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CRISPR–Cas9-mediated gene editing of the *BCL11A* enhancer for pediatric β^0/β^0 transfusion-dependent β-thalassemia

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Gene editing to disrupt the GATA1-binding site at the +58 *BCL11A* erythroid enhancer could induce γ-globin expression, which is a promising therapeutic strategy to alleviate β-hemoglobinopathy caused by *HBB* gene mutation. In the present study, we report the preliminary results of an ongoing phase 1/2 trial (NCT04211480) evaluating safety and efficacy of gene editing therapy in children with blood transfusion-dependent β-thalassemia (TDT). We transplanted *BCL11A* enhancer-edited, autologous, hematopoietic stem and progenitor cells into two children, one carrying the β^0/β^0 genotype, classified as the most severe type of TDT. Primary endpoints included engraftment, overall survival and incidence of adverse events (AEs). Both patients were clinically well with multilineage engraftment, and all AEs to date were considered unrelated to gene editing and resolved after treatment. Secondary endpoints included achieving transfusion independence, editing rate in bone marrow cells and change in hemoglobin (Hb) concentration. Both patients achieved transfusion independence for >18 months after treatment, and their Hb increased from 8.2 and 10.8 g dl^{-1} at screening to 15.0 and 14.0 g dl^{-1} at the last visit, respectively, with 85.46% and 89.48% editing persistence in bone marrow cells. Exploratory analysis of single-cell transcriptome and indel patterns in edited peripheral blood mononuclear cells showed no notable side effects of the therapy.

β-Thalassemia is an inherited hemolytic disease that is prevalent worldwide and is one of the most common monogenic diseases[1]. Over 200 mutations in the *HBB* gene, which encodes the β-subunit of hemoglobin A (HbA), result in β-thalassemia. These mutations can cause reduced (β^+) or loss (β^0) of β-chains of Hb synthesis, which leads to an imbalanced ratio of the α-chain and β-chain globin that constitutes Hb, disruption of red blood cell (RBC) generation, chronic hemolysis and microcytic anemia[2–4]. Due to the severe lack of functional β-globin, a considerable number of patients require regular transfusion of blood cells to survive, resulting in TDT.

As a result of the limited blood resources and the high cost of iron chelators, the survival rate of TDT patients in China is much lower than that in developed countries (~1% aged >20 years in China[5] versus ~55% aged >30 years in the UK[6]). Even the remaining 1% of patients are not eligible for hematopoietic stem cell transplantation because of worsening conditions such as iron overload. In many developing countries, including China, survival is better in children. As pediatric and adolescent patients have better tolerance to treatment-related toxicity of hematopoietic stem cell transplantation (HSCT), eligible patients should receive HSCT as early as possible (aged 2–7 years)[7]. The exploration of gene-editing therapy in children with TDT is critical and urgent for those unmet medical needs of children in developing and underdeveloped regions.

In some patients with β-thalassemia, continuous expression of fetal Hb (HbF) to adulthood (for example, hereditary persistence of HbF) can alleviate the symptoms of anemia, which suggests that using HbF to replace functionally impaired HbA may be a feasible treatment for β-thalassemia[8–10]. Genome-wide association studies and animal experiments have confirmed the negative regulatory function of *BCL11A* on the expression of γ-globin[9,11,12]. Cas9:single guide (sg)RNA ribonucleoprotein (RNP)-mediated disruption of the GATA1-binding site at the +58 *BCL11A* erythroid enhancer results in the reduction of *BCL11A* expression and the induction of fetal γ-globin, which is a practicable therapeutic strategy for treating β-thalassemia and sickle cell disease[13–17]. Recently, the results of two clinical studies have shown that gene editing or lentiviral small hairpin (sh)RNA for downregulation of *BCL11A* can successfully induce HbF expression, thereby curing β^0/β^+ TDT and sickle cell disease[16,18]. However, it is not clear whether the HbF induced by the *BCL11A* enhancer editing is sufficient to cure the most severe β^0/β^0 patients lacking β-globin chain expression, nor is this therapy safe for children. In the present study, we transplanted autologous hematopoietic stem and progenitor cells (HSPCs) with a clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9-edited *BCL11A* enhancer into two children with β-thalassemia (patient 1: β^0/β^0; patient 2: β^+/β^+), resulting in durable HbF induction. Moreover, we performed single-cell RNA-sequencing (scRNA-seq) of peripheral blood mononuclear cells (PBMCs) for comprehensive analysis of the transcriptomes of the blood lineages reconstituted from unedited or edited HSPCs, indicating that *BCL11A* enhancer editing did not lead to substantial transcriptional changes in

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Table 1 | Characteristics of patients and gene-editing outcomes

Patient no.	Patient 1	Patient 2
Age (years)	7	8
Sex	male	male
Genotype	41-42M/17M (β^0/β^0)	IVS2-654M and -28M (β^+/β^+)
Race	Asian–Chinese	Asian–Chinese
Transfusion frequency (d) and volume before transplantation (U)	~20, 2	~15–20, 2
Gene-editing efficiency (%)	93.50	94.50
Number of CD34$^+$ cells in infusion (per kg)	11.8×10^6	3.89×10^6
Neutrophil implantation time (days after transplantation)	14	17
Platelet implantation time (days after transplantation)	52	40
Discharge time (d)	52	40
Time of last blood transfusion after transplantation	Day 29	Day 19

nonerythroid cells. Overall, our data suggested that reactivation of HbF by CRISPR–Cas9 gene editing has curative potential even for β^0/β^0 thalassemia patients.

Patient demographics and clinical outcome measurement

Previously, it was demonstrated that highly efficient *BCL11A* enhancer editing in CD34$^+$ HSPCs by electroporation of SpCas9 RNP was a realistic and scalable strategy for durable HbF induction in treating β-hemoglobinopathies[12,14,19]. On the basis of these results, we designed a gene therapy clinical trial for patients with TDT using CRISPR–Cas9-mediated disruption of the +58 *BCL11A* erythroid enhancer, resulting in induction of fetal γ-globin. In the trial, appropriate patients were enrolled if they had received a diagnosis of β-thalassemia major with either homozygous or compound heterozygous mutations. Eligibility criteria were designed to select patients, and additional details about the inclusion and exclusion criteria are provided on the ClinicalTrials.gov database (NCT no. NCT04211480). The study was approved by the Medical Ethics Committee of Xiangya Hospital of Central South University in China. From December 2019 to December 2021, two patients with β-thalassemia were treated in the present study (Table 1 and Supplementary Table 1). The duration of follow-up after transplantation was 18 months. Patient 1 was aged 7 years and 41-42M/17M (mutations in *HBB*: codons 41-42, -TTCT; codon 17 A-T) with an annualized red-cell transfusion history of about 36 units per year over 2 years before consent. Patient 2 was aged 8 years and IVS2-654M/-28M (mutations in *HBB*: IVS2-654 C-T; -28 A-G) with an annualized red-cell transfusion history of about 42 units per year over 2 years before consent (see Table 1 for more patient information). The serum ferritin levels before treatment were 1,190 μg l^{-1} in patient 1 and 2,143 μg l^{-1} in patient 2. Neither patient underwent splenectomy.

The present study began on 30 December 2019, when both patients and their legal guardians first provided their signed written informed consent. Patient 1 was enrolled on 10 January 2020 and patient 2 on 13 March 2020. With regard to the six patients who were recruited but did not receive gene-editing therapy, three did not meet the criteria due to testing positive for Epstein–Barr virus (refer to the description of inclusion/exclusion criteria in the protocol in Supplementary information for details). Another failed to meet the required number of cells after two rounds of mobilization and chose a haploidentical HSCT on the advice of the attending physician. On the other hand, taking into consideration safety, we planned to arrange for the patients to receive gene-editing treatment in batches. With regard to the other two enrolled patients, their parents were unwilling to wait because the observation period of the first batch of subjects was long. They changed their mind before mobilization, quit the study and opted for a haploidentical HSCT. All six patients who quit the study received haploidentical HSCT and are currently alive and under follow-up.

We performed the present study to evaluate the safety and feasibility of transplantation starting with isolation of CD34$^+$ HSPCs from mobilized peripheral blood of two TDT patients (patient 1 with 41-42M/17M (β^0/β^0) and patient 2 with IVS2-654M/-28M (β^+/β^+)). Autologous CD34$^+$ HSPCs were isolated from patients by apheresis after mobilization with filgrastim. More than 5×10^6 CD34$^+$ cells per kg were harvested after mobilization with filgrastim (a granulocyte–colony-stimulating factor (G-CSF)). Subsequently, CRISPR–Cas9-edited CD34$^+$ HSPCs were manufactured from these isolated CD34$^+$ cells by electroporation with CRISPR–Cas9 and a modified synthetic sgRNA (2′-*O*-methyl-3′-phosphorothioate modifications in the first and last three nucleotides). Both Sanger DNA-sequencing (DNA-seq) and deep DNA-seq were used to evaluate the percentage of allelic editing at the on-target site. Finally, CRISPR–Cas9, gene-edited, autologous CD34$^+$ cells were infused into these two patients. Both patients received single-agent, pharmacokinetically adjusted, intravenous busulfan myeloablation for 4 consecutive days before the infusion. The patients received a single intravenous infusion of edited autologous CD34$^+$ HSPCs. Additional information about the production and infusion of CRISPR–Cas9-treated CD34$^+$ HSPCs is provided in Table 1.

