BRIEF REPORT

CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia

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SUMMARY

Transfusion-dependent β-thalassemia (TDT) and sickle cell disease (SCD) are severe monogenic diseases with severe and potentially life-threatening manifestations. BCL11A is a transcription factor that represses γ-globin expression and fetal hemoglobin in erythroid cells. We performed electroporation of CD34+ hematopoietic stem and progenitor cells obtained from healthy donors, with CRISPR-Cas9 targeting the *BCL11A* erythroid-specific enhancer. Approximately 80% of the alleles at this locus were modified, with no evidence of off-target editing. After undergoing myeloablation, two patients — one with TDT and the other with SCD — received autologous CD34+ cells edited with CRISPR-Cas9 targeting the same *BCL11A* enhancer. More than a year later, both patients had high levels of allelic editing in bone marrow and blood, increases in fetal hemoglobin that were distributed pancellularly, transfusion independence, and (in the patient with SCD) elimination of vaso-occlusive episodes. (Funded by CRISPR Therapeutics and Vertex Pharmaceuticals; ClinicalTrials.gov numbers, NCT03655678 for CLIMB THAL-111 and NCT03745287 for CLIMB SCD-121.)

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This article was published on December 5, 2020, and updated on December 7, 2020, at NEJM.org.

DOI: 10.1056/NEJMoa2031054

Transfusion-dependent β-thalassemia (TDT) and sickle cell disease (SCD) are the most common monogenic diseases worldwide, with an annual diagnosis in approximately 60,000 patients with TDT and 300,000 patients with SCD.[1-3] Both diseases are caused by mutations in the hemoglobin β subunit gene (*HBB*). Mutations in *HBB* that cause TDT[4] result in reduced (β^+) or absent (β^0) β-globin synthesis and an imbalance between the α-like and β-like globin (e.g., β, γ, and δ) chains of hemoglobin, which causes ineffective erythropoiesis.[5,6] Sickle hemoglobin is the result of a point mutation in *HBB* that replaces glutamic acid with valine at amino acid position 6. Polymerization of deoxygenated sickle hemoglobin causes erythrocyte deformation, hemolysis, anemia, painful vaso-occlusive episodes, irreversible end-organ damage, and a reduced life expectancy.[5]

Treatment options primarily consist of transfusion and iron chelation in patients with TDT[7] and pain management, transfusion, and hydroxyurea in those with SCD.[8] Recently approved therapies, including luspatercept[9] and crizanlizumab,[10] have reduced transfusion requirements in patients with TDT and the incidence of vaso-occlusive episodes in those with SCD, respectively, but neither treatment addresses the underlying cause of the disease nor fully ameliorates disease manifestations. Allogeneic bone marrow transplantation can cure both TDT and

SCD, but less than 20% of eligible patients have a related human leukocyte antigen–matched donor.[11-13] Betibeglogene autotemcel, a lentiviral vector–based gene-addition product, is approved in the European Union for the treatment of patients with TDT who have non-β^0 mutations and who do not have a matched sibling donor[14] and is being studied in patients with β^0/β^0 TDT genotypes and in those with SCD.[15,16] In addition, an erythroid-specific knockdown of BCL11A delivered by a lentiviral-encoded, microRNA-adapted short hairpin RNA molecule has been shown to reactivate the γ-globin gene and is in early clinical development.[17-19]

Elevated levels of fetal hemoglobin (consisting of two alpha and two gamma chains) are associated with improved morbidity and mortality in patients with TDT and SCD.[20-22] The production of fetal hemoglobin is developmentally regulated so that the level of γ-globin that is produced in utero decreases postnatally as the production of β-globin and adult hemoglobin (consisting of two alpha and two beta chains) increases. Neonates and infants with TDT or SCD are typically asymptomatic while their fetal hemoglobin levels remain high and become symptomatic during the first year of life when the synthesis of fetal hemoglobin declines[23,24] (Fig. 1A). Patients with TDT or SCD who co-inherit hereditary persistence of fetal hemoglobin, in which fetal expression continues into adulthood, have little or no disease.[25]

Genomewide association studies have identified single-nucleotide polymorphisms (SNPs) associated with increased expression of fetal hemoglobin in adults.[26] Some of these SNPs are located in the *BCL11A* locus on chromosome 2 and are associated with a lower severity of both TDT and SCD.[27] BCL11A is a zinc finger–containing transcription factor that represses γ-globin expression and fetal hemoglobin in erythroid cells; the SNPs that are associated with fetal hemoglobin are in an erythroid-specific enhancer, downregulate *BCL11A* expression, and increase the expression of fetal hemoglobin.[1,23]

