Title: Lentiviral Hematopoietic Stem Cell Gene Therapy for X-linked Severe Combined Immunodeficiency

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Abstract

SCID-X1 is a profound deficiency of T-, B- and NK- cell immunity caused by mutations in *IL2RG* encoding the common chain (γc) of several interleukin receptors. Gamma-retroviral (γRV) gene therapy of SCID-X1 infants without conditioning restores T-cell immunity without B- or NK-cell correction, but similar treatment fails in older SCID-X1 children. Here, we employed a novel lentivirus gene therapy approach to treat 5 SCID-X1 patients with persistent immune dysfunction despite haploidentical-hematopoietic stem cell transplant (HSCT) in infancy. Follow-up data from 2 older patients demonstrates that lentivirus vector γc transduced autologous HSC gene therapy following non-myeloablative busulfan conditioning achieves selective expansion of gene marked T-, NK- and B-cells that is associated with sustained restoration of humoral responses to immunization and clinical improvement at 2-3 years after treatment. Similar gene marking levels have been achieved in 3 younger patients, albeit with only 6-9 months of follow-up. Lentiviral-gene therapy with reduced-intensity conditioning appears safe and can restore humoral immune function

to post-haploidentical transplant older patients with SCID-X1. (163 words)

One Sentence Summary: Lentiviral gene therapy with conditioning achieves multi-lineage marking and humoral reconstitution in older SCID-X1 patients post haploidentical HSCT.

Introduction:

Haploidentical hematopoietic stem cell transplantation (HSCT) without conditioning for treatment of SCID-X1 infants achieves ≥70% long-term survival. However, while donor T-cells engraft and are functional, ~2/3 of such patients lack B- and NK-cell reconstitution, which may ultimately lead to progressive clinical deterioration (*1-3*). Gamma-retroviral (γRV) gene therapy without conditioning effectively corrects the T-cell lineage with no transduced B- or NK- cells in SCID-X1 infants (*4*), but fails in post-haploidentical HSCT older children, possibly due to age-
related thymic damage (*5, 6*).

Leukemias occurred in γRV-gene therapy for SCID-X1, Wiskott-Aldrich syndrome (WAS) and Chronic Granulomatous Disease (CGD) attributable to preferential integration near oncogenes *(7-10)*. Adding a self-inactivating element (SIN) in γRV gene therapy for SCID-X1 infants resulted in a similar integration pattern as earlier γRV trials although less clustering near oncogenes is observed at 38 months follow-up (*11*). Unlike murine gRV, lentivirus vectors (LV) do not preferentially integrate near enhancers and promoters and successful SIN-LV gene therapy employing marrow conditioning of WAS and Metachromatic Leukodystrophy (MLD) (*12, 13*) suggests that SIN-LV may be applicable to SCID-X1.

In this study, we used a codon-optimized SIN-LV (Cl20-i4-EF1a HγcOPT) where the elongation factor 1α (EF1α) core promoter element drives production of γc with an additional

safety feature of a 400-bp chicken β-globin chromatin insulator element (cHS4) (Fig. S10) (*14, 15*). Pre-clinical studies demonstrated safety and efficacy in animal models (*16, 17*). We report successful SIN-LV gene therapy of older SCID-X1 patients who had significant immune and functional problems following previous haploidentical HSCT. Novel features of our clinical trial include first use of SIN-LV to treat SCID-X1, first use of busulfan conditioning for gene therapy of SCID-X1, and first use in patients of SIN-LV manufactured from a stable LV producer cell line (*18*). Reduced intensity conditioning has proven beneficial in gene therapy for adenosine deaminase deficient(ADA)-SCID including the development of gene-marked B- and NK-cells (*19, 20*) .

Results:

Patient characteristics

Five male patients with SCID-X1, aged 23, 22, 7, 16 and 10 years (P1-P5), with persistent disease following one or more haploidentical HSCT (Table 1) were treated in a phase I/II clinical trial. This report describes the course of P1 and P2 through 36 months and 24 months and P3-P5 through 9, 6, and 6 months following autologous SIN-LV (EF1a-hgcOPT)-transduced CD34+ HSC transplant, with a total of 6mg busulfan /kg *i.v.* pre-conditioning. Busulfan levels were drawn on day 1 after the first 3mg busulfan /kg dose, and ranged from 2519.6 to 4528.9 min*umol/L (Table 1). Results of the AUC levels were not available in time to allow dose adjustment. The patients recovered their absolute cell numbers without need of blood product support for discharge within one month (Fig. S2) and monitored per Protocol Schedule (Table S1).