After the transplantation, engraftment and AEs were monitored. Neutrophil engraftment occurred on day 14 for patient 1 and day 17 for patient 2, respectively. Platelet engraftment occurred on days 52 and 40 for patients 1 and 2, respectively, after transplantation (Table 1). The time of neutrophil and platelet engraftment of gene-edited HSPCs was comparable with that of autologous stem cell transplantation[20]. White blood cell (WBC) and lymphocyte counts increased after transplantation (Fig. 1a,b), accompanied by the recovery of the cell counts of other subtypes, and were stabilized in a normal range (Fig. 1c–f and Extended Data Fig. 1). Full donor chimerism was achieved at week 4 after transplantation and persisted through the most recent time point, 18 months after transplantation (Table 2). Hepatic toxicity or veno-occlusive disease was not observed. Both patients had grade 4 neutropenia lasting for 6 and 8 d and platelet transfusion-supported grade 4 thrombocytopenia. The patients were discharged from the hospital 52 and 40 d after transplantation and were clinically well at the last visit. Chemotherapy-related toxicity was mild, consisting of grade 1–2 mucositis, grade 1 skin rash and grade 2 epistaxis. No serious infections were recorded. One patient developed catheter-related thrombosis on day 50, which resolved after treatment with rivaroxaban for 2 weeks (Extended Data Tables 1 and 2). Immune thrombocytopenic purpura (ITP), a rare but well-recognized post-autologous HSCT complication[21], was diagnosed from 82 d post-infusion for patient 1. It was classified as a serious AE because the platelet count dropped down to a low of 29×10^9 l^{-1} on day 89 post-infusion. There were no bleeding symptoms. The diagnosis of ITP was supported by a positive antiplatelet antibody test, normal platelet morphology by blood smear and an abundance of megakaryocytes by bone marrow aspiration examination. The platelet

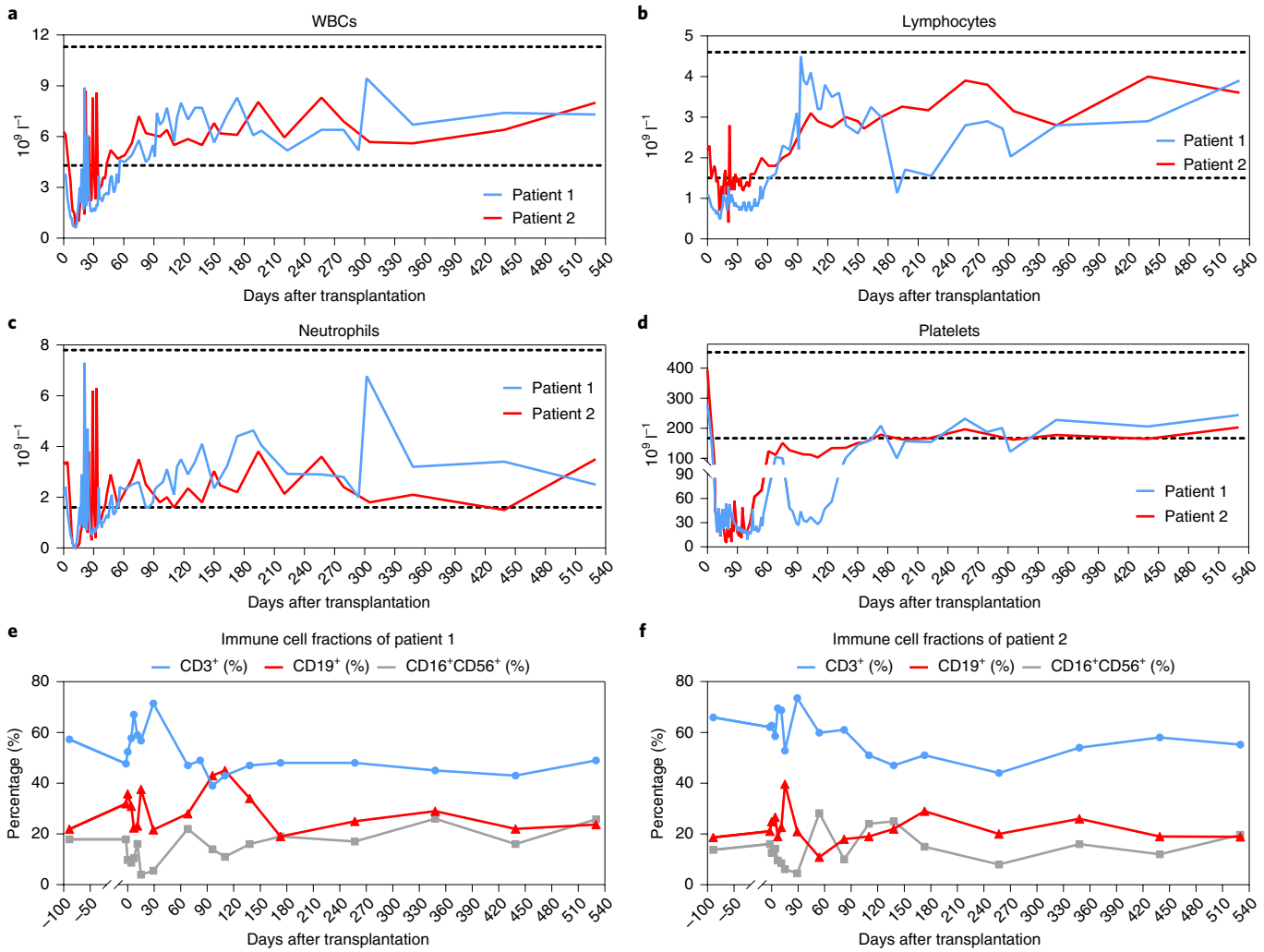

Fig. 1 | Hematological data before and after transplantation. a–d, The cell counts of WBCs (**a**), lymphocytes (**b**), neutrophils (**c**) and platelets (**d**) in the peripheral blood of each patient over time, with blue indicating patient 1 and red patient 2. The dashed lines indicate the normal range for each value. **e**,**f**, The fluctuations in the immune cell fraction for each patient before and after transplantation are shown below with patient 1 in **e** and patient 2 in **f**.

counts recovered and stabilized in a normal range after treatment with prednisone (Fig. 1d and Extended Data Table 1). A proposed mechanism for post-HSCT ITP in patients with thalassemia is that they may have greater pre-HSCT antigen exposure secondary to transfusion burden[22].

Allelic editing of the *BCL11A* enhancer and the HbF level in the peripheral blood of both patients were monitored and analyzed. Peripheral blood of patients 1 and 2 was obtained monthly in the early stage after infusion. Tests were conducted including total Hb, Hb fractions analyzed with high-performance liquid chromatography (HPLC) and the percentage of HbF$^+$ cells (F-cells) from circulating erythrocytes defined by flow cytometry analysis, and the fraction of total DNA that was edited at the on-target site in PBMCs was measured by Sanger sequencing and deep DNA-seq. Deep sequencing was performed to measure indels in CIRCLE-seq identified or computationally predicted, candidate off-target regions and to assess the risk of off-targeting editing.

Exploratory and nonprespecified analyses were performed, including indel patterns analysis of editing outcome in PBMCs, nonhomologous end-joining (NHEJ)/microhomology-mediated end-joining (MMEJ) repair category, transcriptional analysis of *BCL11A* enhancer editing and genotyping analysis of patient single CD34$^+$ cell expansions.

Efficacy of the *BCL11A* enhancer-editing therapy

After transplantation, the number of RBCs and overall Hb levels in both patients began to increase steadily at about day 45 until they reached healthy levels at about day 75 (Fig. 2a,b). To evaluate whether *BCL11A* enhancer-edited HSPCs could result in clinically meaningful γ-globin induction in our patients, we calculated the Hb levels. For patient 1, levels of HbF increased rapidly from 0.26 g dl^{-1} at baseline to 9.70 g dl^{-1} at month 2, 12.01 g dl^{-1} at month 3 and 15.98 g dl^{-1} at month 18 (Fig. 2c). F-cell expression increased from 8% at baseline to 99.4% at month 18 (Fig. 2c). For patient 2, levels of HbF increased from 0.18 g dl^{-1} at baseline to 7.64 g dl^{-1} at month 2, 12.25 g dl^{-1} at month 3 and 14.27 g dl^{-1} at month 18 (Fig. 2b,c). F-cell expression increased from 10% at baseline to 99.6% at month 18 (Fig. 2d). The patients received their last transfusion of packed RBCs 29 d and 19 d, respectively, after the infusion of edited CD34$^+$ cells. Their Hb level normalized to >12.00 g dl^{-1} at month 3 and remained normal through month 18, their most recent study visit. Reverse phase-HPLC analysis of globin chains was conducted in two patients at months 9, 15 and 18. It was found that the two patients had equal amounts of α- and non-α-chains in their blood after treatment compared with those of a healthy donor (Extended Data Fig. 2). Both patients were under iron chelation, which was stopped 1 week before transplantation, and patient 2

Table 2 | Key laboratory values before and after transplantation

Measure	Reference interval	Patient 1					Patient 2					
		Screening	After transplantation				Screening	After transplantation				
			Mo1	Mo2	Mo3	Mo15	Mo18		Mo1	Mo2	Mo3	Mo15	Mo18
WBCs (10^9 l^{-1})	4.3–11.3	4.8	1.8	4.5	4.8	7.4	7.3	5.7	4.1	4.9	6.8	6.4	8
RBCs (10^{12} l^{-1})	4.2–5.7	2.84	2.79	3.47	3.94	5.69	5.92	3.92	3.09	4.14	4.58	4.88	5.19
Hb (g l^{-1})	118–156	82	88	105	117	145	150	108	92	124	139	131	140
Platelet count (10^9 l^{-1})	167–453	276	21	70	27	206	244	293	18	124	151	165	203
Hematocrit (%)	36–46	–	25.4	31.2	32.8	40.3	42.7	33	27	38	37.9	37.8	40.5
Neutrophil count (10^9 l^{-1})	1.6–7.8	2.7	0.8	2.3	2.3	3.4	2.5	1.8	2	2.2	3.03	1.5	3.5
Lymphocyte count (10^9 l^{-1})	1.5–4.6	1.7	0.8	1.5	2.2	2.9	3.9	3.4	1.5	1.8	2.9	4	3.6
Eosinophil count (10^9 l^{-1})	0–0.68	0.1	0.03	0.14	0.01	0.46	0.35	0.1	0	0.29	0.37	0.37	0.37
Basophil count (10^9 l^{-1})	0–0.07	0	0.01	0.02	0.01	0.05	0.05	0	0	0.04	0	0.04	0.05
Monocyte count (10^9 l^{-1})	0.13–0.76	0.3	0.2	0.5	0.2	0.6	0.5	0.4	0.5	0.7	0.5	0.5	0.6
Mean corpuscular volume (fl)	77–92	83.8	90.9	89.9	83.2	70.8	72.1	84.2	87.4	91.8	82.7	77.5	78
Mean corpuscular Hb (pg)	25–34	28.9	31.4	30.3	29.7	25.5	25.3	27.6	29.9	30	30.3	26.8	27
Mean corpuscular Hb concentration (g l^{-1})	310–355	345	345.6	337	357	360	351	327	342	326	367	347	346
RBC volume distributing width (%)	<15.0	11.9	13.1	19.9	18.9	19.2	20.3	15.7	13.2	18.5	17.3	17.5	18.8
Plateletcrit (%)	0.18–0.22	0.31	0.02	–	–	0.19	0.21	0.29	0.02	–	0.13	0.16	0.17
Mean platelet volume (fl)	7.6–13.2	11.1	7.59	–	–	9.4	8.8	9.9	9.23	–	8.4	9.6	8.6

