(71) Applicant: THE UAB RESEARCH FOUNDATION
[US/US]; 1720 2nd Avenue South, Administration Build-
ing 770, Birmingham, Alabama 35924-0107 (US).

(72) Inventors: TOWNES, Tim; 4687 Bridgewater Rd., Birm-
ingham, Alabama 35243 (US). CHANG, Chia-Wei; 2220
Ivy Trace, Vestavia, Alabama 35243 (US). DING, Lei;
1133 Winward Ln, Vestavia, Alabama 35216 (US).

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(54) Title: CRISPR/CAS9 COMPLEX FOR INTRODUCING A FUNCTIONAL POLYPEPTIDE INTO CELLS OF BLOOD CELL LINEAGE

(57) Abstract: Provided herein are CRIS-
PR/Cas9 complexes and methods of using
same.

FIGURE 10
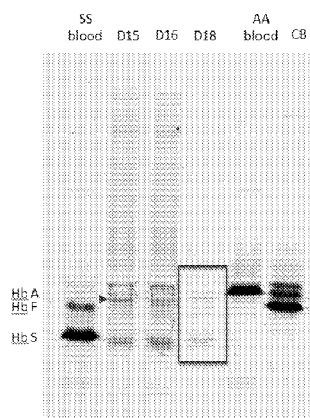
Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))

— as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))

— of inventorship (Rule 4.17(iv))

CRISPR/CAS9 COMPLEX FOR INTRODUCING A FUNCTIONAL POLYPEPTIDE INTO CELLS OF BLOOD CELL LINEAGE

This application claims the benefit of U.S. Provisional Application No. 62/181,145, filed June 17, 2015, which is hereby incorporated in its entirety by this reference.

5   **BACKGROUND**

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems (CRISPR-Cas9 systems) are used for gene editing at desired genomic sites in mammalian cells. In CRISPR-Cas9 systems, a Cas9 nuclease is targeted to a genomic site by complexing with a guide RNA that hybridizes to a target site in the genome. This results in a

10   double-strand break that initiates either non-homologous end-joining (NHEJ) or homology-directed repair (HDR) of genomic DNA via a double-strand or single-strand DNA repair template. However, repair of a genomic site via HDR is inefficient.

SUMMARY

Provided herein are complexes and methods for introducing a functional polypeptide

15   into cells of blood lineage. Provided herein is a method of making tumor-specific T-cell precursor cells the method comprises introducing into a population of T-cell precursor cells a complex comprising: (a) a guide (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of the T cell precursor cells and a second nucleotide sequence that interacts with a site-directed nuclease; (b) a recombinant site-

20   directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the gRNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA to create a double stranded break; and (c) a donor nucleic sequence comprising a third nucleotide sequence that encodes a chimeric antigen receptor (CAR) and a

25   fourth nucleotide sequence that hybridizes to a genomic sequence flanking the double stranded break in the target DNA, wherein the complex is introduced into the T-cell precursor cells under conditions that allow homology-directed repair (HDR) and integration of the third nucleotide sequence into the target DNA to form modified T-cell precursor cells that express the CAR. The method further provides for a high rate of cell survival in the modified T-cell

precursor cells. Also provided are complexes for making tumor-specific T-cell precursor cells.

DESCRIPTION OF THE FIGURES

Figures 1A-1C show that *in vitro* differentiation of JAK3 C1837T patient iPSCs recapitulates SCID phenotypes. Figures 1A and 1B show flow cytometry of iPSC-derived T cells. JAK3 WT iPSCs (Control) and JAK3- deficient iPSCs (JAK3 C1837T) were differentiated into CD34+ cells on OP9 stromal cells and, subsequently, into T cells on OP9-DL4 monolayers. T-cell differentiation from JAK3-deficient iPSCs was absent compared to controls; no CD3+ T cells or CD3-CD16+CD56+ NK cells were observed (Figure 1A), and no CD4+CD8+ double positive (DP), CD4+ single positive (SP), or CD8+ single positive (SP) T cells were detected (Figure 1B). Figure 1C shows the results of RT-qPCR assays for transcripts of key genes that regulate early events during specification of the T cell lineage. RNA levels are shown relative to GAPDH expression.

