Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Mamcarz E, Zhou S, Lockey T, et al. Lentiviral gene therapy combined with low-dose busulfan in infants with SCID-X1. N Engl J Med 2019;380:1525-34. DOI: 10.1056/NEJMoa1815408

Lentiviral Gene Therapy with Low Dose Busulfan for Infants with X-SCID

Contents

Figure S1: Overview of cell processing and transduction.

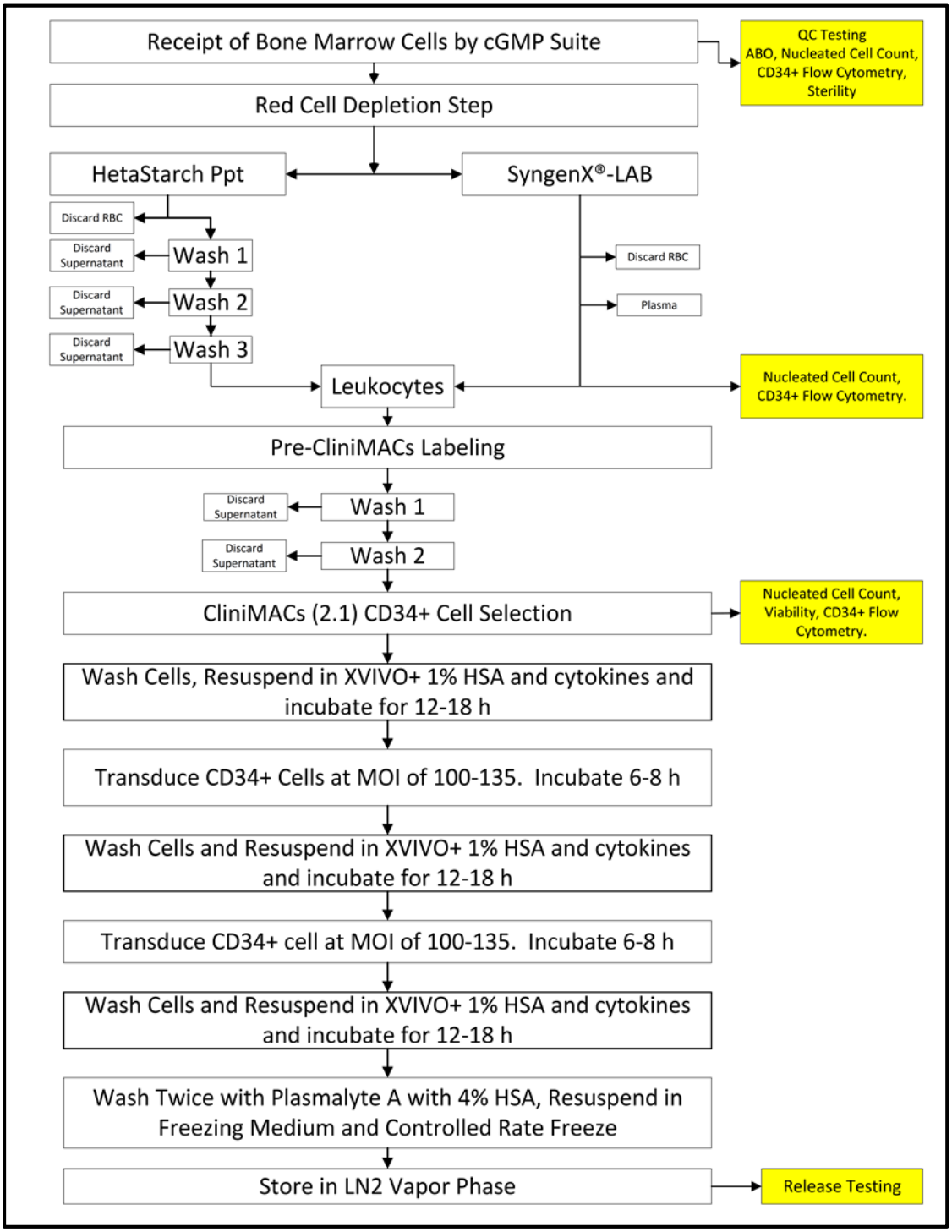


Figure S1: Overview of cell processing and transduction. The GMP flow diagram for bone marrow cell processing and transduction is shown. After the bone marrow cells are received from the operating room, red cell depletion is performed using either a HetaStarch procedure as shown, or the SyngenX™ device according to manufacturer's instructions. RBC-depleted leukocytes are then labelled with CD34 antibody provided with the CliniMACS® device and magnetically selected using the Enrichment 2.1 program according to manufacturer's instructions. The enriched $CD34^+$ cells are then pre-incubated on RetroNectin® coated plates overnight at $1x10^6$ cell/ml in X-VIVO™ 10 medium containing 1% human serum albumin and 100ng/ml each of rhSCF, rhTPO and rhFLT3-L. The next morning, vector is added to the coated flask to achieve a total multiplicity of infection (MOI) of 100-135 and cells are transduced for 6-8 hours. The cells are then washed to remove vector supernatant and resuspended in fresh medium and cultured overnight in the coated flask. The following morning, the 2^{nd} hit of vector is added for 6-8 hours (MOI of 100-135) before washing and a third overnight culture. On the 4^{th} morning, the cells are washed and cryopreserved in medium containing DMSO and kept in vapor phase of liquid nitrogen freezer. The cells are typically held for at least 5 days for all release testing to be complete. After release testing allows certification, the cells are brought frozen to the bedside, thawed in a 37° Celsius water bath, and infused immediately without washing.

Figure S2: Myelosuppression with subablative busulfan conditioning.

