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Lentiviral globin gene therapy with reduced-intensity conditioning in adults with β-thalassemia: a phase 1 trial

Farid Boulad[1,2], Aurelio Maggio[3], Xiuyan Wang[2,4], Paolo Moi[5], Santina Acuto[3], Friederike Kogel[2], Chayamon Takpradit[1,6], Susan Prockop[1], Jorge Mansilla-Soto[2], Annalisa Cabriolu[2], Ashlesha Odak[2], Jinrong Qu[4], Keyur Thummar[4], Fang Du[4], Lingbo Shen[4], Simona Raso[3], Rita Barone[3], Rosario Di Maggio[3], Lorella Pitrolo[3], Antonino Giambona[3], Maura Mingoia[5], John K. Everett[7], Pascha Hokama[7], Aoife M. Roche[7], Vito Adrian Cantu[7], Hriju Adhikari[7], Shantan Reddy[7], Eric Bouhassira[8], Narla Mohandas[9], Frederic D. Bushman[7], Isabelle Rivière[2,4] and Michel Sadelain[2] ✉

β-Thalassemias are inherited anemias that are caused by the absent or insufficient production of the β chain of hemoglobin. Here we report 6–8-year follow-up of four adult patients with transfusion-dependent β-thalassemia who were infused with autologous CD34⁺ cells transduced with the TNS9.3.55 lentiviral globin vector after reduced-intensity conditioning (RIC) in a phase 1 clinical trial (NCT01639690). Patients were monitored for insertional mutagenesis and the generation of a replication-competent lentivirus (safety and tolerability of the infusion product after RIC—primary endpoint) and engraftment of genetically modified autologous CD34⁺ cells, expression of the transduced β-globin gene and post-transplant transfusion requirements (efficacy—secondary endpoint). No unexpected safety issues occurred during conditioning and cell product infusion. Hematopoietic gene marking was very stable but low, reducing transfusion requirements in two patients, albeit not achieving transfusion independence. Our findings suggest that non-myeloablative conditioning can achieve durable stem cell engraftment but underscore a minimum CD34⁺ cell transduction requirement for effective therapy. Moderate clonal expansions were associated with integrations near cancer-related genes, suggestive of non-erythroid activity of globin vectors in stem/progenitor cells. These correlative findings highlight the necessity of cautiously monitoring patients harboring globin vectors.

β-Thalassemias are hereditary anemias caused by over 400 mutations that affect the β-globin gene (*HBB*), resulting in absent or insufficient production of the β chain of hemoglobin (Hb)[1–3] (https://www.ithanet.eu/db/ithagenes). Treatment of the severe form of the disease, known as transfusion-dependent β-thalassemia (TDT), requires lifelong transfusions to supply Hb-replete red blood cells (RBCs), which the thalassemic bone marrow is unable to produce[4,5]. This treatment is life-saving but fraught with medical complications[6–8]. The only curative therapy is the transplantation of allogeneic hematopoietic stem cells (HSCs) harboring functional globin genes, but this option is not available to the vast majority of patients with thalassemia, for whom a suitably matched related donor cannot be found[9]. Although bone marrow transplantation from a matched related donor carries a low risk of morbidity and mortality[10,11], few patients opt for alternative transplant modalities owing to the potentially serious risks associated with matched-unrelated or mismatched transplants[12]. These medical risks, together with their socio-economic cost[13], warrant the pursuit of alternative curative therapies[9]. Gene therapy strategies in autologous cells offer the prospect of a safe transplant without the allo-immune complications of graft rejection or graft-versus-host disease[14–17].

The goal of globin gene transfer is to enable the patients' own HSCs to generate RBCs that contain enough Hb to obviate transfusion therapy[18,19]. We previously showed that globin gene therapy is curative in two mouse models of β-thalassemia intermedia and Cooley's anemia, using a lentiviral vector encoding the human β-globin gene and selected regulatory elements that enabled therapeutic globin expression[20–23]. This vector, termed TNS9, corrected the anemia in β-thalassemic mice and produced an average increase of 4–6 g dl^{-1} of Hb per vector copy in steady-state peripheral blood. These findings were confirmed and extended by others in various models of thalassemia and sickle cell disease (SCD) using variant lentiviral vectors also harboring the HS2, HS3 and

[1]Stem Cell Transplant and Cellular Therapy Service, Department of Pediatrics, Memorial Sloan Kettering Cancer Center, New York, NY, USA. [2]Center for Cell Engineering, Memorial Sloan Kettering Cancer Center, New York, NY, USA. [3]Department of Hematology and Rare Diseases, Azienda Ospedaliera Ospedali Riuniti Villa Sofia-Cervello, Palermo, Italy. [4]Cell Therapy and Cell Engineering Laboratory, Memorial Sloan Kettering Cancer Center, New York, NY, USA. [5]Ospedale Pediatrico Microcitemie 'A.Cao', A.O. 'G.Brotzu', Cagliari, Italy. [6]Department of Pediatrics, Siriraj Hospital, Mahidol University, Bangkok, Thailand. [7]Department of Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA. [8]Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York, NY, USA. [9]Laboratory of Red Cell Physiology, New York Blood Center, New York, NY, USA. ✉e-mail: m-sadelain@ski.mskcc.org

Table 1 | Patient characteristics

Patient ID	Patient 1	Patient 2	Patient 3	Patient 4
Age at cell infusion (years)	23	18	39	18
Sex	F	F	M	M
Genotype	β^0/β^+	β^0/β^+	β^0/β^0	β^0/β^+
Mutation	cod39/IVSI-110	cod39/IVSI-6	cod39/cod39	cod39/IVSI-110
Transfusion requirement (ml per kg per year)	203	214	177	271
Chelation	DFX	DFO + DFP	DFP	DFO
Serum ferritin (ng ml^{-1})	1,217	2,477	838	1,739
Cardiac T2* by MRI (ms)	41	44	32	35
LIC (mg Fe per g dw)	14.8	15.6	11.7	15.8
Liver fibrosis	Minimal	No	No	No
Splenectomy	No	Yes	No	No
HCV	Negative	Negative	Serology positive, RNA negative	Negative

Transfusion requirements before infusion are expressed as volume in milliliters (ml) per body weight (in kg) per year, mean annual volume over the last 5 years before gene therapy. Liver fibrosis evaluation: liver biopsy in Patient 1 and via FibroScan in Patients 2–4. DFO; desferrioxamine; DFP, deferiprone; DFX, deferasirox; F, female; HCV, hepatitis C virus; LIC, liver iron concentration in milligrams (mg) of iron (Fe) per gram (g) of dry weight (dw), estimated by T2* liver MRI; M, male.

HS4 elements of the β-globin locus control region (LCR) to regulate the vector-encoded β-, γ- or mutated β-globin gene (reviewed in refs. [18,19,24,25]). To enable the clinical translation of globin gene therapy, we conducted a $CD34^+$ cell mobilization study in five adult patients with TDT, which showed safe and efficient granulocyte colony-stimulating factor (G-CSF)-induced hematopoietic stem and progenitor cell (HSPC) mobilization and globin vector transduction efficiency, achieving a mean vector copy number (VCN) of 0.2–0.6 in bulk CD34 cells[26].

Based on these encouraging results and after a public review by the National Institutes of Health's Recombinant DNA Advisory Committee (RAC) (https://osp.od.nih.gov//wp-content/uploads/RAC_Minutes_Jun_2007.pdf), we proceeded to a phase 1 clinical trial to evaluate the safety and level of globin gene transfer after RIC in adults with TDT. RIC was chosen because (1) this approach had been used previously and successfully in the context of gene therapy and could be sufficient for the engraftment of gene-transduced cells; and (2) it was a safer first approach. This trial had a median follow-up of 90 months, and we report here on the four patients treated on the protocol.

Results

Patient characteristics. Four patients with TDT were infused with TNS9.3.55-transduced autologous $CD34^+$ cells between November 2012 and May 2015 (Table 1). Patients were enrolled from the Cervello Hospital in Palermo, Italy ($n=3$), and the Microcitemie Hospital in Cagliari, Italy ($n=1$). All patients underwent successful HSPC mobilization, cytoreduction and cell infusion at Memorial Sloan Kettering Cancer Center (MSKCC). One patient who had undergone successful mobilization and transduction eventually opted not to proceed to cytoreduction and cell infusion. The four infused patients included two females and two males, aged 18–39 years at the time of infusion. One patient had a β^0/β^0 genotype, and three patients had a β^0/β^+ genotype, two of which bore the severe β^+ IVSI-110 mutation. All four patients were on RBC transfusion programs, receiving transfusion volumes of 177–271 ml per kg per year before enrollment in this study (Table 1). One patient was splenectomized at age 9. All patients received chelation with deferasirox ($n=1$), desferrioxamine and deferiprone ($n=1$), deferiprone ($n=1$) or desferrioxamine ($n=1$). All patients had increased liver iron concentrations at enrollment, ranging from 11.7 to 15.8 mg g^{-1} of dry weight. The median follow-up for these four patients was 90 months (range, 72–96 months).

$CD34^+$ cell collection and transduction. A median of 16.0×10^6 $CD34^+$ cells per kg (range, $10.3–30.7\times10^6$ $CD34^+$ cells per kg) was collected via apheresis after G-CSF mobilization (Table 2), which was well tolerated (Supplementary Table 1). A backup of 2×10^6 $CD34^+$ cells per kg was cryopreserved for each patient. The mean vector copy number (VCN) in the drug products (DP) ranged from 0.09 to 0.15 (median, 0.15). Patients received a median total TNS9.3.55-transduced dose of 9.0×10^6 $CD34^+$ cells per kg (range, $7.08–11.71\times10^6$ $CD34^+$ cells per kg). Cell infusions were administered in two successive aliquots[27].

Conditioning, engraftment and toxicities. The cumulative busulfan exposure (39.8–59.7 mg × L h^{-1}) was in the non-myeloablative range[28] for all four patients (Supplementary Table 2). Neutropenia (absolute neutrophil count (ANC) <500 µl) occurred on day 11 (median; range, days 10–13) and persisted for 8 d (median; range, 5–11 d). Neutrophil engraftment occurred on day 19 (median; range, days 16–24). Substantial thrombocytopenia (platelet count <20,000 µl) with transfusion dependence occurred in three patients on day 11 (median; range, days 9–12) and lasted for 6 d (median; range, 5–14 d) with platelet engraftment on day 19.5 (median; range, day 14–25) after transplant (Supplementary Table 2). Patient 2 developed severe metrorrhagia and received platelet transfusions to maintain a platelet count greater than 50,000 µl. The minimal platelet count observed in this patient was 43,000 µl. She received the last platelet transfusion on day 18 (Extended Data Fig. 1).

