BRIEF REPORT

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

H. Frangoul, D. Altshuler, M.D. Cappellini, Y.-S. Chen, J. Domm, B.K. Eustace, J. Foell, J. de la Fuente, S. Grupp, R. Handgretinger, T.W. Ho, A. Kattamis, A. Kernytsky, J. Lekstrom-Himes, A.M. Li, F. Locatelli, M.Y. Mapara, M. de Montalembert, D. Rondelli, A. Sharma, S. Sheth, S. Soni, M.H. Steinberg, D. Wall, A. Yen, and S. Corbacioglu

The authors' full names, academic degrees, and affiliations are listed in the Appendix. Address reprint requests to Dr. Frangoul at the Sarah Cannon Center for Blood Cancer at the Children's Hospital at TriStar Centennial, 330 23rd Ave. N., Suite 450, Nashville, TN 37203, or at haydar.frangoul@hcahealthcare.com; or to Dr. Corbacioglu at Children's Hospital Regensburg, University of Regensburg, Franz-Josef Strauss Allee 11, 93053 Regensburg, Germany, or at selim.corbacioglu@mac.com.

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

N Engl J Med 2021;384:252-60.
DOI: 10.1056/NEJMoa2031054

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.)

A Quick Take is available at NEJM.org

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).

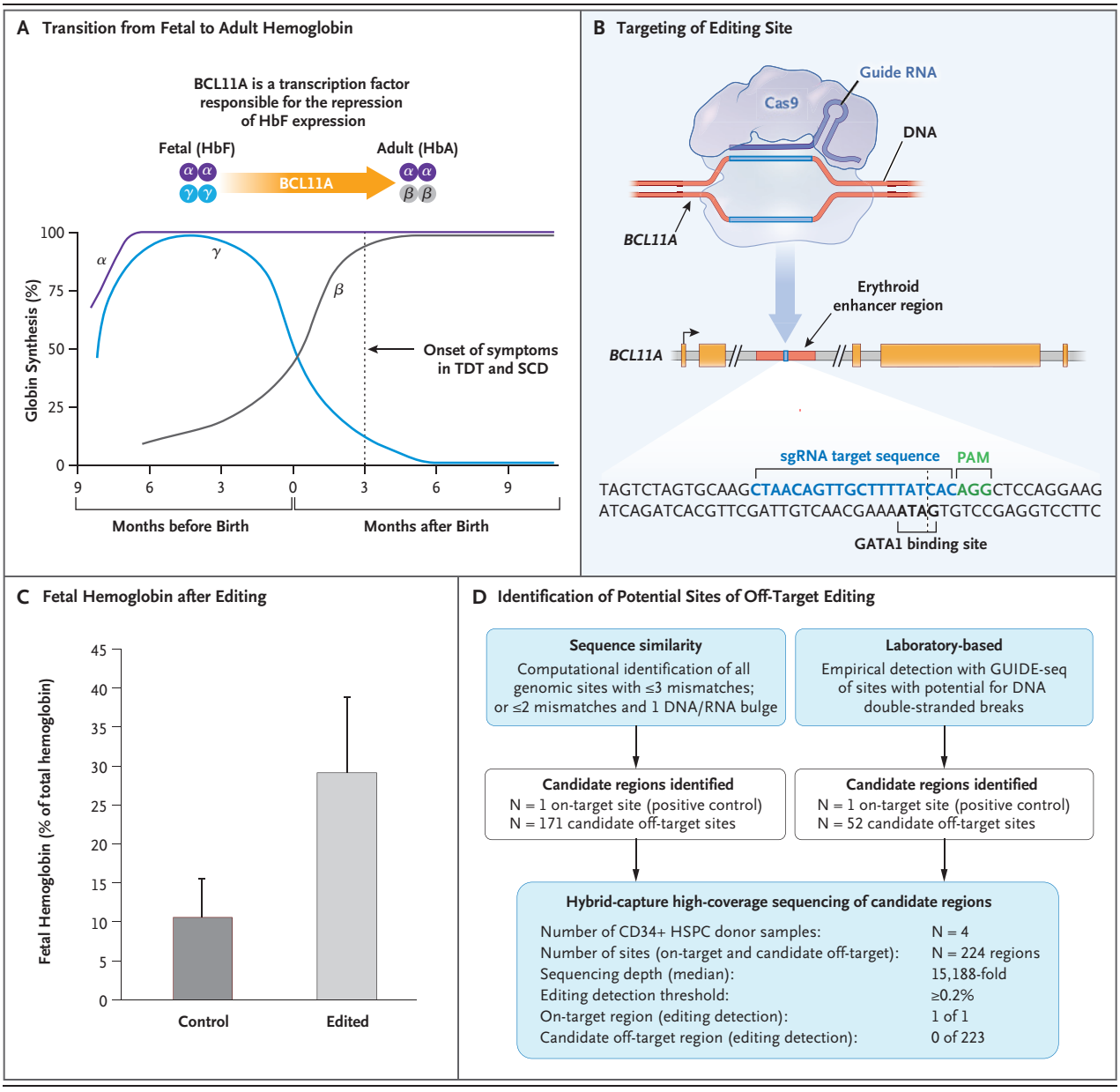
METHODS

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

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]

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 genomewide 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, in-

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.

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.

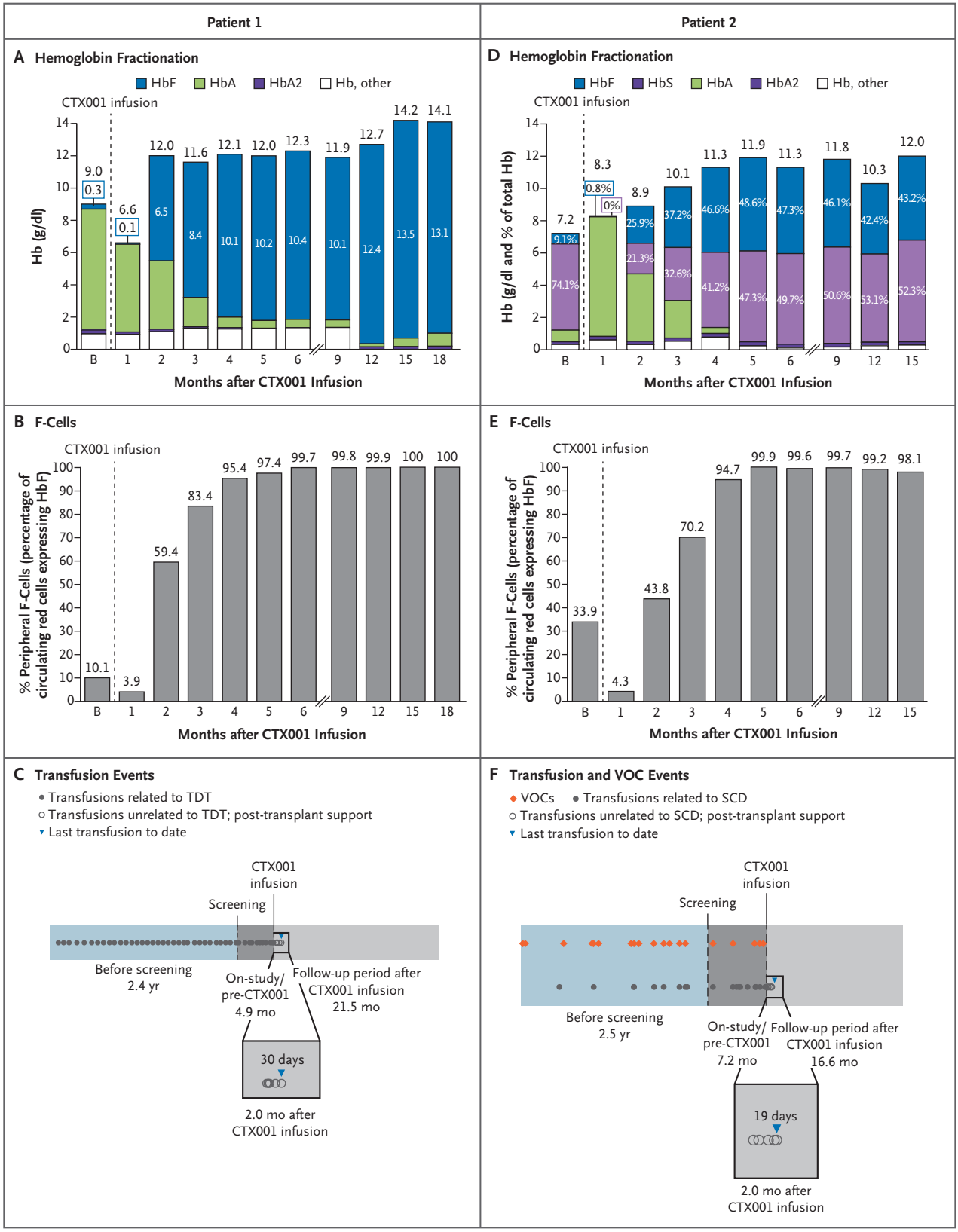
cluding the 18-month study visit, at the time of this report.