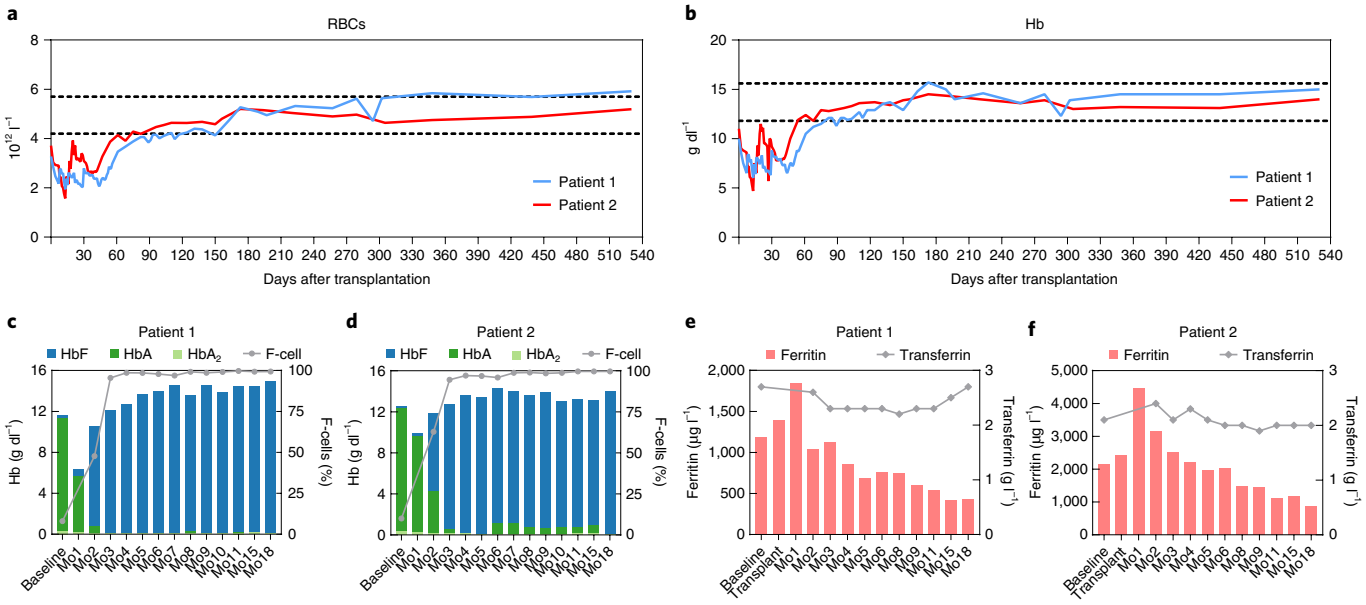

Fig. 2 | Key laboratory values of clinical outcomes. a, The counts of RBCs over time for patient 1, who was β^0/β^0 (indicated in blue), and patient 2, who was β^+/β^+ (indicated in red). **b**, About 2 months after transplantation, consistent levels of Hb were observed, with blue indicating patient 1 and red patient 2. The dashed lines indicate the normal range for each value. **c**,**d**, Representation of Hb adducts (left *y* axis) and the changes in the percentages of F-cells (circulating red cells that express HbF) over time (right *y* axis) for patient 1 (**c**) and patient 2 (**d**). **e**,**f**, The progressive decrease in the ferritin level (left *y* axis) and change of transferrin level (right *y* axis) before and after transplantation are shown in **e** for patient 1 and in **f** for patient 2.

was treated with iron chelation for a short time on days 55–79 after transplantation (Extended Data Table 3). After transplantation, iron metabolism was substantially improved in both patients (Fig. 2e,f). Serum ferritin levels in patient 1 had decreased from 1,190.0 µg l^{-1} before treatment to 432.8 µg l^{-1} at 18 months after transplantation, which was close to the normal interval of 16–400 µg l^{-1}. Serum ferritin levels in patient 2 had decreased by 58.81% at 18 months compared with those before treatment, from 2,143.0 µg l^{-1} to 882.6 µg l^{-1}.

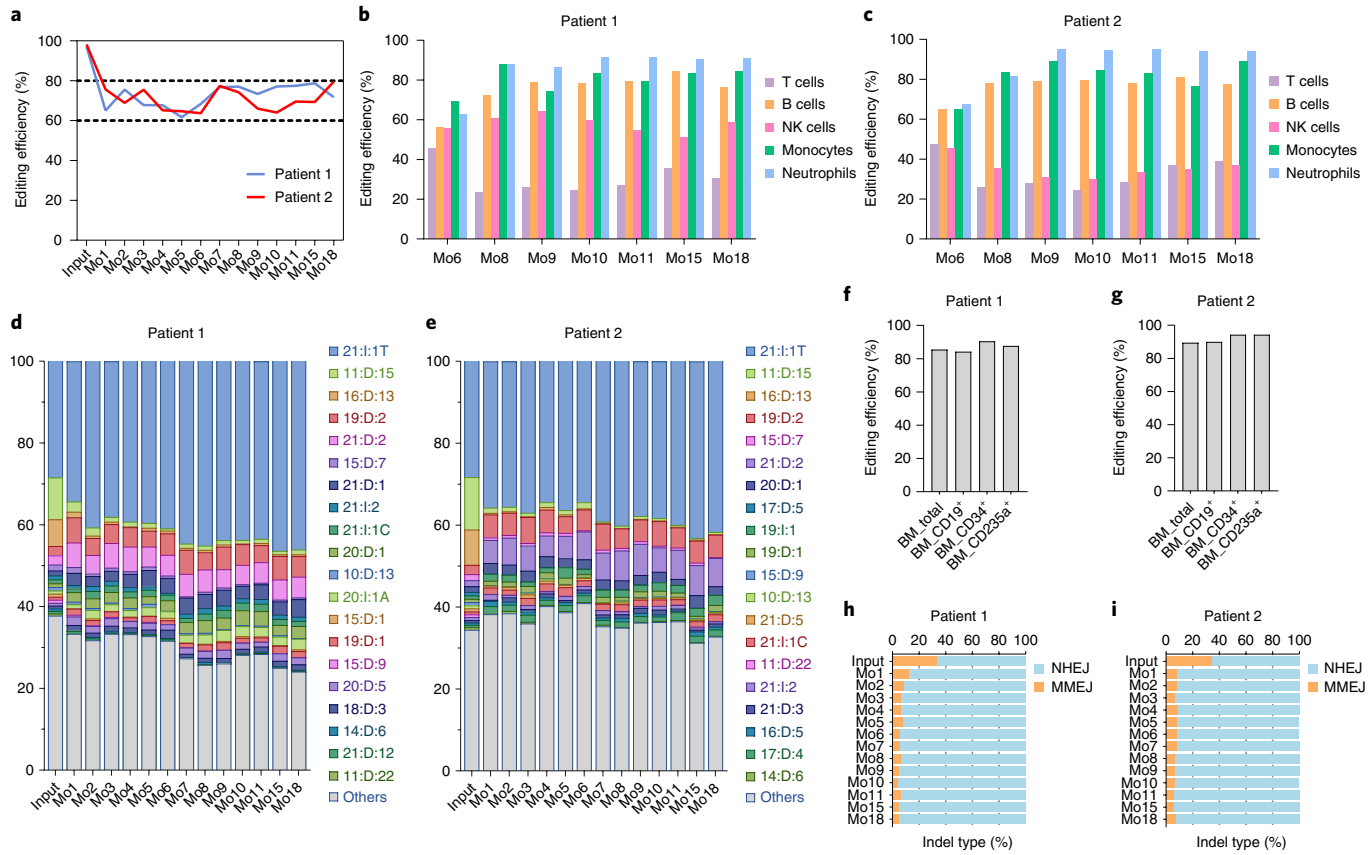

Fig. 3 | Gene-editing efficiency and outcome analysis. a, Frequencies of allelic editing in input and nucleated peripheral blood cells at various times after transplantation, with blue indicating patient 1 and red patient 2. **b**,**c**, Frequencies of allelic editing in cells of various types from peripheral blood samples obtained 6, 8, 9, 10, 11, 15 and 18 months after transplantation in **b** (for patient 1) and **c** (for patient 2). **d**,**e**, Percentage stacked histograms show the distribution of top 20 indel patterns in PBMCs from patient 1 (**d**) and patient 2 (**e**) over time. (They are shown as position: deletion (D) or insertion (I): base count and name; protospacer adjacent motif position is defined as 21–23.) **f**,**g**, The editing frequency of various types of cells in the bone marrow of patients 1 (**f**) and 2 (**g**) at Mo9 after transplantation. **h**,**i**, Relative loss of edited alleles repaired by MMEJ (−9 to −20 bp) and gain of edited alleles repaired by NHEJ (−8 to +6 bp) in transplanted cells over time (for patient 1 in **h**, patient 2 in **i**).

The transferrin content of patients fluctuates in the normal range (2.0–4.0 g g l^{-1}) most of the time.

BCL11A enhancer gene editing

The RNP editing rates of the *BCL11A* enhancer were 97.17% for patient 1 and 98.22% for patient 2, as indicated by deep sequencing. CIRCLE-seq and in silico prediction were previously used to identify 24 potential off-target sites (Supplementary Table 2)[14]. Amplicon deep sequencing of each of these 24 off-target sites from CD34$^+$ HSPCs edited with Cas9 RNP or edited PBMCs at month 6 (Mo6) from the patients did not identify any that were off-target at the limit of detection of 0.01% allele frequency (Supplementary Tables 2 and 3).

Consequently, the proportion of *BCL11A* enhancer-edited alleles in the genome of PBMCs ranged from 61.72% to 79.55% during the 18-month long-term engraftment (Fig. 3a). The representative types of *BCL11A* enhancer mutation are shown in Extended Data Fig. 3. Deep sequencing was used to determine the indel rates in multiple hematopoietic lineages, including T cells, B cells, natural killer (NK) cells, monocytes and neutrophils (Extended Data Fig. 4). As myeloablative conditioning did not remove the original T cells and NK cells in the thymus and spleen, and it has been reported previously that the first phases of hematopoietic reconstitution are most probably sustained by lineage-committed progenitors that are possibly primed during in vitro manipulation[23–25], the editing frequencies of circulating T cells and NK cells were lower than the other lineages and fluctuate during early hematopoietic reconstitution (Fig. 3b,c). The gene-editing frequency of each lineage became stable after Mo6, indicating that steady-state hematopoiesis is reached at 6–12 months[23]. We closely monitored the changes of the top 20 indel patterns of input edited HSPCs in the progeny cells of edited HSPCs in PBMCs and found no substantial pattern expansion, suggesting that clonal expansion may not occur in the 18 months after transplantation (Fig. 3d,e). High frequencies of edited *BCL11A* enhancer alleles were detected in multiple blood lineages sampled from PBMCs and bone marrow cells (Fig. 3b,c,f,g). We then obtained single CD34$^+$ cells by limiting dilution and subjected these single cells to in vitro erythroid differentiation and genotyping. It was found that 88.3% ($n=77$) and 79.8% ($n=104$) of the CD34$^+$ cells of the two patients were biallelic editing (Extended Data Fig. 5), which is consistent with the result that the vast majority of erythrocytes showed strong HbF expression (Fig. 2c and Extended Data Fig. 6). These results collectively showed that CRISPR–Cas9-edited HSPCs successfully engrafted and differentiated into multiple lineages that retained the gene editing. It has been reported that NHEJ may be favored relative to MMEJ repair in the long term, repopulating HSPC populations relative to the bulk HSPC population by mice engraftment analysis[14]. We found similar results by analyzing the indel spectrum in PBMCs from the patients. Although the indel patterns were diversified in the total PBMCs or other subpopulations, the fraction of 15-bp and 13-bp

deletions (together comprising 25% of alleles of the input HSPCs) decreased substantially in the engrafted PBMCs. These data further support engrafted HSCs appearing to favor NHEJ compared with MMEJ repair (Fig. 3h,i and Supplementary Figs. 1 and 2).