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 nuclease system, a bacterial immune system that can cleave bacteriophage or plasmid DNA, enables programmable targeting of insertions or deletions (indels) at a specific genomic DNA site.[28,29] In an attempt to recapitulate the phenotype of hereditary persistence of fetal hemoglobin, we used CRISPR-Cas9 gene-editing techniques in hematopoietic stem and progenitor cells (HSPCs) at the erythroid-specific enhancer region of *BCL11A* to reduce BCL11A expression in erythroid-lineage cells, restore γ-globin synthesis, and reactivate production of fetal hemoglobin[30,31] (Fig. 1B).

Here, we describe the first two patients, one with TDT and the other with SCD, who were infused with CTX001 (autologous CRISPR-Cas9–edited CD34+ HSPCs that were genetically edited to reactivate the production of fetal hemoglobin) and enrolled in CLIMB THAL-111 (for the patient with TDT) and CLIMB SCD-121 (for the patient with SCD).

METHODS

STUDY OVERSIGHT

The study sponsors (CRISPR Therapeutics and Vertex Pharmaceuticals) designed the study protocols, with oversight provided by the study steering committees and an independent data monitoring committee. A representative of each of the sponsors wrote the first draft of the manuscript. All the authors had access to the data and approved the decision to submit the manuscript for publication. All the authors vouch for the accuracy and completeness of the data generated at their respective sites, and the representatives of Vertex Pharmaceuticals and CRISPR Therapeutics vouch for the fidelity of the trial to the protocol, which is available with the full text of this article at NEJM.org.

TRIAL DESIGN AND ELIGIBILITY

In the CLIMB THAL-111 and CLIMB SCD-121 trials, patients with TDT and SCD, respectively, received a single intravenous infusion of CTX001. In the two trials, eligibility was limited to patients who were between the ages of 18 and 35 years. In the CLIMB THAL-111 trial, patients could participate if they had received a diagnosis of β-thalassemia (including the hemoglobin E genotype) with either homozygous or compound heterozygous mutations and had received transfusions of packed red cells consisting of at least 100 ml per kilogram of body weight (or 10 units) per year during the previous 2 years. In the CLIMB SCD-121 trial, patients could participate if they had a documented $\beta S/\beta S$ or $\beta S/\beta^0$ genotype and had a history of two or more severe

vaso-occlusive episodes per year (as determined by an independent end-point adjudication committee) during the previous 2 years. Additional details regarding the inclusion and exclusion criteria are provided in the Supplementary Appendix, available at NEJM.org.

CTX001 PRODUCTION AND INFUSION

CD34+ HSPCs were collected from patients by apheresis after mobilization with either filgrastim and plerixafor (in the patient with TDT) or plerixafor alone (in the patient with SCD) after a minimum of 8 weeks of transfusions of packed red cells targeting a level of sickle hemoglobin of less than 30% (in the patient with SCD). CTX001 was manufactured from these CD34+ cells by editing with CRISPR-Cas9 with the use of a single-guide RNA molecule (Fig. 1B).[30] We used DNA sequencing to evaluate the percentage of allelic editing at the on-target site. Patients received single-agent, pharmacokinetically adjusted busulfan myeloablation before the infusion of CTX001. Additional information regarding the production and infusion of CTX001 is provided in the Supplementary Appendix.

ASSESSMENTS OF CLINICAL OUTCOMES

Patients were monitored for engraftment, adverse events, total hemoglobin, hemoglobin fractions on high-performance liquid chromatography, F-cell expression (defined as the percentage of circulating erythrocytes with detectable levels of fetal hemoglobin), laboratory indexes of hemolysis, requirements for transfusion of packed red cells, and (in the patient with SCD) the occurrence of vaso-occlusive episodes. Bone marrow aspirates were obtained at 6 and 12 months after infusion, and DNA sequencing was used to measure the fraction of total DNA that was edited at the on-target site in CD34+ bone marrow cells and in nucleated peripheral-blood cells. (Details regarding these measures are provided in the Supplementary Appendix.)