Non-hematologic busulfan toxicity was limited to grades 0–3 (Supplementary Table 3). Febrile neutropenia and metrorrhagia (grades 1–2) occurred in three and two patients, respectively. Notably, no veno-occlusive disease (VOD) or grade 4–5 serious adverse events (SAEs) were observed. At the time of neutrophil engraftment (day 15), Patient 1 developed symptoms consistent with engraftment syndrome and received methylprednisolone intravenously for three consecutive days. G-CSF was administered to Patient 1 and Patient 2 during the nadir period. Patients were discharged to the outpatient clinic on days 20–25 (median, 22) and returned home to Italy on day 30. All patients had their medical follow-up and care, including transfusions, at their home

Table 2 | Collection, transduction and dosage of TNS9.3.55-transduced CD34⁺ cells

Patient ID	Patient 1	Patient 2	Patient 3	Patient 4	Median
Number of mobilization cycles	1	1	1	2	
Number of CD34⁺ cells collected (×10⁶ per kg)	30.7	10.25	13.6	7.2 11.2	16.0
DP VCN (Average VCN in all CFCs)	0.15	0.09	0.15	0.17 0.12	0.15
VCN from liquid EC	0.37	0.20	0.28	0.39 0.22	0.28
CD34⁺ cell infusion dose (×10⁶ per kg)	11.71	7.08	10.60	3.87 3.50	9.0
% CD34⁺ EOP cells	99.9	98.3	97.3	99.4 94.3	97.8

EOP, end of production.

thalassemia center, with intermittent follow-up at MSKCC for specific examinations, such as bone marrow aspiration.

Gene marking and multi-lineage engraftment. The VCN per diploid genome was measured by quantitative polymerase chain reaction (PCR) in bone marrow and peripheral blood, including sorting of lymphoid (CD3 and CD19) and myeloid (CD14 and CD15) cells at multiple time points (Fig. 1). Twelve months after infusion, the median VCN in whole bone marrow (wBM) was 0.04 copies (range, 0.02–0.15) and 0.03 (range, 0.01–0.08) in peripheral blood mononuclear cells (PBMCs). At last follow-up, the median VCN in PBMCs was 0.03 (range, 0.01–0.11). The engraftment patterns were overall similar in the three younger adults and noticeably diminished in Patient 3. The highest VCNs were observed in one of the youngest and only splenectomized patient (0.11, 94 months after infusion).

Replication-competent lentivirus (RCL) assays were performed before infusion, followed by testing of all patients at 3, 6 and 12 months after infusion thereafter. No RCL was detected at any time point. On the basis of those results, and according to US Food and Drug Administration (FDA) guidelines, patients no longer need RCL testing and can be followed by a yearly review of medical history for up to 15 years after infusion (https://www.fda.gov/media/113790/download).

Transfusion requirements and transgene expression. All patients required continued transfusions after transplant. Transfusion target levels were temporarily brought down after transplant and then re-adjusted to pre-transplant levels. Based on the latter, transfusion requirements were durably decreased compared to baseline by 35–57% in Patients 2 and 4. Patient 2 was an 18-year-old female with a β^0/β^+ genotype. She received 7.08×10^6 transduced CD34⁺ cells per kg, the lowest number of transduced cells in the cohort. Her cell infusion product had a DP VCN of 0.09. The VCN in PBMCs at last follow-up was 0.11. The mean transfusion requirement dropped from a pre-infusion requirement of 214 ml per kg per year (range, 198–225 ml per kg per year) to 101 ml per kg per year 8 years after infusion (mean of 93 ml per kg per year), representing an overall reduction of 57% in transfusion volume (Fig. 2). Patient 4 was an 18-year-old male with a β^0/β^+ genotype. He received two cell products for a total count of 7.37×10^6 CD34⁺ cells per kg (3.87×10^6 CD34⁺ cells per kg with a DP VCN of 0.17 and 3.5×10^6 CD34⁺ cells per kg with a DP VCN of 0.12). The VCN in PBMCs at his last follow-up was 0.06. The mean RBC transfusion requirement decreased from 271 ml per kg per year (range, 265–280 ml per kg per year) to 183 ml per kg per year 5 years after infusion with a minimum of 145 ml per kg per year (mean, 174 ml per kg per year), accounting for an overall volume reduction of 35% (Fig. 2).

As we could not distinguish the vector-encoded and endogenous β chains, and because of continued transfusions, we turned to hematopoietic colony analysis to assess vector function. Hematopoietic colonies were established 1 year after cell infusion from bone marrow harvests. The paucity of hematopoietic colonies harboring a single vector copy and the limited material retrieved from individual colonies (needed for both VCN and high-performance liquid chromatography (HPLC) assays) made these studies technically challenging and exceedingly labor-intensive, only allowing a limited study in one patient (Patient 2). In a small analysis, the β/α expression ratio determined by HPLC increased from a mean of 0.11 to 0.39 in burst-forming units-erythroid colonies (BFU-Es) harboring a single copy of the integrated vector (Extended Data Fig. 2), corresponding to a β chain output of 55% (0.28/0.50) relative to a normal endogenous allele, in line with our earlier assessments[26]. We further attempted to evidence correction of erythropoiesis in bone marrow samples from Patient 2. The pathologic accumulation of polychromatophylic erythroblasts and marked deficit of orthochromatic erythroblasts and reticulocytes relative to normal bone marrow[29] improved at month 24, contemporaneously with a reduction in transfusion requirements (Extended Data Fig. 3). In all four patients, we found that the VCN was slightly higher in glycophorin⁺ bone marrow cells compared to the CD45⁺ fraction (Extended Data Fig. 4).

Analysis of integration site distribution and clonal abundance. We analyzed integration site distributions and assessed possible insertional mutagenesis associated with outgrowth of gene-modified clones. We focused our analyses on whole blood samples available over 5.75 years or more (up to 7 years for Patient 1). No integration site that mapped uniquely on the genome showed clonal abundance exceeding 20% at any time point (Fig. 3a). A targeted study of integration sites in genomic repeat sequences also did not uncover clones exceeding 20% abundance at any time point. Population structure of gene-modified cells was quantified using Gini coefficients, Shannon index and UC50 (Extended Data Fig. 5 and Supplementary Table 4). Populations of transduced cells were generally highly diverse.

Some clones were expanded in relative abundance (Fig. 3b), raising the question of whether insertional mutagenesis might have contributed. We, thus, compared integration site distributions in the initial transduction product to distributions at late time points (~6 years) after transduction, taking account of clonal abundance. The frequency of integration sites was elevated near cancer-associated genes (Supplementary Report and Supplementary Table 5), regardless of which of several lists of cancer-associated genes were used. For Patient 4, the most expanded clone (17% of total at the last time point) harbored an integration site near the cancer-associated gene *UBR2* (ref. [30]). More broadly, genes associated with extracellular exosome, protein serine/threonine kinase activity, endosome and signal transduction were modestly enriched in samples from patients ~6 years after transduction (Supplementary Table 6; all *P* values were significant only before correction for multiple comparisons).

We also assessed clustering of vector integration sites as a potential indication of insertional mutagenesis. Clusters selectively present in post-infusion samples potentially mark locations where insertional mutagenesis promoted cell growth and survival. Conversely, clusters present in pre-infusion samples might mark genes that favor growth in culture but that are less important for

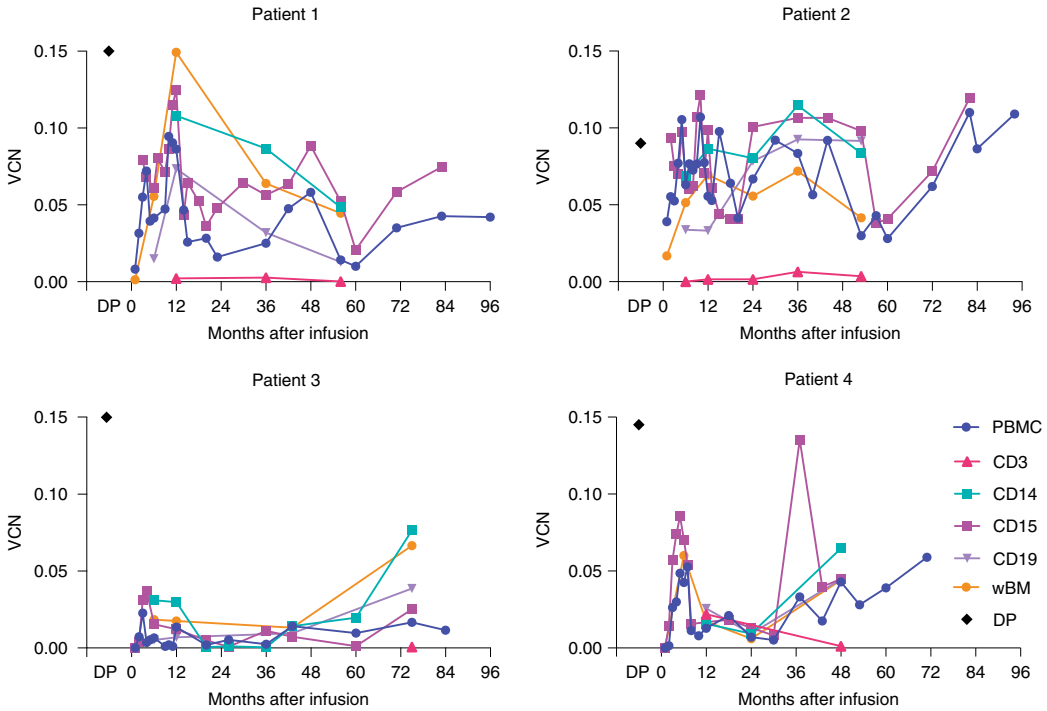

Fig. 1 | Multilineage engraftment of transduced cells in peripheral blood and bone marrow. VCN per diploid genome in different lymphoid (CD3 and CD19) and myeloid (CD14 and CD15) lineages sorted from PBMCs of patients and wBM. DP VCNs were evaluated on infused cells.