Transcriptional impact of *BCL11A* enhancer editing

To study the transcriptional impact of *BCL11A* enhancer gene editing, we performed scRNA-seq on PBMCs. Using microfluidics-based scRNA-seq, we obtained in total six peripheral blood single-cell

Fig. 4 | Transcriptional impact of *BCL11A* enhancer editing in PBMCs from treated patients at single-cell resolution. a, The uniform Manifold Approximation and Projection (UMAP) plot representing 13 color-coded cell clusters identified in merged single-cell transcriptomes of PBMCs from two heathy donors, one patient sample before treatment and three patient samples at different time points after treatment. Cluster names were manually assigned. **b**, Stacked bar plots showing distribution of the identified clusters (**a**) across samples. **c**, Violin plots showing *BCL11A* expression (log(transformed)) for the B-cell or pDC clusters from edited and unedited groups, both showing no difference (B cells, Wilcoxon's two-sided test, $P=0.51$; pDCs, Wilcoxon's two-sided test, $P=0.58$). **d**, Heatmap depicting average expression of selected genes related to B-cell development and proliferation in the B-cell cluster. Expression values were normalized per gene with 0 reflecting the lowest expression and 2 the highest expression. **e**, Plot showing differential gene expression with fold-change ($\log_2$(FC)) between unedited ($n=3$) and edited ($n=3$) plotted versus $-\log_{10}$(FDR). Aggregation of single-cell transcriptome data to pseudo-bulk data for each respective sample was used for analysis. No differentially expressed genes (false discovery rate (FDR) < 0.5) were identified. **f**, Violin plots showing *IGHD* and *IGHM* expression (log(transformed)) for the B-cell cluster from the edited and unedited groups. Both show no significant difference (*IGHD*, Wilcoxon's two-sided test, $P=0.11$; *IGHM*, Wilcoxon's two-sided test, $P=0.016$). **g**, At Mo9 after transplantation, the relative expression of *BCL11A* gene in B cells ($CD19^+$) and erythroid cells ($CD235a^+$) in the bone marrow of both patients.

transcriptomes from two heathy donors (10× indicated data downloaded from 10 Genomics shared dataset), one patient sample before treatment and two patient samples after treatment. Genes detected per donor were 19,620 in the total PBMCs. After filtering out RBCs and cells with low-quality transcriptomes, we obtained transcriptome datasets from 18,536 cells of six samples (Supplementary Table 4). Clustering of merged PBMCs was inferred from the annotation of cluster-specific genes as demonstrated previously. Based on the marker gene expression, we identified $CD4^+$ T cells (interleukin-7 receptor (IL-7R) and CD4), activated $CD8^+$ T cells (CD8a; CD8B and CCL5), nonactivated $CD8^+$ T cells (CD8na; CD8B and CCR7), regulatory T cells (FOXP3 and CTLA4), NK-cell cluster (GNLY and NKG7), B-cell cluster (MS4A1), plasma blasts (plasma; CD38 and TNFRSF17), myeloid lineage cells (LYZ) separated into myeloid dendritic cell (mDC) cluster (FCER1A, CD1C and CLEC10A), granulocytes (S100A8 and S100A9), monocyte cell cluster (CD14) and plasmacytoid DCs (pDCs; TCF4 and TNFRSF21) (Fig. 4a and Extended Data Fig. 7a). We found frequency variations of the subpopulations in each PBMC sample, but they were in the normal range such as lymphocytes (T cells, B cells and NK cells), being present in the range 70–90%, whereas B cells were in the range 5–20% and DCs in the range 1–2% as reported (Fig. 4b and Extended Data Fig. 7b).

BCL11A encodes for a zinc-finger transcription factor that plays an important role in B-cell and pDC development[26,27]. Although the +58 *BCL11A* enhancer we disrupted was reported as an erythroid specific enhancer[9], we needed to further evaluate the potential downregulation of *BCL11A* in other subpopulations of edited PBMCs. By analyzing the B-cell and pDC subpopulations, we found comparable expression of the *BCL11A* transcript in edited B cells and pDCs in comparison with cells from healthy donors or a patient before treatment (Fig. 4c). In addition, differential expression analysis was performed comparing B lymphocytes from the unedited group (two samples from heathy donors, a sample from the patient before treatment) and the edited group (three samples from patients after treatment) using a gene set of B-cell development and function (Fig. 4d and Extended Data Fig. 7c). By statistical analysis, we did not find any genes with significant changes in the gene-edited group (Fig. 4e). An immunoglobulin (Ig) is the antigen-recognition molecule of B cells. We therefore looked more closely at the expression of Ig heavy constant delta (*IGHD*) and Ig heavy constant mu (*IGHM*) and found comparable expression in the gene-edited and -unedited groups (Fig. 4f). By analyzing the percentage of edited alleles in each subpopulation, the B-cell populations ($CD19^+$) also showed editing rates similar to the editing rates of total PBMCs (Fig. 3a–c), indicating that the proliferation and development of B cells with disrupted *BCL11A* enhancer was not impaired. The reverse transcription quantitative PCR (RT–qPCR) results of bone marrow cells from patients at Mo9 after transplantation also showed that *BCL11A* expression was downregulated in $CD235a^+$ cells, but not in $CD19^+$ cells, compared with healthy individuals (Fig. 4g). Overall, these data suggested that autologous HSPCs with *BCL11A* enhancer editing do indeed support blood lineage reconstitution after infusion without undesirable impacts.

Discussion

Our results provide preliminary evidence that the CRISPR–Cas9-based *BCL11A* enhancer-editing strategy would benefit even the patients with the most severe β^0/β^0 TDT, indicating that this treatment strategy has the potential to address the underlying cause of TDT, regardless of genotype.

In conclusion, our study provides proof of principle that transplantation and long-term engraftment of CRISPR–Cas9-edited autologous HSPCs can be achieved, and the sustained elevations in HbF levels were substantial enough to ameliorate the severity of TDT, even for the β^0/β^0 genotype with no β-globin chains. Although both patients in the present study successfully induced robust γ-globin expression and got rid of transfusion dependence, longer follow-up of a larger cohort of patients will provide further evidence on the long-term efficacy http://ClinicalTrials.gov and safety of this gene-editing strategy, confirming whether CRISPR–Cas9-mediated gene editing of the *BCL11A* +58 enhancer can cure pediatric TDT patients.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41591-022-01906-z.

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Method

Study design. We conducted a nonrandomized, single-dose, open-label clinical trial of gene-editing therapy in children with TDT at Xiangya Hospital of Central South University. The study was approved by the Medical Ethics Committee of the Xiangya Hospital of Central South University (no. 201912006) and registered with ClinicalTrials.gov (NCT04211480). Informed written consent was provided by all patients (and their legal guardians) in accordance with the Declaration of Helsinki (2013). The local institutional review board (IRB) reviewed all appropriate study documentation to protect the rights, safety and well-being of the patients, as recognized by local applicable laws and regulations. No safety monitoring board was involved with the present study.

For the healthy controls involved in the present study, the donors signed the informed consent for biological sample donation and data sharing before surgery, with agreement that the samples would be used for biomedical research.

In addition, clinical data management was conducted by the specific data manager and supervised by the IRB. The full clinical trial protocol is included in Supplementary information. Patients were monitored for routine blood test, editing frequency, engraftment, AEs, total Hb, Hb fractions on HPLC, F-cell percentage (defined as the percentage of circulating erythrocytes with detectable levels of HbF), laboratory indices of iron overload, requirements for transfusion of packed red cells and editing pattern shift in PBMCs. Bone marrow aspirates were obtained at 9 months after infusion for editing frequency. Primary outcome measures included proportion of subjects with engraftment, overall survival, incidence of AEs and serious AEs post-transplantation; secondary outcome measures included the proportion of subjects achieving transfusion independence for at least 6 months (TI6) or 12 months (TI12), proportion of alleles with intended genetic modification in bone marrow cells, change in total Hb concentration, change from baseline in annualized frequency and volume of packed RBC transfusions. Safety assessment included time of neutrophil engraftment after edited HSPC infusion, time of platelet engraftment, safety and tolerability assessments based on AEs, clinical laboratory values and vital signs, transplantation-related mortality (TRM) within 3 months of transplantation, TRM within 1 year of transplantation and all-cause mortality. Due to the small sample size ($n=2$), the data summaries are primarily descriptive. All statements about before and after comparisons are qualitative and based on inspection of the data and do not imply statistical significance.

The follow-up schedule for the present study included a treatment follow-up period (2 months to 24 months) and a survival follow-up period (2 years to 15 years). After the end of the examination on day 60, patients entered the treatment follow-up period. Researchers had to monitor relevant indicators according to the study protocol (Annex 1 Schedule of events), including DNA-seq for determination of gene-editing rates and detection of Hb level. After the end of the clinical study (2 years after autologous stem cell transplantation), patients entered the survival follow-up period. Physicians examined subjects every 6 months by on-site visit or phone call to assess survival and disease progression, collect data on subsequent treatment and assess the risk of tumorigenicity, which will continue for 15 years or until the death of subject.

Compared with the original protocol, the main revisions on the current one include concretization of endpoint descriptions, modification in the statement of inclusion–exclusion criteria, as well as several typos (Supplementary Table 5). We assure readers that these revisions do not pose additional risks to subjects and have been reviewed and approved by the Ethics Committee.

Patient enrollment and eligibility. From December 2019 to December 2021, a total of eight TDT patients had provided informed consent: three did not meet eligibility criteria, two withdrew before undergoing mobilization on their own decisions, one withdrew after mobilization and apheresis due to poor collections after the physician's suggestion and two received intervention and had >18 months of follow-up; the preliminary analysis of the present study was based on these last two patients (Table 1). Participant compensation included 200 yuan per time of blood sampling nutritional subsidy and 200 yuan per time of transportation subsidy.

The inclusion criteria included full understanding and voluntary signing of informed consent, age 5–15 years, at least one legal guardian and/or subjects to sign informed consent, clinically diagnosed as β-thalassemia major, phenotypes including $\beta^0\beta^0$, $\beta^+\beta^0$, $\beta E\beta^0$ genotype, subjects with no infection with Epstein–Barr virus, human immunodeficiency virus, cytomegalovirus, treponema pallidum, or hepatitis A virus (HAV), HBV and HCV, and subject's body condition eligible for autologous stem cell transplantation.

