Supplementary Methods

In Vitro Cell Culture. *Methylcellulose Cultures of CD34+ cells.* Samples of transduced and non-transduced CD34+ cells were plated in 0.9% methylcellulose medium (MethoCult GF H4034, Stem Cell Technologies, Vancouver Canada) at 500 and 1000 per ml onto 35 mm plates and incubated for 14 days at 37°C. Plates were scored for Blast-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte/monocyte (CFU-GM), and multi-potent colony-forming unit (CFU-GEMM) by morphology. *Bulk Liquid Culture.* Transduced and non-transduced cell samples were cultured in Iscove's modified Dulbecco's medium (IMDM) with 20% FBS, 2mM L-Glutamine, 100ng/mL each of GM-CSF, IL3, IL6, SCF, Flt-3L, 3 IU/mL of erythropoietin, and 10ng/mL of TPO for up to 4 weeks. Cultures were demi-depleted and fresh medium added 2-3 times weekly. *Stromal co-culture.* A murine stromal cell line (AC6.21) was used to support the growth of B-lymphoid lineages from patient CD34+ cells. Cultures were maintained in IMDM as described but with only SCF and IL-6 cytokines. Samples were taken from the culture weekly for phenotypic analysis, cell sorting, and PCR analysis. *Limiting dilution assay.* Approximately 5 $x10^5$ transduced and non-transduced CD34+ cell products were retained on the day of infusion for limiting dilution analysis. Cells were counted and resuspended in IMDM medium (as described above for bulk liquid cultures). The diluted cells were seeded in 96-well plate to have 50, 10 or 5 cells per well in a volume of 200 µl. The 96-well plates were culture for up to 4 weeks with demi-depletion and replacement of 50-100 µl of fresh medium weekly. All growth positive wells were harvested but only those wells that had grown to >500 cells (as estimated by PCR analysis of a single copy gene Apolipoprotein B - ApoB) were considered evaluable. Wells with >0.75 WPRE/ApoB ratio were considered positive for the transgene.

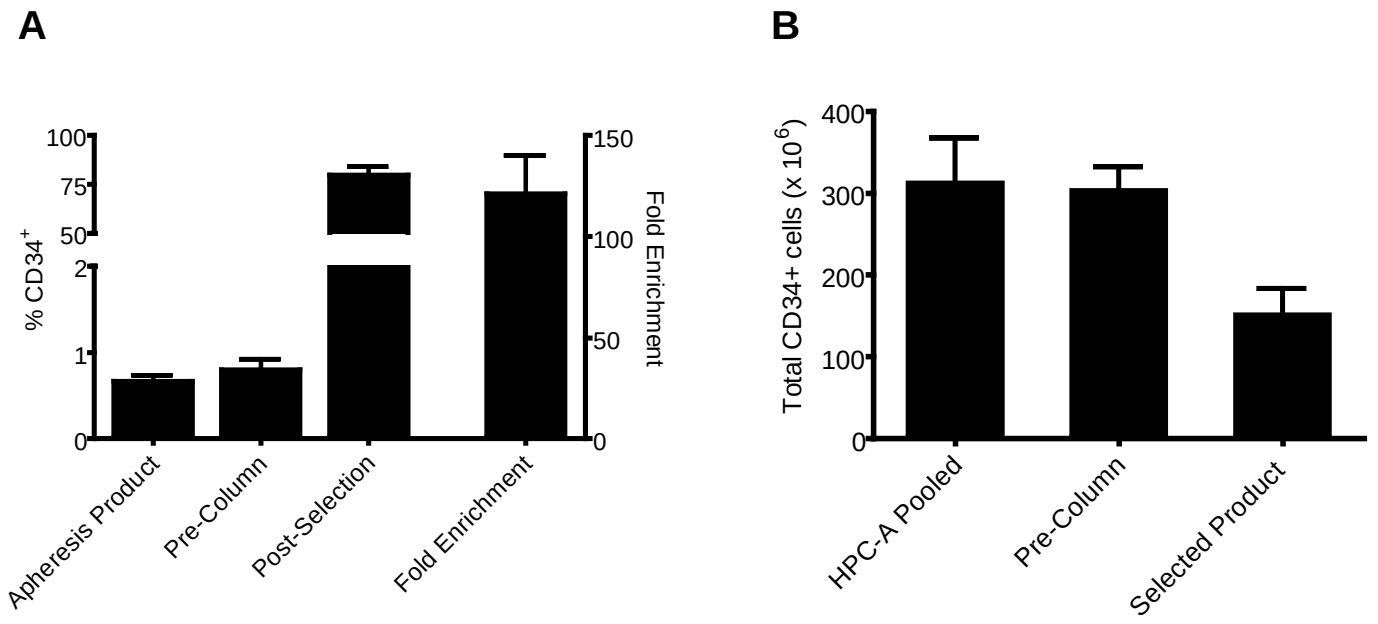
Cell Phenotype and Analysis. Aliquots of cells from bulk culture were taken weekly for phenotypic analysis with antibodies to lineage specific cell surface antigens. Fluorochrome-conjugated antibodies included CD38 FITC, CD34 PE, Glycophorin A PC5, CD33 FITC, CD15 PE, CD14 PC5, CD10 FITC, CD19 PE, and CD3 PC5 (BD Biosciences, San Jose, CA). Cells were washed 3 times in staining buffer (Dulbecco's Phosphate Buffer Saline with 0.1% Bovine serum albumin (BSA)) followed by incubation with fluorochrome-labeled antibody for 20 minutes on ice. After antibody incubation, the cells were washed again 3 times with staining buffer and resuspended in buffer +1μg/mL of propidium iodine (PI) to discriminate live and dead cells. Phenotypic data was collected on an FC500 flow cytometer (Beckman Coulter, Fullerton, CA) and analyzed with FCS Express™ V3 software (De Novo Software, Ontario, Canada). Samples labeled as described were also sorted based on surface marker expression to >98% purity using a MoFlo™ cell sorter (Dako/Cytomation, Ft. Collins, CO) for subsequent analysis of DNA marking and gene expression. Fluorescence activated cell sorting was performed on a Beckman Coulter MoFLo XDP using CD3, CD14, CD19 antibodies as listed above and a purity sort mode.

