Supplemental Methods

Animals

Specific pathogen-free rhesus macaques (*Macaca mulatta*) were used following a National Heart, Lung, and Blood Institute (NHLBI) Animal Care and Use Committee approved protocol (approved protocol no: H-0136R4).

Rhesus peripheral blood mononuclear cell (PBMC) and CD34+ hematopoietic stem/progenitor cell (HSPC) derivation

Rhesus PBMCs were collected using Ficoll-Paque PLUS density gradient media (GE Healthcare, Sweden) using the manufacturer's instructions. CD34+ HSPCs were purified from mobilized peripheral blood (PB) as described previously (1). Briefly, cytokine mobilization consisted of a five-day course of 15 mcg/kg Granulocyte-Colony Stimulating Factor (GCSF, Amgen, Thousand Oaks, CA) SQ and a single dose of 1 mg/kg SQ of AMD3100 (Sigma-Aldrich, St. Louis, MO) on the morning of the fifth day, 3-4 hours prior to leukapheresis. As previously described, a small volume leukapheresis procedure was performed using a CS3000 Cell Separator (Baxter Fenwal, Round Lake, IL) and CD34+ HSPCs were collected by immunoselection from the leukapheresis product using rhesus CD34+ antibody (#Clone 12.8, Fred Hutchinson Cancer Research Center) and anti-mouse IgM bead (Miltenyi Biotech, Auburn, AL). The purity of the selected cells were determined using CD34+ antibody (#550761, #Clone 563, BD biosciences, CA). Selected CD34+ HSPCs were used fresh or frozen for subsequent studies.

Ribonucleoprotein (RNP) electroporation

Frozen or fresh rhesus CD34+ HSPCs were pre-stimulated in X-VIVO 10 media (Lonza, MD) supplemented with human stem cell factor (SCF, R&D Systems, Minneapolis, MN), human thrombopoietin (TPO, R&D Systems), and recombinant human Flt3-ligand (Flt3-L, R&D

Systems) (100 ng/ml each). The chemically modified synthetic sgRNAs (2) (2′-*O*-methyl-3′-phosphorothioate modifications in the first and last three nucleotides) #1617 (CUAACAGUUGCUUUUAUCAC) and #AAVS1 (CUCCCUCCCAGGAUCCUCUC) were purchased from Synthego (Synthego Co., CA). The concentrations of sgRNAs were calculated according to the OD reporting method, which is 3-fold higher than the full length product reporting method (2). Sterile glycerol (30%, Thermo Fisher Scientific, CA) was added to 2×-NLS SpCas9 (University of California, Berkeley, CA) or 3×-NLS SpCas9 (Scot Wolfe laboratory, University of Massachusetts Medical School, Worcester, MA, USA (2)) (200 pmol for 20 µl Nucleocuvette Strips [V4XP-3032], 1000 pmol for 100 µl Nucleocuvettes [V4XP-3024]) protein to a final concentration of 2% (v/v). sgRNAs (600 pmol by OD method for 20 µl Nucleocuvette Strips [V4XP-3032, Lonza], 3000 pmol by OD method for 100 µl Nucleocuvettes [V4XP-3024, Lonza]) were added to SpCas9+Glycerol mix and incubated 15 minutes at room temperature to allow RNP formation. Rhesus CD34+ HSPCs (5×10^4 cells for 20 µl strips, 1.5-5 $\times10^6$ cells for 100 µl Nucleocuvettes) were suspended in P3 solution, mixed with RNP and transferred to respective cuvettes for electroporation with EO-100 program. After electroporation, cells were added XVIVO-10 media with growth factors (80 µl for 20 µl strips, 500 µl for 100 µl Nucleocuvettes) and incubated for 10 minutes at room temperature. Cells were transferred to Eppendorf tubes and centrifuged at 500×g for 5 minutes. Cell pellets were transferred to cell culture plates in XVIVO-10 media containing growth factors at a concentration of 0.5-1$\times10^6$/ml and incubated for 48 h. At the end of the incubation period, cells were either frozen or used for erythrocyte differentiation. For viability analysis, cells were counted 48 h after electroporation and the viability was given as electroporated cell number divided by mock control without electroporation.

Red blood cell (RBC) differentiation of rhesus CD34+ HSPCs

To analyze γ-globin induction, the electroporated cells were differentiated towards erythrocytes using our previously published protocol (3). In short, 1×10^5 cells/ml were inoculated into tissue culture plates (Corning, ME) coated with irradiated OP9 cells (ATCC, Crl-2749) in proliferation media containing IMDM (Iscove's Modified Dulbecco's Medium; Mediatech Inc.) with 10 ng/mL SCF, 1 ng/ml interleukin (IL)-3 (R&D Systems), 2 U/ml erythropoietin (EPO, Amgen), 10^{-6} M dexamethasone (DEX, VETone), 10^{-6} M estradiol (E2, Pfizer) and 20% fetal bovine serum (FBS, Gibco) for 6 days. Then, the media was replaced with maturation media including IMDM containing 10 ng/ml insulin (INS, Lilly), 500 mg/ml holo-transferrin (Sigma Aldrich), 2% bovine serum albumin (BSA, Roche), 2 U/ml EPO and 20% FBS, and cells were incubated for additional 8 days. At the end of the differentiation process, cells were collected for RNA, DNA, and protein analysis.

Hemoglobin (Hb) electrophoresis

Differentiated rhesus CD34+ HSPCs (day 14) were examined for their Hb content using cellulose acetate membranes and alkaline buffer solution according to the manufacturer's instructions (Helena Laboratories, TX).

Reverse-phase high-performance liquid chromatography (RP-HPLC)

To evaluate relative protein levels of globin chains, fresh rhesus RBCs ($3\text{-}5\times10^6$ cells) or *ex vivo* differentiated cells ($0.5\text{-}3\times10^6$ cells) were washed with phosphate-buffered saline (PBS, Corning Cellgro) three times. After lysing RBCs in 100 µl HPLC grade water, cells were centrifuged at 16,000×g for 10 minutes. The supernatant was transferred to a new Eppendorf tube and added 10 µl of 100 mmol/L TCEP (Thermo Fisher Scientific). After 5 minutes of incubation at room temperature, the mixture was added 85 µl of 0.1% TFA/32% acetonitrile, and the solution was pulse-vortexed (3×) for 10 seconds. The resulting solution (10-40 µl) was analyzed in 0.7

mL/min flow for 50 min using the Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA) equipped with a reverse-phase column, Aeris 3.6 µm Widepore C4 200 (250 × 4.6 mm, Phenomenex, Torrance, CA) using solvent A (0.12% TFA in water) and solvent B (0.08% TFA in acetonitrile) with a gradient for the separation of globin protein of 35% solvent B, followed changes in percent of solvent B as follows: 3 min at up to 41.2%, 3 min at up to 41.6%, 5 min at up to 42%, 4 min at up to 42.4%, 6 min at up to 42.8%, 6 min at up to 44.4%, 6 min at up to 47%, 7 min at up to 75% and re-equilibrated for 10 min at 35%. The globin chain peaks were detected at 215 nm and confirmed by Agilent HPLC-6224 mass spectrometer equipped with an ESI interface and a time-of-flight mass detector (Agilent Technologies) as described previously (4, 5).

Flow cytometry

Enucleation analysis in rhesus cells was completed after staining cells with the cell-permeable DNA dye Hoechst 33342 (Life Technologies, NY) for 10 min at 37 °C at a concentration of 2 µg/ml. The Hoechst 33342 negative cells were further gated for cell size analysis with Forward Scatter (FSC) A parameter. To analyze indel frequencies in PB and BM cell lineages of editing animals, mononuclear cells (MNCs) in PB and bone marrow (BM) were collected using Ficoll-Paque density gradient separation. Cells were incubated with antibodies against CD3-APC (#130-099-690, #Clone 10D12, CloneMiltenyi Biotec, Auburn, CA), CD14-FITC (#555397, #Clone M5E2, BD Bioscience), CD20-PE (#555623, #Clone 2H7, BD Bioscience), CD34-APC (#561209, #Clone 563, BD Bioscience), CD45-V450 (#561291, #Clone D058-1283, BD Bioscience), and CD71-PE (#GTX43030, #Clone DF1513, GeneTex, Irvine, CA) overnight. In addition to these fractions, granulocyte and monocyte fractions were sorted using FSC A and SSC A as shown previously (6) using FACSAria II Cell Sorter (BD Biosciences).