Figures 2A-2C show that BCL2 partially rescues T cell developmental defects in JAK3–deficient, *in-vitro* derived cells. Figure 2A shows apoptosis of JAK3-deficient, iPSC-derived T cells compared to JAK3 WT controls. Annexin V-positive cells were analyzed at T cell induction day 10 (TD10) and 17 (TD17). Four independent experiments were performed with control JAK3 WT cells (Control) and 5 independent experiments were performed with JAK3-deficient cells (JAK3 C1837T). *$P < 0.005$. Figure 2B shows the results of RT-qPCR assays for anti-apoptotic BCL2 and proapoptotic BAX expression in two lines (1 and 2) from JAK3 WT (Control) and JAK3-deficient cells (JAK3 C1837T). ND, not determined (due to insignificant JAK3 qPCR signal). RNA levels are shown relative to GAPDH expression. Figure 2C shows flow cytometry of JAK3–deficient iPSCderived T cells transduced with BCL2-2A-GFP lentivirus to assess effects on NK (CD16+56+) and T cell (CD3+) development and DP (CD4+CD8+) to SP (CD4+ or CD8+) T cell maturation.

Figures 3A-3D show that CRISPR/Cas9 enhanced correction of the JAK3 C1837T mutation in patient-specific iPSCs. Figure 3A depicts the strategy for genome modification using CRISPR/Cas9 to induce double-strand breaks in the JAK3 locus and a template for homology directed repair. Top line, structure of the JAK3 gene. Open boxes, exons. Asterisk, C1837T mutation. Arrows, guide RNAs. Figure 3B, top, shows PCR analysis demonstrating homologous recombination; primers for 5' and 3' analysis are indicated. (Lower Left) RT-PCR analysis demonstrating JAK3 mRNA expression in JAK3 WT (Control), JAK3-

deficient (JAK3 C1837T), and corrected (JAK3 Corrected) T cells. (Lower Right) Western Blot analysis demonstrating JAK3 protein expression in JAK3 WT (Control), JAK3-deficient (JAK3 C1837T), and corrected (JAK3 Corrected) T cells. Figure 3C provides a summary of targeting efficiencies of guide RNAs. (Figure 3D) Sanger sequencing of the PCR amplicons from parental JAK3 iPSCs (Left), heterozygous corrected (Middle) and homozygous corrected iPSCs (Right). The two heterozygous clones were corrected with gRNA2 + wild type Cas9, and the homozygous clone was corrected with gRNA1 + gRNA2 + nickase Cas9 (D10A).

Figures 4A-4C show *in vitro* differentiation of JAK3 corrected patient iPSCs produces T cells with phenotypic and functional characteristics of mature T cells. Figure 4A shows the expression of T cell developmental markers in JAK3 WT (Control, n=3), JAK3-deficient (JAK3 C1837T, n=5) and JAK3- corrected (JAK3 Corrected, n=6) T cells. Cells were stained with indicated antibodies and analyzed by flow cytometry at T cell induction Day 14, 21, 28 and 35 (TD 14, 21, 28 and 35). Figure 4B shows T cell receptor (TCR) Vβ analysis of JAK3-corrected T cells. A highly diverse repertoire of TCR Vβ is represented in T cells derived from corrected SCID patient iPSCs. Figure 4C shows flow cytometry demonstrating T cell activation in JAK3-corrected T cells. T cells derived from JAK3 WT (Control) and JAK3-corrected iPSCs were stimulated with anti-CD3/28 beads for 3 days before analysis of activation markers CD25 and CD69. The data were gated on CD3+ populations.