RESULTS

PRECLINICAL STUDIES OF *BCL11A* EDITING

We assessed the frequency of gene editing associated with CTX001 in CD34+ HSPCs obtained from 10 healthy donors. High frequencies of allelic editing (mean [±SD], 80±6%) were observed and maintained across all subpopulations of CD34+ cells, including long-term hematopoietic stem cells. In edited CD34+ HSPCs that were isolated and edited at clinical scale and differentiated toward the erythroid lineage, fetal hemoglobin levels increased to a mean of 29.0±10.8%, as compared with 10.5±5.2% in unedited control cells (Fig. 1C). In immunocompromised mice, there was equivalent engraftment for control and single-guide RNA-edited cells, and the persistence and pattern of edits were stable at 16 weeks, with maintenance of the diversity of edits (Tables S2 and S3 and Fig. S1 in the Supplementary Appendix).

We identified potential sites of off-target editing using sequence similarity (computational) and laboratory-based methods by means of genome-wide unbiased identification of double-stranded breaks enabled by sequencing (GUIDE-seq). These sites were evaluated with the use of high-coverage, hybrid-capture experiments by means of deep next-generation sequencing of edited CD34+ cells obtained from 4 healthy donors. There was no evidence of off-target editing (Fig. 1D).

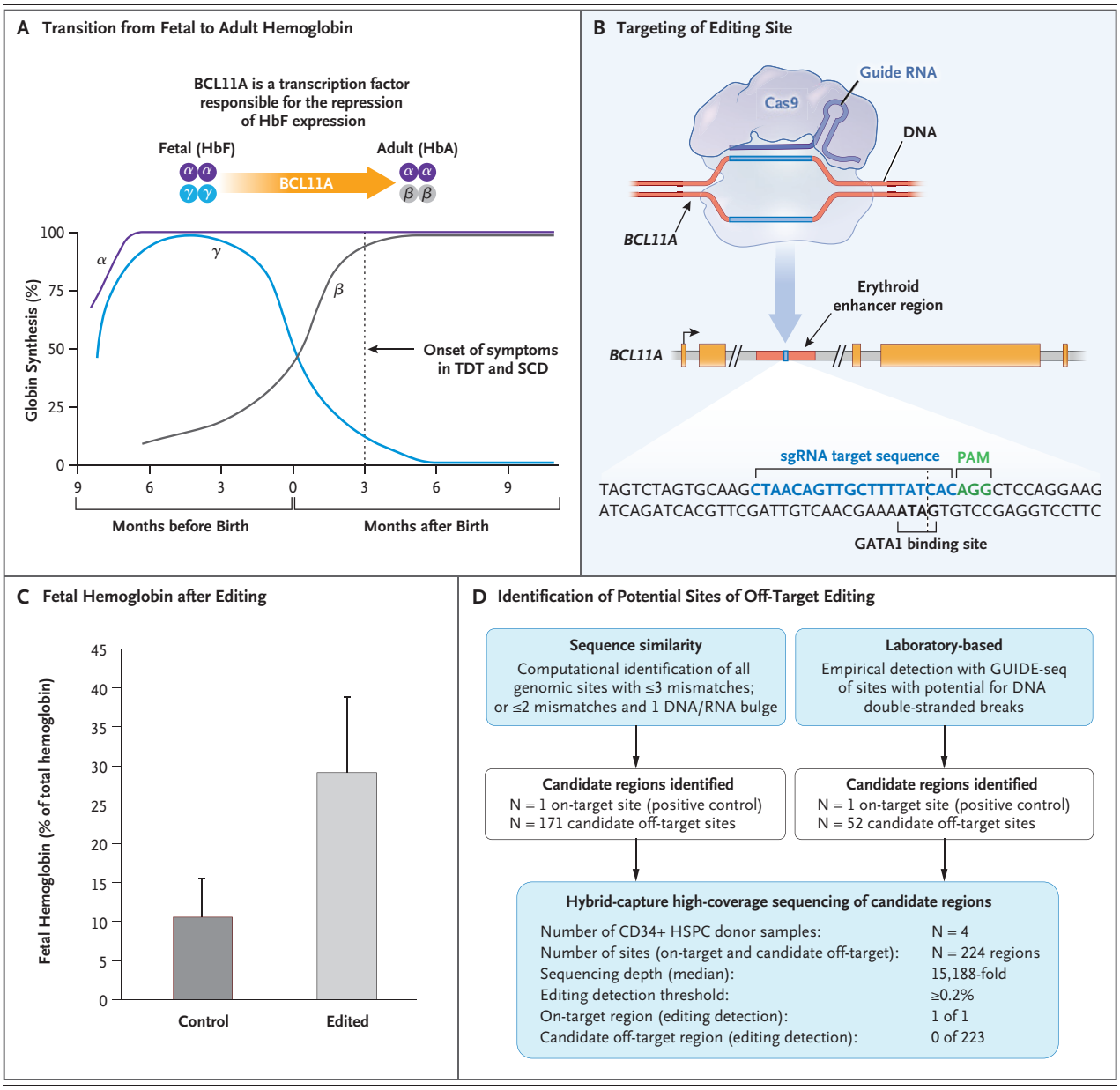
PATIENT DEMOGRAPHICS AND OUTCOMES

Patient 1

Patient 1 was a 19-year-old female with the β^0/β^+ (IVS-I-110) genotype of TDT. Before enrollment, she had received 34 units of packed red cells per year, annualized over a 2-year period. She had received such transfusions since birth, and iron chelation was initiated when she was 2 years old. Her medical history included iron overload, inactive hepatitis C, splenomegaly, and osteonecrosis of the skull. After treatment with CTX001, the patient had been followed for 21.5 months, including the 18-month study visit, at the time of this report.

Patient 1 underwent myeloablative conditioning and was infused with CTX001 (16.6×10^6 CD34+ cells per kilogram) on day 1; neutrophil engraftment was reported on day 33 and platelet engraftment on day 37. (Additional details about the course of treatment are provided in the Supplementary Appendix.) The CTX001 drug product had an allelic editing frequency of 68.9%. After the administration of CTX001, high levels of edited alleles in bone marrow CD34+ cells and nucleated peripheral-blood cells were maintained (Table 1).

Levels of fetal hemoglobin increased rapidly



from 0.3 g per deciliter at baseline to 8.4 g per deciliter at month 3, 12.4 g per deciliter at month 12, and 13.1 g per deciliter at month 18 (Fig. 2A). F-cell expression increased from 10.1% at baseline to 99.7% at month 6 and was maintained through month 18 (Fig. 2B).