Hematopoietic colonies

Colony-forming unit (CFU) ability of rhesus blood progenitor cells was analyzed by inoculating 2×10^4 BM MNCs or 5×10^3 CD34+ HSPCs per 1.1 ml of MethoCult semi-solid media (#04444, STEMCELL Technologies, MA) following the manufacturer's instructions. On day 14, the colonies were counted and collected for indel analysis.

Measurement of indel frequencies

BCL11A enhancer DHS +58 functional core was amplified with KOD Hot Start DNA Polymerase (Novagen Inc, Madison, WI) and corresponding primers (*BCL11A*_enhancer_forward: CCCATGGGCAAACCAGACTA. *BCL11A*_enhancer_Reverse: GTGGACAGACCGACAGATCA. AAVS1_forward: CCATGGCCACTTCAGGACAA. AAVS1_reverse: GCGGCTACTGGCCTTATCTC) using the following cycling conditions: 95 degrees for 3 min; 35 cycles of 95 degrees for 20 s, 60 degrees for 10 s, and 70 degrees for 10 s; 70 degrees for 5 min. Resulting PCR products were subjected to Sanger sequencing. Sequencing traces were imported to TIDE software for indel frequency measurement with a 40 bp decomposition window.

RT-qPCR quantification of γ-globin induction

Total RNA isolation from differentiated rhesus cells was conducted using RNeasy columns (#74106, Qiagen, Chatsworth, CA), followed by reverse transcription with iScript cDNA synthesis kit (#170-8890, Bio-Rad, Hercules, CA). RT–qPCR was performed using iQ SYBR Green Supermix (#170-8880, Bio-Rad) to determine β-like globin expression percentages using primers amplifying γ-globin (HBG1/2) or β-globin (HBB) cDNA (*HBG*_qRT_forward: AGAAACCCTGGGAAGGCTC. *HBG*_qRT_reverse: TGGCATCTCCCAAGGAAGTC. *HBB*_qRT_forward: TGCCTTTAGTGATGGCCTGA. *HBB*_qRT_reverse: ATCCACATGCAGCTTGTCAC). The data were normalized to α-globin (HBA)

(*HBA*_qRT_forward: CTGGCGAGTATGGTGCGG. *HBA*_qRT_reverse: CCCTTAACCTGGGCAGAGC) expression in respective groups.

Amplicon deep sequencing

For indel frequencies with deep sequencing, *BCL11A* enhancer or *AAVS1* loci were amplified with corresponding primers (either *BCL11A*_enhancer_seq_forward: GCTGATTCCACTGCAAAGTCC, *BCL11A*_enhancer_seq_reverse: GAGGTCTGCCAGTCCCCTTC or *BCL11A*.F – 5'-GCAGCTAGACAGGACTTGGG-3' and *BCL11A*.R – 5'-CTCCATCAGCAAGAGAGCCT-3'), (either *AAVS1*_seq_forward: ACCCCCACCTCCTTTTAAGC, and *AAVS1*_seq_reverse: CAGAGCAGCTCAGGCTCC or *AAVS1*.F-ACCCCCACCTCCTTTTAAGC-3' and *AAVS1*.R – 5'-CAGAGCAGCTCAGGCTCC-3'). After another round of PCR with primers containing sample-specific barcodes, amplicons were sequenced using 2×250 paired-end reads with the MiSeq Sequencing System (Illumina) and 2×150 paired-end reads with the MiniSeq Sequencing System (Illumina) with 10% PhiX. Sequencing adaptors were trimmed with Trimmomatic (*v. 0.36, PE CROP:150 MINLEN 10).* The deep sequencing data were analyzed using CRISPResso2 software (*v.2.0.31 CRISPRessoBatch --quantification_window_center -3 --quantification_window_size 1 --min_average_read_quality 27 --ignore_substitutions TRUE)*. Samples for which paired-end sequences failed to merge due to low sequencing quality in the read proximal to a G-rich homopolymer were analyzed as single-end reads, which were sufficient to cover the spacer+PAM. Following alignment in CRISPResso2, all reads were collapsed by edits overlapping the quantification window. Deletions $\geq$8 bp were categorized as edits generated by microhomology-mediated end joining (MMEJ), and all other indels were considered results of nonhomologous end joining (NHEJ).

Off-target evaluation

The 28 top off-target sited were predicted by CIRCLE-seq (**Supplemental Table 4**). 26 of these candidate off-target genomic sites could successfully be amplified using rhAMPSeq assays designed and produced by IDT (**Figure 4E**). The amplicons were sequenced using 2×150 paired-end reads on a MiniSeq Sequencing System (Illumina), and post-run trimming was performed using Trimmomatic (*v. 0.36, PE CROP:150 MINLEN 10).* Paired-end reads were merged and aligned in CRISPResso2 (*v.2.0.31 CRISPRessoPooled --quantification_window_center -3 --quantification_window_size 1 --ignore_substitutions TRUE --min_reads_to_use_region 10).* Post-alignment, all reads were collapsed by edits overlapping the quantification window.

Transplant

Cytokine mobilization consisted of a five-day course of 15 mcg/kg G-CSF SQ and a single dose of 1 mg/kg SQ of AMD3100 on the morning of the fifth day, 3–4 hours prior to leukapheresis. As previously described, a small volume leukapheresis procedure was performed using a CS3000 Cell Separator and CD34+ HSPCs were collected by immunoselection from the leukapheresis product (1). The CD34+ HSPCs were pre-stimulated and electroporated as mentioned before. Detailed materials and protocols for each transplant are presented in **Table 1**. After the efficiency of gene editing was confirmed *ex vivo*, the CD34+ HSPCs were thawed, and reinfused into the recipient following a two-consecutive day course of total body irradiation (TBI) monkey at a dose of 5 Gy (5 Gy x 2) with a dose rate of approximately 0.6 Gy/min using a large, wet source storage panoramic Cobalt 60 irradiator (Armed Forces Radiobiology Research Institute, MD). Following the transplant, the animals received supportive care as described previously (7).

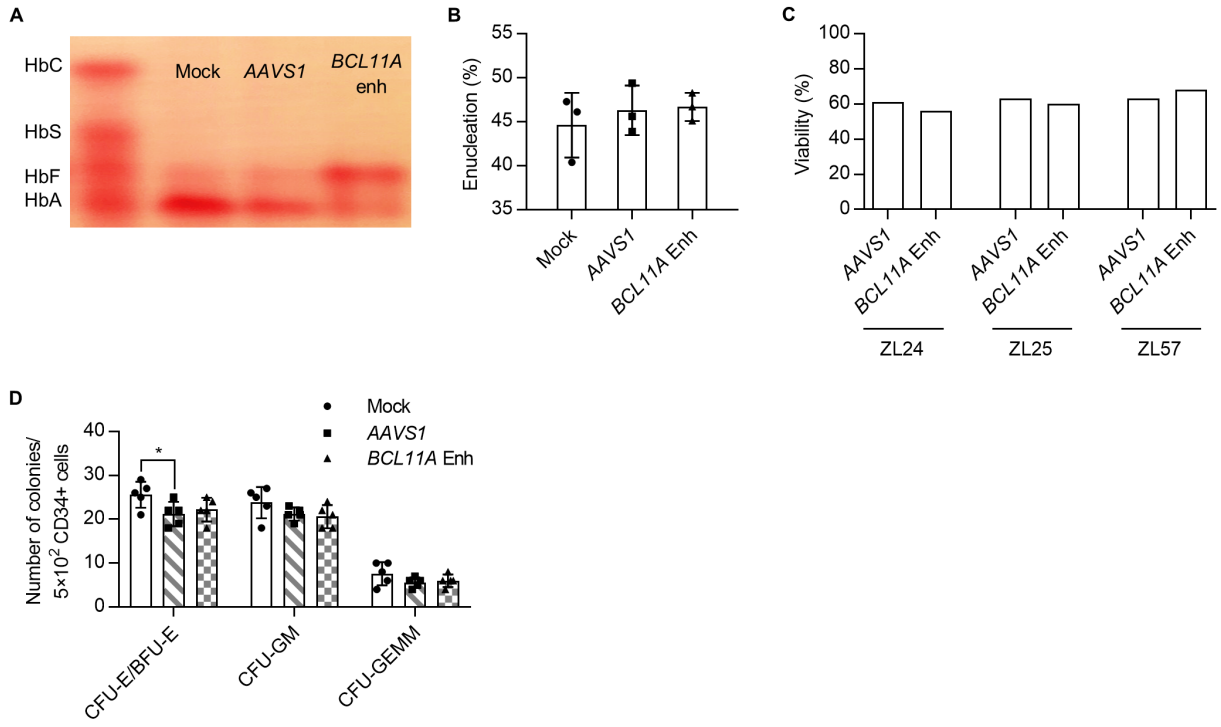
Phlebotomy

To maintain a hematocrit (HCT) of ~25%, 20% of total blood volume (estimated at 60mL/kg) was collected in citrate two times per week for 6 weeks with volume replacement using Normosol-R (Hospira, Inc., Lake Forest, IL). Complete blood count (CBC) and serum blood chemistries were performed using the phlebotomized blood. Just prior to the blood draws, an iStat was used to determine HCT. If the HCT was 20% or higher, the 20% BV was drawn with volume replacement. Bodyweight, body temperature, and heart and respiratory rate were monitored. Throughout the period of phlebotomy, these remained within normal limits. When iron levels trended to fall below normal limits, a single dose of iron dextran (10mg/kg) and folic acid (0.25mg/kg) IM was administered.

