Supplemental Materials and Methods

Retroviral Vector Supernatant Production and Characterization: Both vectors were packaged using the PG13 packaging cell line which confers the Gibbon Ape Leukemia Virus (GALV) pseudotype. High titer clones with intact vector proviruses were identified and used to produce Master Cell Banks at NIH (GCsapM-ADA) and Baylor College of Medicine (MND-ADA). Clinical lots of vector supernatant were collected in X-VIVO 10 medium with 1% human serum albumin either at the National Human Genome Research Institute, NIH or at the Indiana University Vector Production Facility. Aliquots of the unprocessed supernatant were cryopreserved at -80°C and demonstrated to be free of microbiological contaminants, adventitious viruses, and replication-competent retrovirus (RCR), following FDA guidelines. Vector titers of GCsapM-ADA and MND-ADA were approximately $1.6 \times 10^6$/mL and $5.0 \times 10^5$/mL, respectively. Identities of each vector were determined by sequence analysis of 250-300 bp segments of vector proviral integrants in transduced cells and potencies of the vector supernatants were determined annually by measuring conferred ADA enzyme activity in transduced cells.

CD34+ cell isolation, ex vivo transduction and characterization: Bone marrow (10-15 ml/kg) was harvested from subjects under general anesthesia from the posterior iliac crests and collected in Plasma-Lyte and heparin (10 IU/ml final concentration) +/- sodium citrate (1:8 vol/vol). For subjects receiving busulfan, a fraction of the marrow containing $5 \times 10^7$ total nucleated cells/kg of subject body weight was cryopreserved as safety back-up without additional manipulation. Mononuclear cells were enriched from the remaining bone marrow by centrifugation and/or Ficoll-Hypaque gradient separation and CD34+ cells were then selected using the Isolex 300i (Baxter Therapeutics Inc., Irvine, CA). The CD34+ cell enrichment procedures yielded preparations of CD34+ cells that were 74.5-98.5% pure from starting bone marrow harvests that contained between 0.26-6.92% CD34+ cells.
For ex vivo transduction, CD34+ cells were divided into two aliquots, seeded into cell culture bags or flasks that had been coated with recombinant fibronectin fragment CH-296 (2µg/cm2) (Takara Shuzo, Inc, Otsu Japan), pre-cultured for 40-48 hours at 37°C in X-VIVO 15 serum-free medium (Lonza, Walkersville, MD) containing ckit ligand (50ng/ml) (SCF, Amgen Inc, Thousand Oaks, CA), Flt-3 ligand (300ng/ml) (Immunex Corp, Seattle WA) and Megakaryocyte Growth and Differentiation Factor (50ng/ml) (MGDF, Amgen, Inc) and 1%HSA. Pre-cultured CD34+ cells were then transduced with either the GCsapM-ADA or MND-ADA retroviral vectors (for subject 201C, only the GCsapM-ADA vector was used). Every 24 hours for three cycles, cells were pelleted by centrifugation, resuspended in an equal mixture of freshly thawed retroviral supernatant and 2X cytokine-containing culture medium, and returned to the original culture bags or flasks. At the end of the 72 hour transduction period, cells were harvested and washed x3 with Plasma-Lyte 1%HSA and suspended in 10-25ml Plasma-Lyte1%HSA as the Final Cell Product.

Release criteria for the Final Cell Product for clinical infusion included: viability >70%, negative in-process bacterial and fungal stains and cultures, and endotoxin <5 EU/kg by Limulus Amebocyte Lysate Kit (Associates of Cape Cod, Inc., East Falmouth, MA) or using the Endosafe®-PTS Kit (Charles River Laboratories, Charleston, SC). Additional tests performed on portions of the Final Cell Product to complete the Certificate of Analyses included mycoplasma test (agar cultivable and non-agar cultivable, BioReliance, Rockville, MD), sterility, measurement of ADA enzyme activity, and quantification of vector copies per cell using qPCR (Figure 2). For all ten enrolled subjects, the Final Cell Products met release criteria and were administered without incident.