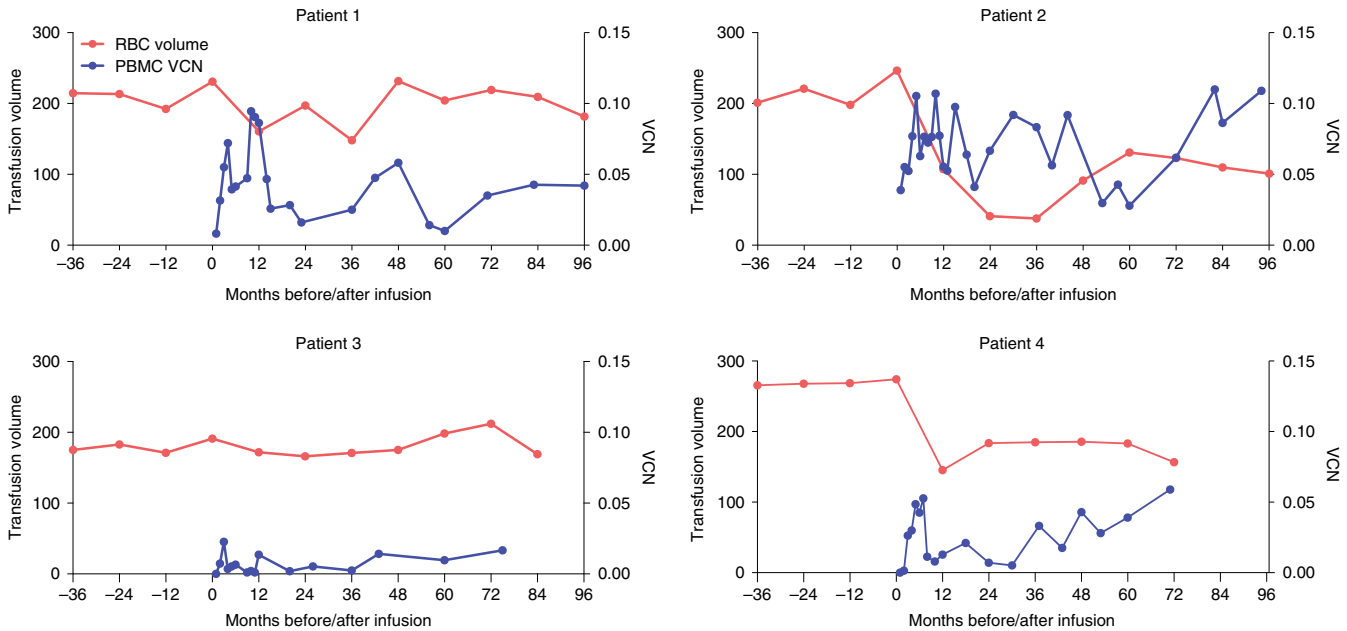

Fig. 2 | Transfusion requirements and VCN in PBMCs. Annual blood transfusion volume (ml per kg per year) and VCN (copy/diploid genome) in PBMCs before and after infusion with TNS9.3.55-transduced CD34⁺ HSPCs.

growth after transplantation. We identified clusters of integration sites using scan statistics as described in ref. [31], comparing pooled pre-transplant samples to pooled ~year-6 samples (Supplementary Table 7). One notable cluster was seen in a 13-kb interval on chromosome 17 (Extended Data Fig. 6) encoding the first intron of the *STAT3* gene (six integration sites were detected at year 6, four of which were in the same transcriptional orientation as *STAT3*, and zero were detected before cell infusion). HIV integration in the first intron of *STAT3* in the same transcriptional orientation was previously reported to be associated with clonal proliferation in T cells[32] and a B cell lymphoma in a patient who was positive for HIV[33].

Additional clusters of integration sites were seen in additional genes of potential interest, including *ASH1L*, encoding a histone lysine methyltransferase important in growth control in multiple cell types, including the hematopoetic cell lineage, and *DNMT1*, encoding a DNA methyl transferase important in gene

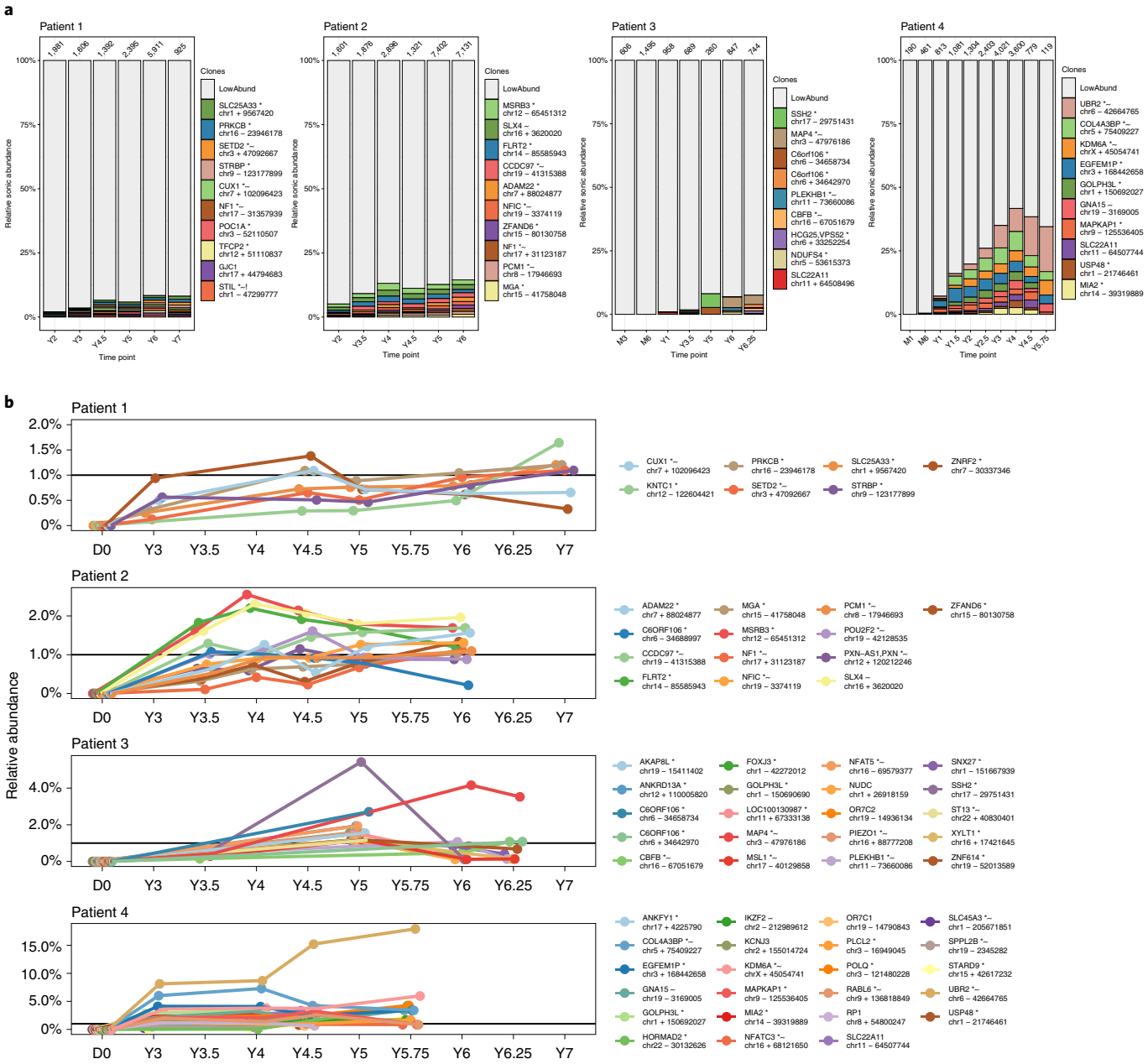

Fig. 3 | Long-term clonal abundance and succession in all patients. a, Longitudinal relative abundance of the ten most abundant cell clones in whole blood as marked by lentiviral integration sites. The different colors (horizontal bars) indicate the top ten most abundant cell clones, whereas the remaining sites are binned as low abundance (LowAbund; gray). The *x* axis indicates time points after infusion, and the *y* axis is scaled by proportion of the total cells sampled. The total number of genomic fragments used to identify integration sites is listed atop each plot. A key to the sites, named for the nearest gene, is shown on the right side of the graphs. The nearest genes possess additional annotations: *site is within a transcription unit; ~site is within 50 kb of human cancer-related genes. **b**, Cell clones surpassing 1% of relative abundance over time. Longitudinal representation of relative abundance of cell clones in whole blood as marked by lentiviral integration sites. D, day; Y, year.

regulation. Thus, these data, together with the observed expansion of the *UBR2*-associated clone (Fig. 3b), suggest potential examples of insertional mutagenesis influencing subsequent cell growth[34], although all, to date, are clinically benign.

Discussion

We report here on the safety and efficacy of globin gene therapy for β-thalassemia using the TNS9.3.55 lentiviral vector, with a median follow-up of 90 months. Autologous CD34$^+$ cell products were administered after RIC in four adult patients with severe β-thalassemia genotypes. No SAEs or unexpected safety issues related to the cell product or the conditioning were observed (Supplementary Table 3). Late effects included gonadal failure. Cumulative busulfan area under the curve (AUC) exposures ranged from 39.8 to 59.7 mg × L h^{-1}, within the non-myeloablative range[28]. All other recent and ongoing globin gene therapy trials in patients with TDT rely on myeloablative conditioning, using either busulfan or treosulfan–tiotepa[35–37].