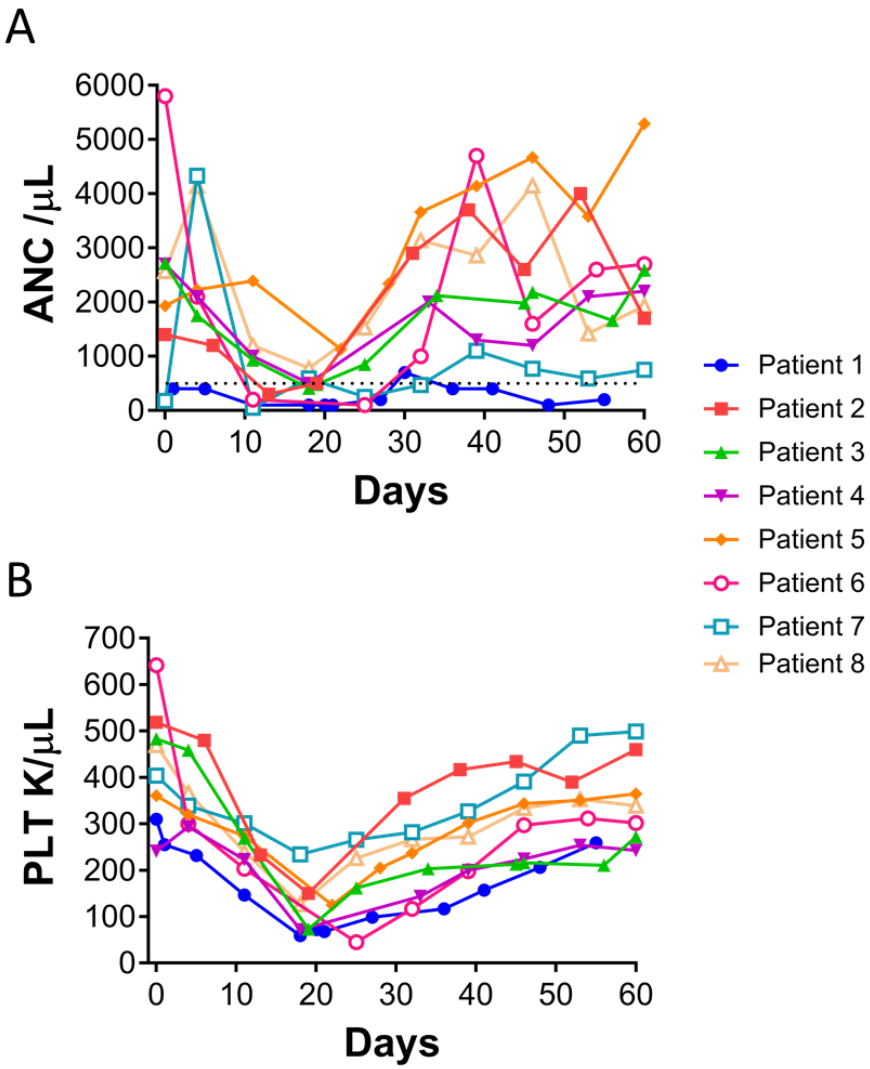


Figure S2: Myelosuppression with subablative busulfan conditioning. Neutrophil counts (panel A) and platelet counts (panel B) are shown for each case with days after $CD34^+$ cell infusion shown on the X-axis. Neutrophil count nadirs were characterized by 5-15 days of absolute neutrophil counts (ANC) below 500 prior to spontaneous recovery. Note that patient 1 had severe pre-existing neutropenia both before and after busulfan. No neutrophil growth factors were given with the exception of case 1. (B) Platelet count nadirs were also mild and spontaneously resolved in all cases without the need for platelet transfusion in any cases.

Figure S3: Growth velocity after gene therapy.