DNA Analysis of in vitro-derived cells. Samples from in vitro culture were analyzed weekly for the presence of the Woodchuck post-transcriptional regulatory element (WPRE) and a housekeeping gene (Apolipoprotein B – ApoB) by real time quantitative DNA PCR (qPCR). Samples from culture were lysed with PCR lysis buffer at 60°C for 1 hour, 90°C 10 minutes. 2 μl of lysate was used in 20μl of PCR reaction containing SYBR Green (iQ™ SYBR Green, BIO-RAD), 0.5pmole of primer pairs. Primers used for detection of WPRE transgene were: 5'-CCGTTGTCAGGCAACGTG -3' (sense) and 5'-AGC TGACAGGTGGTGGCAAT-3' (antisense). Primers used for detection of house keeping gene Apolipoprotein B (ApoB) as internal control for cell number were: 5'-TGAAGGTGGAGGACATTCCTCTA-3' (sense) and 5'-CTGGAATTGCGATTTCTGGTAA-3' (antisense). DNA isolated from known numbers of human peripheral blood mononuclear cells (PBMC) was used to create standard curves for estimating cell number. A standard curve of pHIV7-shI-TAR-CCR5RZ plasmid DNA spiked into genomic DNA from PBMC was used to quantify the number of integrated copies of vector per cell in each assay.