Self-inactivating Lentivector from Stable Producer and HSC Transduction

The lentivector (LV) used in the study is self-inactivating (SIN) and utilizes an EF1α promotor to drive a codon-optimized human γc cDNA, flanked by a 400bp chicken insulator (*14, 15*). Unlike γRV that is produced by stable cell lines, LV used for clinical gene therapy trials thus far rely on transient, four-plasmid transfections of packaging cells. The quantity of high-titer clinical grade vector produced using this approach is generally insufficient to treat larger (adult) subjects. In contrast, the stable SIN- EF1a-hγcOPT producer can make up to 150 liters of vector at 5×10^6 Infectious Units (i.u.)/ml for each production run. The downstream process and quality controls resulted in vector at $4\text{-}7 \times 10^8$ i.u. /ml as previously described (*15*).

CD34+ HSCs were mobilized with G-CSF and pleraxifor (*21*) and were collected by apheresis and cryopreserved. Autologous HSCs ($1\text{-}2 \times 10^7$ CD34+ HSC/kg) were pre-stimulated with stem cell factor, FLT-3L, and thrombopoietin (100ng/ml) for 16 hours before two daily 7-hour transductions at a MOI of 100-150 with vector concentration ~30% of culture volume (Fig. S1). On day 3, transduced cells were washed and infused following confirmation of quality and safety criteria. Between 17.0 to 57.7% of colonies derived from the bulk transduced cells were gene marked (Table 1).

Multi-lineage Gene Marking and Immune Reconstitution following gene therapy

Peripheral blood cells from patients were purified by density fractionation and magnetic bead-based immunoselection into lineages including polymorphonuclear cells (PMN), CD14, CD3, CD19, and NK cells (see (SM&M)). Gene marking was monitored over 2-3 years in P1 and P2 and demonstrated an increase in gene marking for all lineages tested (Fig.1A). Myeloid marking in PMN and CD14+ cells appeared by 2 weeks and stabilized by 6 months at 0.08-0.1 vector copy number (VCN) per cell in both P1 and P2. Vector marking of B- and NK-cells appeared later but the levels exceeded that of myeloid cells at 0.30-0.40 and 0.56-0.77 VCN, respectively. T-cell marking lagged until 5-6 months post-treatment but steadily increased to 0.13-0.57 VCN. CD14+ and B-cell marking occurs early in all five patients, particularly in P4 and P5 who received relatively larger doses of corrected HSC (Fig. 1B). Importantly, gene

marking was also evident in circulating peripheral blood CD34+ HSC from P1 and P2 at 1 year and at 4 months, respectively, approaching the myeloid levels (Fig. 1A).

Emergence of Gene-corrected autologous T-cells

To preserve donor T-cells, patients did not receive T-cell depleting agents. Autologous T-cells, identified by microsatellite DNA fingerprinting chimerism analysis, increased about 10 months post-treatment (Fig. 1C) corresponding to the increase in T-cell vector marking and T-cell numbers in P1 (Fig. 1D). In P2, increased gene marking in T-cells with concomitant increased autologous T-cell chimerism without significant changes in his total number of T-cells likely reflected a replacement of donor T-cells by the autologous gene-corrected T-cells (Fig. 1D). A possible explanation for the relative survival advantage of autologous gene-corrected T-cells may be better cross-talk between autologous T-cells with the other immune cells, or constitutive expression of γc, although no increases in expression of γc or its downstream signaling component JAK3 was found in earlier gene therapy trials (*22*). T-cell receptor excision circles (TRECs), a measure of thymic-derived T-cells, increased from <25 TREC/μg to 90-120 TREC/μg DNA in both P1 and P2, where the lower limit of normal range for this assay is 75 TREC/μg DNA (Fig. S4). *In vitro* T-cell functional responses to mitogenic stimulation were significantly improved following treatment (Fig. 2A). Due to a lag in the appearance of gene marked immune cells, a less substantial increase is expected in P3-5 at this early stage (Fig. S5).

Gene-corrected Transitional B-cells and Humoral Reconstitution

Gene-corrected B-cells emigrating from bone marrow first appear in peripheral blood as immature/transitional T1 (CD10++/CD21lo) B-cells, which increased in P3 from 2.6% pre-treatment to 25% at 8 weeks post treatment (Fig. 2B, C), subsequently differentiating into