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 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 ($\beta S/\beta S$ 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 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 pneu-

monia 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 preclinical 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

The authors' full names and academic degrees are as follows: Haydar Frangoul, M.D., David Altshuler, M.D., Ph.D., M. Domenica Cappellini, M.D., Yi-Shan Chen, Ph.D., Jennifer Domm, M.D., Brenda K. Eustace, Ph.D., Juergen Foell, M.D., Josu de la Fuente, M.D., Ph.D., Stephan Grupp, M.D., Ph.D., Rupert Handgretinger, M.D., Tony W. Ho, M.D., Antonis Kattamis, M.D., Andrew Kernytsky, Ph.D., Julie Lekstrom-Himes, M.D., Amanda M. Li, M.D., Franco Locatelli, M.D., Markus Y. Mapara, M.D., Ph.D., Mariane de Montalembert, M.D., Damiano Rondelli, M.D., Akshay Sharma, M.B., B.S., Sujit Sheth, M.D., Sandeep Soni, M.D., Martin H. Steinberg, M.D., Donna Wall, M.D., Angela Yen, Ph.D., and Selim Corbacioglu, M.D.

The authors' affiliations are as follows: the Sarah Cannon Center for Blood Cancer at the Children's Hospital at TriStar Centennial, Nashville (H.F., J.D.), and St. Jude Children's Research Hospital, Memphis (A.S.) — both in Tennessee; Vertex Pharmaceuticals (D.A., B.K.E., J.L.-H., A.Y.) and Boston University School of Medicine (M.H.S.), Boston, and CRISPR Therapeutics, Cambridge (Y.-S.C., T.W.H., A. Kernytsky, S. Soni) — both in Massachusetts; the University of Milan, Milan (M.D.C.), and Ospedale Pediatrico Bambino Gesù Rome, Sapienza, University of Rome, Rome (F.L.); the University of Regensburg, Regensburg (J. Foell, S.C.), and Children's University Hospital, University of Tübingen, Tübingen (R.H.) — both in Germany; Imperial College Healthcare NHS Trust, St. Mary's Hospital, London (J. de la Fuente); Children's Hospital of Philadelphia, Perelman School of Medicine at the University of Pennsylvania, Philadelphia (S.G.); the University of Athens, Athens (A. Kattamis); BC Children's Hospital, University of British Columbia, Vancouver (A.M.L.), and the Hospital for Sick Children–University of Toronto, Toronto (D.W.) — both in Canada; Columbia University (M.Y.M.) and the Joan and Sanford I. Weill Medical College of Cornell University (S. Sheth), New York; Necker–Enfants Malades Hospital, Assistance Publique–Hôpitaux de Paris, University of Paris, Paris (M.M.); and the University of Illinois at Chicago, Chicago (D.R.).

REFERENCES

1. Bauer DE, Orkin SH. Hemoglobin switching's surprise: the versatile transcription factor BCL11A is a master repressor of fetal hemoglobin. Curr Opin Genet Dev 2015;33:62-70.
2. Piel FB, Steinberg MH, Rees DC. Sickle cell disease. N Engl J Med 2017;376:1561-73.
3. Saraf SL, Molokie RE, Nouraie M, et al. Differences in the clinical and genotypic presentation of sickle cell disease around the world. Paediatr Respir Rev 2014;15:4-12.
4. Cao A, Galanello R. Beta-thalassemia. Genet Med 2010;12:61-76.
5. Pasricha SR, Drakesmith H. Hemoglobinopathies in the fetal position. N Engl J Med 2018;379:1675-7.
6. Shah FT, Sayani F, Trompeter S, Drasar E, Piga A. Challenges of blood transfusions in β-thalassemia. Blood Rev 2019;37:100588.
7. Engert A, Balduini C, Brand A, et al. The European Hematology Association Roadmap for European Hematology Research: a consensus document. Haematologica 2016;101:115-208.
8. Platt OS, Orkin SH, Dover G, Beardsley GP, Miller B, Nathan DG. Hydroxyurea enhances fetal hemoglobin production in sickle cell anemia. J Clin Invest 1984;74:652-6.
9. Cappellini MD, Viprakasit V, Taher AT, et al. A phase 3 trial of luspatercept in patients with transfusion-dependent β-thalassemia. N Engl J Med 2020;382:1219-31.
10. Ataga KI, Kutlar A, Kanter J, et al. Crizanlizumab for the prevention of pain crises in sickle cell disease. N Engl J Med 2017;376:429-39.
11. Baronciani D, Angelucci E, Potschger U, et al. Hemopoietic stem cell transplantation in thalassemia: a report from the European Society for Blood and Bone Marrow Transplantation Hemoglobinopathy Registry, 2000-2010. Bone Marrow Transplant 2016;51:536-41.
12. Eapen M, Brazauskas R, Walters MC, et al. Effect of donor type and conditioning regimen intensity on allogeneic transplantation outcomes in patients with sickle cell disease: a retrospective multicentre, cohort study. Lancet Haematol 2019;6(11):e585-e596.
13. Gluckman E, Cappelli B, Bernaudin F, et al. Sickle cell disease: an international