RIC aims to minimize transplant-related morbidity and mortality related to severe short-term toxicities, such as prolonged

cytopenia, mucositis and, in case of busulfan conditioning, VOD. It is well established that the risk of VOD decreases when lowering busulfan administration[38–40]. None of our four patients developed VOD, in contrast to seven cases reported from globin gene therapy trials relying on myeloablative busulfan conditioning (Supplementary Table 8). Sub-myeloablative conditioning also shortened hospitalization and time to platelet engraftment (Supplementary Table 8), which was similar to that recorded in the GLOBE trial (14–25 d and 10–24 d, respectively). The time to platelet engraftment is particularly important in patients with thalassemia, who are often refractory to platelet transfusion and at risk for severe bleeding while in aplasia[41]. RIC is especially appealing in patients with underlying health conditions, such as patients with hemoglobinopathies. RIC has been shown to result in fewer median hospital days and a lower overall cost compared to myeloablative conditioning[42,43].

Alkylating agents also expose to the long-term risk of secondary malignancies. Although few secondary malignancies have been reported in patients with thalassemia who underwent allogeneic HSCT after myeloablative conditioning[44], recent reports of two patients who developed myelodysplastic syndromes/acute myeloid leukemia after lentiviral vector-mediated globin gene therapy for SCD (NCT02140554) raised concern regarding the risk of myeloid neoplasms in patients with SCD[45]. Several potential causes, such as insertional mutagenesis[45], alkylating agents[46] or a generally increased risk for myeloid neoplasms in SCD due to the constant hematopoietic hyperplasia[46–49], have been proposed to increase the chance of somatic mutations and result in transformation of myeloid precursors.

Long-term engraftment of up to 8 years of follow-up was remarkably stable in all patients, albeit with a moderate discrepancy between the DP VCN and the VCN in the engrafted cells (Fig. 1). Gene marking stabilizing at a lower level than the VCN measured in the cell infusion product is common in gene therapy trials. An ongoing phase 1/2 study in SCD recently showed, in three patients, that RIC allows for successful engraftment of HSCPs transduced with a lentiviral vector encoding a modified γ-globin gene[50]. Interestingly, a third patient who was given better transduced CD34$^+$ HSPCs showed 2–4× higher gene marking of PBMCs after 6 months, supporting our interpretation that it is the transduction efficiency rather than the conditioning intensity that precluded achieving transfusion independence in our cohort.

Therapeutic efficacy of globin gene therapy for β-thalassemia aims to achieve durable transfusion independence, which we did not reach in these four patients. The cell product size (median, 9.0×10^6 CD34$^+$ cells per kg; Table 2) and rapid engraftment after conditioning (Extended Data Fig. 1) argue against cell dosing being limiting. The vector function was robust, based on extensive preclinical evaluation[20–22,26] and limited measurements of vector performance in BFU-E and bone marrow erythropoiesis (Extended Data Figs. 2, 3b and 4). These findings argue, as discussed below, that rate limiting transduction was, therefore, the most likely explanation for not achieving transfusion independence in this cohort.

Three other phase 1/2 clinical trials investigating the globin lentiviral vectors BB305 and GLOBE recently reported on clinical outcomes and provided VCN measurements. Both vectors are variants of TNS9.3.55, with GLOBE omitting the HS4 LCR element. In HGB-204/-205, 15 of the 22 patients infused with BB305-transduced autologous CD34$^+$ cells became transfusion independent[35]. In the GLOBE trial, four of the six pediatric patients became transfusion independent but none of the three adult patients did[51,52]. The patients in whom transfusion therapy was suspended all received a cell product with a VCN greater than 0.6 in the HGB trials and greater than 0.7 in the GLOBE study. We did not achieve the same level of transduction, owing to impurities in the vector lot that precluded increasing the multiplicity of infection (our vector stock, produced earlier in time, was not chromatography purified), only achieving a median DP VCN of 0.15.

Based on VCN measurements in bone marrow and PBMCs, the lowest gene marking associated with transfusion independence was 0.8 in the GLOBE study[52] and 0.3 in the BB305 trials[35]. In our patients, the highest in vivo VCN reached in PBMCs was 0.11. In the two patients who sustained a VCN greater than 0.05 for extended periods, transfusion requirements were diminished but not abrogated.

The minimum VCN requirement, however, depends on the severity of the β chain deficit, determined by the patient's genotype. In the HGB-204/-205 studies, 12 of the 13 patients with a non-β^0/β^0 genotype stopped transfusions after treatment, whereas only three of the nine patients with a severe genotype (β^0/β^0 or IVSI-110/IVSI-110) discontinued transfusions. Three of the four patients in our trial had a severe genotype—one patient with β^0/β^0 mutations and two patients with a β^0/β^+ genotype of equivalent severity (IVSI-110). Patient 2 had a milder β^0/β^+ genotype (IVSI-6) and showed the best response. The combined data from the HGB, GLOBE and MSKCC studies suggest that a minimum PBMC VCN of at least 0.3 for vectors encoding HS2, HS3 and HS4 LCR elements is required in patients with milder TDT to achieve transfusion independence and that a VCN of 0.05–0.3 will only result in a reduction of transfusion needs in patients with severe genotypes. The GLOBE study suggests that a VCN greater than 0.8 might be necessary for vectors that only encode HS2 and HS3.

Additional factors other than VCN, β chain output per VC and genotype likely determine response to therapy. In the GLOBE trial, the only patients who achieved transfusion independence were the pediatric patients. Of three patients who received a cell product with a VCN of 0.7, only the pediatric patient, but not the two adults, achieved transfusion independence. In our trial, Patient 2, who had the best response, was one of the youngest patients (age 18 years). In addition to the patient's age, splenectomy might be another contributing factor. The patient with the best clinical response in our trial was the one who was previously splenectomized. Nearly half of the patients in the HGB-204/-205 studies were also splenectomized. It is conceivable that diminished splenic entrapment might result in improved engraftment, which might also be enhanced by intra-osseous cell infusion[52].

The regular monitoring of vector integration sites in peripheral blood samples showed a polyclonal profile without extensive (>20%) clonal dominance. However, several clones accounting for more than 1% of circulating genetically modified cells were detected in all patients (Fig. 3b).

Integration site mapping revealed significant skewing toward cancer-related genes, as has been previously observed in persisting clones of either hematopoietic or T cell origin[34]. The most prominent clone, accounting for up to 17.5% of all genetically marked cells in Patient 4 after 5.75 years, harbored a vector integration in proximity to *UBR2*, which encodes an E3 ubiquitin ligase implicated in hyperproliferation, chromosome instability and hypersensitivity to DNA damage-inducing reagents and was found to be overexpressed in multiple cancers[30]. Clustering of integration sites was observed in the first intron of *STAT3*, a location previously implicated in insertional mutagenesis and clonal expansion in studies of HIV[32,33]. Integrations reported in six patients from the HGB and GLOBE trials showed a lower frequency of integrations near cancer-related genes, but those were reported at earlier time points (1.5–2 years after infusion) relative to ours after 4–7 years (Supplementary Table 9).

A case of insertional mutagenesis[53] was previously documented in a patient with thalassemia who was treated with the HPV569 vector, a precursor to BB305 (ref. [54]). In this patient, an expanded clone harboring the globin vector integrated in the *HMGA2* locus showed a 10,000-fold increase in expression of HMGA2 transcripts in erythroblasts, resulting from a combination of deregulated

post-transcriptional regulation and increased transcription[53]. *HMGA2* overexpression was later shown to expand mouse lineage-negative bone marrow HSCs as well as human umbilical cord stem and progenitor cells in mice transplanted with these constructs[55–57]. Some LCR elements, in particular HS1 and HS2, are active in hematopoietic progenitor cells[58,59], which provides a potential mechanism for the trans-activation of cancer-related genes. Additional studies in murine hematopoietic chimeras will be useful to assess the non-erythroid transcriptional activity of regulatory LCR elements contained in globin vectors.

Although our associations with clonal expansion are correlative, *UBR2* and *STAT3*, like *HMGA2*, might thus have been activated by the non-erythroid transcriptional activity of globin vector elements, accounting for their favored capture in our integration site analysis.

In summary, our studies establish that a DP VCN of 0.15 is not sufficient to achieve transfusion independence in TDT. Other studies have shown that a DP VCN of at least 0.3 is needed in patients with mild genotypes. Our study, which, to our knowledge, is the only one to not have relied on myeloablative conditioning to maximize $CD34^+$ cell engraftment, further suggests that reduced-intensity conditioning is sufficient to achieve stable, long-term HSC engraftment in patients with TDT and could be potentially curative, provided that higher VCNs are achieved. This approach should be prospectively evaluated. Finally, our analyses of integration site distributions emphasize the importance and urgency of carefully monitoring clonal expansions in patients with TDT or SCD who have been treated with lentiviral globin vectors.

Online content

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Methods

Study design and oversight. This is an open-label, phase 1 clinical trial (NCT01639690) for the treatment of TDT with autologous CD34+ HSPCs transduced with TNS9.3.55, a lentiviral vector encoding the normal human β-globin gene. This protocol was reviewed and approved by the National Institutes of Health Recombinant DNA Advisory Committee in 2007, approved by MSKCC's institutional review board (IRB) in 2010 and approved by the FDA in 2012. Patients were enrolled from 19 September 2012 (Patient 1) to 29 January 2014 (Patient 4). The clinical trial was initiated in 2012. Study enrollment, mobilization, collection and transduction of peripheral blood HSPCs, patient conditioning and transplantation were performed at MSKCC. Patients are followed at their center of origin at the Cervello Hospital in Palermo, Italy, and the Microcitemie Pediatric Hospital in Cagliari, Italy.

The primary endpoints of this phase 1 trial are the safety and tolerability of (1) the infused autologous CD34+ hematopoietic cells transduced with lentiviral vector TNS9.3.55, as defined by the insertional oncogenesis and the generation of an RCL, and (2) the low-dose non-myeloablative conditioning regimen.

Secondary endpoints included the level of engraftment of the genetically modified autologous CD34+ cells, the expression of the transduced β-globin gene and the post-transplant transfusion requirements. Clinical information and in vivo biological material from all patients treated in this trial were collected and analyzed during the years 2012–2021 in adherence to informed consent. Primary and secondary endpoints are further detailed in Supplementary Table 10.

Patients. Patients were screened for the inclusion criteria as specified on the IRB-approved protocol, and eligible patients signed a consent form. All volunteers were evaluated with a medical history and physical examination. Baseline laboratory tests, including complete blood count and differential, reticulocyte count, iron, total iron-binding capacity, transferrin saturation, ferritin, coagulation profile, blood chemistry studies (electrolytes and renal function tests), ABO blood typing and testing for infectious disease markers, were performed. Female patients of childbearing age underwent a serum pregnancy test. Adverse events during and after therapy were assessed according to the National Institutes of Health Common Terminology Criteria for Adverse Events, version 4.0.