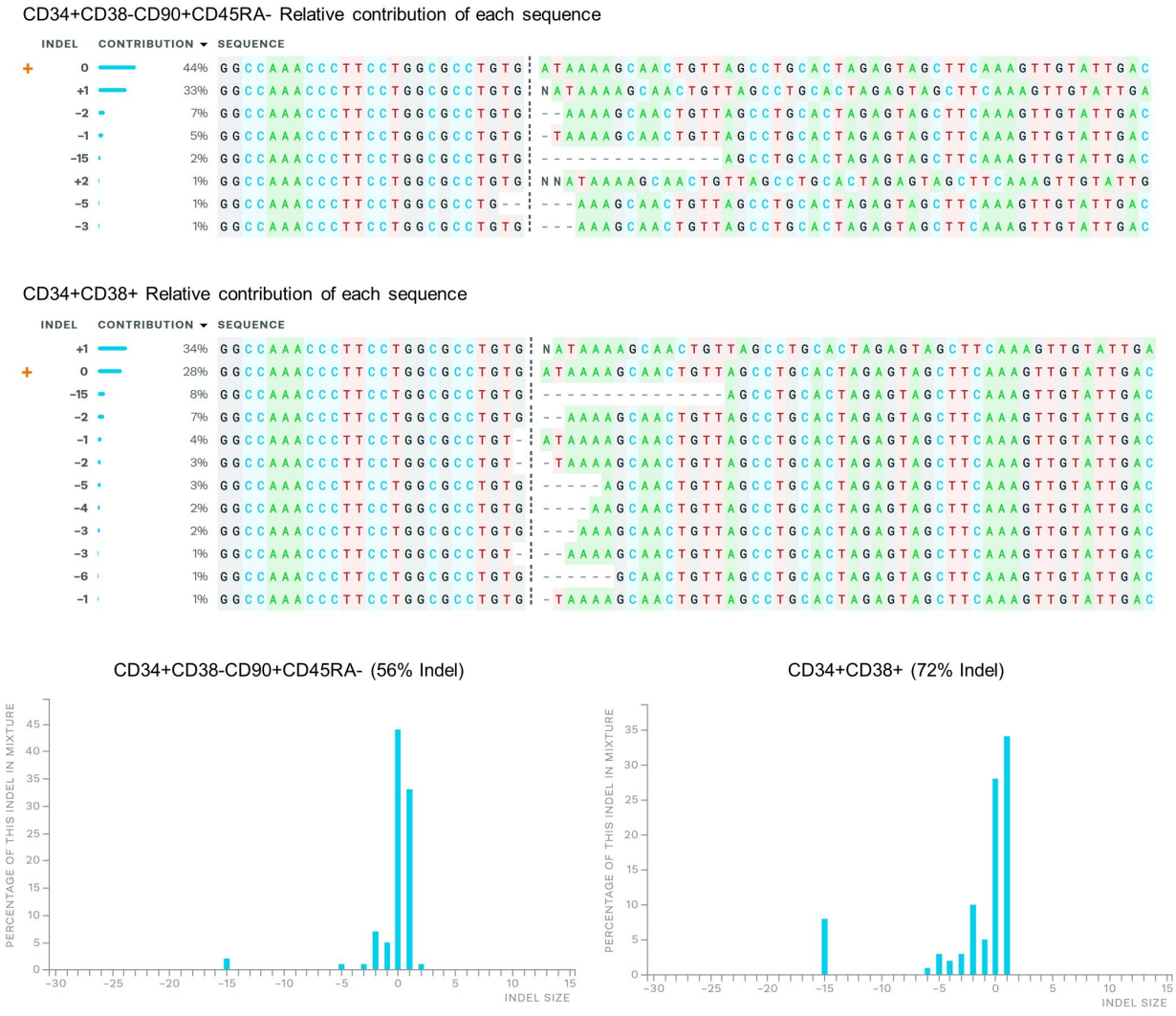
Statistical analysis

Standard errors of the mean are given as error bars in all figures. The data were statistically analyzed with one-way analysis of variance (ANOVA) followed by the Tukey post hoc test using Graphpad Prism 7 software (GraphPad Software, Inc, CA). Data were represented as mean ± SD and considered significantly different at $p < 0.05$. Clonality index and lineage data analysis were performed by means of R statistical software (8) and packages vegan and ggplots.