survey of results of HLA-identical sibling hematopoietic stem cell transplantation. Blood 2017;129:1548-56.
14. European Medicines Agency. Zynteglo: summary of product characteristics (https://www.ema.europa.eu/en/documents/product-information/zynteglo-epar-product-information_en.pdf).
15. Thompson AA, Walters MC, Kwiatkowski J, et al. Gene therapy in patients with transfusion-dependent β-thalassemia. N Engl J Med 2018;378:1479-93.
16. Ribeil JA, Hacein-Bey-Abina S, Payen E, et al. Gene therapy in a patient with sickle cell disease. N Engl J Med 2017;376:848-55.
17. Brendel C, Guda S, Renella R, et al. Lineage-specific BCL11A knockdown circumvents toxicities and reverses sickle phenotype. J Clin Invest 2016;126:3868-78.
18. Brendel C, Negre O, Rothe M, et al. Preclinical evaluation of a novel lentiviral vector driving lineage-specific BCL11A knockdown for sickle cell gene therapy. Mol Ther Methods Clin Dev 2020;17:589-600.
19. Esrick EB, Lehmann LE, Biffi A, et al. Post-transcriptional genetic silencing of *BCL11A* to treat sickle cell disease. N Engl J Med 2021;384:205-15.
20. Musallam KM, Sankaran VG, Cappellini MD, Duca L, Nathan DG, Taher AT. Fetal hemoglobin levels and morbidity in untransfused patients with β-thalassemia intermedia. Blood 2012;119:364-7.
21. Platt OS, Brambilla DJ, Rosse WF, et al. Mortality in sickle cell disease: life expectancy and risk factors for early death. N Engl J Med 1994;330:1639-44.
22. Powars DR, Weiss JN, Chan LS, Schroeder WA. Is there a threshold level of fetal hemoglobin that ameliorates morbidity in sickle cell anemia? Blood 1984;63:921-6.
23. Sankaran VG, Orkin SH. The switch from fetal to adult hemoglobin. Cold Spring Harb Perspect Med 2013;3(1):a011643.
24. Canver MC, Orkin SH. Customizing the genome as therapy for the β-hemoglobinopathies. Blood 2016;127:2536-45.
25. Steinberg MH. Fetal hemoglobin in sickle cell anemia. Blood 2020;136:2392-400.
26. Bauer DE, Kamran SC, Lessard S, et al. An erythroid enhancer of *BCL11A* subject to genetic variation determines fetal hemoglobin level. Science 2013;342:253-7.
27. Uda M, Galanello R, Sanna S, et al. Genome-wide association study shows *BCL11A* associated with persistent fetal hemoglobin and amelioration of the phenotype of β-thalassemia. Proc Natl Acad Sci U S A 2008;105:1620-5.
28. Bak RO, Dever DP, Porteus MH. CRISPR/Cas9 genome editing in human hematopoietic stem cells. Nat Protoc 2018;13:358-76.
29. Hendel A, Bak RO, Clark JT, et al. Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. Nat Biotechnol 2015;33:985-9.
30. Canver MC, Smith EC, Sher F, et al. *BCL11A* enhancer dissection by Cas9-mediated in situ saturating mutagenesis. Nature 2015;527:192-7.
31. Wu Y, Zeng J, Roscoe BP, et al. Highly efficient therapeutic gene editing of human hematopoietic stem cells. Nat Med 2019;25:776-83.
32. Dalle JH, Giralt SA. Hepatic veno-occlusive disease after hematopoietic stem cell transplantation: risk factors and stratification, prophylaxis, and treatment. Biol Blood Marrow Transplant 2016;22:400-9.
33. Bomberger C, Singh-Jairam M, Rodey G, et al. Lymphoid reconstitution after autologous PBSC transplantation with FACS-sorted CD34+ hematopoietic progenitors. Blood 1998;91:2588-600.
34. Nachbaur D, Kropshofer G, Heitger A, et al. Phenotypic and functional lymphocyte recovery after CD34+-enriched versus non-T cell-depleted autologous peripheral blood stem cell transplantation. J Hematother Stem Cell Res 2000;9:727-36.
35. Corbacioglu S, Cappellini MD, Chapin J, et al. Initial safety and efficacy results with a single dose of autologous CRISPR-CAS9 modified CD34+ hematopoietic stem and progenitor cells in transfusion-dependent β-thalassemia and sickle cell disease. Presented at the virtual European Hematology Association Congress, June 11–14, 2020. abstract.
36. Frangoul H, Bobruff Y, Cappellini MD, et al. Safety and efficacy of CTX001 in patients with transfusion-dependent β-thalassemia or sickle cell disease: early results from the CLIMB THAL-111 and CLIMB SCD-121 studies of autologous CRISPR-CAS9-modified CD34+ hematopoietic stem and progenitor cells. Presented at the American Society of Hematology Annual Meeting, December 5–8, 2020. abstract.

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Frangoul H, Altshuler D, Cappellini MD, et al. CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. N Engl J Med 2021;384:252-60. DOI: 10.1056/NEJMoa2031054

Supplementary Appendix to:

CRISPR-Cas9 Gene Editing for SCD and Transfusion-Dependent β-Thalassemia

Haydar Frangoul, M.D.,[1] David Altshuler, M.D., Ph.D.,[2] Maria Domenica Cappellini, M.D.,[3] Yi-Shan Chen, Ph.D., [4] Jennifer Domm, M.D., [1] Brenda K. Eustace, Ph.D.,[2] Juergen Foell, M.D., [5] Josu de la Fuente, M.D., Ph.D.,[6] Stephan Grupp, M.D., Ph.D.,[7] Rupert Handgretinger, M.D., [8] Tony W. Ho, M.D.,[4] Antonis Kattamis, M.D.,[9] Andrew Kernytsky, Ph.D.,[4] Julie Lekstrom-Himes, M.D.,[2] Amanda M. Li, M.D.,[10] Franco Locatelli, M.D.,[11] Markus Y. Mapara, M.D., Ph.D.,[12] Mariane de Montalembert, M.D.,[13] Damiano Rondelli, M.D.,[14] Akshay Sharma, M.B.B.S.,[15] Sujit Sheth, M.D.,[16] Sandeep Soni, M.D.,[4] Martin H. Steinberg, M.D.,[17] Donna Wall, M.D.,[18] Angela Yen, Ph.D.,[2] and Selim Corbacioglu, M.D.[5]

[1]Sarah Cannon Center for Blood Cancer at The Children's Hospital at TriStar Centennial, Nashville, TN, USA; [2]Vertex Pharmaceuticals Incorporated, Boston, MA, USA; [3]University of Milan, Milan, Italy; [4]CRISPR Therapeutics, Cambridge, MA, USA; [5]University of Regensburg, Regensburg, Germany; [6]Imperial College Healthcare NHS Trust, St Mary's Hospital, London, UK; [7]Children's Hospital of Philadelphia, Perelman School of Medicine at the University of Pennsylvania, PA, USA; [8]Children's University Hospital, University of Tübingen, Tübingen, Germany; [9]University of Athens, Athens, Greece; [10]BC Children's Hospital, University of British Columbia, Vancouver, Canada; [11]Ospedale Pediatrico Bambino Gesù Rome, Sapienza, University of Rome, Italy; [12]Columbia University, New York, NY, USA; [13]Necker-Enfants Malades Hospital, Assistance Publique-Hôpitaux de Paris (AP-HP), University of Paris, Paris, France; [14]University of Illinois at Chicago, Chicago, IL, USA; [15]St. Jude Children's Research

Hospital, Memphis, TN, USA; [16]Joan and Sanford I Weill Medical College of Cornell University, New York, NY, USA; [17]Boston University School of Medicine, Boston, MA, USA; [18] The Hospital for Sick Children/University of Toronto, Toronto, Canada

Contents

List of Investigators

The CLIMB THAL-111 Study Group, as of October 01, 2020, includes Monica Bhatia, Pediatric Hematology, Oncology and Stem Cell Transplantation, Columbia University Herbert Irving Comprehensive Cancer Center, New York NY; Ami Shah, Pediatrics Stem Cell Transplantation, Lucile Packard Children's Hospital, Palo Alto, CA; Markus Mapara, Columbia University, New York, NY, USA; Haydar Frangoul, Sarah Cannon Center for Blood Cancer at The Children's Hospital at TriStar Centennial, Nashville, TN; Rupert Handgretinger, Children's University Hospital, University of Tübingen, Tübingen, Germany; Franco Locatelli, Ospedale Pediatrico Bambino Gesù Rome, Sapienza, University of Rome, Italy; Selim Corbacioglu, University of Regensburg, Regensburg, Germany; Josu De La Fuente, Imperial College Healthcare NHS Trust, St Mary's Hospital, London, UK; Donna Wall, University of Manitoba, Winnipeg, Canada; Amanda Li, BC Children's Hospital, University of British Columbia, Vancouver, Canada.