Exclusion criteria included subjects acceptable for allogeneic HSCT who have an available, fully matched, related donor, active bacterial, viral or fungal infection, treated with erythropoietin for 3 months, immediate family member with any known hematological tumor, subjects with severe psychiatric disorders who are unable to cooperate, recently diagnosed with malaria, a history of complex autoimmune disease, persistent aspartate transaminase, alanine transaminase or total bilirubin values >3× the upper limit of normal, subjects with severe heart, lung and kidney diseases, serious iron overload with serum ferritin >5,000 mg ml^{-1}, any other condition that would render the subject ineligible for HSCT, as determined by the attending transplant physician or investigator, subjects who are receiving treatment from another clinical study or have received another gene therapy, subjects or guardians who had resisted the guidance of the attending doctor and subjects whom the investigators do not consider appropriate for participating in the present clinical study.

Mobilization and apheresis for participants with TDT (NCT04211480). Participants were assessed by the study researcher to ensure qualification to proceed with apheresis before G-CSF administration. The apheresis-experienced physician or nurse determined whether a central line was required. G-CSF was administered subcutaneously (10 μg kg^{-1} every 24 h for 5–6 d). After G-CSF mobilization, CD34$^+$ HSPCs were harvested in accordance with standard operating procedures of the COBE Spectra apheresis system. The target number of cells to be collected was ≥10×10^6 CD34$^+$ cells kg^{-1}. This may have required performing more than one apheresis procedure using standard methods.

Myeloablative conditioning. Myeloablative conditioning with intravenous busulfan (6 d before infusion; 3.2 mg kg^{-1} once a day without pharmacokinetic monitoring and dose adjustment) was performed for 4 consecutive days. This was followed by a minimum 48-h wash-out period.

Preparation of *BCL11A* enhancer-edited autologous HSPCs. *BCL11A* enhancer-edited autologous HSPCs consist of autologous CD34$^+$ HSPCs edited with CRISPR–Cas9 RNP at the +58 erythroid specific enhancer region of the *BCL11A* gene. The sequence of sgRNA targeting the *BCL11A* enhancer is 5′-CTAACAGTTGCTTTTATCAC-3′. The modified synthetic sgRNA (2′-*O*-methyl-3′-phosphorothioate modifications in the first and last three nucleotides) was synthesized followed by purification. Cas9 protein was expressed in *Escherichia coli* Rosetta (DE3) pLysS cells (EMD Millipore) and purified with Ni-NTA (nitriloacetic acid) resin, cation exchange chromatography and size-exclusion chromatography on a Hiload 16/600 Superdex 200-pg column.

PBMCs were collected from patients at the hospital by apheresis and sent to the manufacturing sites. CD34$^+$ HSPCs were separated from PBMCs using the CliniMACS System (Miltenyi Biotec). Cell counting (Countstar Rigel S5) and flow cytometry (BD Aria II) were performed to record the live cell numbers and percentage of CD34$^+$ cells. CD34$^+$ HSPCs were then cultured in X-VIVO 15 (Lonza, catalog no. 04-418Q) supplemented with 100 ng ml^{-1} of human stem cell factor, 100 ng ml^{-1} of human thrombopoietin and 100 ng ml^{-1} of recombinant human Flt3-ligand. The RNP complex was prepared by mixing Cas9 and sgRNA and incubating for 15 min at room temperature immediately before electroporation. The RNP complex and cells were mixed in electroporation solution and transferred to a cuvette for electroporation. The electroporated cells were resuspended with X-VIVO media with cytokines and stored in liquid nitrogen after cryopreservation.

Subsequently, the detection of CD34$^+$ cell purity, confirmation of editing rates by TIDE analysis[28], and deep sequencing, cell counting and microbial testing were performed. The frozen cells were shipped to the hospital and thawed before infusion following the protocol.

Definition and assessment of neutrophil and platelet engraftment. Neutrophil engraftment was defined as the first day of 3 consecutive days with measurements of absolute neutrophil count (ANC) ≥ 500 μl^{-1}, achieved within 42 d post-γ-globin-reactivated autologous HSC infusion, without use of the unmodified (back-up) CD34$^+$ cells after reaching the nadir, defined as ANC < 500 μl^{-1}. Platelet engraftment was defined as the first day of 3 consecutive measurements of platelets ≥20,000 μl^{-1} (TDT) on 3 different days after γ-globin-reactivated autologous HSC infusion, without platelet transfusion support over the last 7 d, after reaching the nadir, defined as platelet count <20,000 μl^{-1} (TDT).

Off-target analysis using deep sequencing. We performed deep sequencing to detect 24 potential off-target editing sites (20 off-target editing sites detected by CRICLE-seq and 4 additional in silico, computationally predicted off-targets from previous work)[14] in PBMCs from two patient donors and a healthy donor. Specifically, potential off-target loci were amplified with the corresponding primers first. After another round of PCR with primers containing the adapter, amplicons were sequenced for 2×150 paired-end reads with the MiSeq Sequencing System (Illumina). The on-target site was used as an internal positive control and was identified in the edited samples of every donor with >10,000 deep-sequencing reads. The deep-sequencing data were analyzed by CRISPResso2 software (v.2.1.3)[29]. In particular, we used a minimum alignment identity of 75%, window size of 2 bp around the cleavage site to quantify indels and an average PHRED quality score of 30 and excluded substitutions to limit potential false positives.

On-target editing efficiency. The on-target allelic editing efficiency was assessed using deep sequencing. The genomic (g)DNA from unedited healthy donor was used as an internal negative control. The deep-sequencing data (with >10,000 reads) were analyzed using CRISPResso2 software (v.2.1.3).

Hb analysis. Hemolysates were prepared from the peripheral blood of two patients in different periods after transplantation and analyzed with HPLC using clinically calibrated standards for the human hemoglobins.

F-cell percentage analysis using flow cytometry. Intracellular staining was performed as described previously. Briefly, cells were fixed with 0.05% glutaraldehyde (Sigma-Aldrich) for 10 min at room temperature and then permeabilized with 0.1% Triton X-100 (Life Technologies) for 5 min at room temperature. Anti-human HbF antibodies (clone HbF-1 with FITC; Life Technologies, catalog no. MHFH01) were used to stain cells for 30 min in the dark. Antibody dilution was determined according to the manufacturer's instructions, and blood samples from healthy donors were used as positive controls. Control cells without staining were used as negative controls. Before FACS analysis, cells were washed to remove unbound antibodies. BD FACSDiva Software v.8.0.1 was used for data collection and FlowJo v.10 for data analysis.

T-, B- and NK-cell analysis by flow cytometry. Patient PBMCs were stained with FITC anti-human CD3 Antibody (BioLegend, clone no. UCHT1, catalog no. 300406), PE CD16 Antibody (BioLegend, clone no. 3G8, catalog no. 302008), PE CD56 Antibody (BioLegend, clone no. 5.1H11, catalog no. 362508), APC CD19 Antibody (BioLegend, clone no. HIB19, catalog no. 302212) and PerCp CD45 Antibody (BioLegend, clone no. HI30, catalog no. 304026) and sorted using BD FACSArial II (BD Bioscience). Antibody dilution was determined according to the manufacturer's instructions. The cells were further gated into T-cell ($CD45^+CD3^+$), B-cell ($CD45^+CD3^-CD19^+$) and NK-cell ($CD45^+CD3^-CD16/56^+$) subgroups. BD FACSDiva Software v.8.0.1 was used for data collection and FlowJo v.10 for data analysis.

ScRNA-seq analysis. Droplet-based scRNA-seq was utilized on the Chromium Single-Cell Controller platform (10× Genomics). The library was prepared using the Chromium Single Cell 3′ Reagent Kit v.2 and sequenced with HiSeq × Ten system (Illumina). Raw Illumina sequencer base call files were processed using the Cell Ranger analysis set (v.3.1.0: https://github.com/10XGenomics/cellranger) with default parameters. Briefly, pipelines involved sample demultiplexing, barcode decoding, filtering low data, aligning to human genome v.GRCh38 and gene counting. The output was a clean gene–barcode matrix composed of unique molecular modifier counts for each gene. Gene–barcode matrices were processed using Seurat (v.3.2.2: https://satijalab.org/seurat) R package. Abnormal cells and RBCs in all matrices were filtered, with outliers detected via gene number, a higher mitochondrial gene ratio or Hb expression. Subsequently, all samples were merged using an integration anchor algorithm. We performed scaling, principal component analysis, variable gene identification and clustering on the integrated dataset. Differentially expressed genes for each cluster, compared with all remaining cells, were found using Wilcoxon's rank-sum test; only positive changes were reported. Using canonical markers and the list of genes differentially expressed in defined subpopulations from PBMCs, 13 cell types were identified[30].

RT–qPCR quantification of *BCL11A* expression. Bone marrow cell samples from patients 9 months after transplantation and lineage cells after sorting were used to determine *BCL11A* expression. RNA was isolated using RNAsimple Total RNA Kit (DP-419, TIANGEN), reverse transcription with ReverTra Ace qPCR RTMaster Mix with gDNA Remover Kit (FSQ-301, BioRad) and RT–qPCR analyzed with FastStart Universal SYBR Green Master (Rox; Roche, catalog no. 04 913 914 001) according to the manufactures' suggested protocols. All gene expression data represent the mean of at least three technical replicates.

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

Data availability

All amplicon deep-sequencing data generated in this article can be found at the National Center for Biotechnology Information's Sequence Read Archive (accession no. PRJNA839164). Single-cell transcriptome data generated in this article can be found at the Gene Expression Omnibus database with accession no. GSE204688. A shared dataset used in this article for 10,000 PBMC single-cell transcriptome data from a healthy donor can be downloaded from: https://cf.10xgenomics.com/samples/cell-exp/4.0.0/SC3_v3_NextGem_SI_PBMC_10K/SC3_v3_NextGem_SI_PBMC_10K_raw_feature_bc_matrix.tar.gz.

Individual participant data (IPD) that underlie the results reported in published article will be shared, after de-identification (text, tables, figures and appendices). Other available documents include the study protocol. IPD sharing will start at 6 months and end at 36 months after article publication. IPD will be shared with investigators for individual data meta-analysis, after their proposed use of the data has been approved by an independent review committee. Proposals should be directed to yxwu@bio.ecnu.edu.cn and fu.bin@csu.edu.cn. To gain access, data requestors will need to sign a data access agreement. Source data are provided with this paper.

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Author contributions

B.F., D.L., M.L. and Y.W. designed and led the study. B.F. was site principal investigator. J.L. and S.C. conducted experiments and analyzed data with the help of W.L., Q.W., F.Y., S.H., Y.J. and L.W. B.F., J.H. and F.C. were involved in patient care, testing and data presentation. B.F., J.L., S.C. and Y.W. wrote the manuscript. All authors contributed to the manuscript and approved its final version.

Competing interests

The authors declare the following competing interests: W.L., F.Y., D.L., M.L. and Y.W. are employees of Bioray Laboratories. The remaining authors declare no competing interests.