Figures 5A-5C show *in vitro* generation of CD34+ HSCs from hiPSCs by co-culture with human bone marrow stromal cells (hMSC). Human iPSCs were cultured on hMSCs for 18 days before analysis for hematopoietic markers, CD34 and CD43 (Figure A). CD34+ cells were purified on beads and differentiated into T cells (Figure B), erythroid and myeloid cells (Figure C). To generate T cells, purified CD34+ cells were plated on OP9-DL4 cells for 3 to 4 weeks. For the CFC assay to generate myeloid and erythroid cells, purified CD34+ cells were plated in MethoCult H4434 Classic medium according to the manufacturer's protocol. These data demonstrate that hiPSC can be efficiently differentiated into multipotent HSC after co-culture on hMSC.

Figure 6A-6C show *in vitro* generation of T cells by culturing hiPSC derived CD34+ cells with hMSC-DL4. To generate CD7+ T progenitor cells, hiPSC derived CD34+ cells were co-cultured on hMSC-DL4 for 3 to 4 weeks (Figure 6A). When CD7+ cells from Figure 6A were purified on magnetic beads and co-cultured on OP9-DL4, fully mature CD4+/CD8+/CD3+/TCR-αβ+ cells were produced in 10 days or less (Figures B and C).

These data demonstrate that hiPSC can be efficiently differentiated into CD7+ lymphoid progenitors after co-culture on hMSC-DL4.

Figure 7 shows *in vitro* generation of γδ T cells from hiPSC. Human iPSC were transduced with a lentiviral vector carrying a pre-rearranged human V γδ1 cDNA linked with a 2A-GFP cDNA fragment. After co-culture with OP9 for 18 days, hiPSC derived CD34+ cells were purified on magnetic beads. These cells were subsequently plated on OP9-DL4 cells for T cell differentiation. Cells were harvested at Day 32 and T cell surface markers were analyzed by FACS. The GFP+ population represents Vδ1-2A-GFP lentiviral transduced cells. A high percentage of these GFP positive cells expressed Vδ1 (66%). A low percentage of GFP negative cells expressed Vδ1 (1%). These results demonstrate that Vδ T cells expressing recombinant T Cell Receptors (TCR) can be efficiently produced from genetically modified iPSC. Production of Vδ T cells expressing recombinant T Cell Receptors (TCR) specific for tumor antigens provides a powerful cellular therapy for many types of cancer.

Figure 8 shows that a correction complex including guide RNA, a modified Cas9 and a single stranded oligonucleotide donor sequence (ssODN) can correct a sickle cell mutation. The complex was introduced into sickle iPSC by nucleoporation, and 2 days later genomic DNA was analyzed by digital PCR (ddPCR) and sequenced. Over 65% of the cells contained at least one corrected gene. The results were confirmed as follows. Two days after introduction of the correction complex, the cells were plated in culture dishes, and 43 individual iPSC colonies were isolated. Genomic DNA was isolated from these colonies and the beta-globin gene was sequenced. Sixty-five percent of the colonies contained at least one corrected beta-globin gene (S corrected to A).

Figure 9 shows that introduction of a sickle cell correction complex (gRNA-modified recombinant Cas9-ssODN) into patient primary bone marrow CD34+ cells can correct a sickle cell mutation. After twelve days of *in vitro* differentiation, DNA was analyzed by digital PCR (ddPCR) and sequenced. Approximately equal amounts of betaA and betaS mRNA were observed.

Figure 10 is an isoelectric focusing (IEF) gel of *in vitro* differentiated red blood cells from the corrected sickle patient CD34+ cells of Figure 9, showing an HbA (normal hemoglobin) to HbS (hemoglobin with sickle cell mutation) ratio of about 1:3, which is sufficient to inhibit sickling and treat sickle cell anemia.

DETAILED DESCRIPTION

Provided herein are CRISPR/Cas9 complexes for genomic modification of cells. Methods of using the complexes provided herein result in increased efficiency of modification, an increased cell survival ratio and/or an increased ratio of HDR to NHEJ in the cells. These complexes and methods can be used for therapeutic purposes, for example, to correct a mutation in cells, wherein the mutation is associated with a disease or disorder.

Provided herein is a complex for correcting a mutation in the genome of a cell comprising (a) a guide RNA (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of a cell, wherein the target DNA comprises a mutation, and a second nucleotide sequence that interacts with a site-directed nuclease; (b) a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the guide RNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA to create a double stranded break; and (c) a single-stranded donor oligonucleotide (ssODN) that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and integrates into the target DNA to correct a mutation in the target DNA.