The CLIMB SCD-121 Study Group, as of October 01, 2020, includes Selim Corbacioglu, University of Regensburg, Regensburg, Germany; Franco Locatelli, Ospedale Pediatrico Bambino Gesù Rome, Sapienza, University of Rome, Italy; Josu de la Fuente, Imperial College Healthcare NHS Trust, St Mary's Hospital, London, UK; Donna Wall, The Hospital for Sick Children/University of Toronto, Toronto, Canada; Michael Eckrich, Methodist Healthcare System of San Antonio/Methodist Children's Hospital; Alina Ferster, Hopital Universitaire des Enfants Reine Fabiola (HUDERF); Monica Bhatia, Columbia University Medical Center; Ami Shah, Lucile Packard Children's Hospital (Stanford University), Palo Alto, Domiano Rondelli, University of Illinois at Chicago, Chicago, IL; Markus Mapara, Columbia University, New York, NY; Haydar Frangoul, Sarah Cannon Center for Blood Cancer at The Children's Hospital at TriStar Centennial, Nashville, TN; Akshay Sharma, St. Jude Children's Research Hospital, Memphis, TN; Stephen Grupp, Children's Hospital of Philadelphia, Perelman School of Medicine at the University of Pennsylvania, PA.

Supplementary Methods

I. Mobilization and Apheresis for Participants with TDT in CLIMB THAL-111 (NCT03655678)

Mobilization

Before starting administration of plerixafor and G-CSF (e.g., filgrastim), participants were assessed by the study investigator to confirm eligibility to proceed with apheresis (as per local guidelines). Decisions on whether a central line was required was made by the apheresis-experienced nurse or physician. G-CSF (e.g., filgrastim) was administered subcutaneously or intravenously at a dose of 5µg/kg/dose every 12 hours for 5-6 days. Plerixafor (0.24 mg/kg, subcutaneous injection) was administered for 4 days before leukapheresis. G-CSF (e.g., filgrastim) could be adjusted based on local practices in the presence of significant leukocytosis (e.g., >70 x 10^9/L).

TDT Apheresis Procedure

CD34+ human hematopoietic stem and progenitor cells (HSPCs) were collected per clinical site protocols.

II. Mobilization and Apheresis for Participants with SCD in CLIMB SCD-121 (NCT03745287)

Mobilization

Red blood cell (RBC) exchange transfusions were conducted for a minimum of 8 weeks prior to first mobilization. Before starting administration of plerixafor, participants were assessed by the study investigator to confirm whether they were eligible to proceed with apheresis (as per local guidelines). Each participant underwent stem cell mobilization with plerixafor (0.24 mg/kg subcutaneous injection) only. Patient 2 (SCD) was admitted to the hospital and observed throughout the mobilization process; hemoglobin was maintained at 10g/dL during mobilization therapy.

SCD Apheresis Procedure

Peripheral blood mononuclear cells (PBMC) were collected by apheresis.
Participants received RBC exchange transfusion 3 (± 1) day before the start of mobilization/ apheresis cycle.

III. Conditioning: Busulfan Administration

Busulfan was administered IV daily at a starting dose of 3.2 mg/kg/day for 4 consecutive days (based on weight collected within 3 to 7 days prior to the first day of busulfan administration). Once-daily dosing was the preferred schedule, but the busulfan dose regimen could be adjusted to be given q6h per site/investigator preference. The average target area under the curve (AUC) for participants at a starting dose of 3.2 mg/kg/day for 4 days was 5000 µM·min (range: 4500 to

5500), equivalent to a target cumulative busulfan exposure of 90 mg·hr/L (range 80 to100 mg ·hr/L). The AUC for participants receiving busulfan q6h for 4 days was 1125 μM·min (range: 900 to 1350) (Table S8; Table S13).

IV. CTX001 Manufacturing and Infusion Procedures

CTX001 is composed of autologous CD34+ HSPCs modified with CRISPR-Cas9 at the erythroid lineage-specific enhancer region of the BCL11A gene. All manufacturing procedures, including gRNA, Cas9, and CD34+ cells, were carried out under GMP manufacturing conditions.

The gRNA was synthesized in the 3' to 5' direction using standard solid support synthesis methods followed by purification. Cas9 protein was produced in recombinant *E.coli* fermentation and purified by cation exchange and hydrophobic interaction column chromatography.

Peripheral patient PBMCs were collected at a qualified clinical site by apheresis, according to the clinical protocol, and shipped to the manufacturing site under controlled conditions. Following platelet reduction, CD34+ HSPCs were isolated from PBMCs using a closed, automated, sterile, micro-bead system (CliniMACS Prodigy System, Miltenyi Biotec). CD34+ enriched HSPCs were sampled for cell count, viability, and post-sort CD34+ HSPC enumeration before incubation in defined culture medium. Following this culture period, the cultures were sampled for cell count and viability, and cells were sampled as unedited controls for allelic editing assays.

The ribonucleoprotein complex (RNP) was prepared in situ prior to electroporation by mixing the gRNA and the Cas9 protein. The electroporation mix containing cells and RNP complex was then loaded into a cassette and electroporated (MaxCyte Gen2 GT). After electroporation, the cells were incubated in defined culture medium prior to cryo-preservation and storage in vapor phase liquid nitrogen. Quality control release assays were performed on CTX001 samples, including CD34+ cell purity analysis by flow cytometry, on-target editing frequency (TIDE; see Section XI), post-thaw cell count and viability, as well as compendial sterility, mycoplasma, and endotoxin testing. CTX001 frozen suspension was shipped to the clinical site where it was stored in liquid nitrogen vapor. CTX001 was thawed just prior to the scheduled infusion utilizing local site SOPs. The single dose of CTX001 was given after the last busulfan dose (Figure S2).

V. Definition and Assessments of Neutrophil and Platelet Engraftment

Neutrophil engraftment was defined as the first day of 3 measurements of absolute neutrophil count (ANC) ≥ 500μL on 3 consecutive days, achieved within 42 days post-CTX001 infusion, without use of the unmodified (back-up) CD34+ cells after reaching the nadir, defined as ANC < 500μL. Platelet engraftment was defined as the first day of 3 consecutive measurements of platelet ≥20,000μL (TDT) or ≥50,000μL (SCD) on 3 different days after CTX001 infusion, without platelet-transfusion support in the past 7 days, after reaching the nadir, defined as platelet <20,000μL (TDT) or <50,000 μL (SCD).

VI. Blood for Biomarker Assessments

Blood samples were collected for evaluation of biomarkers:

- Protein based biomarkers, including but not limited to: (1) hemoglobin fractionation and quantitation in peripheral blood to assess bulk fetal hemoglobin (HbF) levels, and (2) the proportion of circulating erythrocytes expressing HbF (F-cells)
- Proportion of alleles with intended genetic modification present in peripheral blood leukocyte DNA.

VII. Guide RNA Sequence Used in CRISPR-Cas9 Editing of the *BCL11A* Enhancer Region

Guide RNA sequence:

7*5*6 *ACA GUU GCU UUU AUC ACG UUU UAG AGC UAG AAA UAG CAA GUU AAA AUA AGG CUA GUC CGU UAU CAA CUU GAA AAA GUG GCA CCG AGU CGG UGC 5*5*5 *U

Modified positions are shown as numbers (5: 2' OMe-rU; 6: 2' OMe-rA; 7: 2' OMe-rC), phosphorothioate positions are highlighted with an asterisk (*).