transitional T2/T3 (CD10+/CD21hi) B-cells with a concomitant decrease in T1 being noted from 9 weeks (Fig. 3B, C), consistent with previously described phenotypes (*23*)(*14*). The derivation of T1, T2/3 B-cells was confirmed by gene marking in sorted B-cell subsets, with VCN in T1>T2/3>Naïve> myeloid for P3-5 (Fig. 2F). Longer follow up in P1 and P2 demonstrated functional correction in B-cells with class-switching to IgG+CD27+memory B- cells (Fig. S6) and normal IgG (Fig. 2D). In all patients, serum IgM increased starting about 3 months post-therapy (Fig 2E). Interpretation of IgG production in patients receiving IgG supplements is challenging, although IgM levels are not affected by IgG supplementation. Along with the early appearance of gene-marked B-cells, serum IgM levels also increased early with the greatest increases seen in P1 and P4. These subjects had largest proportion of vector positive colonies from the CFU assay performed on the bulk transduced HSCs, especially P4 who also achieved the highest level of busulfan AUC and received one of the most $CD34^+$ cells. A diagnostic workup for monoclonal gammopathy in P1 (including PET-CT) was negative and the increased IgM levels spontaneously declined as the IgG levels increased. Response to IL-21 was demonstrated in *vitro* with peripheral blood cells from P1 at 6 months post-therapy (Fig. 3A). ELISpot analysis of P1 before and after vaccination with the 2013 Flu-vaccine (at 12 months post-therapy) demonstrated significantly increased vaccine-specific IgG+ B-cell frequencies, as well as the total number of IgG+ B-cells, thus confirming reconstitution of humoral immunity (Fig. 3B). Vaccine- induced responses to polio, diphtheria, tetanus, hemophilus were observed in P1 and P2, including a robust response in P1 to anti-rabies vaccination, an FDA-approved antigen, with anti-rabies titer of 11.3 International Units/ml (>0.5 IU/mL represents acceptable response). Of note, the improved B-cell responses may be attributable to interaction of the gene-corrected B-cells with either the donor T-cells or the autologous gene-corrected T-cells.

Gene marking in CD3-CD56+ NK-cells also appeared by 2 months and doubled by 15 months for P2 (Fig. 1A). Post-therapy NK-cells had features of mature NK-cells similar to those observed in

healthy controls (Fig. S7). The increase in P2's NK cell numbers coincided with shrinkage of warts (Fig. 4A).

Clinical Benefits

For P2, extensive molluscum contagiosum and disfiguring warts on both hands improved post-gene therapy (Fig. 4A). Chronic (>2 years prior to treatment) norovirus infections in P1 and P2 cleared within 21 months of therapy and severe protein-losing enteropathy resolved with normalization of albumin, and weight gain indicative of an improved nutritional state (Fig. 4B, 4C). P2 entered the study with bronchiectasis with irreversible airway damage (Fig. S8) complicated by severely impaired pulmonary function (<40% of normal). Worsening lung disease led to a fatal pulmonary bleed at 27 months post-gene therapy.

Vector integration clonality analysis (VISA)

VISA of sorted blood lineages in P1 and P2, using previously described methods (*24, 25*) identified 333,822 integration events, of which 38% were unique integration sites and most clones comprising <1% of total sites (Fig. 5A). Using the Chao estimator for species richness, the greatest diversity was observed in B-cells, followed by myeloid cells, then T- and NK-cells (Table S2)(*26*). In addition to the diversity of the integration site repertoire, our goal is to be able to detect clone(s) that may undergo any significant expansion. Oligoclonality index (OCI)(*27*) may overestimate polyclonality in situations of limited library size or sample material, further described in SM&M. To facilitate comparison of clonal expansion between samples, we determined the number of unique clones comprising the top 50% of the total clones, the Unique Clone Index (UC_{50}) (Fig 5A)(SM&M). UC_{50} for NK-cells suggests a more limited diversity with 79 clones comprising half of all NK-cells for P1 at 30m, and 7 for P2 at 24m (Fig. 5A). Quantitative ddPCR surveillance of the most abundant clones revealed the fluctuations in clonal abundance within each lineage (Fig. 5B). Although individual clones transiently increased in

abundance, in particular those possessing integrations near the *TNFSF12* and the *PIM1* gene, these declined over time.

Vector Integration Site Distribution

A comparison of P1 and P2's VISA datasets to an *in vitro* LV-transduced CD34+ cell library dataset (259,648 unique sites) mapped across the human genome (Fig. 6A) confirms LV's preferential targeting of actively expressed genes and gene-dense regions (79% within genes versus 39% for random control) (*24, 25*)(discussed further in SM&M). Also evident from this comparison is that the *in vivo* IS repertoire is remarkably similar to that of the input clones, highlighting the dominant effect of the vector-specific integration preferences retained in *in vivo* patient samples independent of disease or therapeutic gene (WAS, MLD and ALD) transferred (*12, 13*). A comparison of shared integration sites between different lineages at different time points, or Similarity Index shows highly variable IS in myeloid cells, consistent with the increased cell turnover rates in myeloid compared with T-cells (Fig. S9).