Additional information

Extended data Extended data are available for this paper at https://doi.org/10.1038/s41591-022-01906-z.

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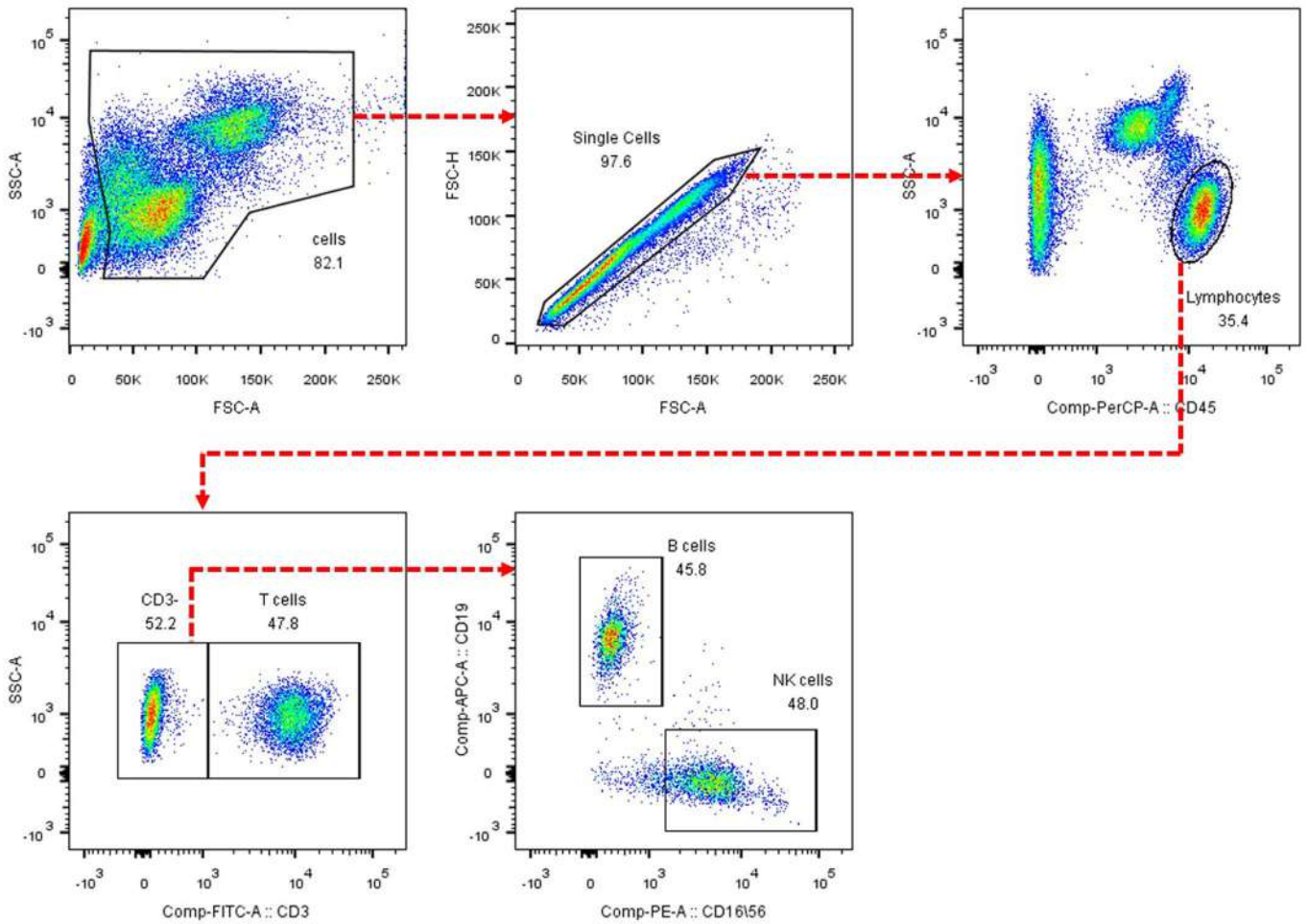
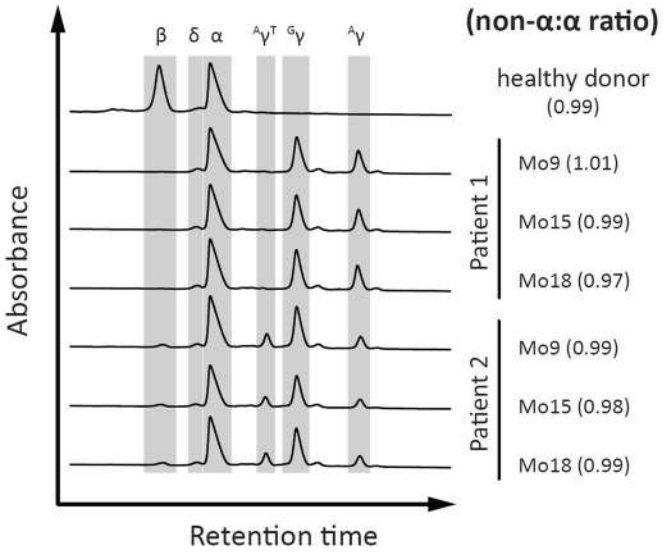
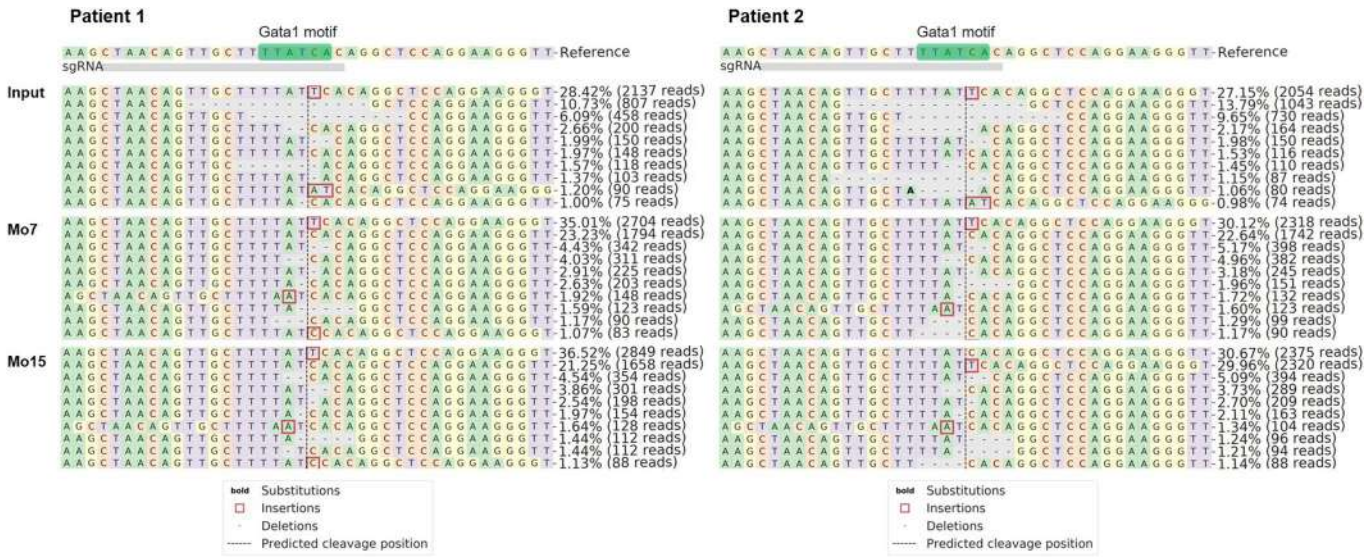


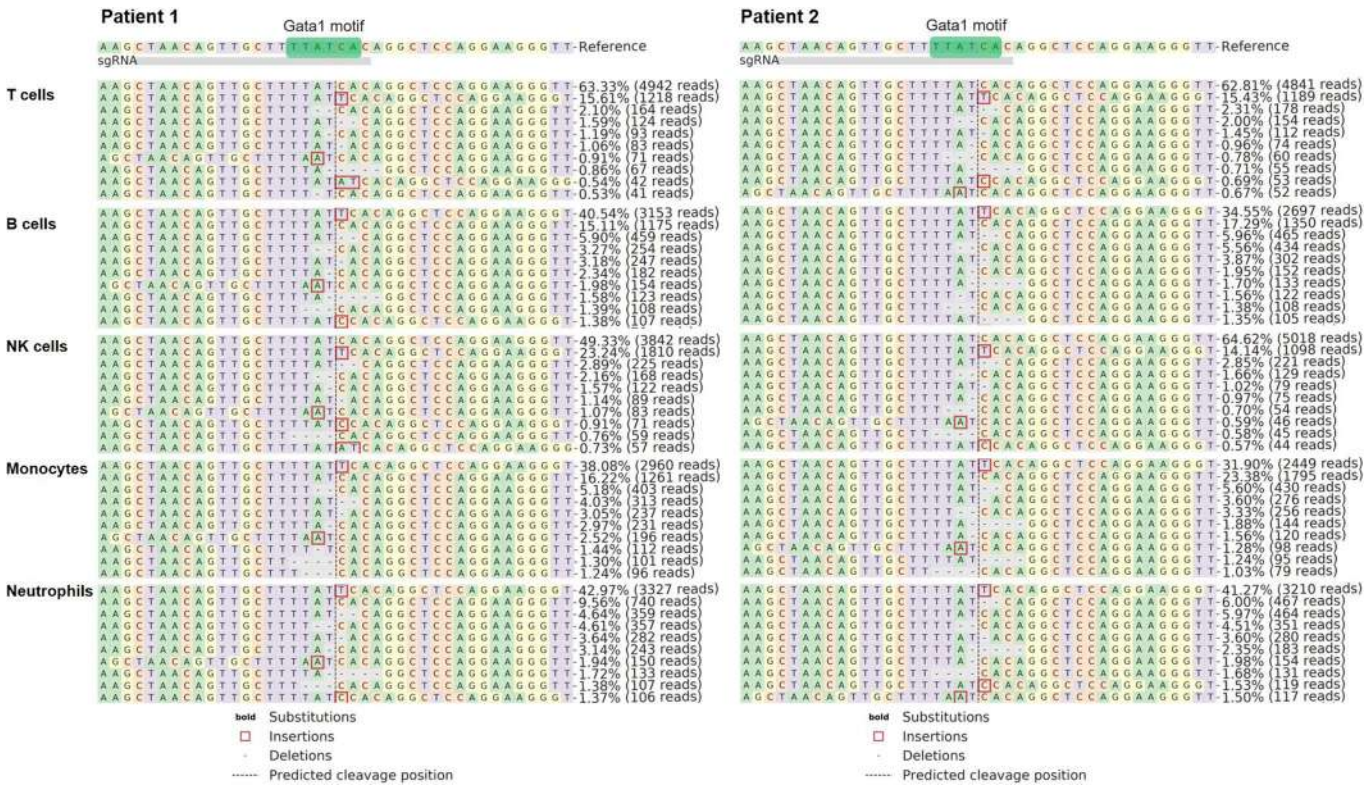
Extended Data Fig. 1 | Representative blood cell subtype cytometry analysis. Representative blood cell subtype cytometry analysis. The gating strategy for blood cell subtype analysis is as shown. Lymphocytes were gated from a single cell population with CD45 high, SSC low. T cells are gated from the CD45+/CD3+ population. B cells are gated from the CD45+/CD3-/CD19+ population. NK cells are gated from the CD45+/CD3-/CD16/56+ population.