During the 21.5 months after receiving CTX001, Patient 1 had 32 adverse events, most of which were considered to be grade 1 or 2 in severity (Table S6). Study investigators classified 2 adverse events as serious: pneumonia in the presence of neutropenia and veno-occlusive liver disease with sinusoidal obstruction syndrome (VOD–SOS), both of which began on study day 13. Pneumonia in the presence of neutropenia had resolved by study day 28. The VOD–SOS reached a severity of grade 3, despite defibrotide prophylaxis. Defibrotide was continued with therapeutic intent along with supportive care, and the VOD–SOS resolved on study day 39. At the time of screening, Patient 1 had a low cardiac and liver iron content (32.9 msec on cardiac magnetic resonance imaging with T2-weighted sequences and a liver iron level of 2 mg per gram of dry weight) and a history of hepatitis C (then inactive). Viral hepatitis is associated with a risk

Figure 1 (facing page). CTX001 Molecular Approach and Preclinical Studies.

Panel A shows the transition from fetal hemoglobin (HbF) to adult hemoglobin (HbA) shortly after birth, along with the role of the transcription factor BCL11A in mediating the repression of γ-globin, a component of fetal hemoglobin. In patients who lack the ability to produce functional or sufficient β-globin, the onset of symptoms occurs coincident with the decrease in fetal hemoglobin levels approximately 3 months after birth. SCD denotes sickle cell disease, and TDT transfusion-dependent β-thalassemia. Panel B shows the target editing site of the single guide RNA (SgRNA) that directs CRISPR-Cas9 to the erythroid-specific enhancer region of *BCL11A*. (The sgRNA sequence is provided in the Supplementary Appendix.) The five *BCL11A* exons are depicted as gold boxes. GATA1 denotes the binding site of the GATA1 transcription factor, and PAM the protospacer adjacent motif (NGG, a specific DNA sequence required to immediately follow the Cas9 target DNA sequence). Panel C shows preclinical data regarding fetal hemoglobin as a percentage of total hemoglobin after editing and the differentiation of erythroid cells. Data are from samples obtained from 10 healthy donors. Error bars indicate the standard deviation. Panel D shows the results from off-target evaluation. GUIDE-seq denotes genomewide unbiased identification of double-strand breaks enabled by sequencing, and HSPC hematopoietic stem and progenitor cell. To nominate sites, GUIDE-seq was performed once independently on each of 3 CD34+ HSPC healthy donor samples. To confirm sites, hybrid capture was then performed on each of 4 CD34+ HSPC healthy donor samples. On-target allelic editing was confirmed in each experiment with a mean of 57%, and no detectable off-target editing was observed at any of the sites nominated by GUIDE-seq and by sequence homology. Panel A was adapted with permission from Canver and Orkin.[24]