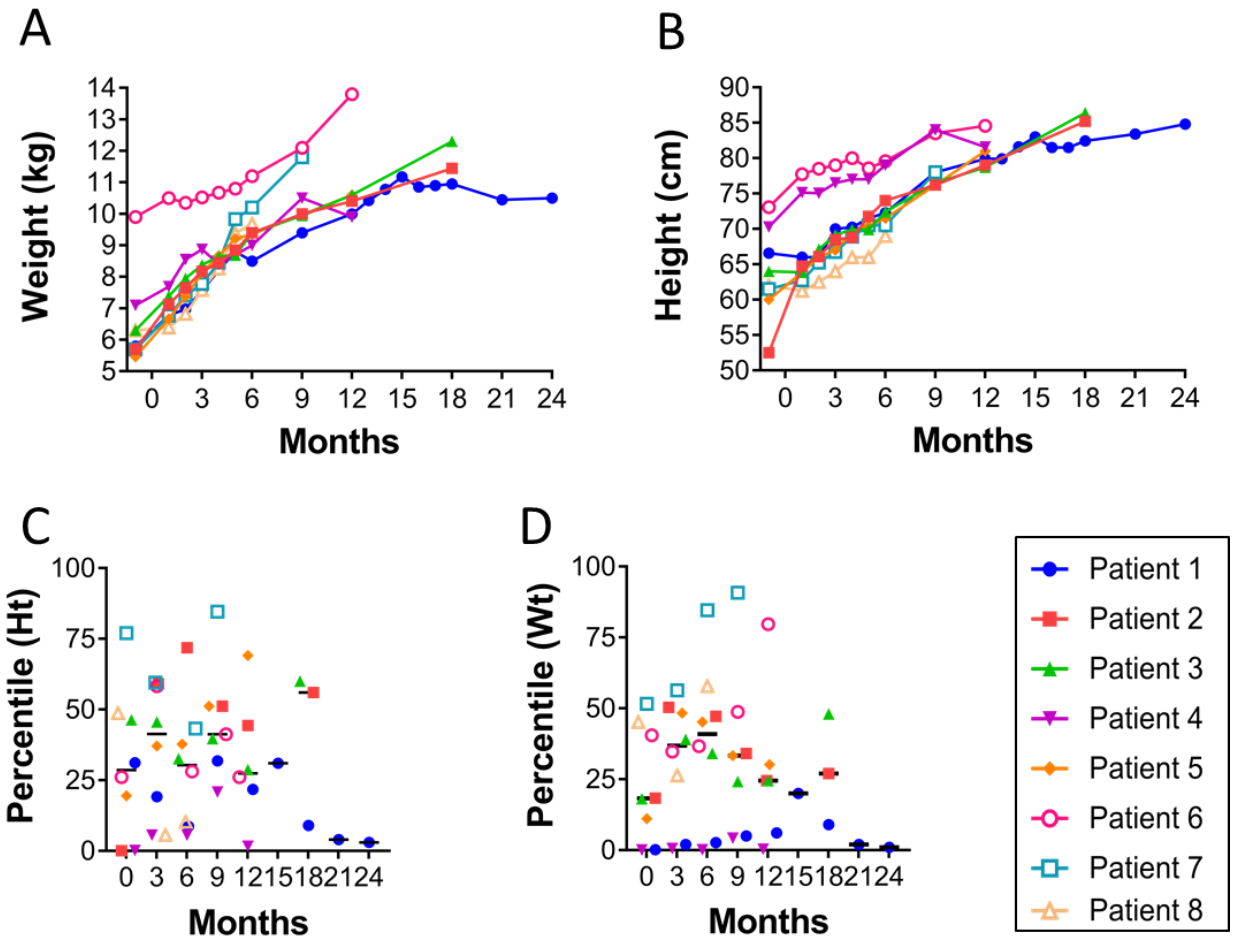


Figure S3: Growth velocity after gene therapy. Body weight and height is shown for all 8 patients prior to (month 0) and post gene therapy. Weight (A) in kilogram (kg) and (C) percentiles (bar: median). Height (B) in centimenters (cm), and (D) percentiles (bar: median). Percentiles were calculated using the CDC online calculator (https://peditools.org/growthinfant/). There were no significant differences between median weight and height percentiles for each time point, indicating that all 8 patients gained weight and grew in length as expected within the first 12 months post gene therapy.

Figure S4: Gene therapy boost in case 1.