VIII. Identification of Candidate Off-target Regions Using GUIDE-seq

We performed GUIDE-seq to nominate potential off-target editing sites in primary CD34+ hematopoietic stem and progenitor cells (HSPCs). We optimized the GUIDE-seq protocol for CD34+ HSPCs to provide high cell viability and sensitivity based on the on-target site. To generate the double-stranded DNA oligo (dsODN), two modified single-stranded GUIDE-seq oligonucletotides (Integrated DNA Technologies) were combined with molecular biology grade water and 10x annealing buffer (New England Biolabs) and heated in a thermocycler. The ribonucleoprotein (RNP) complex (sgRNA and Cas9 protein) and desired concentration of GUIDE-seq dsODN was added to CD34+ HSPCs and electroporated at research scale using the Lonza Amaxa 4D-Nucleofector. All donors had high (> 70%) cell viability at 48 hours post-electroporation of the dsODN and RNP. To generate a DNA sequencing library, genomic DNA (gDNA) was isolated using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturers protocol. gDNA was sheared to 200 bp fragments using a Covaris LE220 Focused-ultrasonicator. All remaining steps for sequencing library preparation were completed following GUIDE-seq methods described in Tsai et al.[1] GUIDE-seq libraries were sequenced on an Illumina NextSeq500 sequencer (Illumina). Sequencing reads were analyzed using a version of the GUIDE-seq analysis pipeline adapted for NextSeq sequencing reads[2] with the human genome build hg38 as the reference genome. All sites identified from the step "identify" were considered candidate off-target Cas9 cleavage sites regardless of the degree of sequence homology with sgRNA sequence. Due to the potential for false positive candidate off-target regions, all candidate GUIDE-seq regions suitable for next-generation sequencing were assessed

with hybrid capture sequencing, with low-complexity regions excluded from hybrid capture sequencing. The on-target site was used as an internal positive control and was identified in the edited samples of every donor with > 8,000 GUIDE-seq reads. In this experiment the number of (within sample control) on-target GUIDE-seq reads was higher than that in the original GUIDE-seq publication[1]. Tests of multiple guide RNAs from other projects using this same GUIDE-seq assay detected candidate off-target sites that were confirmed following hybrid capture.

IX. Identification of Candidate Off-target Regions Using Computational Methods

Computational prediction was used to identify regions with high sequence similarity to the sgRNA sequence. Specifically, regions in the reference human genome build hg38 were identified that contained up to 3 mismatches, or up to 2 mismatches with one DNA or RNA bulge, relative to the target sequence. The optimal NGG PAM was used for all algorithms, as well as the alternate PAMs NAG, NGA, NAA, NCG, NGC, NTG, and NGT when possible.

X. Hybrid-capture High-coverage Sequencing of Candidate Off-target Regions

Candidate regions identified by GUIDE-seq and computational methods were used to generate hybrid capture probes. gDNA from edited CD34+ HSPCs was isolated using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. gDNA was fragmented by acoustic shearing with a Covaris LE220 instrument, and then end repaired, A-tailed, adapter ligated, and amplified with Agilent's SureSelect^{XT} Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library kit (Agilent Technologies) according to manufacturer's protocol. Prepared libraries were hybridized to probes (Agilent) and captured using streptavidin-coated magnetic beads (Dynabeads MyOne Streptavidin T1), amplified, and purified. Libraries were sequenced on Illumina's HiSeq sequencing platform, and sequencing data was subsequently aligned, deduplicated, and indels proximal to the cut site were counted. For each donor, indel frequency in sgRNA-RNP-treated samples and indel frequency in untreated negative controls were compared. For sites showing an indel frequency at least 0.2% higher in the treated sample compared to the matched untreated sample in any donor, we assessed whether indels seen at a potential off-target site were significantly enriched in samples treated with sgRNA-RNP as compared to untreated controls. A candidate site was confirmed to have editing if the statistical test resulted in a significant p-value ($p < 0.05$). No correction for multiple hypothesis testing was applied.

XI. On-Target Allelic Editing Frequency

The frequency of on-target allelic editing was assessed using Sanger sequencing, followed by analysis with a version of the Tracking of Indels by DEcomposition (TIDE) algorithm[3], except as specified in the engraftment study in Supplementary Methods Section XIV.

XII. Hemoglobin Tetramer Analysis

Edited or unedited CD34+ HSPCs were put through a three-step in vitro erythroid differentiation as previously described with some modifications.[4] Briefly, cells were cultured in the absence of an adherent stromal layer, without hydrocortisone, and with 20 ng/ml stem cell factor. Hemoglobin tetramers from in vitro differentiated erythroid cells were analyzed by ion-exchange high performance liquid chromatography with multi-wavelength UV detector (Agilent Technologies). Data were calculated from area under the peaks for fetal hemoglobin (HbF, α2γ2) and adult hemoglobin (HbA, α2β2 and α2d2), and presented as percentage of HbF over total hemoglobin [HbF/Total hemoglobin].

XIII. Subpopulation Analysis by Flow Cytometry

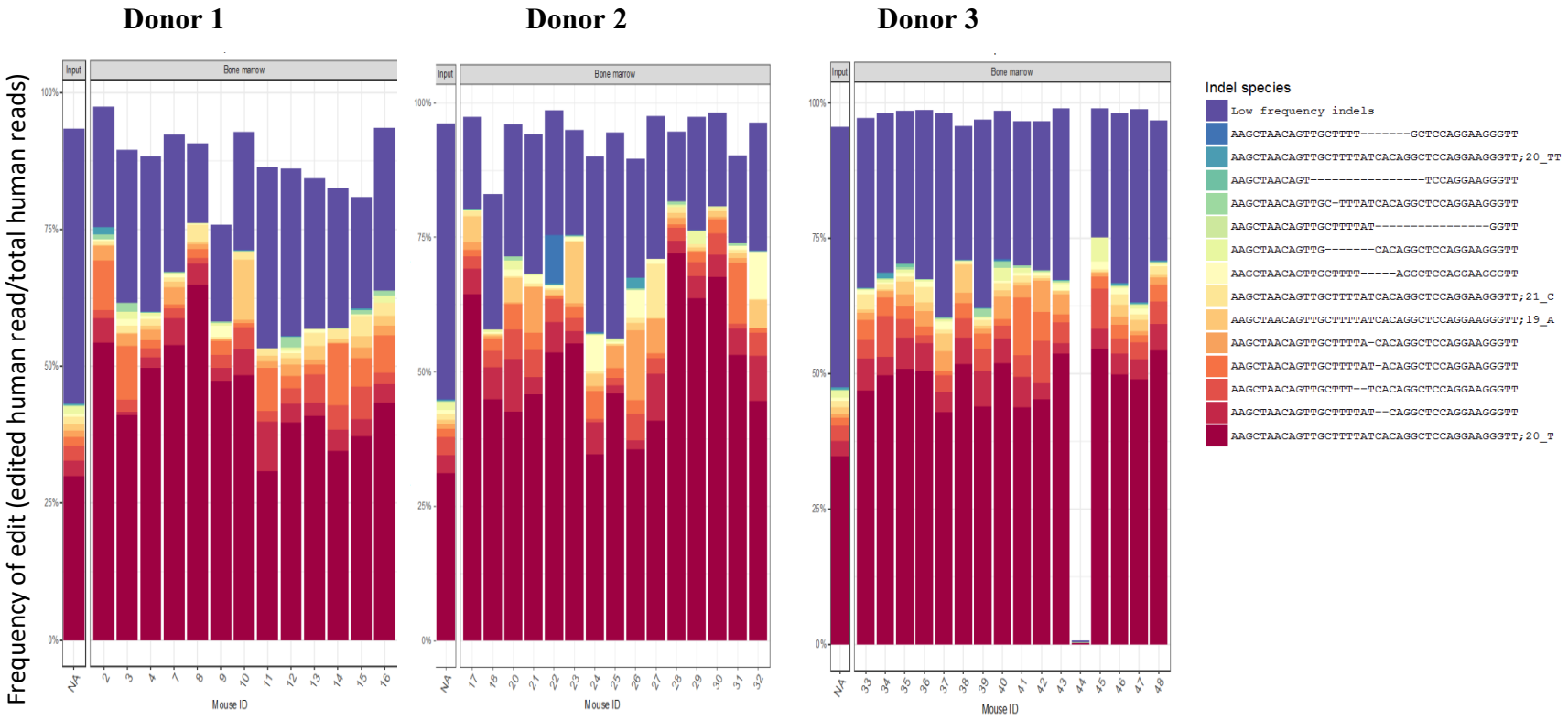
Edited CD34+ HSPCs were stained with CD34 antibody (Clone 581, BioLegend), CD38 antibody (Clone HB7, BioLegend), CD90 antibody (Clone 5E10, BD Biosciences) and CD45RA antibody (Clone HI100, BD Biosciences) and sorted with the BD FACSAria Fusion (BD Biosciences). Subpopulations were sorted based on the cell surface markers: live cells were first divided into two groups based on CD34 and CD38 staining. The CD34+/CD38+ subpopulation was further divided into CD45RA+ (granulocyte-monocyte progenitor cells) and CD45RA– (common myeloid progenitor cells / megakaryocyte-erythroid progenitor cells) populations. The CD34+CD38– subpopulation was further divided into three subpopulations based on CD90 and CD45RA staining patterns: CD90+CD45RA– (LT-HSC), CD90–CD45RA– (multipotent progenitor cells), and CD90–CD45RA+ (multipotent lymphoid progenitor cells). To distinguish between positive and negative staining, fluorescence minus one (FMO) controls were used as references. Allelic editing frequency in each subpopulation was determined as described in Supplementary Methods Section X.