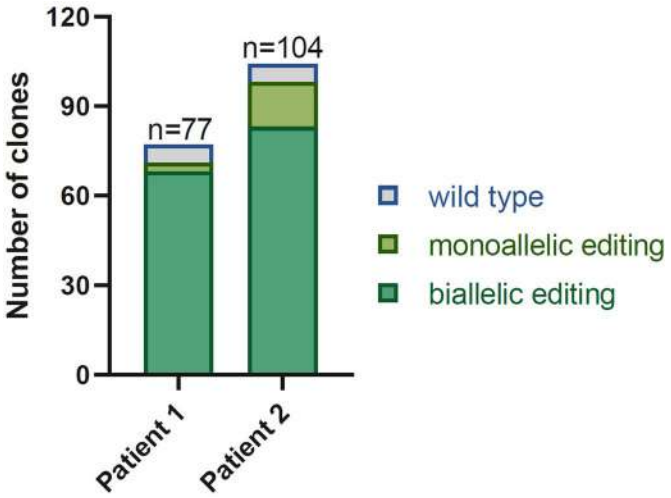
Extended Data Fig. 2 | RP-HPLC analysis of globin chains in treated patients. RP-HPLC analysis of globin chains in healthy donor and two patients (at Month 9, 15 and 18). RP-HPLC chromatograms are reported together with the non-α: α ratio (in brackets). The various globin chains detected were labeled on top and their respective peak shape ranges were marked with a gray background. Gamma globin chains were robustly induced in both patients. The expression of a common AγT chain variant was detected in samples derived from Patient 2. After treatment, the two patients had equal amounts of alpha and non-alpha chains in their blood after treatment as that of healthy donor.



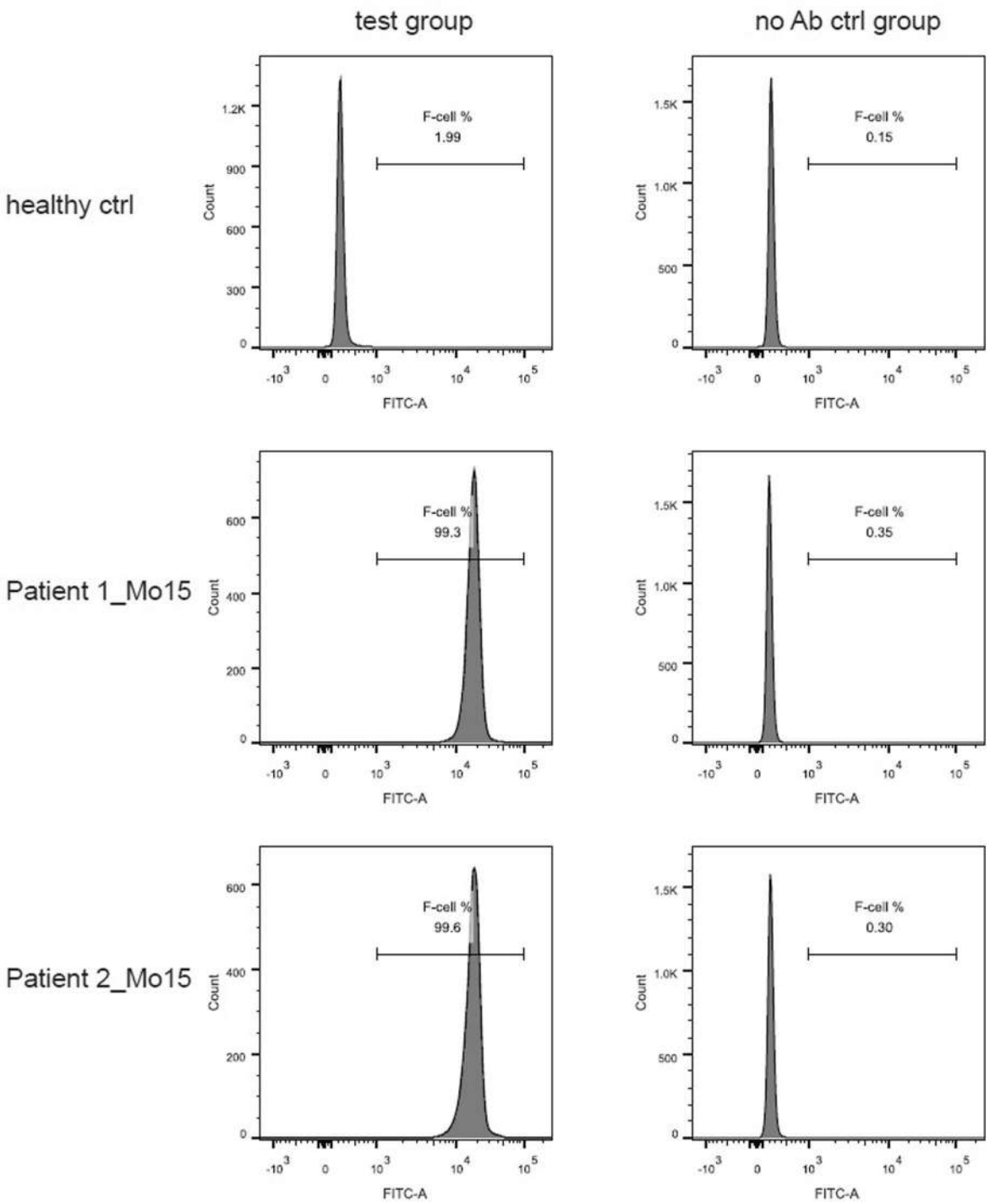
Extended Data Fig. 3 | Representative indel tables of two patients' edited cells. Summary of most frequent indels by deep sequencing of the genome from input CD34+ cells, PBMCs Mo7 after transplantation, and PBMCs Mo15 after transplantation from study patients. The reference sequence at the top of the figure is part of the *BCL11A* enhancer DNase I hypersensitive site (DHS) + 58 functional core. Gata1 motif marked with green box, and the sgRNA targets the complementary strand of the gata1 motif sequence.



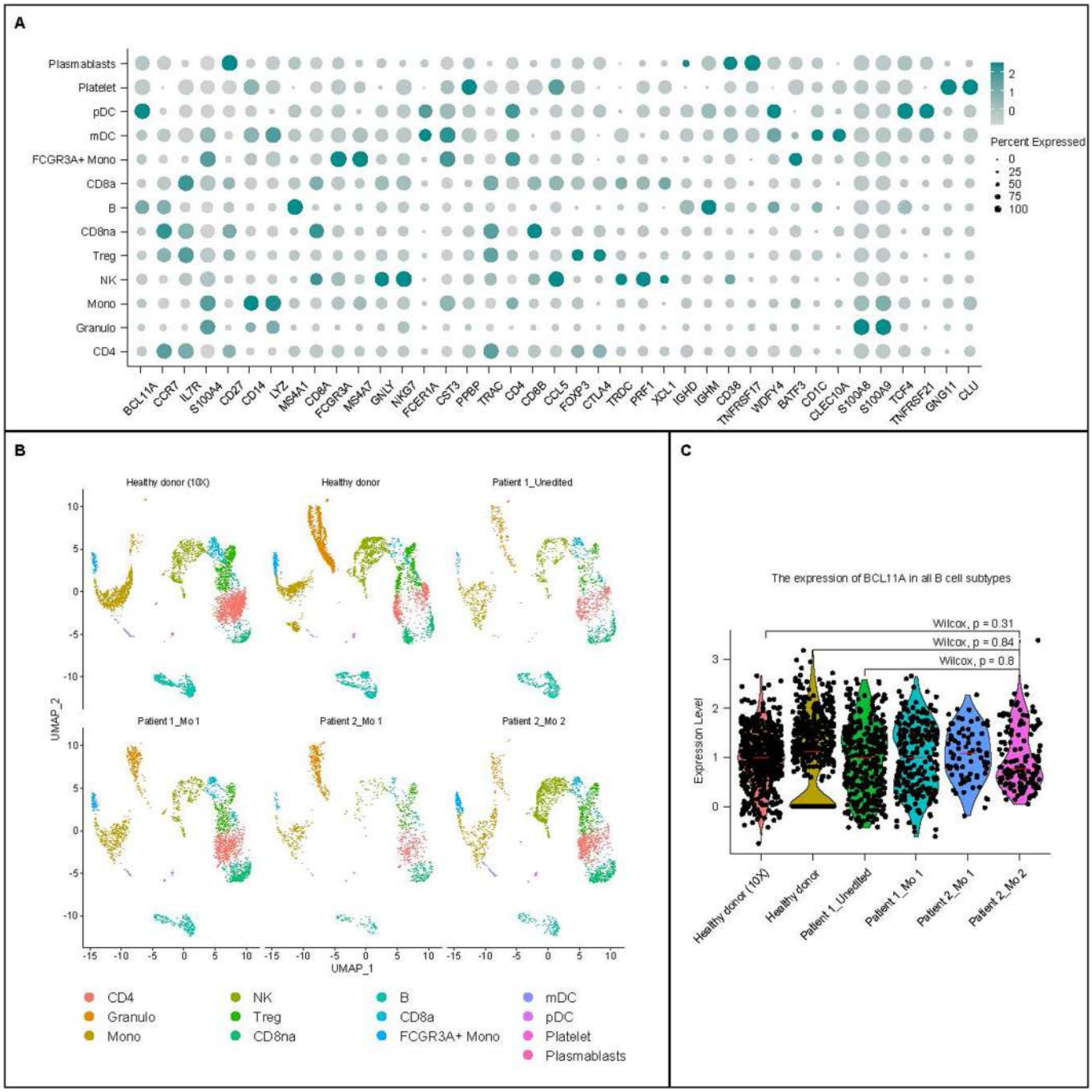
Extended Data Fig. 4 | Representative indel tables of two patients' engrafted cells. Summary of most frequent indels by deep sequencing result of T cells, B cells, NK cells, Monocytes and Neutrophils sorted from peripheral blood at Mo15 post transplantation from study patients. The reference sequence at the top of the figure is part of the *BCL11A* enhancer DNase I hypersensitive site (DHS) + 58 functional core. Gata1 motif marked with green box, and the sgRNA targets the complementary strand of the gata1 motif sequence.