RNA Analysis. STAT-60 was used to extract total RNA according to the manufacturer's instructions (TEL-TEST'B', Friendswood, TX, USA). Residual DNA was digested using the DNA-free kit follow the manufacturer's instructions (Ambion, CA, USA). cDNA was produced using 1µg of total RNA, Moloney murine leukemia virus reverse transcriptase and random primers (Invitrogen, Carlsbad , CA) in a 20µl reaction according to the manufacturer's instructions. Expression of the CCR5RZ was determined by a real-time PCR using 2XiQ SyberGreen Mastermix (Bio-Rad. Hercules, CA) and primers Forward 5'-CGTGATCCATGCGGTTACGG-3' and Reverse 5'-AACGGCCGCTCTAGAGTGTC-3'. Histone gene expression was used for RNA quality control via real-time PCR with the primers H2A-F2 5'-CAACAAGCTG TTGGTAAA-3', H2A-R 5'-AAGTTCAGCCCTTACTTGC-3'. Expression of the tat/rev coding siRNA was analyzed by RT–PCR using the TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems Foster City, CA) product 4366596) according to the manufacturer's instructions. All reaction components were supplied by the manufacturer with the exception of the input RNA and the tat/rev stem-loop RT primer. Each reaction contained: 50 nM tat/rev stem–loop RT primer, 0.25 mM each dNTP, 3.33 U/µl MultiScribe reverse transcriptase and 0.25 U/µl RNase inhibitor in 1X RT buffer for a final reaction volume of 15 µl. Incubation conditions followed the Applied Biosystems protocol (16 °C for 30 minutes for annealing, incubation at 42 °C for 30 minutes for reverse transcription of the tat/rev siRNA template into the first cDNA strand; a five minute incubation at 85 °C to inactivate the RT enzyme). Real-time PCR was performed after the RT reaction by using the TaqMan® MicroRNA Assay kit (product 4324018). Each real-time PCR reaction contained: 1.3µl of the (unpurified) RT reaction, 1X concentration of TaqMan® Universal PCR Master Mix, 0.2 µM TaqMan probe, 1.5 µM tat/rev forward primer and 0.7 µM tat/rev reverse primer. After an initial denaturation step for (10 minutes at 95 °C), each sample was subjected to 40 PCR cycles consisting of: a denaturation step at 95 °C for 30 seconds (sec); an annealing step at 64 °C for 30 sec; and a synthesis step at 72°C for 30 sec. The primers and probe were as follows: Looped RT primer: 5'-GTCGTATCCAGTGCAGGGCCGAGGTATTCGCACTG ATACGACACAGCG-3'; Forward Primer: 5'-GCTGATGAGCTCTTCGTCG-3'; Reverse Primer:5'-GTGCAGGGTCCGAGGT-3';Probe:5'-6-FAM-TCGCACTGGATACGAC ACAGC GACGA–BHQ1 -3'.

Southern Blotting. RT- PCR products were analyzed by gel electrophoresis in a 1% agarose gel. The DNAs were blotted onto nitrocellulose (Hybond-N, Amersham Biosciences, Piscataway, NJ) by capillary transfer. The transferred samples were fixed to the membrane by UV and were hybridized over night in PerfectHyb solution (Sigma-Aldrich, St. Louis, MA) at 37 °C with a gel purified 5'-32P-labeled oligo probe (probe CTGATGAGTCCGTGTGGACGAA). The10 pmol oligo probe was end-labeled with polynucleotide kinase (PNK)-10 units, New England BioLabs, Salem, Mass.) and γ-32P-ATP in a 20 µl reaction for 1h. Following the labeling the probe was purified in a G-25 Sephadex spin-column (GE Healthcare). The hybridized membranes were washed at room temperature once with 6X SSC/0.1% SDS, then twice with 2X SCC/0.1% SDS, before exposure to film (Biomax MS, Kodak, New Haven, CT)

Measurement of in vivo gene marking. The presence of shI-TAR-CCR5RZ-marked cells in peripheral blood and was assessed by qPCR analysis of WPRE sequences. DNA was isolated directly from peripheral blood using Qiagen DNA Blood Mini Kit (Qiagen, Valencia CA) and quantified by OD_{260}. Q-PCR analyses were performed using an MJ Research DNA engine with a Chromo 4TM Continuous Fluorescence Detector Q-PCR module (Bio-Rad Laboratories, Hercules, CA). Each 20-µl reaction mixture contained 50 ng DNA, 10 µl of IQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and 0.5 pmole each primer. The WPRE sense primer had the sequence 5'-ACTGTGTTTGCTGACGCAAC-3' and an anti-sense primer had the sequence 5'-CAACACCACGGAATTGTCAG-3'. The average % WPRE+ DNA in samples was determined using a standard curve generated from DNA isolated from a clone with a single copy integration of the WPRE-containing lentivirus and used in a standard curve to titrate DNA isolated from patient specimens. A parallel set of qPCR amplifications was performed on of all test and reference samples using the sense primer 5' TCCTCTGAAAGCTGACT GCC 3' and the anti-sense primer 5' CTATTTTGTCCTTGGGCTGC 3' specific for the p21 promoter as an internal control.



