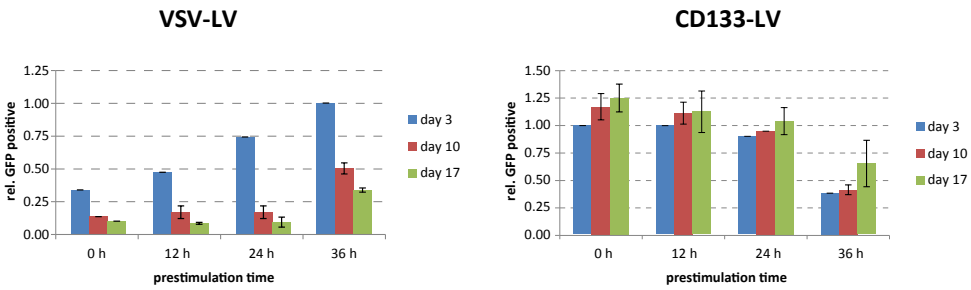

Figure S2. Impact of prestimulation on transduction rate and population dynamics over time. Freshly isolated mPB CD34+ cells were either immediately transduced or prestimulated for 12h, 24h or 36h prior to transduction. FACS analysis was performed 3d, 10d and 17d post transduction. The graph shows the percentage of GFP+ cells normalized to the optimal transduction condition for each vector (36h for VSV-LV, 0h for CD133-LV). Mean + SD.

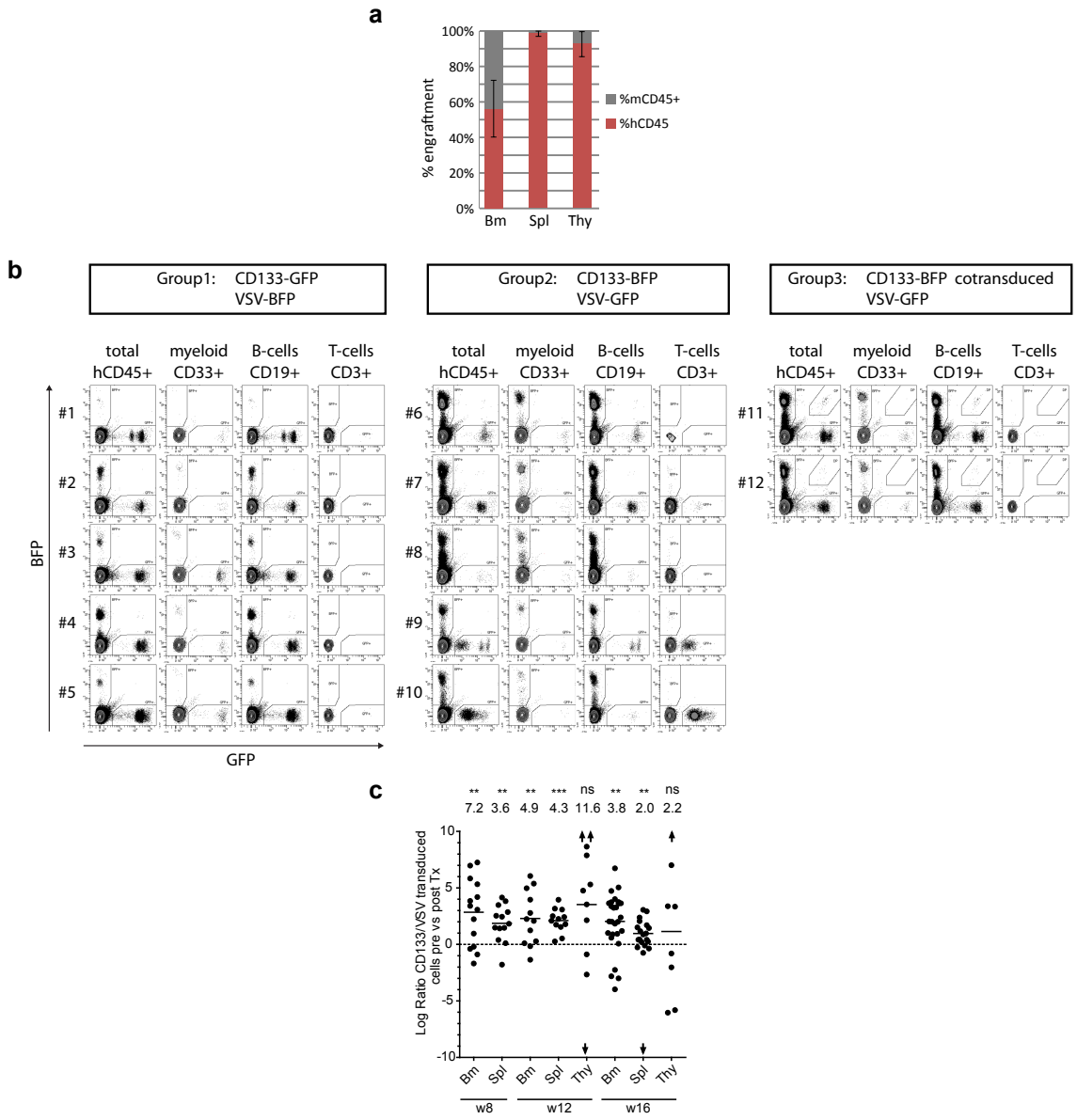

Figure S3. Competitive repopulation experiments in NSG mice.