Many of the integration sites in our patient samples are shared with the common integration sites (CIS) in the lentiviral Wiskott-Aldrich Syndrome trial (*12*) although they do not appear to be enriched compared to the initial *in vitro* CD34+ cell library, suggesting a lack of selective *in vivo* expansion. In contrast, *HMGA2* has multiple unique integrations, most of which are in the same orientation, especially in intron 3 (Fig. 5C). Although over 38-fold enrichment in *HMGA2* contribution is observed *in vivo*, the overall contribution from *HMGA2* integrant clones remained very low in all gene-marked cells (~1%), with no significant expansion of any single clone. In the β-thalassemia study, a single *HMGA2* integrant clone was highly expanded due to an alternative splice acceptor created by the unstable 2 x 250bp cHS4 insulator core that resulted in the overexpression of a truncated and more stable HGMA2 protein (*29*). Using primers targeting the flanking insulators (Fig.S10), we confirmed the integrity of our transgene sequence around the

insulators, without any evidence for rearrangement. To assess for potential biological effects of the low level expansion of *HMGA2* integrant clones, the expression level of *HMGA2* in different lineages of PB from P1 and P2 was determined using ddPCR assays for the 5' (exon 3, before integration sites) and 3' (exon 4/5, after integration site). Expression of HMGA2 in all cell lineages was low (data not shown) and the ratio of the 5'assay/3'assay was close to 1, suggesting no overexpression of a truncated form of HMGA2.

Discussion:

This study describes the first successful SIN-LV gene therapy with low-dose busulfan conditioning of older SCID-X1 patients who had failed previous HSCT. In P1 and P2, with ≥2 years follow-up, significant stable gene marking in multiple hematopoietic lineages coincided with reconstitution of humoral immunity, specific vaccine responses and marked clinical improvement, in contrast to restricted T-cell marking in prior mgRV gene therapy trials without conditioning (4). This was also the first use of SIN-LV made by a stable producer cell line, GPRG-γc, with multiple copies of self-inactivating vector genomes generated by a concatemeric array transfection technique (*18*) that permitted production of high-titer vector (>10^8 IU/ml) sufficient to treat >1 × 10^9 or 2 × 10^7/kg cells for each patient (*17*). Despite reported improved outcomes with myeloid conditioning in ADA-SCID and Wiskott-Aldrich syndrome gene therapy trials, particularly with regards to B- or NK-cell reconstitution, no myelo-conditioning has been used in SCID-X1 to date (*19, 30*). Significant multi-lineage marking in this study underscores the importance of myeloid conditioning to improve engraftment in SCID-X1, although lentivector likely also targets HSC more efficiently, since mγRV gene therapy with busulfan conditioning in Chronic Granulomatous Disease (*18*) and WAS (*19*) did not result in significant B-cell gene marking.

Humoral reconstitution in P1 and P2 corresponded with the eradication of chronic norovirus in P1 and P2, a poorly appreciated medical problem in SCID-X1 patients with incomplete immune reconstitution following prior haplo-HSCT without conditioning. Despite minimal diarrhea, chronic norovirus infection in these patients can result in a protein-losing enteropathy, electrolyte-, mineral-and vitamin-losses and subsequent nutritional and growth failure with endoscopic appearance of Celiac-like flat or blunted villi. It is interesting that the gene-corrected B-cells were able to eradicate the norovirus while both oral IgG (used by some to treat chronic norovirus) and IV IgG failed, suggesting a potentially unique role for gut lamina propria B-cells in the control of norovirus.

We also report the first significant gene marking and correction of NK-cells in SCID-X1 following gene therapy with substantial clinical improvement of warts and molluscum (*31*). In contrast to the rapid expansion of gene-corrected T-cells in SCID-X1 infants following gene therapy, the T-cell marking in adult P1 and P2 increased slowly. Prior to gene therapy, P1 and P2 lacked donor stem cells and, together with the absence of TRECs, this suggests that circulating T-cells were from a long-lived thymic population. The number of gene-corrected autologous cells continues to out-compete donor T-cells as indicated by the T-cell chimerism assays, and the increase in TRECS indicate the presence of at least some residual thymic function. Longer-term follow-up of similarly treated, but younger SCID-X1 patients will shed light on the relative importance of a more preserved thymus. Of note, the increase of all lineages (T-, B- and NK-cells) continues steadily and stable CD34+ cell VCN even at 3 years post-therapy (in P1) and stable CD34+ gene marking (with 0.12 VCN in $CD34^+$ at 1yr), demonstrating the persistence of lentivector-corrected hematopoietic stem cells.