FigureS1. ***Purity and recovery of CD34 cells during processing.*** Samples of HPC-A products were taken at 3 times: immediately after collection, just prior to column separation (after washing and staining) and following selection. Samples were analyzed for total cell number and CD34 content. (**A**) Percent CD34+ cells (left Y axis) and fold enrichment of CD34+ cells (right Y axis) are shown. (**B**) Total CD34+ cells in various fractions prior to and after selection. Means and Standard Deviations are shown; N=5.

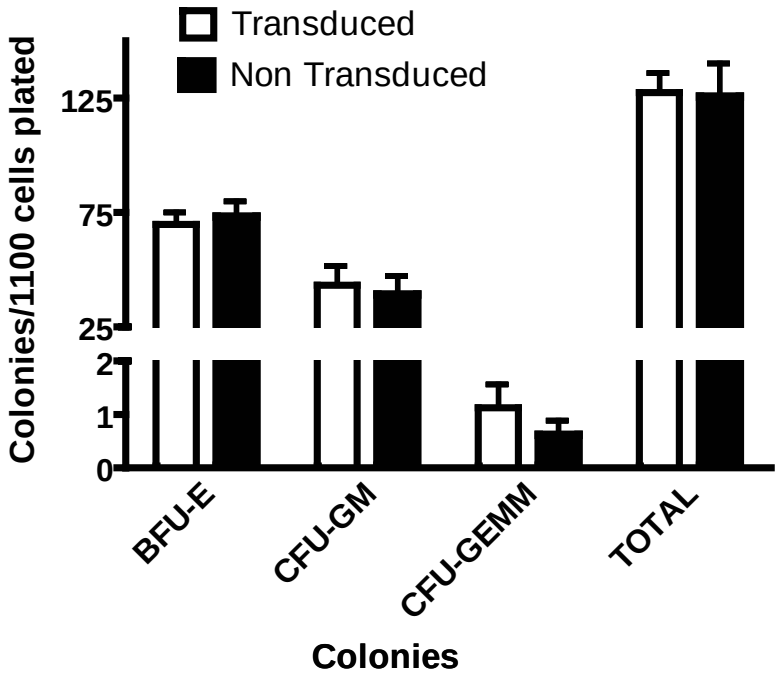


Figure S2. ***Comparison of Colony forming potential in transduced and non-transduced CD34+ cells.*** Samples of CD34+ cells were taken from each patient product following transduction and plated in methycellulose culture (1100 cells per 35 mm plate in triplicate) in the presence of erythropoietin, GM-CSF, Thrombopoietin, and IL-3. The average numbers (+ standard deviation) of Burst forming units – erythroid (BFU-E), Granulocyte/Macrophage (CFU-GM), multipotent progenitors (CFU-GEMM) and total colonies are shown. Open columns – transduced cells, filled columns – control (non transduced) cells; N=5.