Table 1. Frequency of Allelic Editing in CTX001 Cells, Nucleated Peripheral Blood, and Bone Marrow in the Study Patients.*

Allelic Editing	CTX001 Cells	Nucleated Peripheral-Blood Cells	Bone Marrow
		percentage of alleles	
Patient 1 with TDT			
Mobilization cycle 1†	68.9		
Mo 1		48.1	
Mo 2		69.7	
Mo 3		66.4	
Mo 4		62.3	
Mo 5		63.2	
Mo 6		60.0	78.1
Mo 9		62.8	
Mo 12		64.3	76.1
Mo 18		62.9	
Patient 2 with SCD			
Mobilization cycle 1	82.6		
Mobilization cycle 2	78.7		
Mo 1		48.8	
Mo 2		72.0	
Mo 3		68.8	
Mo 4		72.6	
Mo 5		52.6	
Mo 6		69.4	81.4
Mo 9		64.3	
Mo 12		61.9	80.4

* SCD denotes sickle cell disease, and TDT transfusion-dependent β-thalassemia.

† Each mobilization cycle is used to produce a manufacturing lot. In Patient 2, the infusion consisted of two manufacturing lots.

of VOD–SOS in patients with β-thalassemia undergoing hematopoietic stem-cell transplantation and busulfan conditioning.[32]

The patient received her last transfusion of packed red cells 30 days after the CTX001 infusion (Fig. 2C). Her hemoglobin level normalized to 12.1 g per deciliter at month 4 and remained normal through month 18, her most recent study visit (Fig. 2A).

Patient 2

Patient 2 was a 33-year-old female with SCD (βS/βS and a single α-globin deletion). In the 2 years before enrollment, she had averaged seven severe vaso-occlusive episodes per year. She also had 3.5 SCD-related hospitalizations and five RBC transfusions per year, annualized over a 2-year period. Her medical history included chronic pain, cholelithiasis, increased lactic dehydrogenase levels, and decreased haptoglobin levels. At the time of this report, she had been followed for 16.6 months after the CTX001 infusion, which included a 15-month study visit.

After myeloablative conditioning, Patient 2 was infused with CTX001 (3.1×10^6 CD34+ cells per kilogram) and had evidence of neutrophil and platelet engraftment on day 30. (Details regarding the course of treatment are provided in the Supplementary Appendix.) The CTX001 infusion, which consisted of two manufacturing lots, had allelic editing frequencies of 82.6% and 78.7%, respectively. After the administration of CTX001