XIV. Mouse Engraftment Study

~0.5 x 10^6 genetically edited or unedited CD34+ human HSPCs were injected into 6-8 weeks old female NOD/SCID/IL2Rγnull (NSG) mice following a total body irradiation (radiation dose 200 cGy). After a 16-week observation period, bone marrow samples were harvested. Percentage of human chimerism was assessed by flow cytometry using antibodies specific for human CD45RA and mouse CD45 (hCD45RA antibody, clone HI100, BD Biosciences; mCD45 antibody, clone 30-F11, BD Biosciences). gDNA was extracted from mouse bone marrow samples. An amplicon sequencing library was generated for each sample with two rounds of PCR: (i) using human genome target-specific primers and (ii) using Illumina sequencing-compatible index primers. Amplicon libraries were sequenced on Illumina's MiSeq sequencing platform with a 2x150bp configuration. Computational analysis was performed to quantify on-target allelic editing frequency and unique indel species. Sequencing reads were demultiplexed, merged, mapped, and aligned to the human reference genome hg38. Sequencing reads were grouped based on the sequence at the cut site to identify unique indel species and corresponding frequencies. To calculate total allelic editing frequency, frequencies of all observed indel species were summed, and a one-sided Mann-Whitney test was used to test for any significant decrease in total percent editing at 16 weeks post-injection. A p-value less than 0.05 was considered statistically significant.

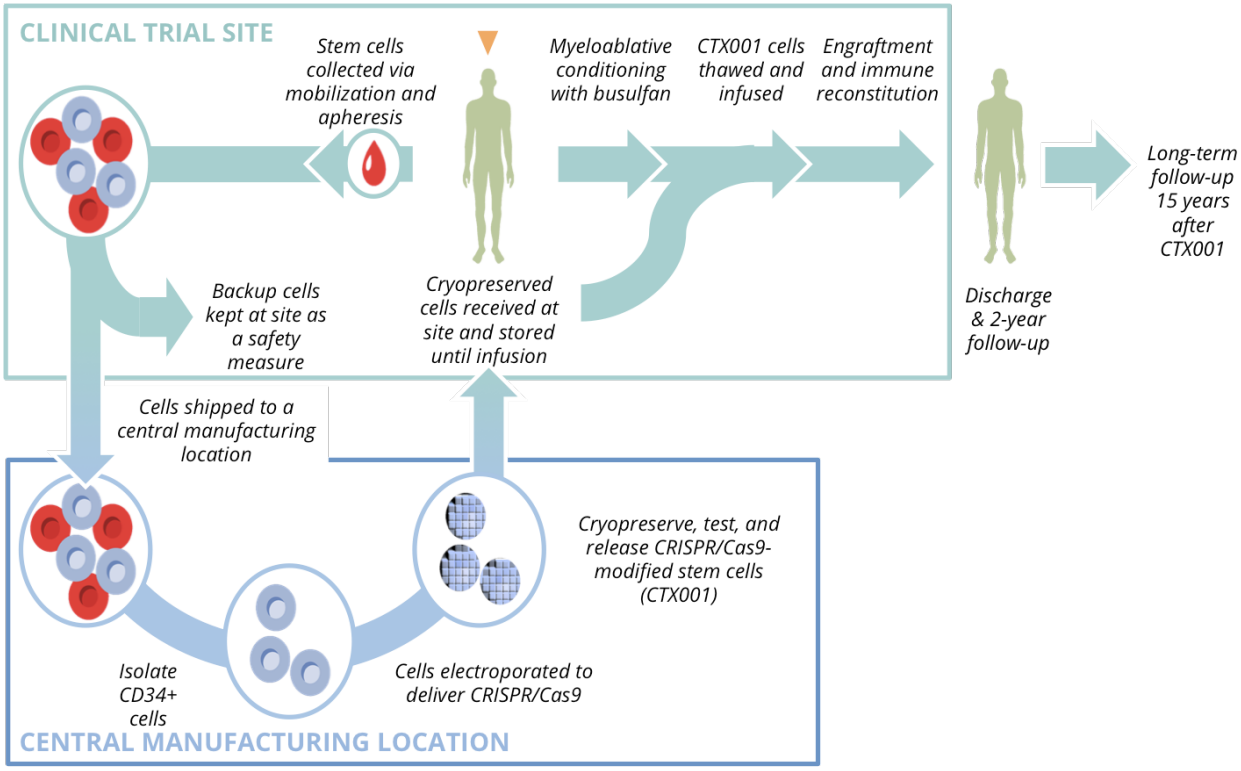
Supplementary Results

Figure S1. Indel Distribution Profile in Input Edited CD34+ Human HSPCs and Engrafted Mouse Bone Marrow



Notes: Indel distributions of CD34^{+} cells in sgRNA-RNP-edited input and mouse bone marrow samples at 16 weeks post-injection for sgRNA-RNP-edited samples from three donors. The top purple stacked bar is a conglomerate of all unique sequences with <8% frequency in all sgRNA-RNP-edited samples. Each color other than the top bar represents a single unique on-target edit (insertion or deletion). Deleted bases are indicated with a dash, whereas insertions are annotated with their position relative to the shown sequence and the sequence inserted, such that "20_T" indicates an insertion of a T at the cut site, centered in the sequence shown (20 bases from the start of the sequence shown). The total proportion of edited sequences is represented by the total height of the stacked bars. Each input edited hHSPC sample and mouse bone marrow sample is represented by a separate bar. Mouse #44 has very low or background levels of allelic editing likely due to experimental error.

Figure S2. Schematic Representation of the CTX001 Manufacturing and Infusion Process



Patients enrolled in CLIMB THAL-111 received a combination of plerixafor and filgrastim for mobilization, while patients enrolled in CLIMB SCD-121 received plerixafor only.

Patients will be followed for 24 months after CTX001 infusion with physical exams, laboratory and imaging assessments, and AE evaluations. All patients who receive CTX001 will be followed for 15 years in a long-term follow-up study (NCT04208529) after completion or withdrawal from CLIMB THAL-111 or CLIMB SCD-121.