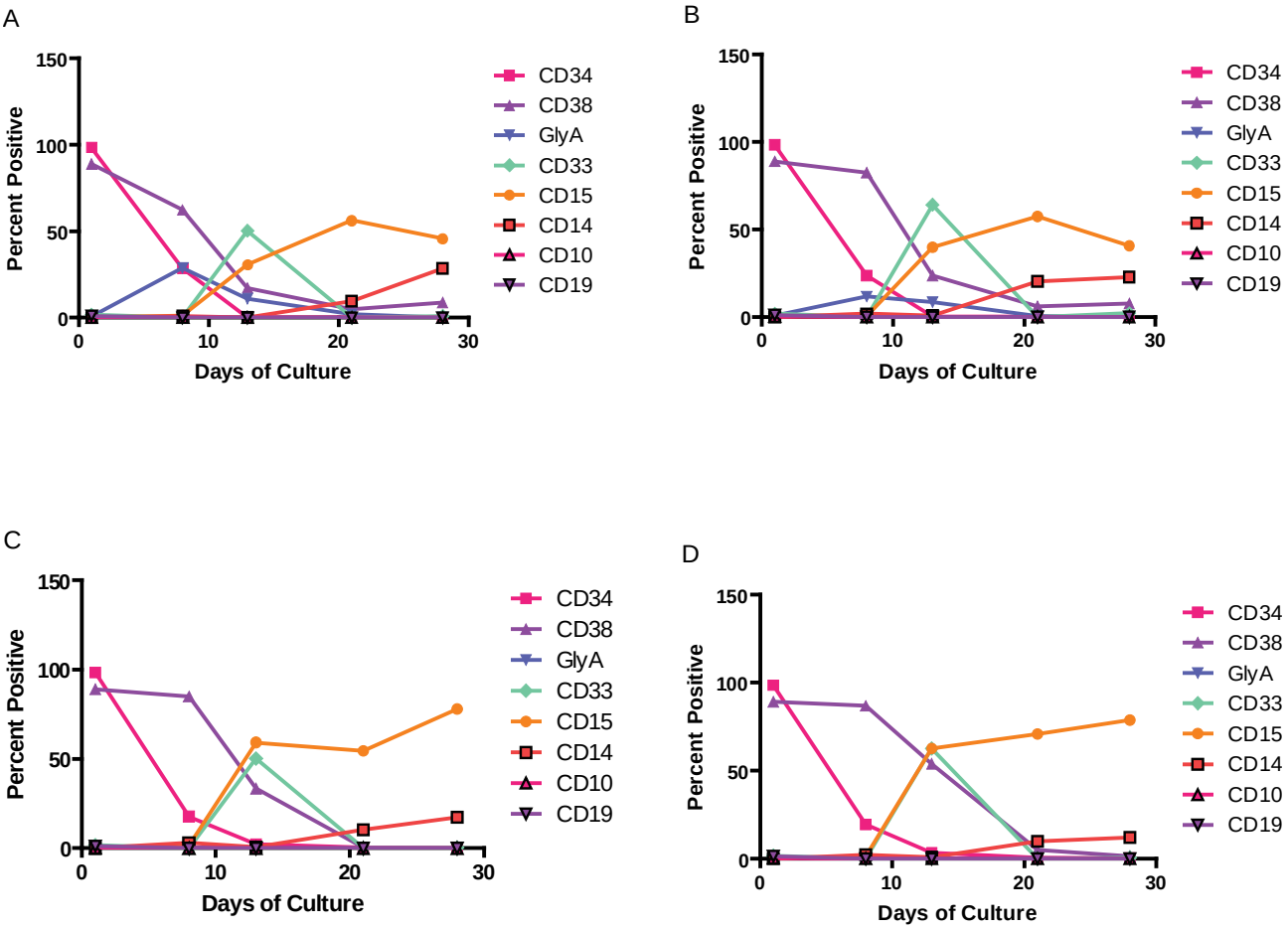


Figure S3. **Kinetics of Lineage Development following lentiviral transduction.** Samples of transduced cells from UPN0305 were cultured in liquid media plus growth promoting cytokines (**A** and **B**) or on a stromal cell line with cytokines (**C** and **D**) for 4 week as described in methods. Flow cytometric analysis of culture phenotype was performed weekly using antibodies to lineage specific antigens. No differences in the frequency or kinetics of production of specific lineages were observed between untransduced (**A** and **C** respectively) and transduced (**B** and **D** respectively) samples.