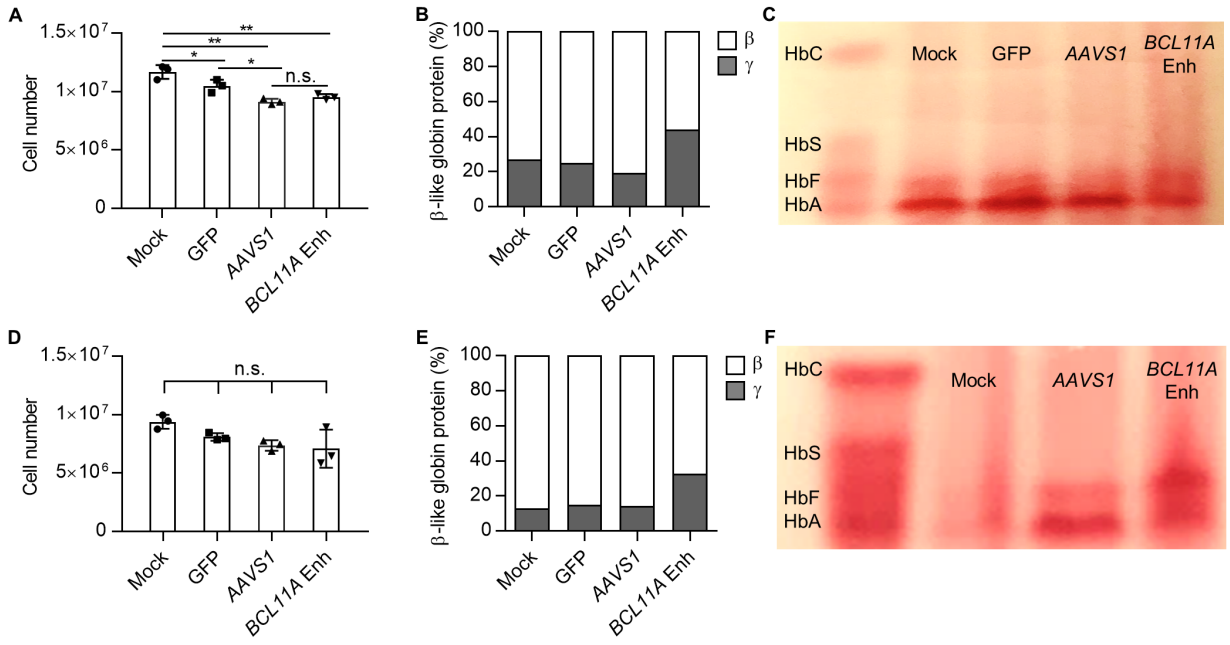
Supplemental Figures



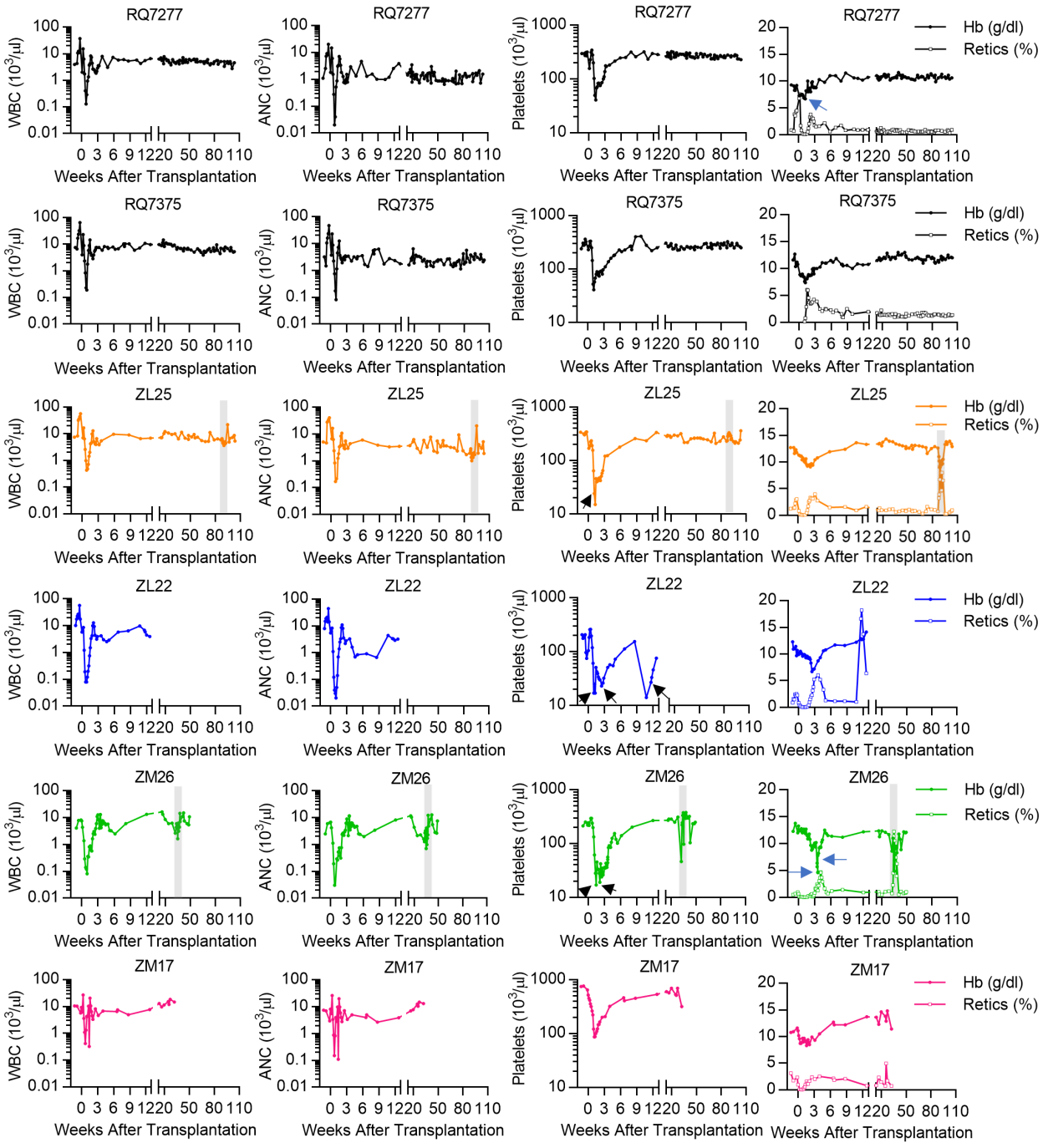
Supplemental Figure 1. *BCL11A* enhancer editing induces γ-globin in rhesus CD34+ HSPCs without affecting colony-forming unit (CFU) formation ability and enucleation. (A) Hemoglobin (Hb) electrophoresis and (**B**) enucleation percentages for ex vivo differentiated red blood cells (RBCs) in non-edited (Mock) and edited (*AAVS1* or *BCL11A* enhancer) rhesus CD34+ HSPCs. (n=3, one-way analysis of variance (ANOVA) followed by the Tukey post hoc test) (**C**) Cell viability of electroporated rhesus CD34+ HSPCs edited for *AAVS1* or *BCL11A* enhancer in small scale (ZL24 and ZL25, 5×10^4 cells, 200pmol for both 2×NLS SpCas9 and sgRNAs) and large scale (ZL57, 1.5×10^6 cells, 1000pmol for both 2×NLS SpCas9 and sgRNAs) electroporation conditions. (**D**) CFU formation ability of CD34+ HSPCs incubated 48 h in ex vivo culture conditions after electroporation with RNP. (n=5, one-way ANOVA followed by the Tukey post hoc test, * $P < 0.05$)



Supplemental Figure 2. Slightly lower indels in the HSC-enriched population with higher indel frequencies repaired by NHEJ compared to committed progenitors. Indel spectrum of rhesus CD34+ HSPCs sorted 2 hours after RNP electroporation for HSC-enriched population (CD34+CD38-CD90+CD45RA-) and committed progenitors (CD34+CD38+). Sanger sequences of the two populations were analyzed using ICE (Synthego).

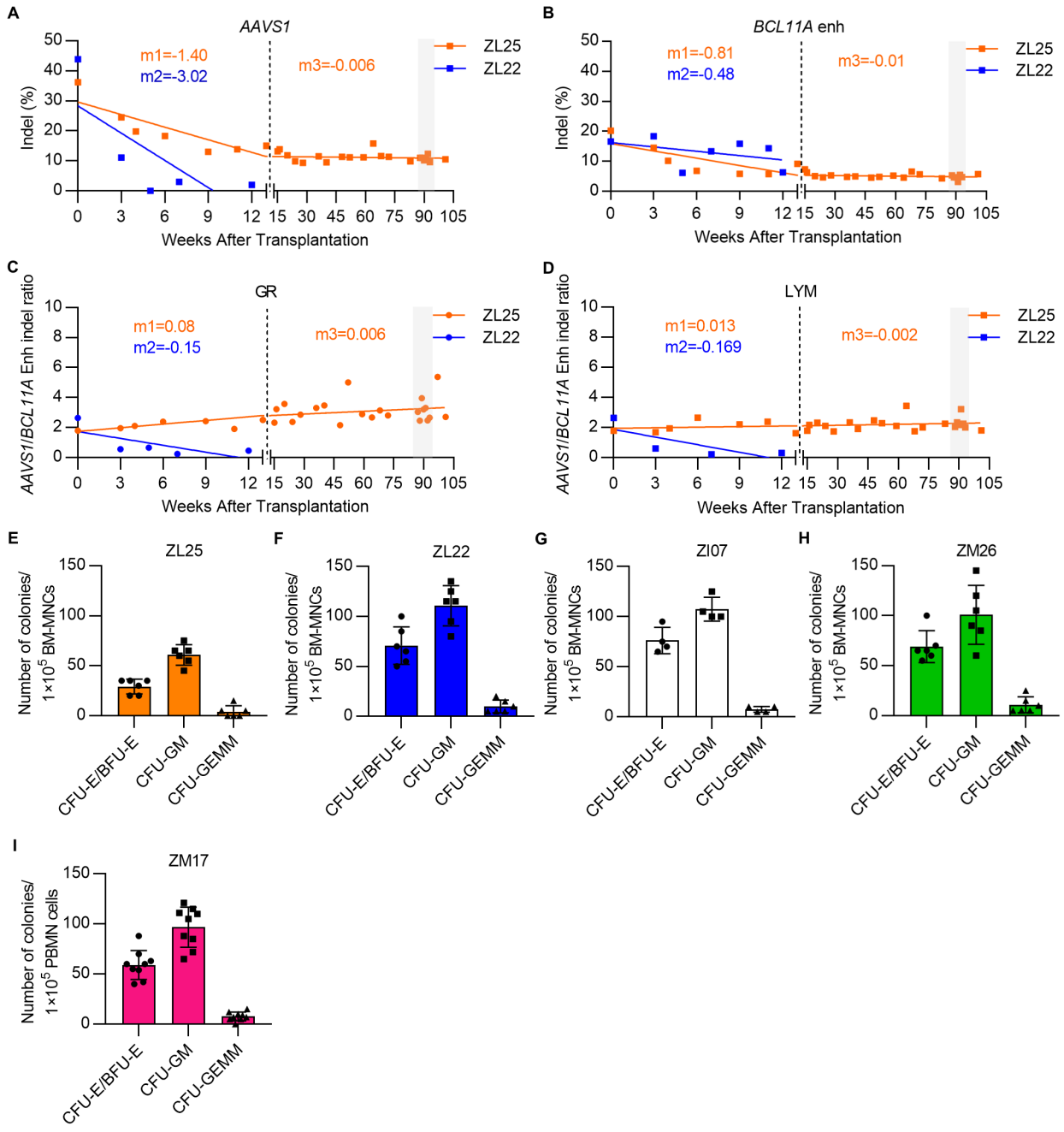


Supplemental Figure 3. Ex vivo γ-globin induction in *BCL11A* enhancer edited rhesus CD34+ HSPCs before transplantation. (A) Cell number (n=3) and β-like globin protein expression by (**B**) reverse phase-high performance liquid chromatography (RP-HPLC) and (**C**) hemoglobin (Hb) electrophoresis in ex vivo differentiated ZL25 CD34+ HSPCs before transplantation. (one-way analysis of variance (ANOVA) followed by the Tukey post hoc test, * $P < 0.05$, ** $P < 0.01$). **(D)** Cell number (n=3) and β-like globin protein expression by (**E**) RP-HPLC and (**F**) Hb electrophoresis in ex vivo differentiated ZL22 CD34+ HSPCs before transplantation.