Patients eligible on this protocol were 18 years or older, of any gender or ethnic background, with a diagnosis of β-thalassemia major of any genotype and with a transfusion requirement of more than 100 ml per kg per year for at least 2 years. Patients with HLA-matched siblings were excluded. Patient eligibility included adequate organ function, with a Karnofsky performance score of more than 70%. The inclusion and exclusion criteria can be found at ClincalTrials.gov (NCT01639690). Informed consent was obtained for all patients. Although the Recombinant DNA Advisory Committee supported enrollment of patients aged 15 and older, the FDA requested initial enrollment of adult patients only (https://osp.od.nih.gov//wp-content/uploads/RAC_Minutes_Jun_2007.pdf).

Trial procedures. HSPC collection was performed by apheresis after G-CSF mobilization, as previously described[26], with a goal of a minimum of 8×10^6 CD34+ cells per kg. CD34+ HSPCs were selected using the CliniMACS Plus system[60], pre-simulated and transduced with current good manufacturing practice (cGMP)-grade TNS9.3.55 vector stocks at the MSKCC Cell Therapy and Cell Engineering Facility. After busulfan conditioning, patients were infused with two graft aliquots, the first aliquot on day 0 and the second on day 2. The cell infusion product consisted of autologous TNS9.3.55-transduced CD34+ cells. A non-selected backup cell dose of a minimum of 2×10^6 CD34+ cells per kg was cryopreserved and stored for rescue in case of graft failure. All treatment-related adverse events occurred as expected in an autologous HSCT setting and were managed according to institutional standards. Additional details regarding the trial procedures are listed at ClinicalTrials.gov (NCT01639690).

Mobilization and apheresis. The entire procedure of mobilization and apheresis was performed in the outpatient clinic. Patients received G-CSF at a dose of 10 μg kg^{-1} once daily subcutaneously for 6 d. Patients underwent specific monitoring of side effects and adverse events and complete blood counts on days 1, 3, 5 and 6 of mobilization. Apheresis was performed via two peripheral veins when available ($n=4$) or via a central venous catheter when intravenous access was insufficient to allow for the insertion of a large-bore intravenous access catheter ($n=1$). Each patient underwent leukapheresis with a collection goal of 8×10^6 CD34+ cells per kg. Leukapheresis were performed on the mornings of the fifth and sixth day of G-CSF treatment on a continuous flow cell separator according to institutional standards, using acid citrate dextrose for anti-coagulation and calcium prophylaxis to prevent citrate toxicity. The volume of blood processed per leukapheresis session was approximately three times the total blood volume as tolerated by the patient. CD34+ cells were positively selected using a CliniMacs Plus system as per standard operating procedures in the Cell Therapy Laboratory at MSKCC. The final number of collected CD34+ HSPCs per patient was measured by cell counting and fluorescence-activated cell sorting (FACS) analysis.

Busulfan regimen. Patients received pharmacokinetic-adjusted, single-agent intravenous busulfan conditioning. Patients 1, 2 and 3 received intravenous busulfan at a planned non-myeloablative dosing of 2 mg per kg per dose q 12 h × 4 doses (planned total dose of 8 mg kg^{-1} over 2 d), with a target first dose AUC of 2,800 μM × min. Patient 4 received 0.8 mg per kg per dose q 6 h × 14 doses (planned total dose of 11.2 mg kg^{-1} over 4 d), with a target first dose AUC of 1,100 μM × min. Busulfan dosing was adjusted in Patients 3 and 4 according to the first-dose pharmacokinetic analysis to achieve the targeted AUC. The cumulative busulfan exposure (mg × L h^{-1}) was calculated by the sum of the daily extrapolated AUC measurements and indicated that all four patients had received non-myeloablative conditioning.

Engraftment criteria. Neutrophil engraftment was defined as ANC exceeding 500 μl for three consecutive days. The first of these three consecutive days was considered the day of engraftment. Platelet engraftment was defined as the first of three consecutive days with a platelet count exceeding 20,000 μl without platelet transfusion support in the past seven consecutive days.

Post-transplant transfusion requirements. Before transplant, all patients were transfused according to the standard clinical practice for thalassemia at their thalassemia center, taking into consideration individual clinical conditions. The target Hb transfusion levels were 9 for Patients 1, 2, and 4, based on their institutional standards, and were 10 for Patient 3, also according to their standards. The transfusion history before and after infusion documented each pre-transfusion Hb, individual transfusions, milliliters of packed RBCs infused and actual patient body weight. The transfusion volume per year was recorded as ml of blood per kg per year, and the mean daily decrease in Hb (%) was calculated.

Patients were screened for deletional and non-deletional hereditary persistence of fetal hemoglobin. As characteristically seen during bone marrow regeneration after hematopoietic stem cell transplantation[61], a moderate, transient increase in fetal hemoglobin (HbF) was observed in all patients in the first 3–14 months after infusion (Supplementary Table 11). Of note, the results of the Hb transfusion reduction described in this manuscript are based on the last follow-up and HbF at pre-infusion levels for all patients.

After transplant, the target Hb transfusion level was temporarily decreased in all patients, as shown in Supplementary Table 12, but eventually changed back to the pre-transplant Hb transfusion levels. The results of the Hb transfusion reduction as reported in this manuscript were based on the Hb levels equivalent to the pre-transplant ones.

Globin lentiviral vector. The TNS9.3.55 lentiviral vector encodes the wild-type β-globin chain under the erythroid-specific transcriptional control of the human β-globin promoter and three segments of the LCR known as HS2, HS3 and HS4 (ref. [20]). Clinical-grade TNS9.3.55 vector stock was produced under cGMP conditions at the Center for Biomedicine and Genetics in Duarte, California. TNS9.3.55 vector stocks were titrated on a quality-controlled cell bank of HeLa cells at various dilutions (in the range of 1/5 to 1/12,500) of the vector. Titers were calculated using consecutive dilutions that yield linear data. The HeLa titer of the cGMP vector lot was 3.5×10^8 transduction units per ml.

Transduction and VCN quantification. CD34+ HPCs were cultured for 18–24 h in serum-free X-VIVO 10 supplemented with human stem cell factor, Flt-3 ligand, thrombopoietin and interleukin-3. Fractions were subsequently cultured for 14–16 d in liquid erythroid cultures (ECs) or hematopoietic colony assays for VCN quantification by quantitative PCR using the Applied Biosystems 7500 real-time PCR system.

RCL analysis. RCL assays were performed at the Indiana University Vector Production Facility according to FDA guidelines by PCR, as previously described[62].

CD34+ HSPC transduction. Selected CD34+ HSPCs were released to the MSKCC Cell Therapy and Cell Engineering Facility where they were either pre-simulated immediately or after overnight storage at 4 °C for 18–24 h in serum-free X-VIVO 10 supplemented with 100 ng ml^{-1} of human stem cell factor (SCF), human Flt-3 ligand, human thrombopoietin and 20 ng ml^{-1} of human IL-3. Before pre-stimulation, a fraction of the cells was also tested for colony formation potential in methylcellulose colony-forming unit (CFU) assays. CD34+ HSPCs underwent two rounds of transduction 18–24 h apart using GMP TNS9.3.55 vector stocks. Cells were subsequently cultured in serum-free X-VIVO 10 with the same cytokines. On day 0 and at the end of the cultures, CD34+ HSPCs were inoculated into methylcellulose for colony formation. Transduced CD34+ HSPCs were cryopreserved on day 2 or day 3. Aliquots were subsequently differentiated for 14–16 d to the erythroid lineage or plated for colony differentiation before extracting genomic DNA (gDNA) for VCN measurement.

Erythroid differentiation and CFU assay. CD34+ cells were seeded at $0.5–1\times10^6$ ml^{-1} in a six-well plate containing Alpha MEM (Sigma), FBS (STEMCELL Technologies), glutamine (Gibco), BSA (Sigma), human erythropoietin (Amgen), β-mercaptoethanol (Gibco), dexamethasone (American Regent), holo-transferrin (American Regent) and human recombinant SCF (Sigma). Cells were counted and fed from day 4 to day 10 with the same EC differentiation medium. This differentiation process was used for in vitro modelling of erythropoiesis and VCN determination. For CD34+ cells before and after transduction, CD34+ cells

were plated at 500 cells per plate in MethoCult H4435 Enriched (STEMCELL Technologies). BFU-E, CFU-M, CFU-G and CFU-GM were distinguished and counted after 14–16 d as per the manufacturers' directions.

VCN quantification. gDNA was extracted using the Gentra Puregene Kit (Qiagen) as per the manufacturer's directions. Next, 100 ng of gDNA was used for real-time PCR reaction. For gDNA extraction from colony-forming cells (CFCs), isolated colonies were aspirated with a pipette tip under the microscope, washed in PBS and suspended into 25 µl of proteinase K (Roche) containing lysis buffer (14 mM Tris HCl, pH 8.3, 2.5 mM $MgCl_2$, 105 mM KCl, 0.3 mg ml^{-1} of gelatin, 0.45% Tween 20, 0.45% NP40 and 0.2 mg ml^{-1} of proteinase K) in 96-well plates. Plates were incubated at 55 °C for 60 min and 95 °C for 15 min to end the reaction and kept at 4 °C until use within 48 h. Similarly to Charrier et al.[63], we found that vector frequency is underestimated in CFUs when compared to liquid EC from which gDNA is extracted with the Puregene Kit. DP VCN in the cell infusion product was assessed in all CFCs. Quantification of the TNS9.3.55 lentiviral VCN was performed using the Applied Biosystems 7500 real-time PCR system. Average TNS9.3.55 VCN per cell was calculated by normalizing to the endogenous *ALB* gene. Analysis was performed using 7500 System SDS software, version 1.4.0 (Applied Biosystems).

Bone marrow studies. The bone marrow studies, agreed to by participating patients on a voluntary basis after month 12, ceased after month 60. We are grateful to the patients for kindly donating bone marrow, and we understand their cessation after 5 years on the study (except Patient 3, who returned once to New York City for a bone marrow aspiration at month 75).