Extended Data Fig. 5 | Genotyping analysis of patient single CD34+ cell expansions. CD34 + cells were sorted by MACS from bone marrow cells of patients 9 months after transplantation, and their single-cell expansions were analyzed for genotype by sanger sequencing. It was found that 88.3% (n=77) and 79.8% (n=104) of the CD34+ cells of the two patients were biallelic editing.



Extended Data Fig. 6 | Representative cytoflow analysis for F-cells proportion in patient RBCs after treatment. The proportion of F-cells in peripheral red blood cells was measured by FACS at 15 months after transplantation in both patients, blood sample from healthy people as control. After treatment, both patients showed robust HBF expression in peripheral red blood cells.



Extended Data Fig. 7 | Sc-RNA seq analysis of edited and unedited patient PBMCs. a, Marker gene expression for subtypes from PBMCs. b, UMAP plots representing 13 color-coded cell clusters identified in single-cell transcriptomes of PBMCs from 2 heathy donors, 1 patient sample before treatment and 3 patient samples at different time points after treatment. c, Violin plots showing *BCL11A* expression (log transformed) for B cell cluster from each sample. Wilcox two-tailed test, with each p-value, is indicated above the comparison group.

Extended Data Table 1 | Summary of Adverse Events for Patient 1 (TDT)

AE Terminology	Occurrence Time (days post transplantation)	Grade*	Resolved
Stomatitis	-5	II	Yes
Nausea	-3	I	Yes
Emesis	-3	II	Yes
Hot flush	0	I	Yes
Febrile neutropenia	3	III	Yes
Neutropenia	3	IV	Yes
Dizzy	4	I	Yes
Abdominal pain	6	I	Yes
Pharyngeal inflammation	9	II	Yes
Stomatitis	11	III	Yes
Epistaxis	14, 16	II	Yes
Thrombembolia	40	II	Yes
Erythra	69	I	Yes
Thrombocytopenia	82	IV	Yes
Oral ulcer	113	I	Yes

*All the grade of AEs was evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0, which was published on November 27 2017.

*All the grade of AEs was evaluated according to the Common Terminology Criteria forAdverse Events (CTCAE) Version 5.0, which was published on November 27 2017.

Extended Data Table 2 | Summary of Adverse Events for Patient 2 (TDT)

AE Terminology	Occurrence Time (days post transplantation)	Grade*	Resolved
Nausea	-1, 4	I	Yes
Emesis	-1, 4	I	Yes
Neutropenia	7	I	Yes
Thrombocytopenia	7	I	Yes
Epistaxis	8	II	Yes
Febrile neutropenia	9	III	Yes
Pharyngeal inflammation	10	II	Yes
Stomatitis	12	III	Yes
Thrombembolia	54	II	Yes

*All the grade of AEs was evaluated according to the Common Terminology Criteria for Adverse Events (CTCAE) Version 5.0, which was published on November 27 2017.

*All the grade of AEs was evaluated according to the Common Terminology Criteria forAdverse Events (CTCAE) Version 5.0, which was published on November 27 2017.

Extended Data Table 3 | Medication History of iron chelation for two patients

	Drug	Administration	Dosage	Start date	End date	Treatment date	Frequency
Patient 1	Deferasirox	po.	500mg	2015/uk/uk	2020/4/22	2020/4/29	qd.
Patient 2	Deferasirox	po.	600mg	2015/5/uk	2020/3/27	2020/4/29	qd.
	Deferasirox	po.	600mg	2020/4/5	2020/4/22	2020/4/29	qd.
	Deferasirox	po.	400mg	2020/6/23	2020/7/17	2020/4/29	qd.
	Deferoxamine	Iv.gtt.	/	2019/6/uk	2019/12/uk	2020/4/29	uk

uk: unknown

uk: unknown.

nature portfolio

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Reporting Summary

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Statistics

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

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐ A description of all covariates tested
☐	☒ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ 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	BD FACSDiva Software Version 8.0.1
Data analysis	FlowJo V10, CRISPResso2 sotware v2.1.3, GraphPad Prism v9.0.0, Cell Ranger analysis set v3.1.0, Seurat v3.2.2

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 Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

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 description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

All amplicon deep sequencing data generated in this article can be found at the NCBI Sequence Read Archive (PRJNA839164). Single cell transcriptome data generated in this article can be found at the GEO database with the accession number GSE204688. A shared dataset used in this article for 10k Peripheral blood mononuclear cells (PBMCs) single cell transcriptome data from a healthy donor can be downloaded from https://cf.10xgenomics.com/samples/cell-exp/4.0.0/SC3_v3_NextGem_SI_PBMC_10K/SC3_v3_NextGem_SI_PBMC_10K_raw_feature_bc_matrix.tar.gz.
Individual participant data (IPD) that underlie the results reported in published article will be shared, after deidentification (text, tables, figures and appendices). Other available documents include study protocol. IPD sharing will begin at 6 months and end at 36 months following article publication. IPD will be shared with investigators for individual data meta-analysis, after their proposed use of the data has been approved by an independent review committee. Proposals should be

directed to yxwu@bio.ecnu.edu.cn and fu.bin@csu.edu.cn. To gain access, data requestors will need to sign a data access agreement.

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	No sample size calculation was performed in this article. From December 2019 to December 2021, a total of 8 TDT patients had provided informed consent: three did not meet eligibility criteria, two withdrew before undergoing mobilization on their own decisions, one withdrew after mobilization and apheresis due to poor collections following physician's suggestion, two received intervention and had more than 18 months follow-up. Taking into considerations of safety, we planned to arrange for the patients to receive gene editing treatment in batches. The screening and enrolment of subsequent thalassemia patients in this clinical study is still in progress, and the data presented in this paper are an preliminary analysis of the clinical data of the first batch treated patients.
Data exclusions	No data were excluded from the analyses.
Replication	Data obtained from a unique biological sample, such as blood, bone marrow cells from a specific period of a specific patient, were successfully performed with more than three technical replicates. All attempts at replication were successful.
Randomization	According to the design of the research protocol, this study is a non-randomized clinical trial. All the enrolled subjects will receive the intervention, and the evaluation of outcomes will refer to the changes of the relevant hematological indicators before and after the intervention. So it does not involve the random assignment of subjects to the experimental group and the control group.
Blinding	Not relevant for objective measures.

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	FITC anti-human HbF antibodies (Life Technologies, clone# HBF-1, cat# MHFH01) FITC anti-human CD3 Antibody (BioLegend, clone# UCHT1, cat# 300406) PE CD16 Antibody (BioLegend, clone# 3G8, cat# 302008) PE CD56 Antibody (BioLegend, clone# 5.1H11, cat# 362508) APC CD19 Antibody (BioLegend, clone# HIB19, cat# 302212) PerCp CD45 Antibody (BioLegend, clone# HI30, cat# 304026)
Validation	Each antibody for the species and application is validated. Validation statements are available on the manufacturer's website. FITC anti-human HbF antibodies (Life Technologies, clone# HBF-1, cat# MHFH01), https://www.thermofisher.com/antibody/product/Fetal-Hemoglobin-Antibody-clone-HBF-1-Monoclonal/MHFH01; FITC anti-human CD3 Antibody (BioLegend, clone# UCHT1, cat# 300406), https://www.biolegend.com/en-us/products/fitc-anti-human-cd3-antibody-863; PE CD16 Antibody (BioLegend, clone# 3G8, cat# 302008), https://www.biolegend.com/en-us/products/pe-anti-human-cd16-antibody-569; PE CD56 Antibody (BioLegend, clone# 5.1H11, cat# 362508), https://www.biolegend.com/en-us/products/pe-anti-human-cd56-ncam-antibody-9958; APC CD19 Antibody (BioLegend, clone# HIB19, cat# 302212), https://www.biolegend.com/en-us/products/apc-anti-human-cd19-antibody-715; PerCp

CD45 Antibody (BioLegend, clone# HI30, cat# 304026), https://www.biolegend.com/en-us/products/percp-anti-human-cd45-antibody-4239 .

Human research participants

Policy information about studies involving human research participants

Population characteristics	Patient 1 is a 7-year-old male and 41-42M/17M with an annualized red-cell transfusion history of 30 units/y over 2 y prior to consent. Patient 2 is a 8-year-old male and IVS2-654M/-28M with an annualized red-cell transfusion history of 20 units/y over 2 y prior to consent.
Recruitment	Informed consent was obtained by all participants. Participant compensation includes 200 yuan/time blood sampling nutritional subsidy and 200 yuan/time transportation subsidy. Patients were recruited out of Xiangya Hospital at Central South University. Eligibility was restricted to patients who had clinically diagnosed as β-thalassemia major, transfusion dependent, including β0β0, β+β0, βEβ0 genotype. Included patients should be 5-15years old and eligible for autologous stem cell transplant. Subjects acceptable for allogeneic hematopoietic stem cell transplantation or have an available fully matched related donor, have active infection, treated with erythropoietin prior 3 months, with serious iron overload (serum ferritin>5000mg/ml), with severe heart, lung and kidney diseases, with persistent aspartate transaminase (AST), alanine transaminase (ALT), or total bilirubin value >3 X the upper limit of normal (ULN) would be excluded. There was no selection bias in recruiting or enrollment of patients in the Phase 1/2 trial.
Ethics oversight	The study protocol was approved by the ethics committee of Xiangya Hospital of Central South University in China.

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	ClinicalTrials.gov number, NCT04211480
Study protocol	The study protocol is provided in the supplemental information.
Data collection	Data was captured using case report forms and data was entered into an electronic data management system. Patients were recruited from Xiangya Hospital at Central South University, starting from December 30, 2019 until December 30, 2021. Data collection was from December 30, 2019 until October 11, 2021.
Outcomes	Since the Phase1/2 trial of γ-globin reactivated autologous hematopoietic stem cells transplantation was a first in children gene editing therapy trial, safety was the primary objective and was defined as the primary outcome. And this is a gene editing therapy hence evaluation of genetic editing durability in edited HSPCs and efficacy were the secondary outcome. Primary Outcome Measures: Proportion of subjects with engraftment; Overall survival. Incidence and severity of adverse events as a measure of safety and tolerability. Adverse events assessed according to NCI-CTCAE v5.0 criteria. Secondary Outcome Measures: Proportion of subjects achieving transfusion independence for at least 6 months (TI6); Proportion of subjects achieving TI12; Proportion of alleles with intended genetic modification in bone marrow cells; Change in total hemoglobin concentration; Change from baseline in annualized frequency and volume of packed RBC transfusions.

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	The sample preparation and biological source of the cells were described in the manuscript.
Instrument	Cell sorting was performed on a FACSAria II machine (BD Biosciences), and flow cytometry analysis was performed on a BD LSR machine (BD Biosciences).
Software	The flow cytometry data were analyzed by flowJo 10 software.
Cell population abundance	The purity of the samples was determined by rerunning with flow cytometry.

Gating strategy | The gating strategy was described in the manuscript and previous publications.

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