Table S1. Allelic Editing Frequency in Subpopulations of Edited CD34+ HSPCs

Sample	Bulk Editing (%)	LT-HSC (%)	MPP (%)	MLP (%)	GMP (%)	CMP/MEP (%)
1	74	78	80	87	85	86
2	77	77	81	79	83	84
3	75	70	71	87	88	87
4	77	65	67	85	83	70
5	80	73	76	90	90	89
6	81	79	80	88	88	79
7	85	85	79	88	89	88
8	86	85	80	89	91	85

LT-HSC: long-term hematopoietic stem cells; MPP: multipotent progenitor cells; MLP: multipotent lymphoid progenitor cells; GMP: granulocyte-monocyte progenitor cells; CMP: common myeloid progenitor cells; MEP: megakaryocyte-erythroid progenitor cells; HSPC: hematopoietic stem and progenitor cells.

Table S2. Persistence of Editing in Mice Engrafted with Edited CD34+ Human HSPCs at 16 Weeks

Sample Type[a]	Number of Samples	Allelic Editing Frequency Mean (Standard Deviation)
Input (Edited CD34+ Human HSPCs)	3	95.1% (1.5%)
Bone marrow from mice engrafted with edited CD34+ human HSPCs	44	91.4% (15.1%)[b]

[a] Genetically edited and unedited CD34+ human HSPCs were injected into NOD/SCID/IL2Rγnull (NSG) mice via a single intravenous (i.v.) injection. After a 16-week observation period, mouse bone marrow samples were collected to assess allelic editing frequency in engrafted human cells.

[b] Using a one-sided Mann-Whitney test to check for a loss of editing after engraftment, the evidence did not reject the null hypothesis that no reduction in editing occurred in bone marrow (P = 0.61), suggesting that there is not a significant reduction in editing over the time span of engraftment.

Table S3. Engraftment of Edited and Unedited CD34+ Human HSPCs at Week 8 and Week 16 in Mice: Analysis of hCD45RA^{+} % Chimerism[a] (Group Averages Across All Donors) Using Flow Cytometry

Tissue	Time Point	Group 1 (No-electroporation) % (SD)	Group 2 (Mock-EP)[b] % (SD)	Group 3 (GFP)[c] % (SD)	Group 4 (sgRNA)[d] % (SD)
Whole Blood	Week 8	43.2 (11.7) n=40	32.0 (10.1) n=47	34.1 (8.6) n=47	32.7 (9.4) n=47
	Week 16	12.9 (7.2) n=41	4.4 (3.5) n=48	4.1 (2.8) n=43	4.4 (3.1) n=44
Bone Marrow	Week 16	64.8 (14.1) n=44	49.5 (12.1) n=45	49.1 (13.0) n=45	46.0 (13.8) n=44
Spleen	Week 16	49.6 (15.5) n=39	28.4 (11.7) n=45	29.5 (11.7) n=43	27.0 (11.2) n=42

Note that all n values refer to the number of flow cytometry samples analyzed.
[a]Percentage of chimerism was determined by flow cytometry and is based on hCD45RA.
% Chimerism = % hCD45RA+ cells in live cells/(% hCD45RA+ cells in live cells + % mCD45+ cells in live cells)
[b] Mock electroporation of cells without any CRISPR/Cas9 editing components
[c] Electroporation of cells with green fluorescent protein (GFP) gRNA and Cas9 protein
[d] Electroporation of cell with sgRNA (used in this study) and Cas9 protein

Table S4. Mobilization Agents and CTX001 Cell Dose Manufactured for Patient 1 (TDT) and Patient 2 (SCD)

Mobilization and cell dose characteristics			
	Mobilization Cycles	**Mobilization Agents**	**Cell dose manufactured, 10^6 cells/kg**
Patient 1, TDT	1	G-CSF/plerixafor	16.6
Patient 2, SCD	2	Plerixafor	3.1

G-CSF, granulocyte colony-stimulating factor; SCD, sickle cell disease; TDT, transfusion-dependent β-thalassemia.

Table S5. Demographics and Pre-Study Characteristics of Patient 1 (TDT)

	Patient 1, TDT
Genotype	β^0/β^+ *β^0 due to the c.118 C>T mutation [also known as Codon 39 (C->T)]in exon 2 of the beta globin gene* β^+ *due to the c.93-21 G>A mutation [also known as IVS-I-110 (G->A)]*
Age at consent, *years*	19
Gender	F
Pre-study pRBC transfusions[a]	
Units/year	34
Transfusion episodes/year	16.5

[a]Annualized number during the 2 years before consenting to study participation. pRBC, packed red blood cells
TDT: transfusion-dependent β-thalassemia; pRBC: packed red blood cells

Table S6. Summary of Adverse Events for Patient 1 (TDT)

Screening to CTX001 infusion	
AEs	12
SAEs	0
After CTX001 infusion	
AEs	32
SAEs	2[a]
Months of follow-up	21.5
AEs by Grade	
Grade 1	31
Grade 2	7
Grade 3	6
Grade 4	0

[a]Pneumonia and venoocclusive liver disease both of which resolved
AE, adverse event; n, number of AE events; SAE, serious adverse event

Table S7. Summary of Laboratory Values for Patient 1 (TDT)[a]

	Normal Range	Baseline	Month 3	Month 6	Month 12
Creatinine (mg/dL)	0.5-1.1	0.54	0.48	0.51	0.68
AST (U/L)	0-34	15	17	21	13
ALT (U/L)	0-34	12	25	19	16
Total bilirubin (µmol/L)	3.4-23.9	85.3	28.6	32.1	27.4
Alkaline phosphatase (U/L)	45-117	80	138	149	158

	Normal Range	Baseline	Month 3	Month 6	Month 12
Hematocrit (%)	34.1-44.9	32.0	33.9	35.5	35.2
Total hemoglobin (g/dL)	11.2-15.7	11.2	11.6	12.5	12.5
MCV (fl)	79.4-94.8	82.9	80.7	80.0	79.6
MCH (PG)	25.6-32.2	29	27.6	28.2	28.3
MCHC (g/dL)	32.2-35.5	35.0	34.2	35.2	35.5
Leukocytes ($*10^6$/L)	3.98-10.00	8.64	4.65	5.14	5.39
Platelets ($*10^9$/L)	182-369	253	115	117	176
$CD19^+$ B-Cells ($*10^6$/L)	100-500	451	304	389	357

[a] Note that all values in table were collected and assayed at local laboratories. ALT: alanine aminotransferase; AST: aspartate aminotransferase; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration

Table S8. Busulfan Administration for Patient 1 (TDT)

Study Day[a]	Busulfan Dose	Observed AUC[b]
Day -7	3.2 mg/kg	3811.40 µmol*min/L
Day -6	5 mg/kg	Not Done
Day -5	5 mg/kg	7490.50 µmol*min/L
Day -4	2.4 mg/kg	Not Done

[a]Study Day 1 is day of CTX001 infusion.
[b]Target range for busulfan dose AUC is 4500-5500 µmol*min/L.
AUC: area under the curve

Table S9. Summary of LDH and Haptoglobin Data for Patient 1 (TDT)

Subject	Serum LDH[a] (U/L)	Haptoglobin[b] (mg/dL)	Reticulocyte Count (10^9/L)
Patient 1	B[c]: 185	B[c]: BDL	B[d]: 13.1
	M1: --	M1: --	M1: 6.3
	M2: --	M2: --	M2: 123.8
	M3: 156	M3: BDL	M3: 83.8
	M4: --	M4: --	M4: 77.2
	M5: --	M5: --	M5: 103.2
	M6: 159	M6: 7.8	M6: 76.8
	M9: 136	M9: 7.8	M9: 105.0
	M12: 159	M12: BDL	M12: 97.2
	M15: 140	M15: 10	M15: 72.7

B: baseline; BDL: below detectable limit; LDH: lactate dehydrogenase; M: Month; NA: not available; TDT: transfusion-dependent β-thalassemia

Note: Measured time points are presented according to study protocol.]