Busulfan administration, serum levels, and area-under-the-curve (AUC) calculation. Subjects who received pre-transplant busulfan were given phenytoin (15 mg/kg IV loading dose the
night before busulfan was administered and 5 mg/kg IV q12 hr x 3 doses) as anti-seizure prophylaxis and ondansetron (0.15 mg/kg IV) as anti-nausea prophylaxis. Busulfan was given as an intravenous infusion of 32.5-45 mg/m² over 2 hours on two successive days (65-90 mg/m² total). Blood samples were taken immediately at the end of the first busulfan infusion and 0.25, 1, 2.5, and 4 hours later for measurement of serum busulfan levels by HPLC (Childrens Hospital Los Angeles Clinical Special Chemistry Laboratory or Mayo Clinic). The busulfan levels were used to calculate the area-under-the-curve (AUC) by trapezoidal estimation.

**ADA enzyme assay:** Cells (2-5 x 10⁵ PBMC or CD34+ cells) were washed with Hank’s Balanced Salt Solution (HBSS), pelleted and frozen at -80°C until use. Pellets were thawed at 37°C and resuspended at 5x10⁶/ml in M-Per Mammalian Protein Extraction Reagent (Pierce, Rockford, IL), vortexed and left on a cell shaker for 30-40 min. Lysates were then cleared of cellular debris by centrifugation and 10 µl aliquots were transferred to 0.5 ml tubes and incubated with 10 µl of 1 mM ¹⁴C-adenosine (50 mCi/ml) at 37°C for 5-40 min. The products of the enzymatic reactions were then separated by thin layer chromatography and the conversion of adenosine into inosine determined by using a phosphoimager (Fuji Medical Systems, Stamford, CT) and expressed as units of ADA (1 unit = 1 nmol of adenosine deaminated per 10⁸ cells per min).

**VCN primers and probe sequences, reaction concentrations, and standard curves:** A common sense primer (5' - tca atg cgg cca aat cta gtt) and a common TAMRA probe (6FAM - tcg acc tgc tct ata aag cct atg gga tgc - TAMRA) were used to detect both MND-ADA and GCsapM-ADA. The anti-sense primer for MND-ADA (5' - tgg act aat cga tac cgt cga c - 3') and the anti-sense primer for GCsapM-ADA (5' - gcc ttg caa atg gcg tta c - 3') were specific to their respective vectors. The primer concentrations used to detect MND-ADA (sense and antisense) were 400 nM and the primer concentrations used to detect GCsapM-ADA (sense and antisense) were 600 nM. The common TAMRA probe concentration was 50 nM in all reactions. All reactions
utilized Universal Master Mix (Applied Biosystems, Inc. (ABI), Fullerton, CA) and were run under default conditions in the 7900 or 7500 Sequence Detector System (ABI). Test results were compared to 350 ng of DNA from a set of copy number standards. The standards were produced using vector-transduced HT29 cell clones in which integrated proviral copies were detected and quantified by Southern blot. MND-ADA/HT29 Clone #5 contains two copies of the MND-ADA provirus and GCsapM-ADA/HT29 Clone #5 contains four copies of the GCsapM-ADA provirus. DNA extracted from either clone was serially diluted into DNA of unmodified HT29 cells for an assay sensitivity of 1:100,000 cells or 0.00002 copies/cell for MND-ADA and 0.00004 copies/cell for GCsapM-ADA.

*Immunological Monitoring:* Immunological assays for lymphocyte subset numbers by immunophenotype, lymphocyte proliferative responses to mitogens and serum immunoglobulin levels were performed in the Clinical Immunology Laboratories at CHLA, the Department of Laboratory Medicine of the Clinical Center, NIH, the Laboratory of Cell-Mediated Immunity of National Cancer Institute, SAIC-Frederick, and the University of California, Los Angeles (UCLA). T-cell receptor (TCR) spectratyping was performed as described² using RNA extracted from cryopreserved PBMC samples. Vector-genomic DNA integration sites were amplified from cryopreserved PBMC DNA using LAM-PCR at the National Gene Vector Biorepository and analyzed by agarose gel electrophoresis and UV illumination. TREC analysis was performed as described³ with the plasmid standard kindly provided by Dr. Dan Douek. Reference range was based on values from Krogstad, 2002.⁴