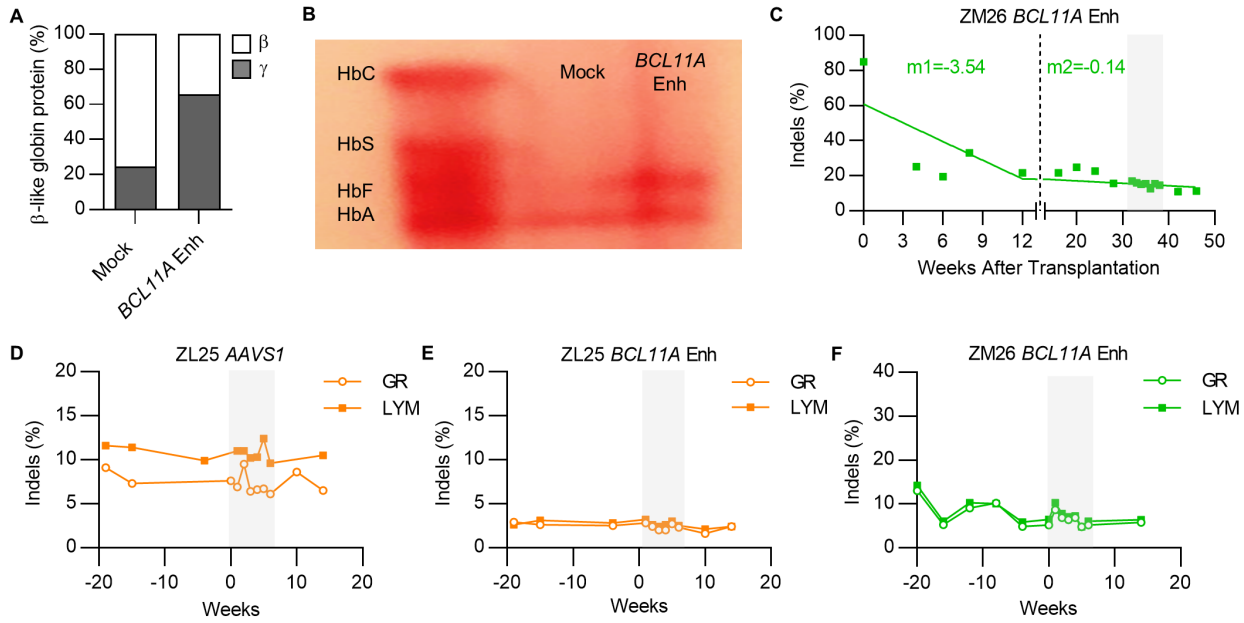
Supplemental Figure 4. Typical autologous reconstitution kinetics following *BCL11A* enhancer gene editing. *BCL11A* enhancer edited CD34+ HSPC transplanted animals (ZL25, ZL22, ZM26, and ZM17) rescued their cell counts and hemoglobin (Hb) levels with typical kinetics similar to control animals transplanted with lentivirus (GFP vector) transduced CD34+

HSPCs (RQ7277 and RQ7375). The black arrow indicates platelet transfusion and the blue arrow indicates whole blood transfusion. The gray rectangle represents the phlebotomy course.

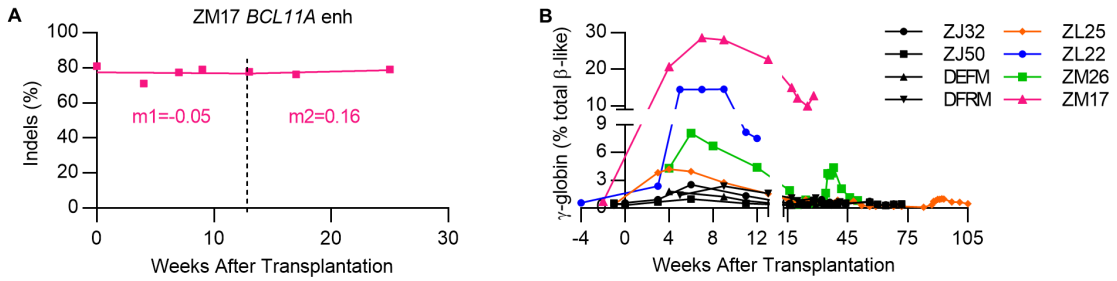


Supplemental Figure 5. **Durable autologous engraftment and colony-forming unit (CFU) potential following *BCL11A* enhancer gene editing.** ZL25 and ZL22 were transplanted with *AAVS1* and *BCL11A* enhancer edited cells (1:1). The gray rectangle represents the phlebotomy course. Editing frequencies in lymphocyte (LYM) fractions for (**A**) *AAVS1* and (**B**) *BCL11A* enhancer in transplanted rhesus macaques. *AAVS1*/1617 editing ratio in (**C**) granulocyte (GR)

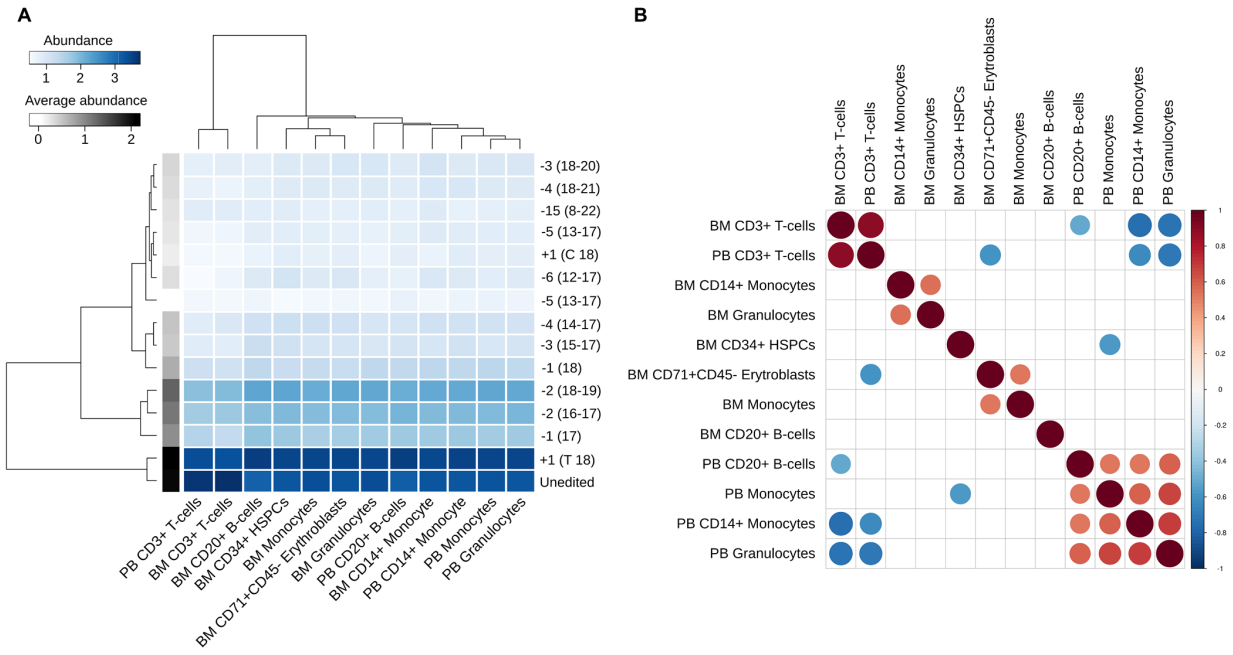
and (**D**) LYM fractions of the transplanted animals. CFU potential of BM MNCs of (**E**) ZL25 at 100 weeks post-transplantation (n=6), (**F**) ZL22 at 13 weeks post-transplantation (n=6), (**G**) a non-transplanted control animal, ZI07 (n=4), (**H**) ZM26 at 38 weeks post-transplantation (n=6), and ZM17 at 28 weeks post-transplantation (n=9).