(**a**) Engraftment of human cells in bone marrow (Bm), spleen (Spl) and Thymus (Thy) of a representative cohort of 12 NSG mice transplanted with 10^6 cells and analyzed twelve weeks after transplantation. (**b**) Representative set of FACS blots from bone marrow of 12 mice showing GFP/BFP expression in different cell types. (**c**) Ratio of CD133-LV / VSV-LV transduced cells before versus post transplantation in total hCD45+ cells from the bone marrow (Bm), spleen (Spl) or thymus (Thy) at 8, 12 and 16 weeks after transplantation. Differences observed between repeated measures of organs at different time points are statistically not significant. Error bars: SD.

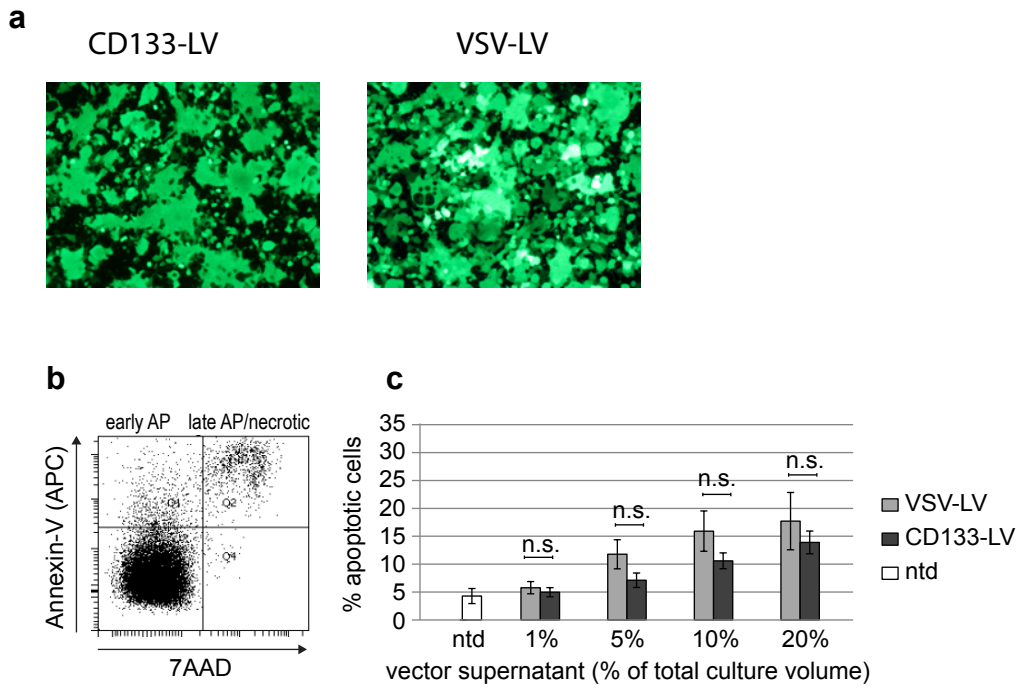

Figure S4. Toxicity of CD133-LV and VSV-LV.

(**a**) Syncytia formation can be observed to a similar degree in packaging cells releasing CD133-LV and VSV-LV. Photographed 48h post transfection. (**b**) Apoptosis induction upon application of vector concentrates to mPB CD34+ cells. VSV-LV or CD133-LV vector supernatants were concentrated 100 fold and various volumes were added to freshly isolated mPB CD34+ cells to final concentrations of 1, 5, 10 or 20% of the final volume. Apoptosis induction mediated by vector toxicity was measured by 7AAD/AnnexinV staining. Viable cells (lower left quartile), early apoptotic (AP) or late apoptotic / necrotic populations are indicated. (**c**) Vector concentration dependent increase in apoptotic cells. ntd: untransduced control; n.s.: not significant; error bars: SEM of 8-10 samples per bar.

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cell dose per mouse		responding / total mice	
CD133-LV	VSV-LV	CD133-LV	VSV-LV
23760	99000	4/5	5/5
11880	49500	5/7	4/7
5940	24750	3/8	2/8
2970	12375	1/7	1/7

Table S1. Results of the limiting dilution competetive repopulation assay shown in Figure 5a.