HPLC analysis. Erythroid colonies were generated and collected as described above. Half of the material was processed for gDNA extraction, and the other half was washed twice with PBS and lysed in water by three rapid freeze–thaw cycles, followed by centrifugation at 16,000*g*. Supernatants were recovered and stored in liquid nitrogen before HPLC analysis. HPLC analysis was performed as previously described[64].

Bone marrow flow cytometry. Bone marrow erythroblasts from Patient 2 were analyzed at three consecutive time points for surface expression of glycophorin A (GPA), α4 integrin and band 3, as previously described[29].

The following flurophore-conjugated antibodies were used: anti-human APC-α4 integrin (clone MZ18-24A9, Miltenyi Biotec, no. 130-124-008, 1:100) and anti-human PE-glycophorin A (GPA or CD235a, clone JC159, DakoCytomation, no. R7078, 1:100). Mouse monoclonal antibodies against the extracellular regions of human band 3 were generated by the New York Blood Center (1:100).

Data were collected using FACSDiva software, version 7.0 (BD Biosciences), on a FACSCanto flow cytometer and analyzed using FlowJo software, version 7.6.5.

Integration site analysis. Integration site analysis was carried out using ligation-mediated PCR essentially as described[65,66]. DNA samples were fragmented by sonication, and then DNA adaptors were ligated to the free DNA ends. Two rounds of PCR were then carried out, amplifying from the adaptor to the LTR of the lentiviral vector. All samples were worked up in quadruplicate to suppress the effects of PCR jackpotting. Sequences were aligned to the hg38 draft human genome assembly. Clonal abundance was quantified using the SonicAbundance method[67]; in this method, the points of linker ligation associated with each unique integration site are quantified and used as an abundance estimate—this is more accurate than counting sequence reads, where abundance is subject to distortion during PCR steps. For the analysis of association with cancer-associated genes, all cells sampled were used for the statistical comparison (sonic abundance), and not the counts of unique sites, to better reflect the clonal structure. Sequence samples studied and their sonic abundance, number of unique sites and population metrics are in the Supplementary Report on the analysis of the frequency of integration near cancer-related genes (database Fisher report). All sequences acquired in this study are available under National Center of Biotechnology Information Sequence Read Archive accession number PRJNA705203.

Statistical analysis. The reported means, medians and ranges are provided for descriptive purpose. The individual transfusion requirements (in ml of blood per kg of body weight per year) were calculated before (mean volume per year over 5 years before cell infusion and after). The transfusion requirement follow-up was updated as of 31 December 2020. Statistics of integration site analysis are outlined in the Results.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. All requests for raw and analyzed data and materials are promptly reviewed by the corresponding author to verify if the request is subject to any intellectual property or confidentiality obligations. Patient-related data not included in this paper were generated as part of clinical trials and might be subject to patient confidentiality. Any data and materials that can be shared will be released via a material transfer agreement.

The following databases were used for oncogene definitions: the 'Bushman lab oncogenes database' (http://www.bushmanlab.org/links/genelists, version 5, June 2021) and four levels of The Cancer Genome Atlas version of the OncoVar database (https://oncovar.org, version 1.2, August 2020).
Sequence data were deposited in the National Center of Biotechnology Information's Sequence Read Archive (SRA BioProject PRJNA705203). For integration site analysis relative to Gene Ontology, the 'GO.db' Bioconductor annotation data package, version 3.13.0, was used.

Code availability

Source code for manuscript analysis has been deposited in an archived format in the Zenodo code base (https://doi.org/10.5281/zenodo.4569099).

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Author contributions

F.B., A.M., P.M., S.A., S.P., S.R., R.B., R.D.M. and L.P. were involved in patient care. X.W., J.Q., K.T., F.D. and L.S. performed transductions and biosafety assays. F.K. and C.T. assembled and analyzed clinical data. J.M.S. and A.C. reviewed vector and sequence data. J.K.E., P.H., A.M.R., V.A.C., H.A., S.R. and F.D.B. conducted vector integration site analyses. J.K.E. and F.D.B. modeled and analyzed IS data. A.G. oversaw HPLC analysis. E.B. oversaw HPLC studies. N.M. oversaw bone marrow FACS analyses. F.B., A.M., I.R. and M.S. designed the study. F.B., A.M., F.K., F.D.B., I.R. and M.S. wrote the manuscript.

Competing interests

The authors declare no competing interests. The TNS9.3.55 vector technology has been granted to Errant Gene Therapy without financial compensation.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41591-021-01554-9.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41591-021-01554-9.

Correspondence and requests for materials should be addressed to Michel Sadelain.

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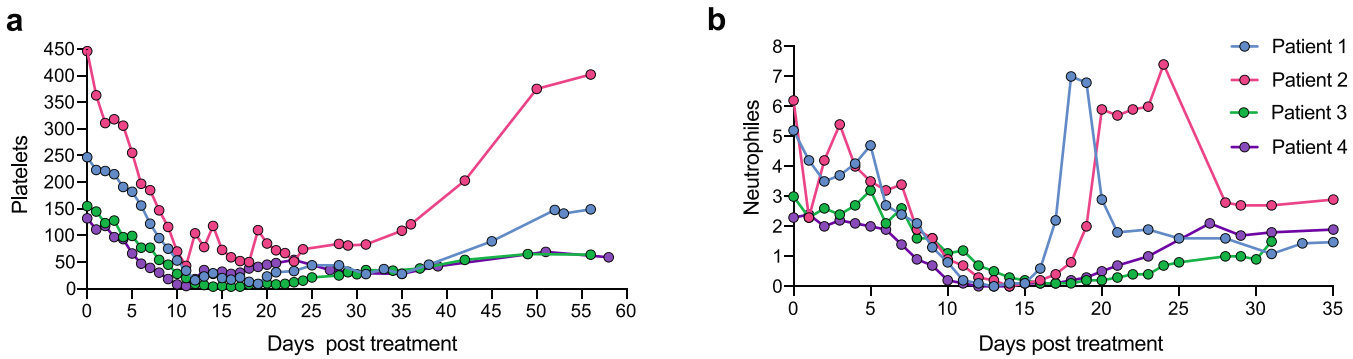
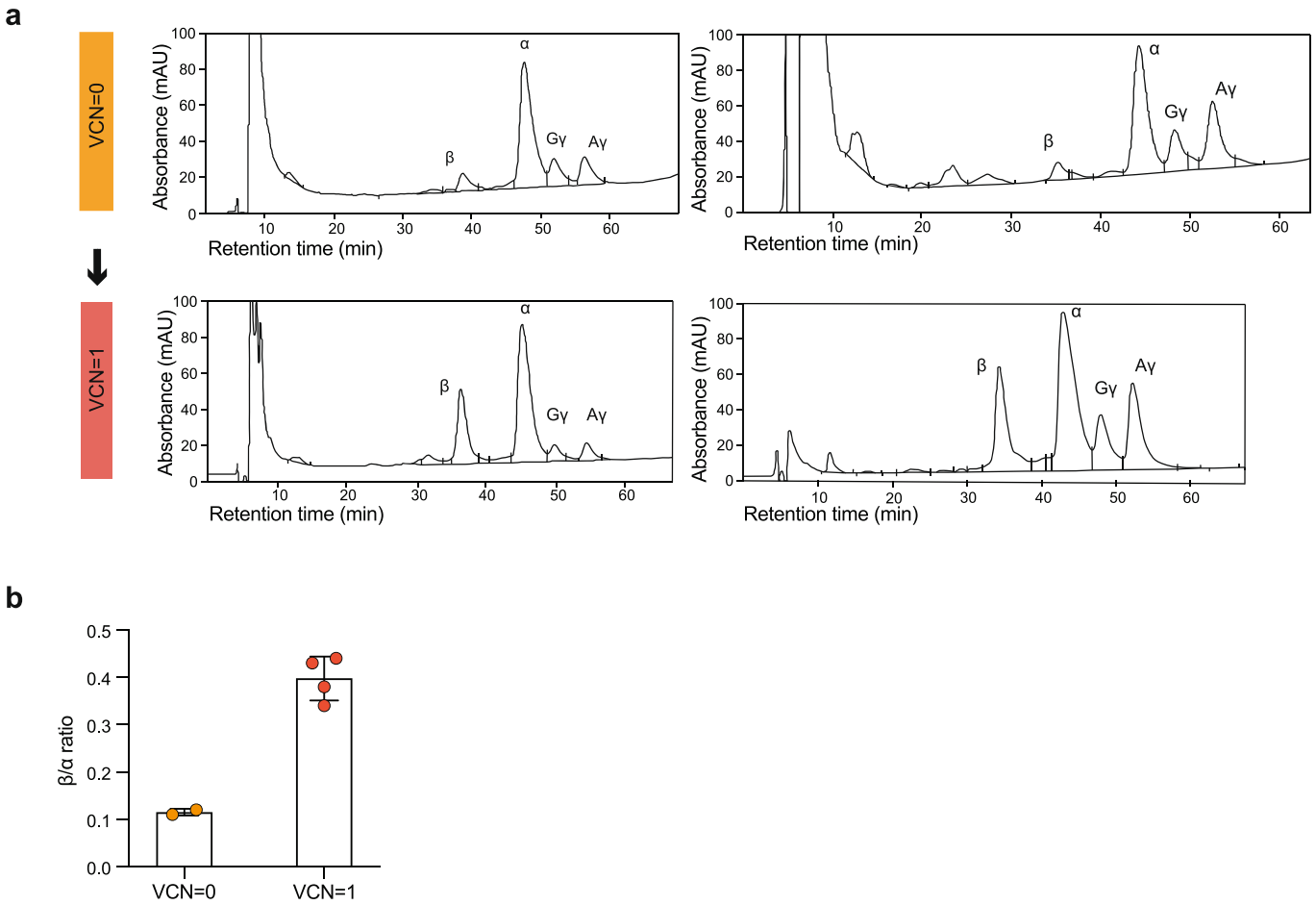
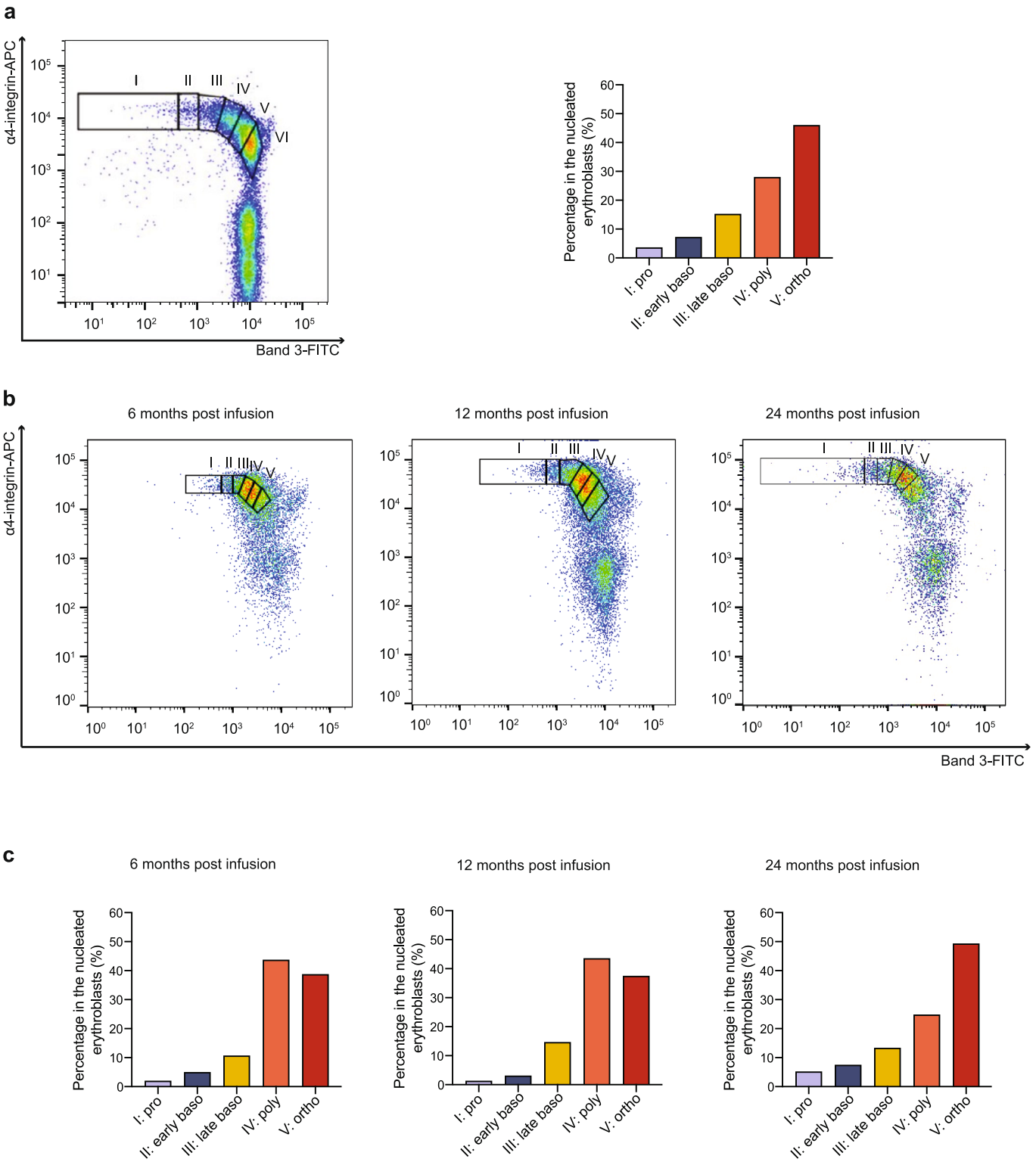