Quantitative ddPCR surveillance of specific clones revealed dynamic fluctuations in clonal contribution such as integrations near *PIM1*, *TNFSF12,* and *HMGA2*; *PIM1* transiently exceeded 20% of marked T-cells but less than 4% of the total lineage. Importantly, these clones decreased spontaneously over time. The integration profile in the patients is likely shaped by two major factors: firstly, the vector-preferred integration pattern at initial transduction, and secondly, selective expansion *in vivo*. Most of the CIS identified in our study and in previous studies simply reflect the initial integration preference of the lentivector, with no significant enrichment in vivo of any of the top CIS. Using a large *in vitro* CD34 integration dataset as a reference baseline of input clones, *in vivo* clonal expansion determined by fold increase over *in vitro* sample revealed *in vivo* expansion of HMGA2, previously described in β-thalassemia lentiviral gene therapy trial(*29*). Multiple independent integration sites in the *HMG2A* gene were found in both P1 and P2, but unlike the single HMGA2 restricted to the myeloid lineage in the b-thalassemia trial, the HMGA2 clones in P1 and P2 were present in all cell lineages (myeloid, T-, B- and NK-cells), suggesting the growth advantage occurred in stem cells. Furthermore, overexpression of the truncated HMGA2 gene selectively occurred in erythroblasts but remained undetectable in granulocytes-monocytes despite presence of the same clone at a greater frequency (*29*). This suggests that the transcription of HMGA2 gene is under tight regulation, with variable expression in specific cell types at specific time points. We hypothesize that the integrations in this study found in the HMGA2 intron 3 provides some growth advantage of host cells transiently by production of the more stable truncated protein at a certain time point, but HMGA2 gene remains under the control and regulation of its native promoters, and can be turned off during the natural cell cycle or cell differentiation, which accounts for the absence of over-expression in P1 and P2 cells. The endogenous expression control for HMGA2 gene likely limits the growth expansion influence from the HMGA2 integration clones. Ongoing

monitoring of HMGA2 and other integration sites will shed further light on this.

Since SCID-X1 is a rare disease, a limitation of this study is the small number of patients treated. Furthermore, all patients have received prior haplo-identical stem cell transplant that resulted in varying degrees of immune reconstitution. Consequently, the study population is heterogenous with a range of complex underlying medical problems that may impact the results. Although only the first 2 older patients have been observed up to 2-3years, early myeloid gene marking corresponds to B-cell gene marking and humoral immunity improvement, providing possible early indicators of outcome. Since vector-related mutagenesis did not appear until 3-4 years following gene therapy in the SCID-X1 infants (*22*), insertion-related mutagenesis cannot be definitively excluded although the diverse repertoire of integrations to date, and the absence of adverse events in the larger experience with LV-gene therapy in other diseases, is reassuring. Our results from the young adult patients suggest that LV-mediated gene therapy with reduced intensity conditioning is a promising approach for treatment of SCID-X1, including in older patients. Importantly, such interventions should be considered early to minimize irreversible organ damage from sub-optimal immune reconstitution, as T-cell reconstitution alone is clearly not sufficient to prevent or reverse progressive injury to lungs.

Materials and Methods:

This is a Phase I/II non-randomized clinical trial of ex vivo hematopoietic stem cell (HSC) gene transfer treatment for X-linked severe combined immunodeficiency (SCID-X1) using a self- inactivating, insulated lentiviral vector. The study is approved by the NIAID Institutional Regulatory Board (Clinical Protocol #11-I-0007). Institutional Biosafety Committee, Food and Drug Administration Agency (IND Drug# 15041) sponsored by NIAID

Regulatory Compliance and Human Subjects Protection Branch (RCHSPB).

Lentiviral Vector

The Cl20i4 EF1 hacOPT used in this clinical trial is the first use of lentivector produced by a previously described stable inducible lentivector-producer cell line GPRTG (*15, 18*). This is a Vesicular stomatitis virus G pseudotyped, 3rd generation SIN vector which uses a promoter fragment from the eukaryotic elongation factor alpha (EF1α) gene to express a codon-optimized human ac cDNA, and contains a 400-bp insulator fragment from the chicken β-globin locus within the self-inactivating long-terminal repeat. Large-scale clinical grade vector was manufactured at St Jude Children's Research Hospital Vector Facility as previously published (Greene). Briefly, producer cells were cultured in WAVE Bioreactor system GE Healthcare Bioscience, Somerset, NJ), and harvests were filtered (Millipore, Billerica, MA), followed by a Mustang Q ion-exchange (Pall, Ann Arbor, MI), and a final concentration by diafiltration (Millipore) (15). Titers of the vector were $4.5\text{-}7.2 \times 10^8$ iu/ml.

Autologous CD34+ hematopoietic stem cells collection and isolation

Patients receive granulocyte colony stimulating factor (G-CSF) at 16mg/kg/day by subcutaneous injection for 5 consecutive days supplemented by Plerixafor (0.24mg/kg SQ 11 hours prior to collection) (NIH protocol 94-I-0073) prior to apheresis at the NIH Clinical Center (22). The products were processed using FDA-approved Isolex immune anti-CD34 magnetic bead system to isolate and enrich CD34+ cells by the NIH Cell Processing Facility, Department of Transfusion Medicine. Purified CD34+ HSCs were cryopreserved until gene therapy.