Supplemental Figure 6. **Stable gene editing dynamics after transplantation and during phlebotomy.** (**A**) Reverse-phase high-performance liquid chromatography (RP-HPLC) and (**B**) hemoglobin (Hb) electrophoresis analysis for β-like globin protein expression evaluation in ex vivo differentiated red blood cells (RBCs) of non-edited (Mock) and *BCL11A* enhancer edited rhesus CD34+ HSPCs of ZM26 before transplantation. (**C**) Indel frequencies in the lymphocyte (LYM) fraction of ZM26 over time. Indel percentages during the phlebotomy course for (**D**) *AAVS1* and (**E**) *BCL11A* enhancer in ZL25, and (**F**) *BCL11A* enhancer in ZM26 for granulocytes (GR) and LYM fractions.

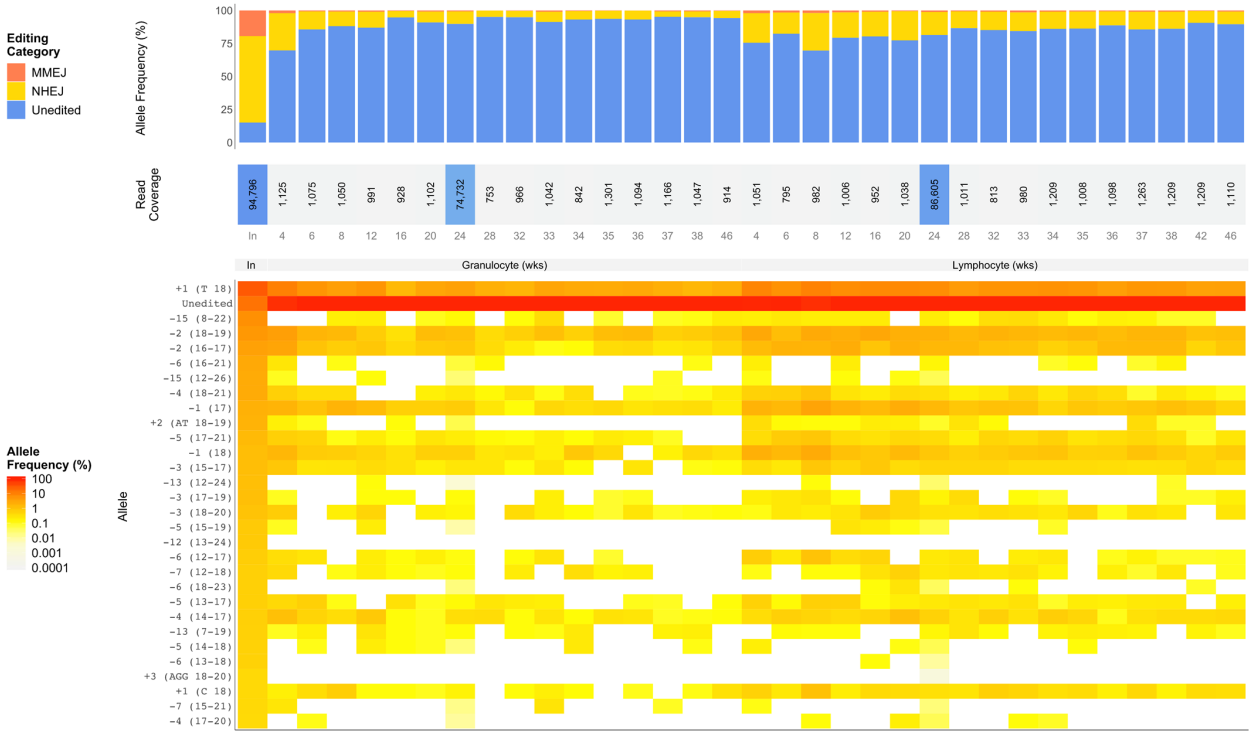


Supplemental Figure 7. Robust *BCL11A* enhancer editing and γ-globin induction. (**A**) Indel frequencies in lymphocyte fraction of ZM17 over time. (**B**) γ-globin protein expression in animals transplanted with lentivirus transduced (GFP/YFP vector) (DEFM, DFRM, ZJ32, and ZJ50) (9), or *BCL11A* enhancer edited (ZL25, ZL22, ZM26, and ZM17) CD34+ HSPCs.



Supplemental Figure 8. Cell lineage gene edit patterns. (A) Heatmap representation of the abundance (log) for the 14 indels with a frequency greater than 1% in at least one lineage plus the unedited allele in BM and PB lineages of ZM17 at 28 weeks post-transplantation. The allele-specific average abundance value (log) is represented by a gray-scale color on the left side of the heatmap. Both columns (lineages) and rows (alleles) are ordered according to their similarity measured by Ward distance. (**B**) The correlation (Pearson's coefficient) among all possible pairs of lineages of ZM17 at 28 weeks post-transplantation based on gene edit distribution. Both circle diameter and color represent Pearson's correlation estimates. Only significant (t-test, alpha = 0.05) correlations are plotted. Columns (and rows) are ordered using Euclidean distance.

Supp 9E. ZM26, *BCL11A* Enhancer



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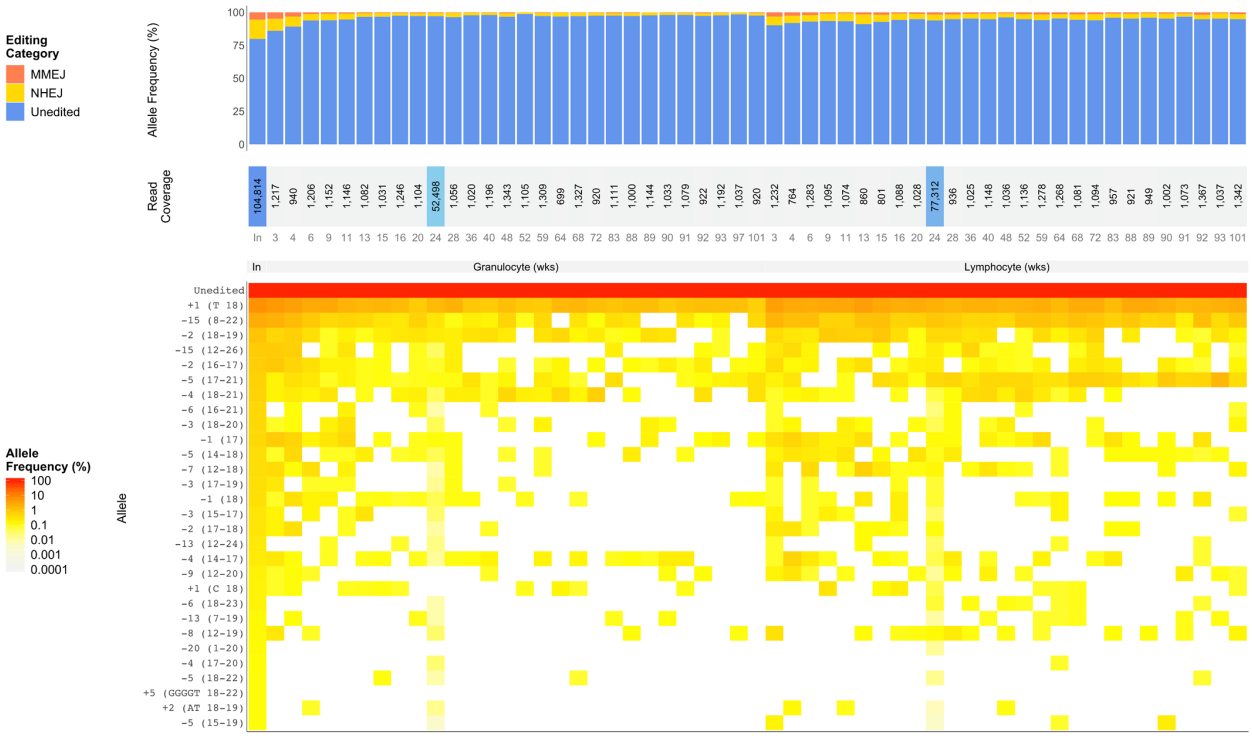
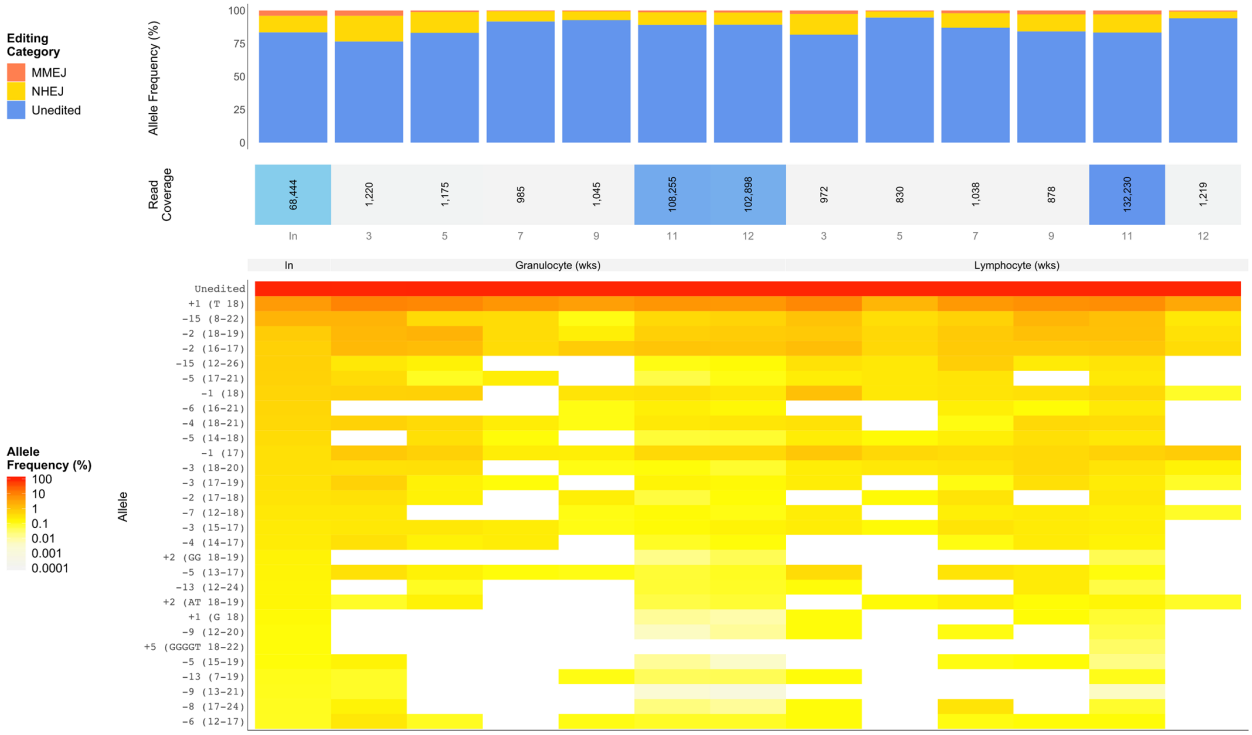