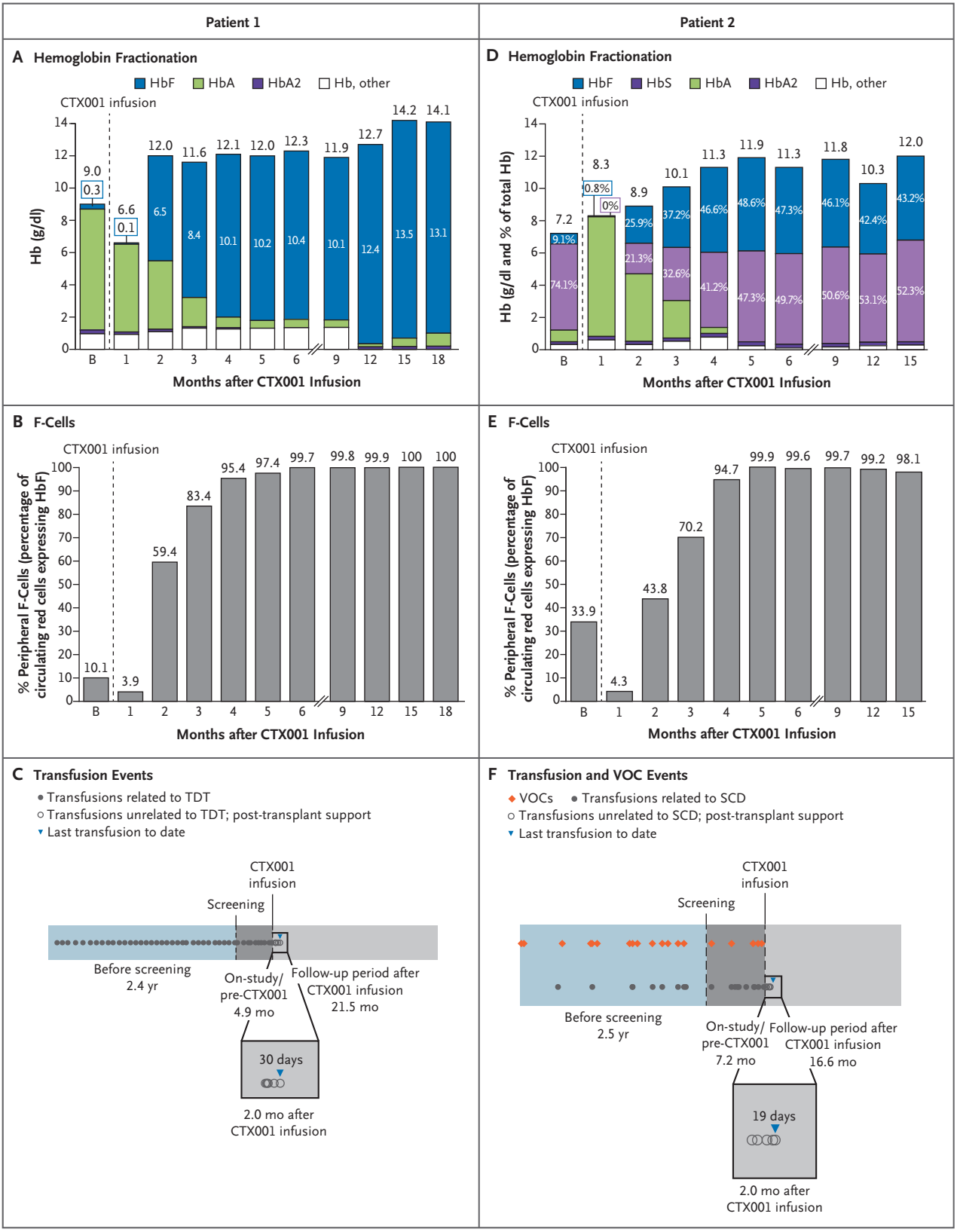


Figure 2 (facing page). Hemoglobin Fractionation, F-Cell Levels, and Transfusion Events in the Study Patients.

The levels of hemoglobin fractionation over time are shown in Patient 1, who was treated with CRISPR-Cas9 for transfusion-dependent β-thalassemia (Panel A), and in Patient 2, who was treated for sickle cell disease (Panel D), including representation of hemoglobin adducts and other variants. The changes in the percentages of F-cells over time are shown in Patient 1 (Panel B) and in Patient 2 (Panel E). The baseline levels of hemoglobin and F-cells were assessed at the premobilization visit. Also shown is the occurrence of transfusion events over time in Patient 1 (Panel C) and vaso-occlusive crises (VOCs) (or episodes) and transfusion events in Patient 2 (Panel F). Exchange transfusions that were performed according to the study protocol and that occurred during the on-study period before the infusion of CTX001 are not shown. B denotes baseline, and HbS sickle hemoglobin.

and over time, high levels of edited alleles in bone marrow CD34+ cells and nucleated peripheral-blood cells were maintained (Table 1).

Her baseline hemoglobin level was 7.2 g per deciliter, which increased to 10.1 g per deciliter at month 3 and 12 g per deciliter at month 15 without transfusion. At baseline, the fetal hemoglobin level was 9.1% and the sickle hemoglobin level was 74.1%. At month 3, the fetal hemoglobin level rose to 37.2% and the sickle hemoglobin level was 32.6%. At month 15, the fetal hemoglobin level rose to 43.2% and the sickle hemoglobin increased to 52.3% (Fig. 2D). F-cell expression was 99.9% at month 5 and was maintained at nearly 100% through month 15, her most recent study visit (Fig. 2E).