Transduction of CD34+ hematopoietic stem cells

Ex vivo culture and transduction of the patient's autologous CD34+ HSC with VSV-G pseudotyped CL20-4i-EF1α-hγc-OPT LV was performed and certified by the NIH Department of Transfusion Medicine Cell Processing Facility. Transduction involves thawing and suspension of patient CD34+ HSC in X-VIVO™ 10 serum-free growth medium (containing 1% human serum albumin plus cytokines (Stem cell factor 50-100 ng/mL; FLT-3 ligand 50-100 ng/mL; Thrombopoietin 50-100 ng/mL; Interleukin-3 5 ng/mL). The cells were cultured in T175 tissue culture flasks coated with the recombinant fibronectin fragment known as Retronectin®, and exposed to lentivector for 6-8 hours each day for 2 consecutive days after an overnight pre-stimulation (Figure 1). Transduced CD34+ HSCs were washed and infused over <30 minutes at the end of culture after required safety testing and quality control testing.

Conditioning Regimen

A dose lower than that used in gRV-gene therapy for CGD (10mg/kg) (18) but higher than the dose used in gRV-gene therapy for ADA (4mg/kg) was chosen, at 6mg/kg given over 2 days. The busulfan levels are shown in Table 1, no dose adjustment was given. No T- or B-cell depleting agents were given.

Clinical course

No adverse events were noted with the infusion of the transduced CD34+ HSC cell product. The nadir for the expected busulfan-related effects of neutropenia and thrombocytopenia occurred at 2-3 weeks post-treatment (Fig. S2), and recovered without requiring any cellular support or intervention. Subjects were discharged home by 3-4 weeks following gene therapy. Three of the 5 patients developed febrile neutropenia that responded to empiric antimicrobial therapy. An overview of the study implementation is shown in Table S1.

Laboratory Evaluation

Cell lineage separation for gene marking and integration analysis

Following polymorphonuclear granulocyte (PMN) separation using histopaque, the mononuclear leukocyte layer was fractionated by magnetic beads per manufacturer's instructions (Dynal Beads, Invitrogen) (Supplementary Materials & Methods (SM&M)). The purity of the bead-sorted PMN from a healthy donor was analyzed (Fig. S3).

B-cell preparations, flow cytometry phenotyping and functional analyses

Immunophenotyping and cell sorting were performed on freshly processed peripheral blood mononuclear cells (PBMCs) or PBMCs that had been cryopreserved with the following anti-human mAbs: allophycocyanin (APC) anti-CD10, APC-H7 anti-CD20, and PE anti-IgG (BD Biosciences); peridinin chlorophyll protein-Cy5.5 (PerCP-Cy5-5) anti-CD19 and PE-Cy7 anti- CD27 (eBioscience); FITC anti-CD21 (Beckman Coulter); VioBlue anti-IgA (Miltenyi Biotec) and Brilliant Violet 510 (BV510) anti-IgM. Cell sorting and immunophenotyping were performed on BD FACSAria II and BD FACSCanto II (BD Biosciences) flow cytometers, respectively. Analyses were performed with FlowJo Version 9.8.5 software (TreeStar). Memory B-cell responses to influenza were performed by ELISPOT as previously described (23) with B- cells isolated by negative magnetic bead-based selection (StemCell Technologies). Phenotyping of immune cells by flow cytometry was also performed by the CLIA-certified Clinical Immunology Laboratory/Department of Laboratory Medicine at the NIH Clinical Center.

B-cell proliferation and class switching in response to IL-21/CD40L

CFSE-based proliferation assays were performed on PBMCs of a healthy donor and P1 as

previously described (24), with following modifications. PBMCs were labeled with CFSE (0.5 μM; Molecular Probes) and cultured with 50 ng/ml IL-21 (PeproTech) and 500 ng/ml CD40 ligand (25). B cell proliferation was determined by CFSE dilution and immunoglobulin class-switch was measured by intracellular expression of IgG and IgM using flow cytometry.

B-cell proliferation and class switching in response to IL-21/CD40L

Peripheral blood mononuclear cells of a healthy donor and P1 were labeled with CFSE (0.5 μM; Molecular Probes) and cultured with 50 ng/ml IL-21 (PeproTech) and 500 ng/ml CD40 ligand (24-26). B cell proliferation was determined by CFSE dilution and immunoglobulin class-switch was measured by intracellular expression of IgG and IgM using flow cytometry.