Supp 9C. ZL22, *AAVS1*

Supp 9D. ZL22, *BCL11A* Enhancer



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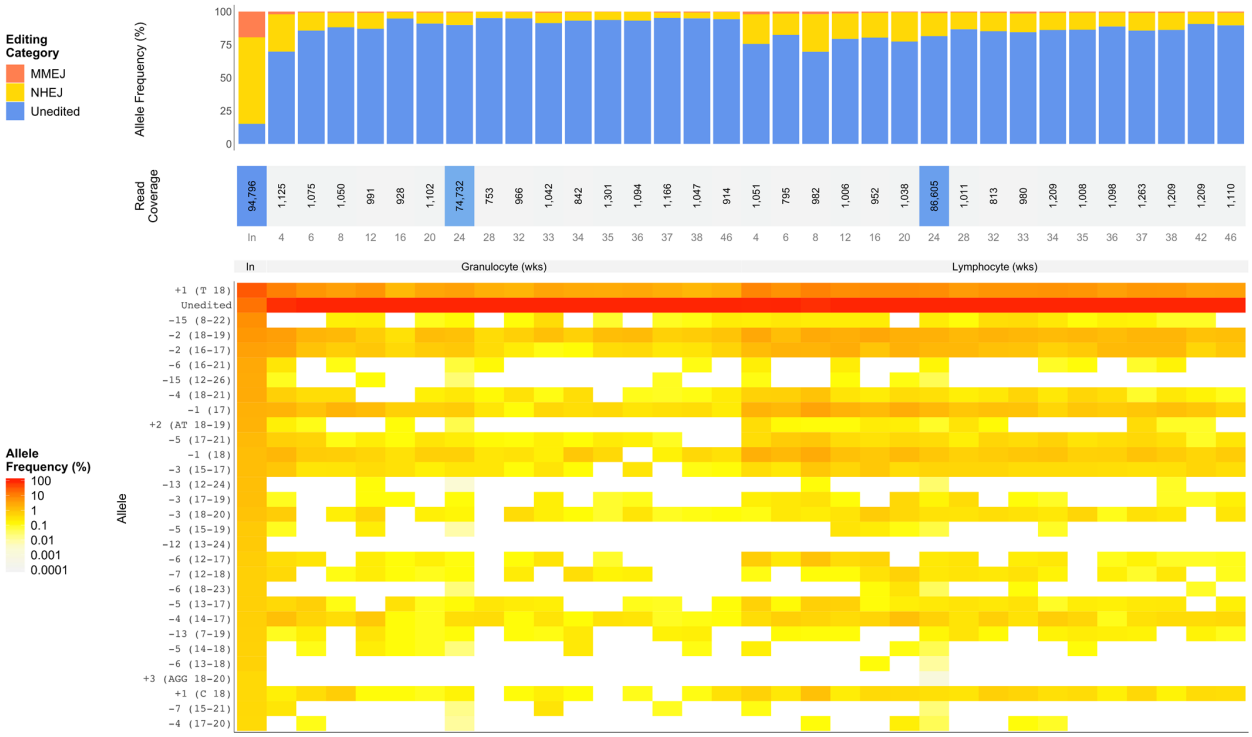
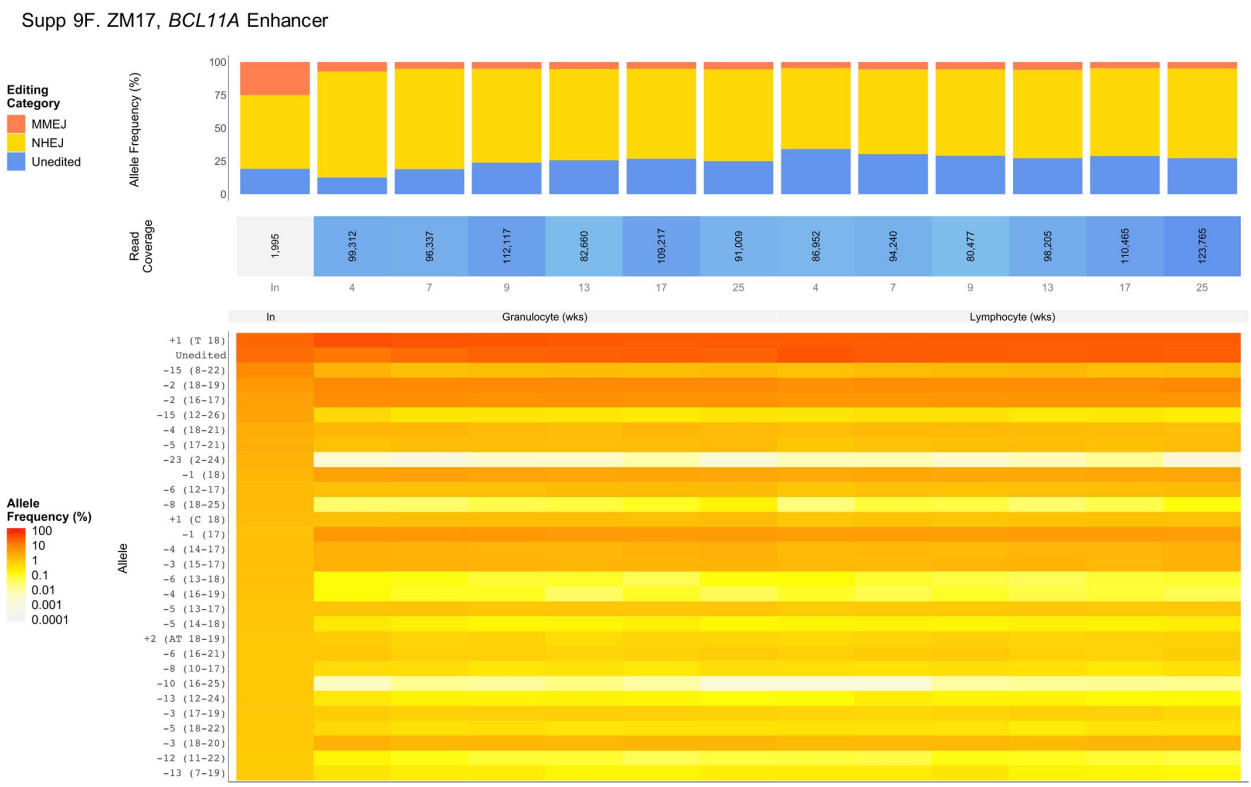
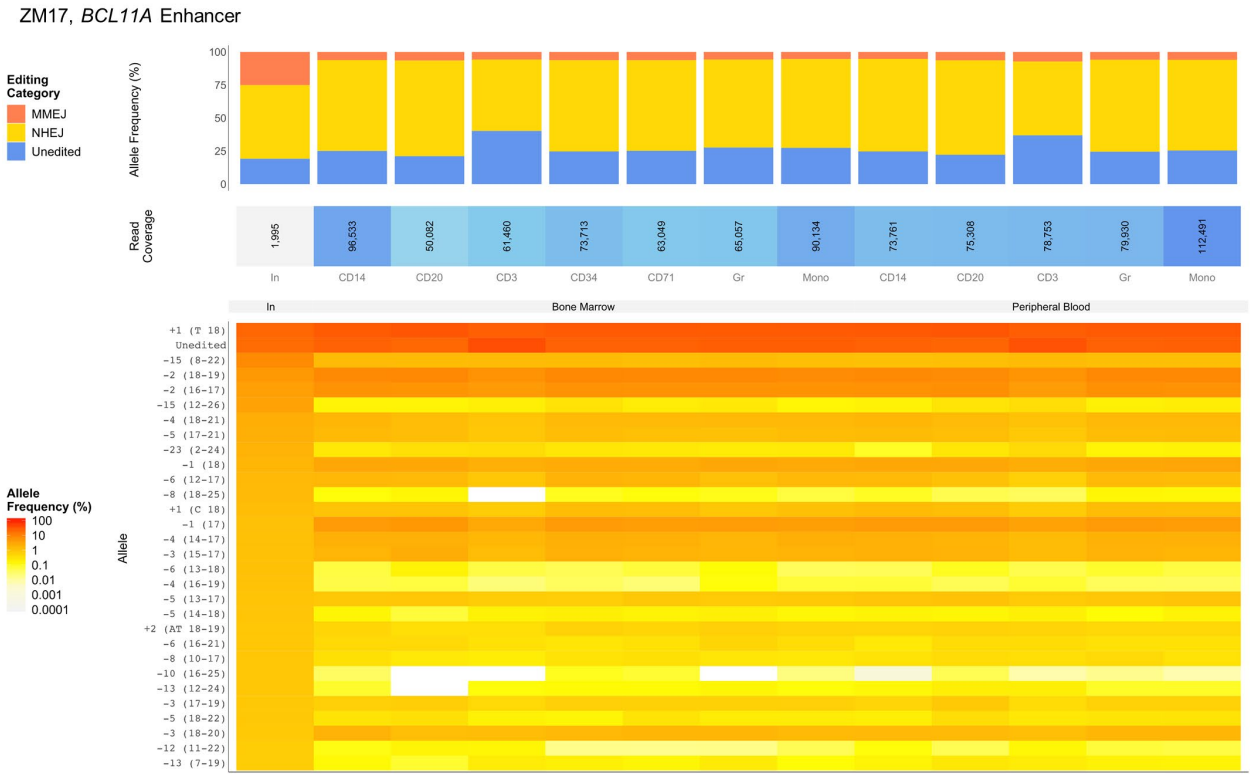
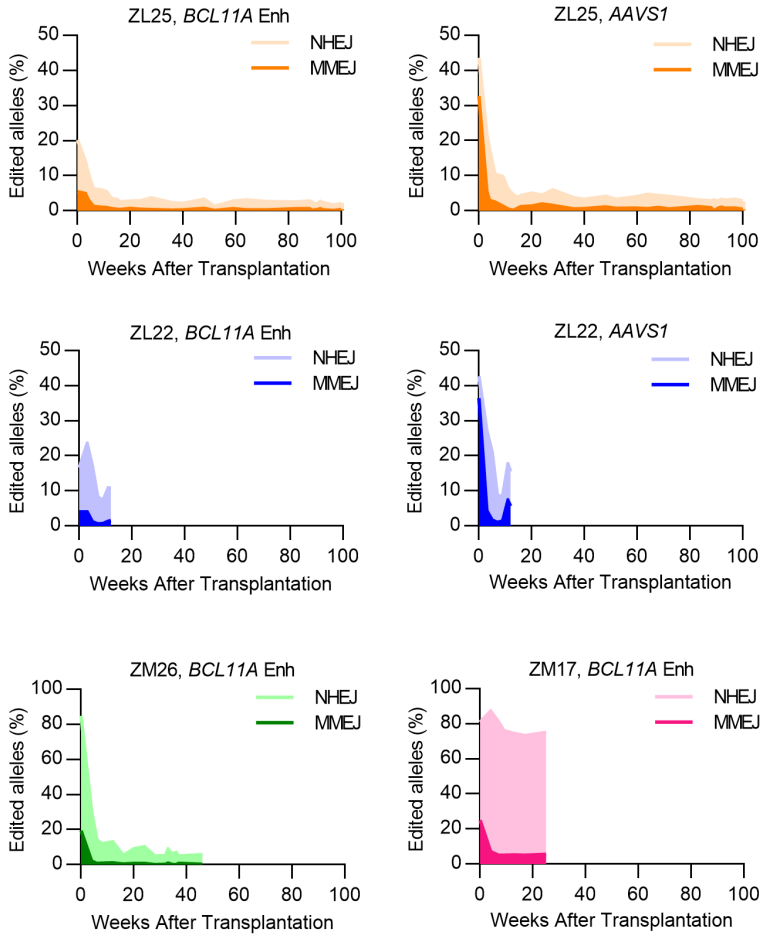




Supplemental Figure 9. Summary of the indel composition in engrafted granulocyte and lymphocyte fractions of peripheral blood over time. Amplicon deep sequencing and analysis was performed to identify (**A**) *AAVS1* edits in ZL25, (**B**) *BCL11A* enhancer edits in ZL25, (**C**) *AAVS1* edits in ZL22, (**D**) *BCL11A* enhancer edits in ZL22, (**E**) *BCL11A* enhancer edits in ZM26, and (**F**) *BCL11A* enhancer edits in ZM17. The 30 alleles with the highest frequency in each sample are shown. In: Infusion product, MMEJ: Microhomology-mediated end joining, NHEJ: Non-homologous end joining. The measured edits in the infusion products for ZL25 and ZL22 were divided by two in these plots to reflect the 1:1 mixing of *AAVS1* and *BCL11A* enhancer edited cells prior to infusion.