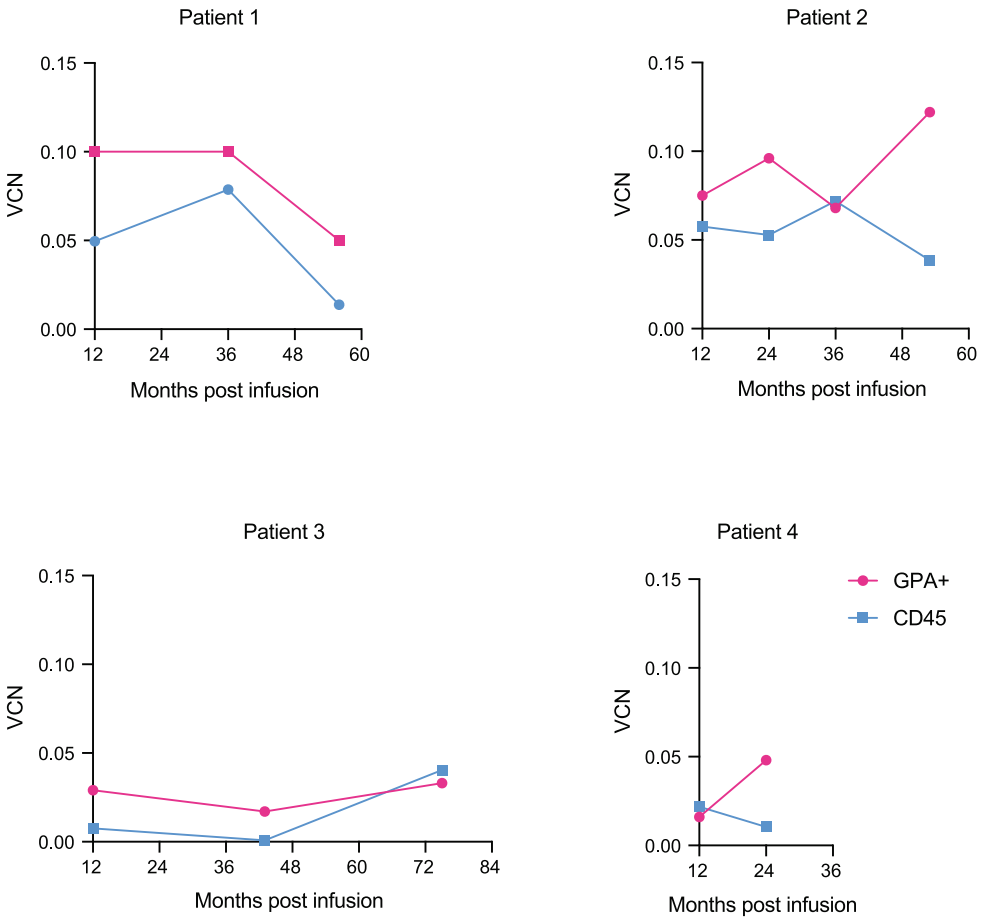
Extended Data Fig. 1 | Cell count recovery after conditioning and infusion of TNS9.3.55 transduced CD34 + HSPCs. a, Platelet count (x10^9/l) after infusion. **b**, Absolute neutrophil count (x10^9/l) after infusion. Granulocyte colony stimulating factor (G-CSF) was administered for Patient 1 (day 13-16) and Patient 2 (day 16-18) during aplasia. Patient 1 received methylprednisolone intravenously for 3 consecutive days (day 16-18) for treating the engraftment syndrome.



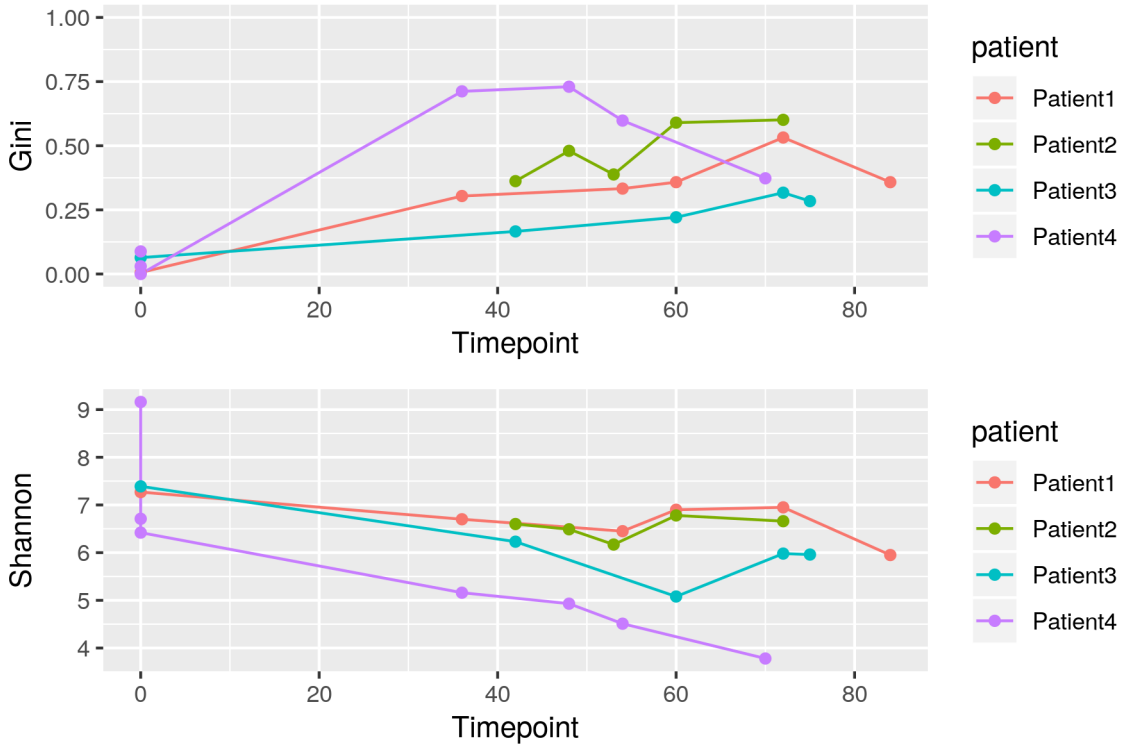
Extended Data Fig. 2 | β-globin expression in Patient 2 at 12 months post infusion. a, HPLC chromatograms illustrating globin production of erythroid cells from four individual BFU-Es derived from bone marrow obtained from Patient 2 at 12 months post infusion. Top chromatograms: two representative examples from two individual, non-transduced BFU-E from Patient 2; lower chromatograms: two representative examples from two individual, transduced BFU-E from Patient 2. **b**, β-globin to α-globin ration in erythroids derived from untransduced and transduced HSCs obtained from Patient 2 at 12 months post infusion. The β/α expression ratio determined by HPLC in single BFU-Es increased from a mean of 0.11 to 0.38 in BFU-Es harboring a single copy of the integrated vector, representing a gain of 0.27. Results from BFU-Es derived from untransduced HSCs (n = 2; VCN = 0) and from BFU-Es derived from transduced HSCs (n = 4; VCN = 1). All data are mean ± s.e.m.