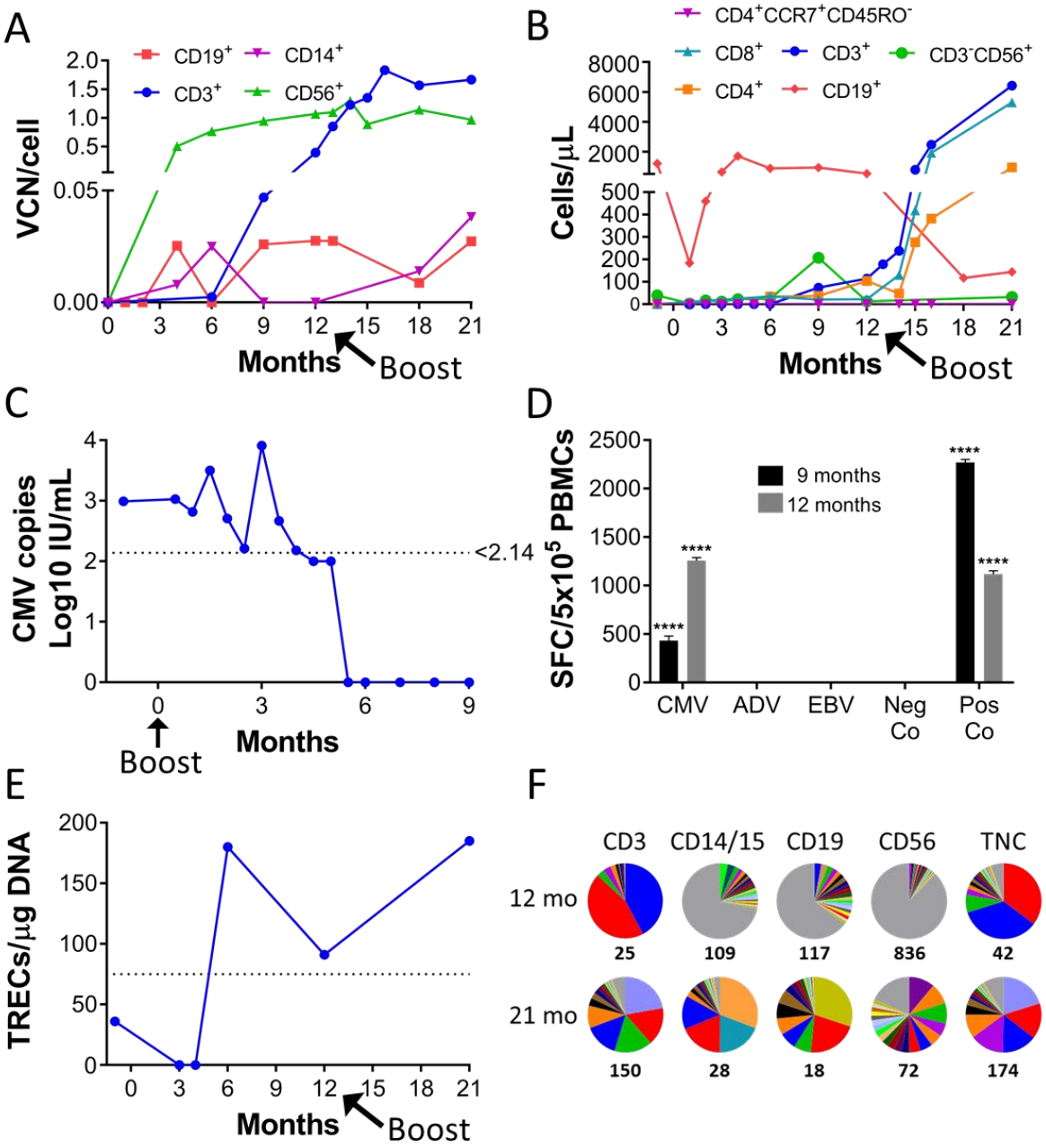


Figure S4: Gene therapy boost in case 1. A second bone marrow harvest and transduction was performed about 12 months after the initial gene therapy in case 1 and indicated by the arrow. (A) The overall VCN by qPCR in sorted $CD3^+$, $CD19^+$ and $CD56^+$ cells pre and post boost. A progressive rise in T-cell marking was noted after the boost. (B) The number of autologous $CD3^+$, $CD4^+$, $CD8^+$, $CD3^+CD4^+CCR7^+CD45RO^-$ (naïve T), $CD19^+$ (B), $CD3^-CD56^+$ (NK) cells in the peripheral blood before and after the boost (Fluorochrome-conjugated antibody against the patient's class I human leukocyte antigen B serotype (HLA-B12) was used to differentiate between maternal and patient lymphocytes). Autologous T-cell production rose sharply 2 months after the gene therapy boost as shown. B cells remained low secondary to administration of rituximab to treat autoimmune hemolytic anemia and thrombocytopenia. (C) CMV infection as determined by genome PCR in peripheral blood both before and after the boost (arrow). The patient had been persistently positive for CMV until 5 months after the boost, at which time the CMV PCR signal became undetectable. (D) Enzyme-linked immunosorbent spot assay (ELISPOT) performed at 9 and 12 months post gene therapy boost. Presence of functional CMV-specific T cells was detected by measuring the production of interferon gamma (IFN-ɣ) in response to pepmixes encoding the CMV proteins pp65 and IE1. No reactivity was observed in the presence of pepmixes encoding Epstein Barr Virus (EBV) or Adenovirus (ADV). Incubation with media served as negative (Neg) control and with staphylococcus aureus enterotoxin as a positive (Pos) control (Co). IFN-ɣ producing cells are presented as SFC (spot forming cell) per $5x10^5$ PBMCs (1-Way ANOVA: **** $p<0.0001$). (E) Absolute number of TRECs in PBMCs until the last follow-up at 21 months (9 months post gene therapy boost). (F) VIS frequency analysis in sorted PB lineages at 12 and 21 months (9 months post gene therapy boost). Restrictive patern in T cell and TNC cells improved post boost and no clonal dominance was noted.

Figure S5: T- cell proliferation.

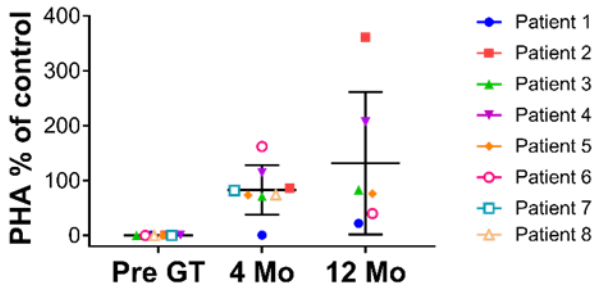


Figure S5:T-cell proliferation. T-cell proliferation in response to phytohemagglutinin (PHA) relative to normal T-cells, measured before and 4 months after gene therapy.

Figure S6: T-cell receptor diversity.

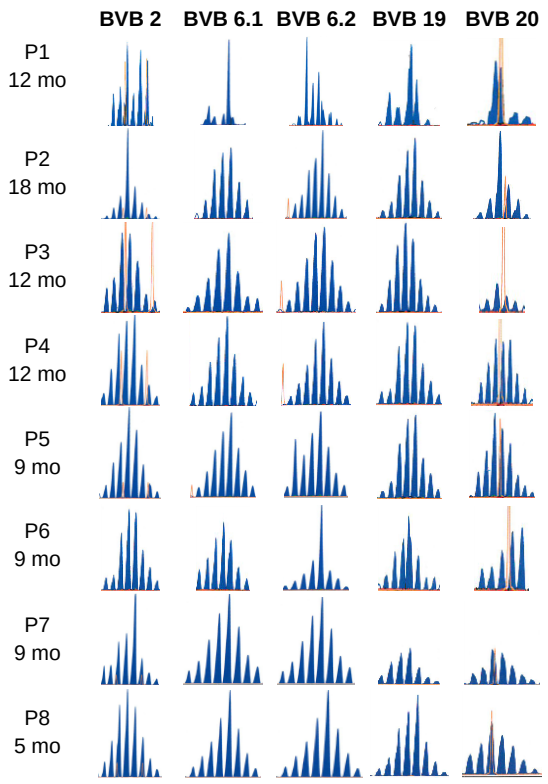


Figure S6: T-cell receptor diversity. T-cell receptor diversity patterns by Vβ-spectratype analysis in 8 patients after gene therapy. The length of complementarity-determining region (CDR3) in each indicated T-cell receptor beta chain variable (TCRBV) gene family was measured after PCR amplification with family-specific primers. The horizontal axis represents the size of the CDR3 peaks, and the vertical axis represents the height of a sequence with a given CDR3 gene family; a Gaussian distribution of CDR3 lengths is indicative of normal diversity.

Figure S7: TCR Vβ complexity score.