Supplemental Figure 10. Similar indel compositions of *BCL11A* enhancer edits in bone marrow (BM) and peripheral blood (PB) lineages. Amplicon deep sequencing and analysis was performed to identify *BCL11A* enhancer edits in BM (CD14+, CD20+, CD3+, CD34+, CD45-CD71+, granulocyte [Gr] and monocyte [Mono]) and PB (CD14+, CD20+, CD3+, granulocyte [Gr] and monocyte [Mono]) fractions of ZM17 over time. For each fraction, amplicon deep sequencing and indel analysis were performed, and the 30 alleles with the highest frequency are shown. In: Infusion product, MMEJ: Microhomology-mediated end joining, NHEJ: Non-homologous end joining.



Supplemental Figure 11. Microhomology-mediated end joining (MMEJ) and non-homologous end joining (NHEJ) indel percentages in edited animals over time. Deletions ≥8 bp were categorized as MMEJ, while all other indels were categorized as NHEJ.

References

1. Donahue R, Kirby M, Metzger M, Sellers S, and Cullis H. Peripheral blood CD34+ cells differ from bone marrow CD34+ cells in Thy-1 expression and cell cycle status in nonhuman primates mobilized or not mobilized with granulocyte colony-stimulating factor and/or stem cell factor. *Blood.* 1996;87(4):1644-1653.
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