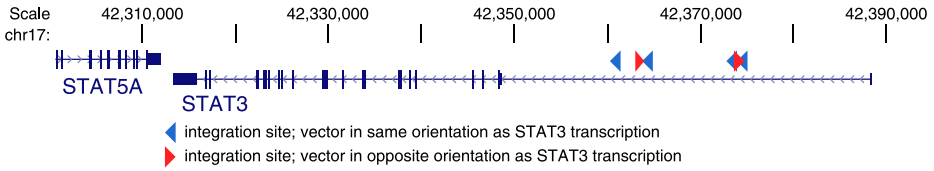
Extended Data Fig. 3 | Erythropoietic maturation in bone marrow (Patient 2). Terminal erythroid differentiation begins with proerythroblasts differentiating into basophilic, then polychromatic, then orthochromatic erythroblasts that enucleate to become reticulocytes. Each distinct stage of terminal human erythroid differentiation can be distinguished using a combination of cell surface markers for glycophorine A (GPA), band 3 and α4 integrin. **a**, Representative flow cytometry plot of band 3 vs α4-integrin of GPA + cells in normal erythropoiesis: proerythroblasts (I), early basophilic (II), late basophilic (III), polychromatic (IV), and orthochromatic erythroblasts (V) and reticulocytes (VI). The box plot represents the quantitation of the proportion of cells at each distinct stage of maturation after normalization based on total nucleated erythroid cells (I-V) as 100% as described in ref. [29]. The left panel is adapted from ref. [29]. **b**, Bone marrow erythroblasts from Patient 2 were analyzed by flow cytometry at 6, 12 and 24 months post infusion stained with GPA, α4-integrin, and band 3. Plot of band 3 vs α4-integrin of GPA + cells represents the quantitation of distinct stages of maturation of erythroblasts as described in a. c, Quantitation of the proportion of cells at each distinct stage of maturation after normalization to total nucleated erythroid cells (I-V).



Extended Data Fig. 4 | Engraftment of transduced cells in bone marrow. Vector copy number (VCN) in erythroid glycophorine A + (GPA +) cells and CD45 + cells sorted from bone marrow of patients.



Extended Data Fig. 5 | Gini and Shannon Index values. Timepoints in months.



Extended Data Fig. 6 | Annotation of the genes *STAT3* and *STAT5A* on chromosome 17. Illustration of a cluster of transgene integrations in the first intron of *STAT3*. Six integration sites were detected at year six, zero were detected pre-transplant. Four out of six integration sites detected were in the same transcriptional orientation as *STAT3*.

nature research

Corresponding author(s): Michel Sadelain

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Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
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☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	7500 System SDS software v1.4.0. (Applied Biosystems) for real-time PCR for VCN quantification. FACSDiva software version 7.0 (BD) on a FACSCanto flow cytometer for flow cytometry data collection.
Data analysis	The FlowJo software version 7.6.5 for flow cytometry analysis. Microsoft Excel Office365 and GraphPad Prism V9.0.1 were used for data analysis and graphical output.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. All requests for raw and analyzed data and materials are promptly reviewed by the corresponding author to verify if the request is subject to any intellectual property or confidentiality obligations. Patient-related data not included in the paper were generated as part of clinical trials and may be subject to patient confidentiality. Any data and materials that can be shared will be released via a material transfer agreement.

The following databases were used for For oncogenes definitions: the "Bushman lab oncogenes database" (http://www.bushmanlab.org/links/genelists, v5 June 2021) and four levels of The Cancer Genome Atlas (TCGA) version of the OncoVar database (https://oncovar.org, v1.2 August 2020).
Sequence data were deposited in the NCBI's Sequence Read Archive (SRA BioProject PRJNA705203). For IS analysis relative to Gene ontology the "GO.db" Bioconductor annotation data package (v3.13.0) was used.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The intended-to-treat (ITT) set included 10 patients. The sample size for this phase I clinical trial was recommended and approved by the NIH's Recombinant DNA Advisory Committee (RAC) and was based on feasibility. The sample size (n=4) reflects all patients treated in this trial. Five Patients were initially enrolled in this trial, with one patient declining to participate after mobilization and apheresis, and four patients receiving busulfan followed by cell product infusion. The vector stock was used up in preparation of these cell products. The patient's clinical characteristics are provided in Table 1. All 4 patients infused in this trial are included in the analysis. Clinical information and in vivo biological material were collected and analyzed during the years 2012-2021 in adherence to informed consent.
Data exclusions	Out of a planned 10 patients, 5 patients were enrolled in this study. All patients enrolled in this study underwent mobilization and apheresis. One patient declined to participate in the study after mobilization and apheresis. All remaining 4 patients were infused with the cell products. This report includes data on all treated patients.
Replication	4 patients were infused with cell products. We included all individual results on all treated patients.
Randomization	This is a phase I single arm clinical study. There was no randomization.
Blinding	This is a phase I single arm clinical study. There was no blinding.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Human research participants
☐	☒ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	The following flurophore-conjugated antibodies were used: anti-human APC-α4 integrin (clone MZ18-24A9, Miltenyi Biotec, #130-124-008, 1:50), anti-human PE-glycophorin A (GPA or CD235a; clone JC159, Dako Cytomation, #R7078, 1:100). Mouse monoclonal antibodies against the extracellular regions of human band 3 were generated by the New York Blood Center (1:50).
Validation	All used antibodies were titrated. All antibodies were validated for use in flow cytometry. Data and references for use of these anti-human antibodies in independent peer-reviewed studies are available on the manufacturer's website and in Hu et al. 2013.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	HeLa cells purchased from ATCC were passaged and transduced with lentiviral vector TNS9.3.55. Transduced cells were cloned and a clone with single TNS9.3.55 lentiviral vector incorporation in the genome was identified and selected as the HeLa single copy control cell line.
Authentication	One genome copy incorporation of TNS9.3.55 vector in the parental HeLa cell genome was verified by Southern Blot analysis. Genomic DNA from HeLa one copy control cell line was digested with enzymes such as EcoR I, Nhe I, or Nar I, or XbaI was hybridized with 32P-labeled vector specific probes
Mycoplasma contamination	Cell lines were routinely tested for mycoplasma and were found to be negative,
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used

Human research participants

Policy information about studies involving human research participants

Population characteristics	The 4 treated patients included 2 females and 2 males, age 18–39 years at the time of cell infusion. All patients were affected by transfusion-dependent β-thalassemia. One patient had a β0/β0 genotype and three patients a β0/β+ genotype.
Recruitment	Patients with severe transfusion dependent β-thalassemia were made aware of this protocol via national and international thalassemia associations and direct presentations by the investigators at hematology and thalassemia meetings. Investigators at the thalassemia centers at the Cervello Hospital in Palermo (N=4) and Microcimetie Hospital in Cagliari (n=1) recruited the four patients enrolled on this trial. All patients interested in participation in the trial received information material and were screened for eligibility criteria as specified on the approved study protocol. Patients were enrolled on the basis of the predefined inclusion and exclusion criteria. Written informed consent was obtained from eligible patients in accordance with the Declaration of Helsinki. There was no participant compensation.
Ethics oversight	The protocol and informed consent documents were reviewed and approved or accepted by the US National Institutes of Health Recombinant DNA Advisory Committee (RAC) in 2007, local Institutional Review Board (IRB) at Memorial Sloan Kettering Cancer Center, and by the Food and Drug Administration (FDA) in 2012.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	NCT01639690
Study protocol	The full protocol is available upon reasonable request to the corresponding author.
Data collection	The lentiviral globin gene therapy, including mobilization, apheresis, conditioning and cell infusion, was performed at the Bone Marrow Transplant Service, at the Department of Pediatrics, Memorial Sloan Kettering Cancer Center. Gene therapy Patient 1, 2 and 4 were treated at their thalassemia center of origin at the Cervello Hospital in Palermo, Italy between 2007 to present. Patient 3 was treated at his thalassemia center of origin at Microcimetie Pediatric Hospital in Cagliari, Italy between 2008 and present. Clinical data, including the transfusion requirement data for each patient pre and post gene therapy were collected by the thalassemia centers to present.
Outcomes	Primary outcomes were safety and tolerability of the infusion of autologous CD34+ hematopoietic cells transduced with TNS9.3.55, measured as the occurrence of insertional oncogenesis and the generation of a replication-competent lentivirus, and safety and tolerability of the low dose non-myeloablative conditioning regimen. Secondary outcome measures were the level of engraftment and post-transplant transfusions requirements. These outcomes were reviewed and approved by by the NIH's Recombinant DNA Advisory Committee (RAC) and represent standard safety considerations in any HSC-based gene therapy trial.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Cells were purified from bone marrow from patient 2 at consecutive timepoints by negative selection by using CD45 microbeads (Miltenyi) according to the manufacturer's instructions. 0.1×10e6 isolated CD45- cells were suspended in 20 µL FACS Buffer, antibodies were added and washed off after the incubation time of 15 minutes on ice. The samples were analyzed within 1 hour after staining.
Instrument	FACSCanto flow cytometer
Software	FACSDiva software (BD), FlowJo software.
Cell population abundance	The progression of erythroid maturation in the bone marrow of patient 2 was assessed based on the expression levels of α4 integrin and band in CD45- primary human bone marrow erythroblasts Quantitation of the proportion of cells at each distinct stage of maturation (proerythroblasts, early basophilic, late basophilic, polychromatic, and orthochromatic erythroblasts) was normalized based on total nucleated erythroid cells as 100%, as shown in Extended Data Fig. 3 and described in Hu et al. 2013.
Gating strategy	CD45- primary human bone marrow erythroblasts were stained with GPA, α4 integrin, and band 3 and gated based on the expression levels of α4 integrin and band 3 as described in Hu et al. 2013.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.