Peripheral blood CD34+ cell purification and expansion for vector copy number and integration site analyses

Peripheral blood mononuclear cells were purified from non-mobilized peripheral blood by Ficoll separation (Lymphocyte Separation Medium; MP Biomedicals). $CD34^+$ cells were isolated from PBMCs using MACS (Miltenyi Biotec) magnetic beads according to manufacturer's protocol. $CD34^+$ cells were expanded for 8 to 12 days in StemSpan II media (Stem Cell Technologies) supplemented with 100 ng/mL each of human stem cell factor, Flt3-ligand, and thrombopoietin (PeproTech). DNA isolated using the DNeasy Blood and Tissue Kit (Qiagen).

Quantitative determination of vector copy number by Digital Droplet PCR

To measure the vector-carrying cells, we used a Digital Droplet PCR (ddPCR, BioRad, Hercules, CA) assay. The ddPCR assay allows the measurement of absolute copy number without using standard curve. The vector specific primers and probes are HIV- F(5'CTG

TTG TGT GAC TCT GGT AAC T3'), HIV-R(5'TTC GCT TTC AAG TCC CTG TT3'), HIV-probe(5'- /56-FAM/AAA TCT CTA/ZEN/GCA GTG GCG CCC G /3IABkFQ/ -3'). We multiplexed a reference gene assay for cell counts in the same reaction (MKL2-F, 5'AGA TCA GAA GGG TGA GAA GAA TG3', MKL2-R, 5'GGA TGG TCT GGT AGT TGT AGT G3', MKL2-probe, 5'-/56-HEX/TG TTC CTG C/ZEN/A ACT GCA GAT CCT GA/3IABkFQ/-3'). Cell number was calculated as a half of the *MKL2* counts because each cell is diploid. Vector copy number was calculated as vectors/cell.

Monitoring clonal expansion of specific integration site. Top expanded clones identified by the VISA sequencing assay were followed up and monitored by specific ddPCR assay. All of our ddPCR assays consist of a common LTR primer, a LTR probe, and a specific primer for genomic DNA junction (5LTR-Rev 5'CTG CAG GGA TCT TGT CTT CTT3', 5LTRjunction-probe, 5'/56-FAM/TGG AAG GGC/ZEN/TAA TTC ACT CCC A /3IABkFQ/3', PIM1-5LTR primer: 5'TCC TAA CAT CCC CAC TGC AT3', TNFSF12-5LTR primer: 5'ACA GTA AAG CAA GAG TGG GAT G3'; 3LTR-F CCC ACT GCT TAA GCC TCA ATA, 3LTRjunction-probe, 5'- /56-FAM/AAG TAG TGT/ZEN/GTG CCC GTC TGT TGT/3IABkFQ/ -3', CDKN1A-3LTR primer: 5'GCA CGA AAT CAC TGC CAT ATT C3'). We multiplexed the integration site specific assay together with the *MKL2* reference gene assay which measures cell counts in the same reaction. The use of ddPCR makes it possible to: 1) monitor the specific integration site accurately, 2) use limited amount of input DNA, 3) make monitoring of expanded clones easy over time and across many different cell lineages.

Evaluation of insulator size in peripheral blood cells from P1

Genomic DNA P1 was used for PCR amplification, using PCR primer sequences for the 5' LTR were 5'-CTG GAA GGG CTA ATT CAC TC-3' (P1) and 5'-TCG CGA TCT AAT TCT CC-3'

(p2); and for the 3' LTR were 5'-GCG GCC GCA TCG ATG CCG TAT AC-3' (P3) and 5'-CTG CTA GAG ATT TTC CAC AC-3' (P4). The expected size of the 5' and 3' LTR PCR fragments with the intact 400bp insulator element was 851bp and 791bp, respectively (The exact length of both LTRs are 649bp without insulator). The sequences of the two PCR fragments were confirmed by direct DNA sequencing of the PCR product. The smaller band (around 500bp) with the P1/P2 primer was TA cloned and sequenced, confirming a mispriming of P2 in the R region which resulted in the artifact, and that the 400bp insulator is intact.

Figure and Table Captions:

Figure Legends:

Figure 1. Immune cell gene marking and numbers after gene therapy.

(A) Gene marking in sorted cell lineages as vector copy number (VCN) per genome following treatment in P1 (to 36 months) and P2 (to 24 months).

(B) Early gene marking in first 6 months in myeloid and B-cells in P1-5.

(C) Percent autologous (corrected) host cells as determined by RFLP assay of T-cell chimerism.

(D) Immune cell numbers in P1 and P2 following treatment.

Figure 2. Functional correction of T- and B-cells with SIN-LV gene therapy.

(A) CD3 T-cell proliferative responses to indicated stimuli.

(B) Emergence of transitional T1 B-cells (CD10++CD21lo) in P3 at 12 weeks, progressing to T2/3 B-cells (CD10+CD21hi) by 16 weeks post gene therapy.