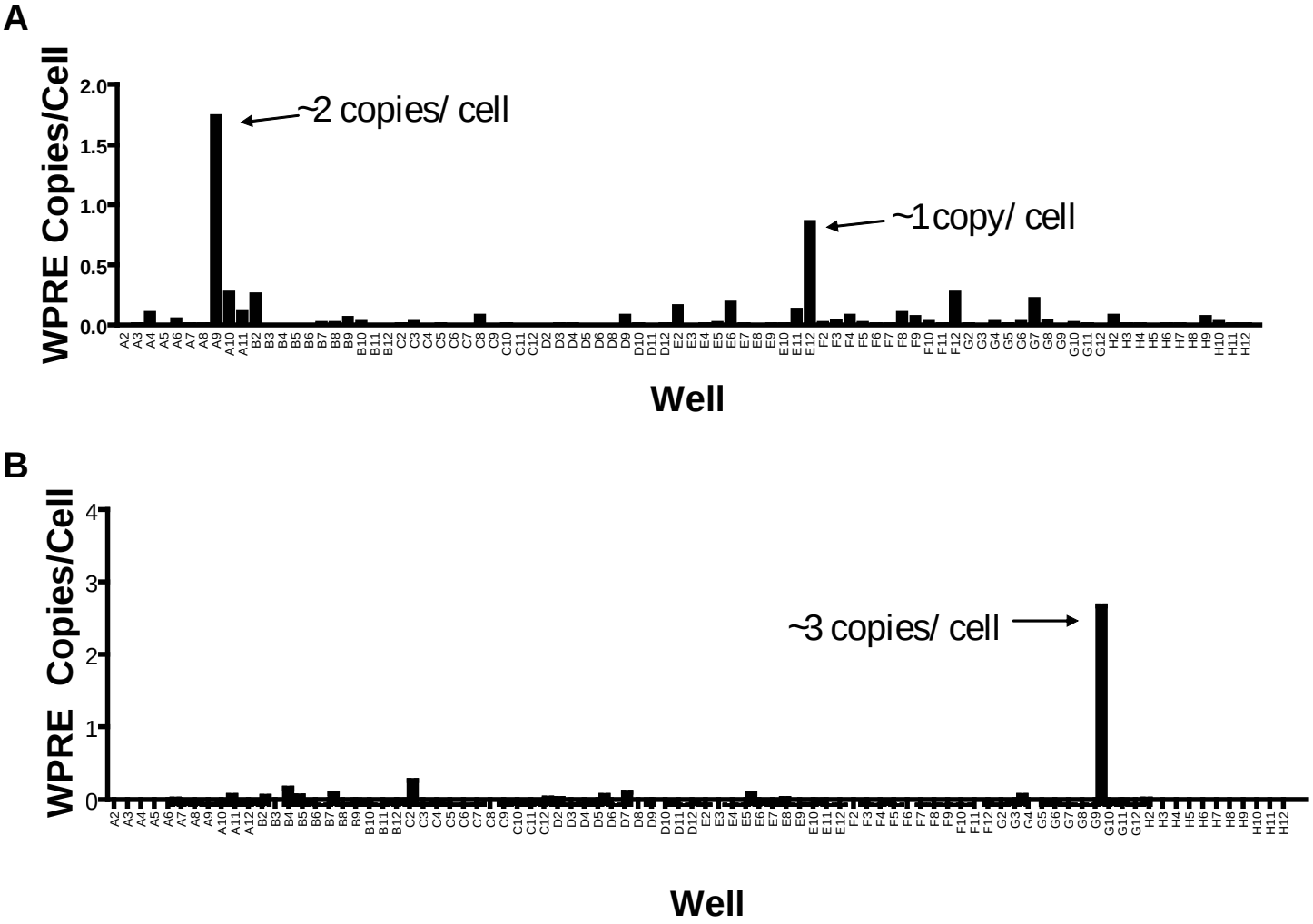


Figure S4. ***Analysis of copies/cell of integrated HIV7-shI-TAR-CCR5RZ in clones derived from patient samples.*** Samples from each patient were placed in liquid culture at 5, 10 or 50 cells well in 96 well plates and grown for 4 weeks. Individual positive wells were harvested and analyzed by DNA qPCR for copies of integrated vector per cell suing primers for the WPRE sequence as described in Methods. Amount of vector DNA was determined against a standard curve with known amounts of pHIV7-shI-TAR-CCR5RZ plasmid spiked into PBMC. Number of cells/sample was determined by qPCR for ApoB against a standard curve of known numbers of cells. The ratio of WPRE/ApoB was used to determine copies/cell. Only those wells with >500 cell equivalents (as estimated by qPCR) were included in analysis and only those samples with a WPRE/ApoB ratio of >0.75 were considered positive for vector marking. (**A**) Samples from patients UPN301 and (**B**) UPN305 are shown.