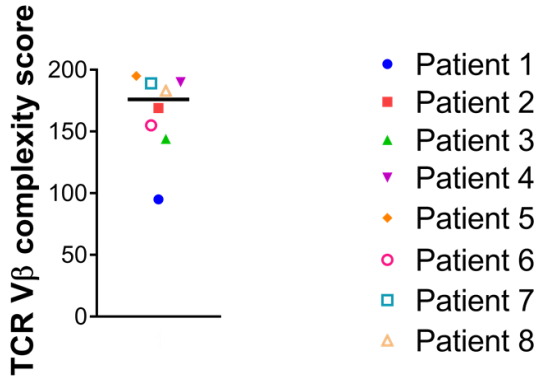


Figure S7: TCR Vβ complexity score. We determined the diversity for 25 of the 26 Vβ gene families by spectratyping as described in the legend for Figure 2I, which shows the graphs for individual patients. The mean TCR Vβ complexity score was determined by counting individual peaks. Eight peaks can be identified per Vβ gene family; thus, the maximum score is 200. The complexity score of patients, who had successful immune reconstitution post busulfan and gene therapy (patients 2 to 8), was 175 (range 144 to 195) for the time points shown in Figure 2J. This compares favorably to a mean TCR Vβ complexity score of 167 (range: 128 to 199) of 20 healthy allogeneic HCT donors at our center, whose TCR Vβ complexity score was determined using the same assay. Patient 1 had a complexity score of 95. The black line represents the mean for all patients.

Figure S8: Function of CD8⁺ T cells and B cells after gene therapy.

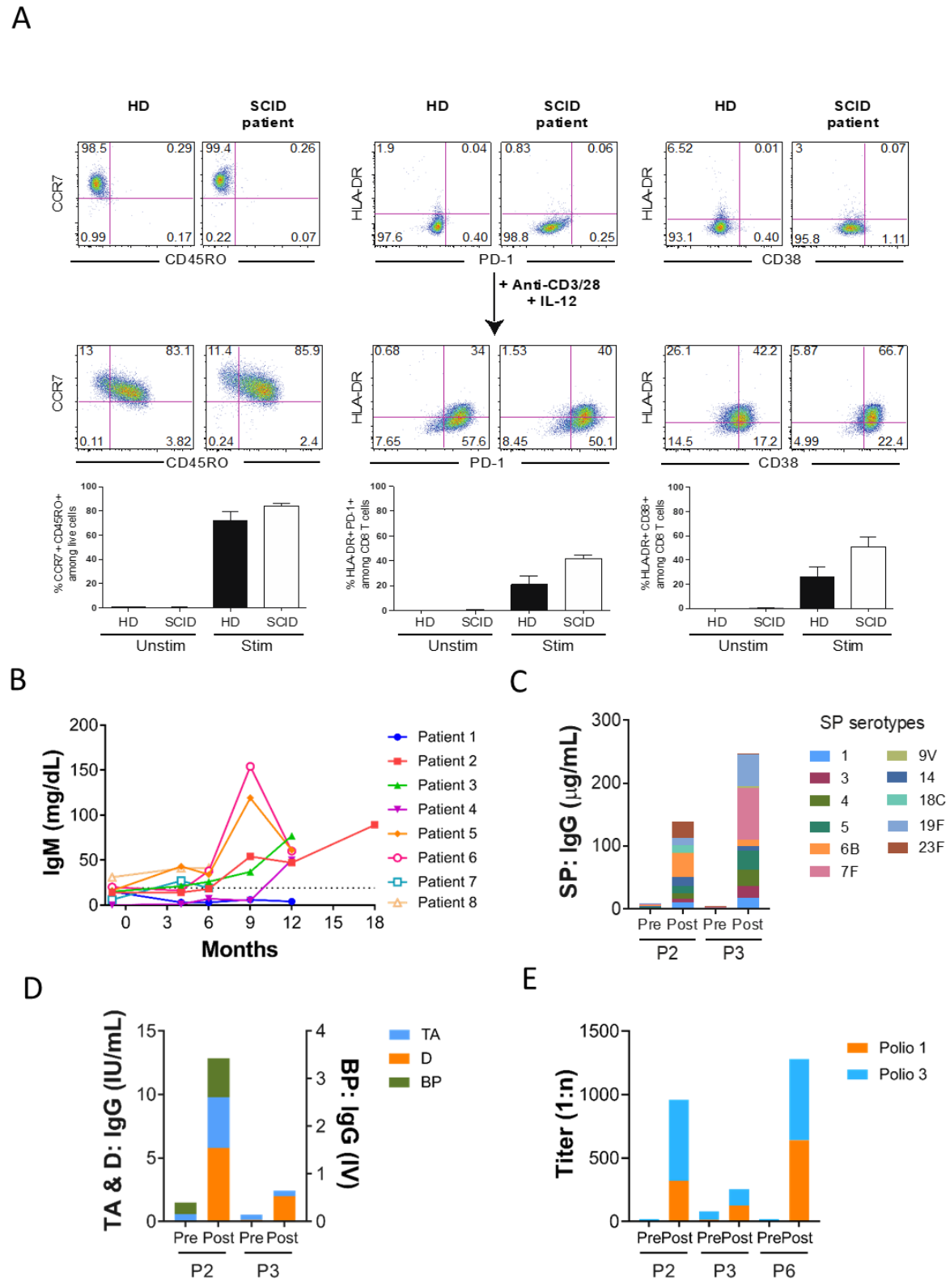


Figure S8: Function of $CD8^+$ T cells and B cells after gene therapy. A) Effector cell differentiation of patient peripheral blood naïve $CD8^+$ T cells. Naïve CD^+8 T cells from gene therapy cases (SCID) and healthy volunteers (HD) were cultured with anti-CD3/CD28 antibodies and IL-12. Following overnight stimulation, cells were harvested and stained for markers of effector cell differentiation (CD38, HLA-DR, and PD-1). Flow cytometry panels show representative data obtained from case 3 at 6 months post infusion. The bar graphs show averaged data from patients 3, 5, and 6 at five months post infusion. B) Serum immunoglobulin M (IgM) levels at various time points after gene therapy in all 8 cases. The dotted line indicates the normal value for IgM (19 mg/dL). C-E) IgG antibody titers to vaccines pre-and post-gene therapy. Vaccination responses to C) Streptococcus pneumonia (SP) IgG titers (µg/mL), D) Bordetella pertussis (BP) IgG titers as index values [IV] (not done for patient 3), and Diphtheria (D) and Tetanus antibody (TA) IgG titers as IU/mL, and E) Polio serotype 1 and 3 titers.