*Measurement of serum levels of human interleukin-7 (IL-7):* Serum IL-7 levels were measured by ELISA (Quantikine® HS Human IL-7 Kit, R&D Systems, Minneapolis, MN). Serum samples were collected at the specified time points, frozen in aliquots and stored at -80°C until analysis at which time sera were thawed and assayed according to the manufacturer's instructions.
Establishment and analysis of T-cell clones from peripheral blood: Feeder cells for the single T-cell clones were derived from the mononuclear cells of two healthy donors and one control human B lymphoblastoid cell line. These cells were treated with 50 ug/ml of Mitomycin-C (Sigma, St. Louis, MO) for 3 hours at 37° C, washed 4 times and cultured overnight in X-VIVO 15 medium + 10% FCS (Hyclone, Logan, UT). The feeder cells (40% Donor 1, 40% Donor 2 and 20% B-cell line) were then resuspended at 2.5–5x10^5 cells/ml in Yssel’s media (Gemini BioProducts, West Sacramento, CA) containing 1% human AB serum or A plasma, 10% FCS, 1 ug/ml phytohemagglutinin (PHA) (Sigma), 10 ng/ml IL-2, IL-4 and IL-7 (Peprotech, Rocky Hill, NJ) (YP247 medium).

Subject PBMC were separated using Ficoll/Hypaque gradients, washed, counted and resuspended at 10^4 cells/ml in YP247 medium. Appropriate dilutions of the cells were made using the feeder cells as diluent to establish 96-well plates containing 33, 10, 3.3, 1, 0.33, and 0.1 PBMC/well. Plates were fed twice a week with YP247 medium, and wells were harvested as growth became apparent. Plates with fewer than 30 wells positive for cell growth were considered to contain single-cell clones.

Cells from each clone were assayed for ADA enzymatic activity and used for DNA extraction using the Puregene DNA kit (Qiagen, Valencia, CA). PCR analysis was performed to determine the presence of MND-ADA and GCsapM-ADA vector sequences using a common forward primer for both vectors (GCG GCC AAA TCT AGT TTC CTC) and reverse primers specific for the MND-ADA (TGG ACT AAT CGA TAC CGT CGA C) or GCsapM-ADA (GCC TTG CAA ATG GCC TTA C) constructs. Cycling conditions were: 95° C for 2 min, followed by 40 cycles of 95° C for 30 sec, 60° C for 30 sec, 72° C for 45 sec. PCR products were then analyzed by agarose gel electrophoresis.
Vector Integration Site Analyses: Genomic DNA was extracted from cryopreserved PBMC. 100-1000 ng of DNA, dependent on availability, was used to perform non-restrictive linear amplification PCR. Briefly, 100 cycles of linear amplification were performed using Accuprime Taq with primers MNDright-linear (Biotin- TGTTTGCATCCGAATCGTGGACT) and GCSAPright-linear (Biotin- TGTGTTGCATCCGAATCGTGGACT). Linear reactions were purified using 1.5 volumes of AMPure XP beads (Beckman Genomics) and captured onto M-280 Streptavidin Dynabeads (Invitrogen Dynal). Captured ssDNA was ligated to read 2 linker (Phos-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC-3C Spacer) using CircLigase II (Epicentre, Madison WI) in a 10 microliter reaction at 65° for 2 hours. PCR was performed on these beads using primers MNDright-PCR (AATGATACGGCGACCACCAGATCTACACTCTCGCTGATCCTTGGGAGG) and GCSAPright-PCR (AATGATACGGCGACCACCAGATCTACACTCTCGCTGATCCTTGGGAGG) and an appropriate indexed reverse primer (CAAGCAGAAGACGGCATACGAGAT-index-GTGACTGGAGTTTACGACGTGT). PCR products were mixed and quantified by probe-based qPCR and appropriate amounts were used to load Illumina v3 flow cells. Paired-end 50bp sequencing was performed on an Illumina HiSeq 2000 instrument using mixed custom read 1 primers (AGATTGATTTGACTGCCCACCTCGGGGGGTCTTTCA and TGAGTGTGACTACCACGACGGGGGTCTTTCA). Reads were aligned to the hg19 build of the human genome with Bowtie and alignments were condensed and annotated using custom Perl and Python scripts to locate vector integrations.
SUPPLEMENTAL REFERENCES


Supplemental Table 1. Busulfan Dosing and Area-Under-the-Curve (AUC).