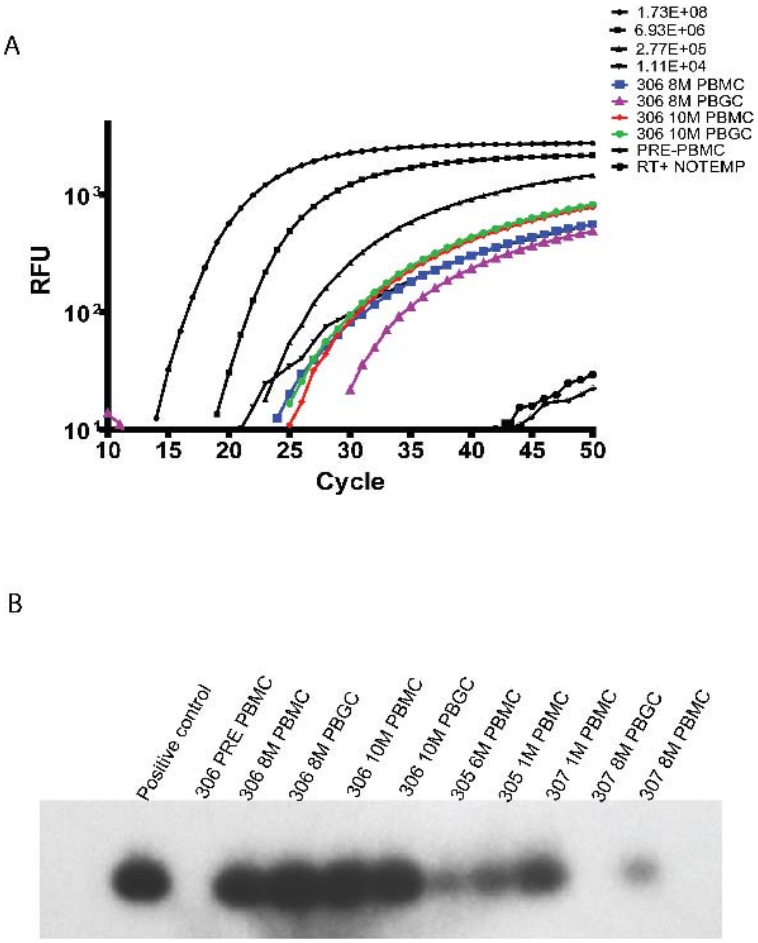


Figure S5. **Analysis of RNA in the peripheral blood of patients following transplantation.** (**A**). RNA was isolated from peripheral blood mononuclear and granulocytic cells (PBMC and PBGC respectively) of patient UPN0306 at 8 and 10 months post transplant and analyzed for expression of siRNA and CCR5 ribozyme (colored lines) using TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Control RNA transcripts of 1.7 $x10^8$, 6.9 x 10^6, 2.7x 10^5 and 1.1 x 10^4 were assayed to establish an RNA standard curve. PBMC samples taken before infusion (Pre-PBMC) and reaction mix with no template (RT+ No Temp) were included as negative controls. (**B**). CCR5 ribozyme RNA expression in the post transplant peripheral blood of patients UPN0305 (1 and 6 months), UPN0306 (8 and 10 months), UPN0307 (1 and 8 months). Cell samples were fractionated into PBMC and PBGC and RNA from each fraction was isolated and amplified by RT-PCR. PCR products were separated on a 1% agarose gel and blotted to nylon membranes and probed with a radiolabeled CCR5-specific probe as described in methods. RNA from a cell line expressing CCR5 was used as a positive control while pre-infusion RNA from UPN0306 was used as a negative control.