Patient 2 had 114 adverse events during the 16.6 months after receipt of the CTX001 infusion (Table S11). Study investigators classified 3 adverse events as serious: sepsis in the presence of neutropenia (on day 16), cholelithiasis (on day 49), and abdominal pain (on day 56). All 3 adverse events resolved with treatment. Study investigators observed intermittent, nonserious lymphopenia, which they considered to be probably due to a delay in T-cell recovery after the infusion of CTX001 and which had resolved by study day 351.

Patient 2 had no vaso-occlusive episodes during the 16.6 months after the infusion of CTX001, and the last transfusion of packed red cells was 19 days after infusion (Fig. 2F). Indexes of hemolysis, including levels of haptoglobin, lactate dehydrogenase, aspartate aminotransferase, and total bilirubin, normalized during the follow-up period after infusion (Tables S12 and S14).

DISCUSSION

We report the investigational use of CRISPR-Cas9–based gene editing to treat two cases of inherited diseases: one in a patient with TDT and the other in a patient with SCD. After the administration of CTX001, both patients had early, substantial, and sustained increases in fetal hemoglobin levels with more than 99% pancellularity during a 12-month period. These findings, which indicate that CRISPR-Cas9–edited HSPCs underwent engraftment that was durably maintained, are consistent with an expected survival advantage of erythrocytes with a high level of fetal hemoglobin. The clinical course of both patients supports our conclusion that CTX001 mimics the phenotype of hereditary persistence of fetal hemoglobin levels.

Adverse events were reported in both patients after the CTX001 infusion. The serious adverse events that were observed were pneumonia in the presence of neutropenia, VOD–SOS, sepsis in the presence of neutropenia, cholelithiasis, and abdominal pain. We also observed the nonserious adverse event of lymphopenia. As a product that is enriched with CD34+ cells, CTX001 may have contributed to the delay in lymphocyte recovery, similar to what has been observed after T-cell–depleted transplantation.[33,34]

Here we report the first two patients who have been treated with CTX001 and followed for at least 1 year. Since the submission of this report, we have administered CTX001 to an additional eight patients (six with TDT and two with SCD) and obtained follow-up data for more than 3 months. Initial efficacy data from these additional patients are broadly consistent with the findings in the two patients described here.[35,36] Of the six patients with TDT who were treated with CTX001 and followed for more than 3 months, one patient had adverse events of hemophagocytic lymphohistiocytosis, acute respiratory distress syndrome, headache, and idiopathic pneumonia syndrome, all of which resolved with treatment. Four other enrolled patients with SCD withdrew from the study before being treated with CTX001 (Table S16).

An additional limitation of our study is that although we performed comprehensive preclini-

cal on-target and off-target analyses and assays of erythrocyte differentiation, we have not performed similar analyses on clinical samples nor characterized their clonal diversity. A fuller analysis of the use of CTX001 therapy in additional patients with longer follow-up will be needed to more extensively characterize the profile of this treatment.

Initial results from the follow-up of the first two patients who were treated with CTX001 have shown the intended CRISPR-Cas9 editing of *BCL11A* in long-term hematopoietic stem cells, with durable engraftment, high levels of fetal hemoglobin expression, and the elimination of vaso-occlusive episodes or need for transfusion. The generalizability of these early results with respect to other patients with TDT and SCD remains to be determined. That being said, the preliminary results reported here support further experimental testing of CRISPR-Cas9 gene-editing approaches to treat genetic diseases.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

A data sharing statement provided by the authors is available with the full text of this article at NEJM.org.

We thank the patients and their families for their participation in these studies; the staff at our study sites for their contributions to the care of the patients; John Wu of BC Children's Hospital and Jane Hankins of St. Jude Children's Research Hospital; Christine Fernandez, Delphine Ranslant, Jennie Webb, Yimeng Lu, Jamie Doyle, Niraj Shanbhag, Sarah Mulcahey, Sorana Ailinca-Luchian, Lisa Moritz, Yael Bobruff, Suzan Imren, Jason Barber, Michael Weinstein, Gary Rea, John Chapin, Ewalina Morawa, Duyen Ngo, Sara Spencer, Parin Sripakdeevong, and Charis Campbell for their contributions to the conduct of these studies; Leah Eardley and Nathan Blow for their editorial support in the preparation of the manuscript; and Complete HealthVizion in Chicago for providing editorial assistance.

APPENDIX

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