Figure S9: Genomic features of vector insertion sites (VIS) in cases 1-5.

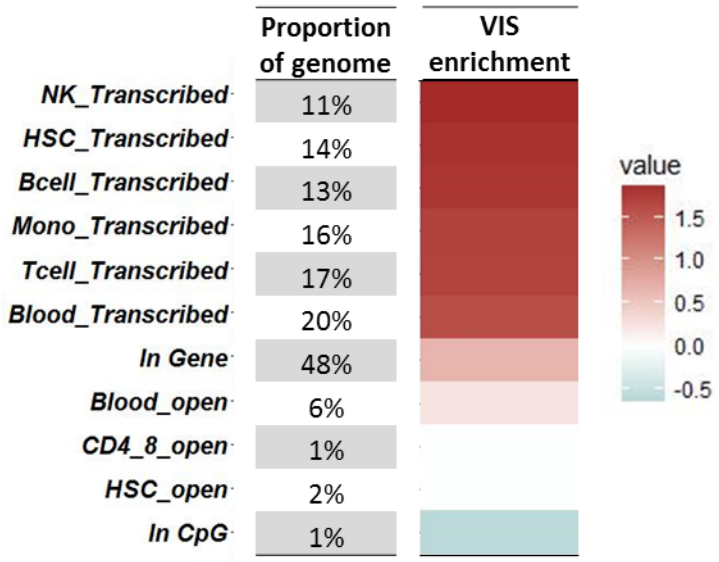


Figure S9: Genomic features of vector insertion sites (VIS) in cases 1-5. The heatmap shows the relative frequency of VIS in the indicated genomic regions, adjusted for their relative proportion within the genome. Red indicates a relative increase in VIS frequency in the indicated genomic areas with a predilection for insertions into regions associated with transcription of blood cell genes and in exons/introns of coding regions. There is a relative depletion of insertions into CpG rich areas typically associated with gene regulator regions.

Figure S10: Vector insertion site analysis.

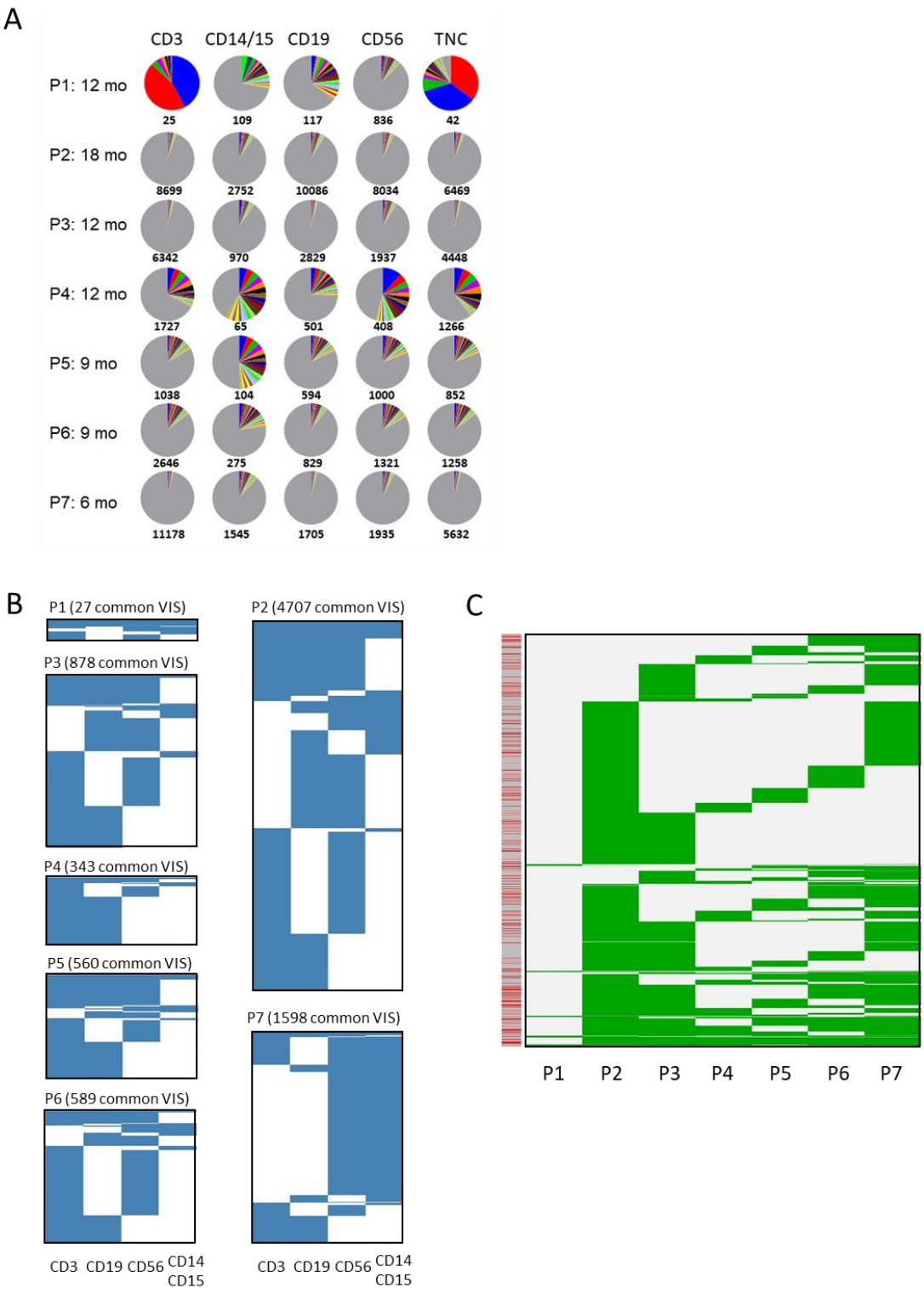
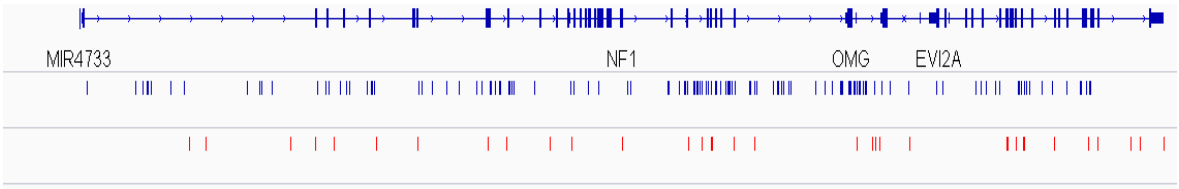