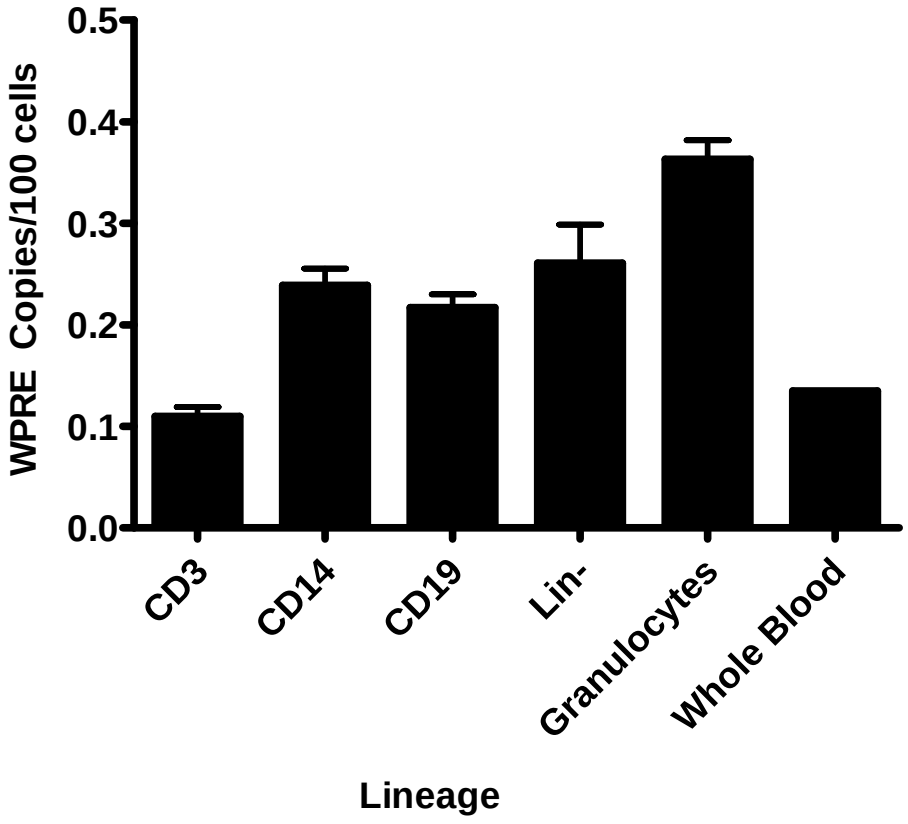


Figure S6. Gene **marking of peripheral blood subsets in UPN0306 at 18 months post infusion.** Peripheral blood cells were harvested and separated into granulocytic and monocytic fractions by density separation and then the mononuclear cell fraction was further divided into lineage specific subsets by flow cytometric cell sorting. Samples of 20,000 cell equivalents of genomic DNA from whole blood and each fraction were analyzed by qPCR for the presence of WPRE gene sequences. WPRE was normalized to cell number as determined by qPCR for a housekeeping gene (ApoB). The number of WPRE copies/100 cells is shown.

UPN	Tests	Screening	Infusion & Follow up										
			Day 0	1M	2M	3M	4M	6M	8M	10M	12M	18M	24M
304	HIV-1 RNA copies/ml	<400*	<400*	<400*	<400*	<400*	<400*	N/A	<48**	<48**	<48** 890***	<48**	<48**
	CD4 cells/μl	366	18	N/A	260	250	233	217	224	308	235	442	577
	CD8 cells/μl	1638	22	N/A	4468	2718	2173	1256	1857	2054	1467	1038	1050
305	HIV-1 RNA copies/ml	<400*	N/A	<400*	<400*	<400*	<400*	<48**	<48**	<48**	<48**	<48**	<48**
	CD4 cells/μl	206	16	99	55	108	75	66	73	N/A	65	90	129
	CD8 cells/μl	178	8	133	97	432	367	381	364	N/A	272	78	142
306	HIV-1 RNA copies/ml	<400*	251**	<48**	<48**	<48**	<48**	<48**	<48**	<48**	<48**	<48**	Pending
	CD4 cells/μl	18	9	304	168	115	128	185	216	418	371	468	Pending
	CD8 cells/μl	70	22	1652	925	910	964	1025	1023	685	791	671	Pending
307	HIV-1 RNA copies/ml	25119**	<48**	<48**	<48**	<48**	N/A	<48**	<400*	<48**	<48**	<48**	Pending
	CD4 cells/μl	577	80	321	399	226	269	301	577	515	614	953	Pending
	CD8 cells/μl	913	80	519	916	452	460	699	913	750	411	238	Pending

Table S1. HIV-CD4/CD8 Observations

HIV gc/ml = genomic copies of HIV virus/ml of blood, CD4/μl = CD4 cells/μl of blood, CD8/μl = CD8 cells/μl of blood
NA = Not available. * Month 15 HIV =970 gc/ml following 3-month drug treatment interruption.