[a] Serum LDH normal range 0-249 U/L.

[b] Haptoglobin normal range 30-200 mg/dL.

[c] Baseline was collected at the most recent nonmissing measurement (scheduled or unscheduled) collected during screening and before the start of mobilization.

[d] Baseline was collected prior to mobilization procedure.

Table S10. Demographics and Pre-Study Characteristics of Patient 2 (SCD)

	Patient 2, SCD[a]
Genotype	β^s/β^s
Age at consent, *years*	33
Gender	F
Pre-study VOCs/year[b]	7
Pre-study hospitilizations/year[b]	3.5
Pre-study pRBC transfusions[b]	
Units/year	5

[a]Patient had received hydroxyurea treatment from 2016 to November 22, 2018 (Study Day −222)

[b]Annualized rate during the 2 years before consenting to study participation.

SCD: sickle cell disease; VOCs: vaso-occlusive crises; pRBC: packed red blood cell

Table S11. Summary of Adverse Events for Patient 2 (SCD)

Screening to CTX001 infusion	
AEs	35
SAEs	11[a]
After CTX001 infusion	
AEs	114
SAEs	3[b]
Months of follow-up	16.6
AEs by Grade	
Grade 1	46
Grade 2	48
Grade 3	53[c]
Grade 4	2[d]

[a]Sickle cell anemia with crisis (n=3), nausea, arthralgia, back pain, pain in extremity, chest pain, neck pain, headache, and abdominal pain, all of which resolved; [b]Sepsis, cholelithiasis, and abdominal pain, all of which resolved; [c]Most common Grade 3AEs occurring ≥2 times after CTX001 infusion: neck pain (n=5), headache (n=5), cholelithiasis (n=4), musculoskeletal chest pain (n=2) and non-cardiac chest pain (n=2), oesophagitis (n=3), and stomatitis (n=2), all of which resolved; [d]Neutropenia and leukopenia, both of which resolved

AE, adverse event; n, number of AE events; SAE, serious adverse event

Table S12. Summary of Laboratory Values for Patient 2 (SCD)[a]

	Normal Range	Baseline	Month 3	Month 6	Month 12
Creatinine (mg/dL)	0.5-1.1	0.73	0.64	0.76	0.45
AST (U/L)	8-46	39	33	70	18
ALT (U/L)	6-50	37	52	95	35
Total bilirubin (µmol/L)	0.0-22.2	15.4	8.6	8.6	8.6
Alkaline phosphatase (U/L)	50-136	203	244	374	186

	Normal Range	Baseline	Month 3	Month 6	Month 12
Hematocrit (%)	36-46	29.9	30.9	34.0	32.3
Total hemoglobin (g/dL)	12-16	10.2	10.3	11.4	11.1
MCV (fl)	80-100	85.3	92	89.8	86.7
MCH (PG)	27-35	29.1	30.6	30.1	29.8
MCHC (g/dL)	31-37	34.1	33.2	33.5	34.4
Leukocytes ($*10^9$/L)	3.9-10.6	11.7	6.2	4.7	3.8
Platelets ($*10^9$/L)	150-450	326	268	256	272
CD-19^+, B-cells ($*10^6$/L)	91-610	595	206	310	392

[a] Note that all values in table were collected and assayed at local laboratories.

ALT: alanine aminotransferase; AST: aspartate aminotransferase; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration

Table S13. Busulfan Administration for Patient 2 (SCD)

Study Day[a]	Busulfan Dose	Observed AUC[b]
Day -6	3.2 mg/kg	5791 μmol*min/L
Day -5	3.2 mg/kg	Not Done
Day -4	2.3 mg/kg	Not Done
Day -3	2.3 mg/kg	Not Done

[a]Study Day 1 is day of CTX001 infusion.
[b]Target range for busulfan dose AUC is 4500-5500 μmol*min/L.
AUC: area under the curve

Table S14. Summary of LDH and Haptoglobin Data for Patient 2 (SCD)

Subject	Serum LDH[a] (U/L)	Haptoglobin[b] (mg/dL)	Reticulocyte Count (10^9/L)
Patient 2	B[c]: 282	B[c]: BDL	B[c]: 251.77
	M1: 155	M1: 377	M1: 29.80
	M2: --	M2: --	M2: 97.92
	M3: 175	M3: 88	M3: 117.60
	M4: --	M4: --	M4: 77.91
	M5: --	M5: --	M5: 56.98
	M6: 193	M6: 90	M6: 94.75
	M9: 173	M9: 124	M9: 94.32
	M12: 138	M12:145	M12: 100.44

B: baseline; BDL: below detectable limit; LDH: lactate dehydrogenase; M: Month; SCD: sickle cell disease
Note: Measured time points are presented according to study protocol.
[a] Serum LDH normal range 100-190 U/L.
[b] Haptoglobin normal range 30-200 mg/dL.
[c] Baseline collected prior to first mobilization procedure.

Table S15. Ratio of Fetal Hemoglobin (HbF) to Sickle Hemoglobin (HbS) in Patient 2 (SCD)

	Screening	Month 3	Month 6	Month 12	Month 15
HbF (g/dL)	0.6552	3.7572	5.3449	4.3672	5.184
HbS (g/dL)	5.3352	3.2926	5.6161	5.4693	6.276
Ratio of HbF/HbS	0.12	1.14	0.95	0.80	0.83

Table S16. Withdrawal from CLIMB SCD-121 Study Prior to CTX001 Dosing

Patient Withdrawn	Reason for withdrawal
001	Early termination: Physician decision, due to worsening SCD, renal disease, and reduction in eGFR that no longer met criteria for having an HSC transplant. Partial drug product (CTX001) was made but not used. Withdrawal was independent of allelic editing.
002	Early termination: Physician decision, after 2 cycles of mobilization and apheresis due to poor collections. Partial drug product (CTX001) was made but not used. Withdrawal was independent of allelic editing.
003	Early termination: Physician decision prior to undergoing mobilization and apheresis due to poor compliance to study visits and poor communication with site.
004	Early termination: Patient personal decision shortly after signing consent prior to undergoing mobilization. No post-enrollment study assessments were conducted with this patient.

Supplementary References

1. Tsai SQ, Zheng Z, Nguyen NT, et al. GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR—Cas nucleases. Nat. Biotechnology. 2015, 33:187-197.
2. Tsai SQ, Topkar VV, Joung JK, Ayree MJ. Open-source guideseq software analysis of GUIDE-seq data. Nat Biotechnol. 2016; 34: 438.
3. Brinkman EK, Chen T, Armendola M, van Steensel B. Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res. 2014; 4(22):e168.
4. Giarratana M-C, Kobari L, Lapillonne H, et al. Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. Nat Biotechnol. 2005; 23(1):69-74.

Data Sharing Statement

Vertex Pharmaceuticals Incorporated and CRISPR Therapeutics are committed to advancing medical science and improving patient health. This includes the responsible sharing of clinical trial data with qualified researchers. Proposals for the use of these data will be reviewed by a scientific board. Approvals are at the discretion of Vertex and CRISPR and will be dependent on the nature of the request, the merit of the research proposed, and the intended use of the data. Please contact CTDS@vrtx.com or medicalaffairs@crisprtx.com if you would like to submit a proposal or need more information.