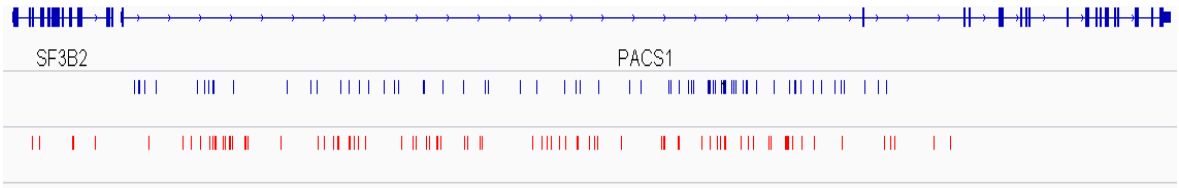
Figure S10: Vector insertion site (VIS) analysis in patients 1 through 7. A) VIS frequency analysis in sorted peripheral blood lineages. Each pie graph shows the top 20 most frequent VIS within the indicated sample as various colored sections. The grey areas show the remaining proportion of all other less frequent VIS in the indicated population. The total number of VIS detected in each sample is shown below each pie diagram. The columns are labeled with the sorting markers used for cell isolation, "TNC" shows total nucleated cells prior to sorting. B) The maps show common VIS present in multiple lineages (blue) within each patient. Sorted lineages are shown on the x-axis. Each horizontal line represents a unique VIS. The total number of VIS analyzed in each sample is shown above each individual map. C) This map shows genes that are commonly targeted in at least 2 of the 5 analyzed cases (green). Each horizontal line represents a single gene and red in the left side color bar indicates that the gene is annotated as a cancer gene.

Figure S11: Selected VIS maps of "hotspot" genes that were frequently targeted in multiple gene therapy cases.

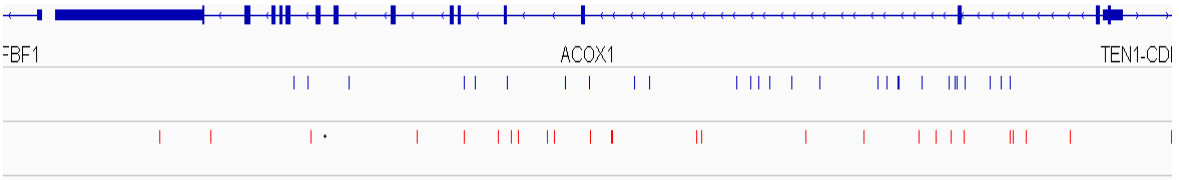
NF1



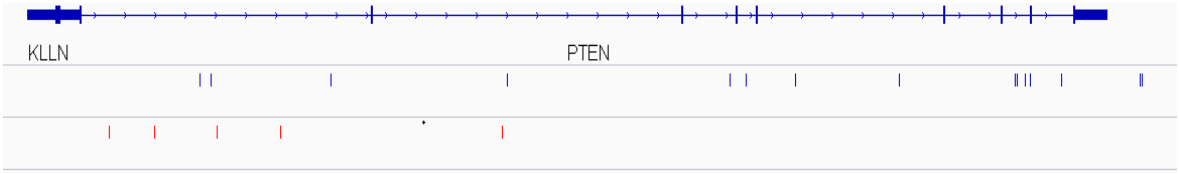
PACS1



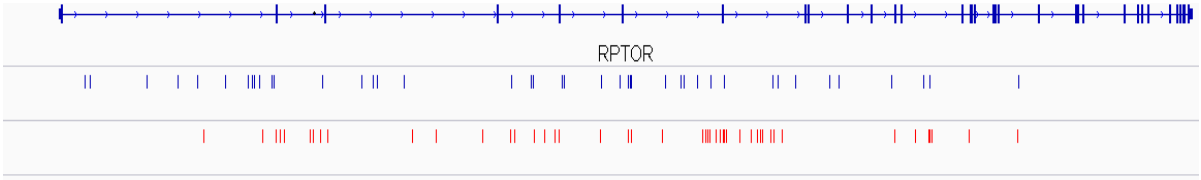
ACOX1



PTEN



RPTOR



TNRC6C

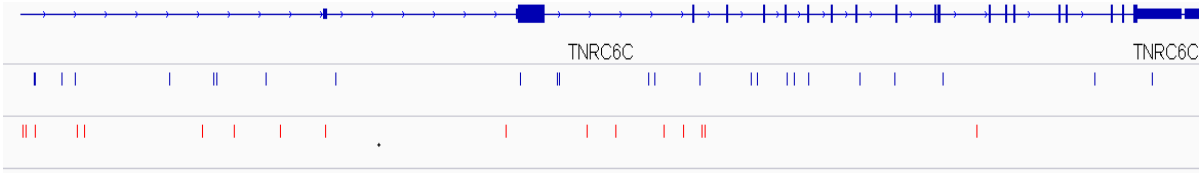


Figure S11: Selected VIS maps of "hotspot" genes that were frequently targeted in multiple gene therapy cases. The gene name is indicated in the upper left hand corner of the gene diagram. The location of gene introns (thin line) and exons (boxes) are indicated along with adjacent genes of interest. Mapped vector insertion sites are shown below as hatched lines, with blue hatchs showing a sense vector orientation relative to the positive DNA strand and red hatchs showing a vector sense orientation relative to the negative strand.