Supplemental Table 2. Analysis of T-Cell Clones from Peripheral Blood for Vector Provirus.
Supplemental Table 1. Busulfan Dosing and Area-Under-the-Curve (AUC).

<table>
<thead>
<tr>
<th>PIN*</th>
<th>Weight (kg)</th>
<th>Busulfan Dosage</th>
<th>Busulfan AUC* (μM*min)</th>
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<tr>
<td></td>
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<td>mg/m²</td>
<td>mg/kg</td>
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<tr>
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<tr>
<td>306N</td>
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*Patient Identification Number

*AUC: Area-Under-the-Curve calculated from plasma busulfan levels measured after administration
## Supplemental Table 2. Analysis of T Cell Clones from Peripheral Blood for Vector Provirus.

<table>
<thead>
<tr>
<th>PIN*</th>
<th>Month After Transplant**</th>
<th>Vector Detected***</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>MND-ADA</td>
<td>GCsap-M-ADA</td>
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<td>4</td>
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</table>

*Patient Identification Number

**T cell clones were isolated from the listed subjects at the indicated times after transplant.

***The clones were analyzed by vector-specific PCR and scored for the presence of MND-ADA or GCsap-M-ADA. The few clones where both vectors were present may have represented bi-clonal populations.
Supplemental Legends for Supplemental Figures

Supplemental Figure 1. Vector Copy Numbers for the 300 Series Subjects. Results from qPCR measurement of vector copy number in PBMC (left column) and granulocytes (right column) are shown, with blue diamonds indicating MND-ADA and red squares indicating GCsap-M-ADA proviral marking. Data was not generated for subject 302C.

Supplemental Figure 2. ADA Enzyme Activity of T Cell Clones. The ADA enzyme activity of the isolated T cell clones was measured. The average ADA enzyme activity for the clones from each of the subjects identified by PCR to have either the MND-ADA or GCsapM-ADA vectors is shown. Error bars indicate the standard deviations.

Supplemental Figure 3. Vector Integration Site Analysis. The distribution of unique vector integration sites was determined in transduced bone marrow CD34+ cells prior to transplant (in vitro) and in peripheral blood mononuclear cells from subjects 6-24 months after gene transfer (in vivo). A. The integration site were mapped relative to transcriptional start sites (TSS) in vitro (n=828 unique sites – gray bars) and in vivo (n=182 – black bars). B. The percentages of unique integration sites near cancer-related genes were determined in vitro (n= 1,740 unique sites) or in vivo (n=321). Integration sites in genes or within 300 kb upstream were considered “near” and cancer-related genes were defined as in Higgins et al (Nucleic Acids Research, 35 (Database issue), D721–6., 2007).

Supplemental Figure 4. Measurements of Lymphocyte Numbers and Function. The values obtained from serial measurements of T, B and NK cell populations, and serum IgA and IgM from the evaluable subjects of the 200 and 300 series are shown.

Supplemental Figure 5. Spectratype Analysis of T-Cell Receptor Vβ Family Usage.
Supplemental Figure 1

PBMC

- 301N
- 303N
- 304C
- 305C
- 306N

Granulocytes

- 301N
- 303N
- 304C
- 305C
- 306N

Vector Copy/Cell

- 301N
- 303N
- 304C
- 305C
- 306N

Months After Transplant
Supplemental Figure 3

A

% Unique Integrants

-10kb to -5kb
-5kb to TSS
TSS to +5kb
+5kb to +10kb

B

% Near Cancer-Associated Genes

In Vitro (n = 1740)
In Vitro (n = 321)