(C) Changes in B-cell subset profiles over time in P3.

(D) Serum IgM and IgG over time in P1 and P2 post treatment. Withdrawal of supplemental IgG is indicated by the arrow. Dotted lines indicate respective normal reference ranges.

(E) Early increases in IgM, comparing P1-5.

(F) A summary of vector copy number in flow-sorted T1, T2/3, Naive B-cells compared to CD3 T cells from P3, P4 and P5.

Figure 3. Restoration of B-cell signaling and specific antibody production

(A) B-cell responses to IL-21 and CD40L stimulation. Peripheral blood mononuclear cells from P1 (bottom) and a healthy control (HC, top) were stained with CFSE and stimulated with IL-21 and CD40 ligand (CD40L). Gated CD3-CD19+ B-cells that have undergone

division are shown in left upper area showing CD27+ (column 1), and IgG+ (column 2), and IgM+ (column 3) expressing B-cells.

(B) ELISPOT of P1 B-cells before and after vaccination. Numbers of Ig antibody secreting cells (ASC) (left), influenza-specific ASC per 10^6 B cells, and influenza-specific as a fraction of total Ig ASCs (right) detected by ELISPOT of peripheral blood B-cells from P1 before and after influenza vaccination to determine memory B-cell responses.

Figure 4. Clinical progress following gene therapy.

(A) Photographs demonstrating Human Papilloma Virus warts (top) and molluscum contagiosum on P2 (bottom) before and 15 months after gene therapy as indicated.

(B) Serial serum albumin for P1-P5 following gene therapy. (Upper and lower reference ranges are indicated by dotted lines.

(C) Body mass measurements for P1-P5 following treatment.

Figure 5. Vector Integration site analysis (VISA).

(A) Total unique integration sites, shown in proportion to their representation of the total diversity in P1 and P2 to 30 and 24 months respectively (left). Clonal composition for sorted cell lineages for P1 and P2 (right). Each horizontal bar represents clonal frequency, from most abundant on the top. The number of unique clones in the top 50% of the cells, UC50, is listed above each sample.

(B) Serial quantitative ddPCR tracking of the 4 most frequent clones (TNFSF12, TNFSF12-TNFSF13, chr 6 and PIM1) in P2 is shown as a percentage of vector-marked cells (left), or of total cells in each lineage (right).

(C) Schematic of unique integrations in *HMGA2* in P1 and P2. Most clones (enumerated next to arrow) are in the same orientation as the gene (blue), with a few in the reverse

orientation (red). Clones are seen in all lineages; CD34, CD14, CD19, NK and PMN, and CD3 (summarized below).

Figure 6.

Circular projection of the human genome with integration sites from P1 and P2 (*in vivo* orange, red), *in vitro* lentivector-transduced CD34 cells (green), and *in vitro* mγRV-transduced CD34 cells (red). The top 100 target genes (*in vitro*) are listed on the outside with top 10 target genes (*in vitro*) in bold.

Tables:

Table 1 Patient characteristics and treatment.

All patients received allogeneic stem cell transplant (HSCT) from haploidentical (haplo) parent donor once or repeated (booster). SQ, subcutaneous injection, CFU, colony forming units; TCR, T-cell receptor; PLE, protein-losing enteropathy.

	P1	P2	P3	P4	P5
IL2RG mutation	823T>G	447 delA	923C>A	c341G>A	31T>A
Age (yrs)	23	22	7	15	10
Prior HSCT	Haplo; booster	Haplo	Haplo: booster	Haplo	Haplo; booster
Immuno-phenotype	↓ T, B, NK	↓ B, NK	↓ T, B, NK	↓T, B, NK	↓T, B, NK
Medical problems	Norovirus, infections PLE, IVIG	Norovirus, infections, IVIG,Warts, molluscum, bronchiectasis, bronchiolitis obliterans	Norovirus, infections, PLE, IVIG, bronchiectasis, growth failure	Norovirus, infections, PLE, IVIG, bronchiectasis	Norovirus PLE, IVIG, Molluscum, bronchiectasis
Busulfan AUC (min*umol/L)	3603.1	4528.9	2519.6	4523.6	3096.6
CD34+ Cells infused ($x10^6$/kg)	18	16	20.4	21.7	25
Bulk CD34 In vitro-CFU (%)	27	17	22	57.7	36.1
Follow-up (months)	36	24	6	3	3
Current status	Cleared norovirus, off IVIG	Cleared norovirus, off IgG supplement, fatal bronchial bleed	stable	stable	stable

List of Supplementary Materials:

Supplementary Figures:

Supplementary Table S8. Gene ontology KEGG_PATHWAY

Supplementary Methods:

References and Notes:

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