Figure S12: Variability in CD34 expression intensity in cases 1 and 6.

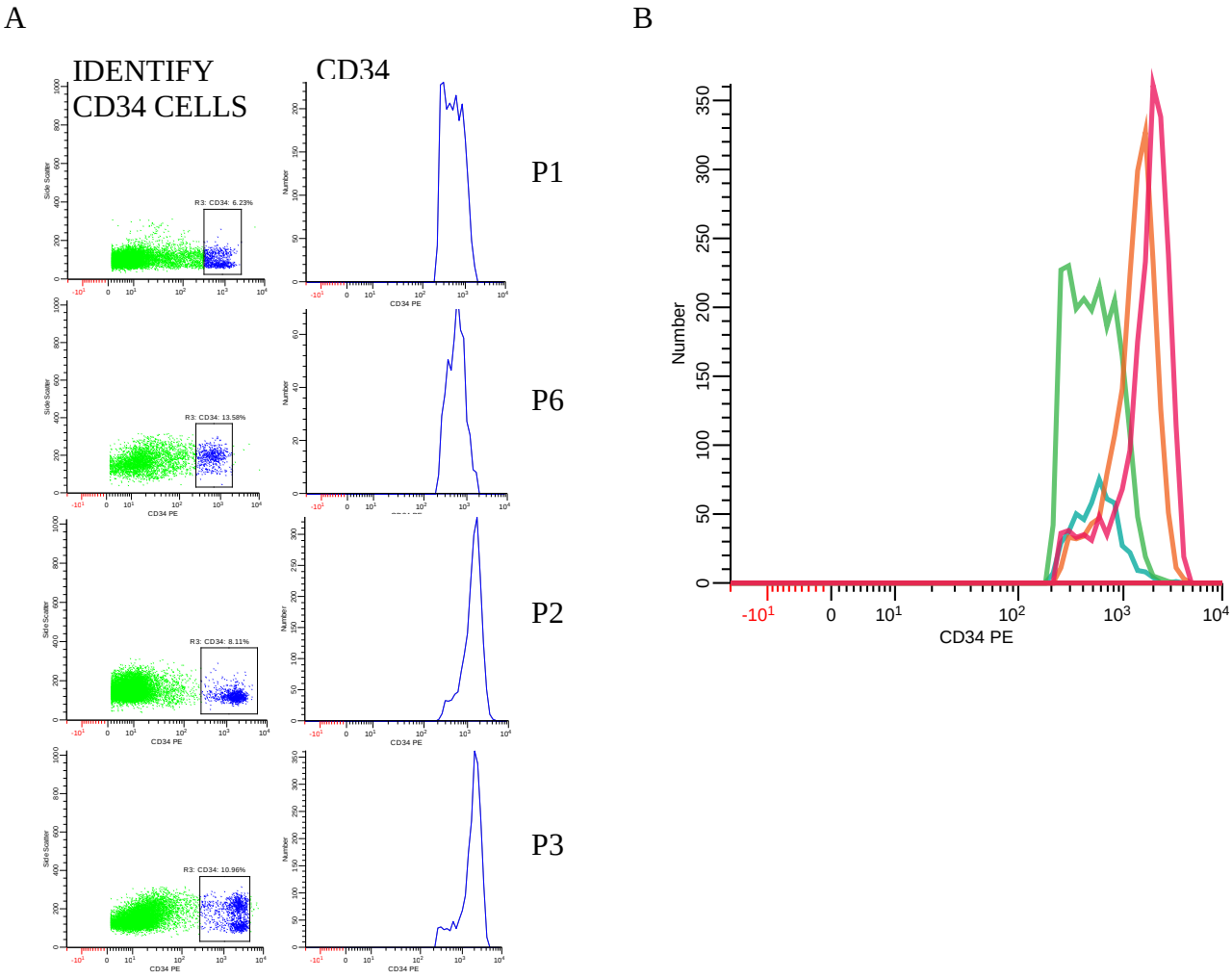


Figure S12: Variability in CD34 expression intensity in cases 1 and 6. (A) Primary flow cytometry data is shown for $CD34^+$ expression in gated mononuclear cells from day 1 of graft processing and prior to transduction. Cases 1 and 6 had the lowest numbers of $CD34^+$cell recovered and the lowest VCN in the series while cases 2 and 3 had the highest numbers in both categories. (B) CD34 mean fluorescence intensity is plotted for case 1 (teal), case 2 (red), case 3 (orange) and case 6 (green). The plot shows log flouresence versus cell number.

Table S1: Busulfan doses and overall exposure by case.

Table S1: Busulfan doses and measured overall exposure by case

Pt No.	Age Mo	1st dose mg/kg	2nd dose mg/kg	Total dose mg/kg	Total AUC mg*hr/L	Adverse Events
1	6	2.59	0.00	2.59	20.0	None
2	3	2.14	2.95	5.09	22.5	None
3	4	2.33	2.39	4.72	20.7	None
4	14	3.08	2.59	5.67	23.0	None
5	3	2.27	2.75	5.02	22.8	Mucositis
6	11	2.59	2.88	5.47	22.9	Mucositis, hair loss
7	2	1.91	0.75	2.66	20.4	None
8	3	2.07	1.56	3.63	22.2	None

The individual 1st and 2nd doses and total dose are shown for each case. The measured cAUC is also shown for each case and was calculated from multiple peripheral blood samples using standard population pharmacokinetic model. Adverse events are listed in cases where such events occurred.