It is understood that the complex comprising a guide RNA (gRNA), a recombinant site-directed nuclease and a donor nucleotide described herein does not occur in nature. The complex, however, provides the elements necessary with the required configuration and stoichiometry to efficiently and effectively modify cells. The gRNA molecule binds to the site-directed nuclease and targets the nuclease to a specific location within the target DNA. A gRNA comprises a first nucleotide sequence that hybridizes to a target DNA in the genome of a cell, wherein the target DNA comprises a mutation, and a second nucleotide sequence that interacts with a site-directed nuclease. The complexes described herein can comprise one or two separate gRNAs. Therefore, the term guide RNA includes both a single guide RNA and a double guide RNA. An example of a guide sequence that can be used to correct a mutation associated with sickle cell anemia is set forth herein as TAACGGCAGACTTCTCCAC (SEQ ID NO: 1). An example of a guide sequence comprising a stem loop for Cas9 binding is provided herein as GTAACGGCAGACTTCTCCACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG CTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTT (SEQ ID

NO: 2). It is noted that the 5'G of SEQ ID NO: 2 was added by T7 during *in vitro* transcription.

In the complexes described herein, the recombinant site-directed nuclease can be an RNA-guided site-directed nuclease, for example, a Cas protein from any bacterial species or a functional fragment thereof. For example, the Cas protein can be a Cas9 protein or a functional fragment thereof. As used herein, the term "Cas9" means a Cas9 protein or a fragment thereof present in any bacterial species that encodes a Type II CRISPR/Cas9 system. See, for example, Makarova et al. *Nature Reviews*, Microbiology, 9: 467-477 (2011), including supplemental information, hereby incorporated by reference in its entirety. For example, the Cas9 protein or a fragment thereof can be from *Streptococcus pyogenes*. Full-length Cas9 is an endonuclease that includes a recognition domain and two nuclease domains (HNH and RuvC, respectively). In the amino acid sequence, HNH is linearly continuous, whereas RuvC is separated into three regions, one left of the recognition domain, and the other two right of the recognition domain flanking the HNH domain. Cas9 from *Streptococcus pyogenes* is targeted to a genomic site in a cell by interacting with a guide RNA that hybridizes to a 20-nucleotide DNA sequence that immediately precedes an NGG motif recognized by Cas9. This results in a double-strand break that is repaired via HDR by a donor nucleotide, for example, a ssODN or a double stranded DNA construct that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and integrates into the target DNA to correct a mutation in the target DNA.

In the complexes provided herein, the molar ratio of gRNA to site-directed nuclease operably linked to a supercharged protein to ssODN can be from about 1:1:0.2 to about 1.5:1:2.0. For example, the molar ratio of gRNA to site-directed nuclease operably linked to a supercharged protein to ssODN can be about 1:1:1, 1.1:1:1, 1:1:1.15, 1:1:1.25, 1:1:1.30; 1:1:1.35; 1:1:1.40; 1:1:1.50, 1.2:1:1, 1.3:1:1. 1.4:1:1, 1.5:1:1, 1.5:1:1.15, 1.5:1:1.25, 1.5:1:1.35; 1.5:1:1.40, 1.5:1:1.45; 1.5:1:1.50; 1.5:1:1.55; 1.5:1:1.60; 1.5:1:1.65; 1.5:1:1.70; 1.5:1:1.75; 1.5:1:1.80; 1.5:1:1.85; 1.5:1:1.90; 1.5:1:1.95; 1.5:1: 2.0 or any ratio in between these ratios. Complexes having these molar ratios can be used in any of the methods described herein. Methods for preparing a complex prior to introducing the complex into a cell or a population of cells are set forth in the Examples.

As used herein, a supercharged protein can be a superpositively charged protein that has an overall positive charge that is greater than its corresponding unmodified protein. For example, the superpositively charged protein can be a superpositively charged green fluorescent protein (GFP) that has an overall positive charge from about +5 to about +40. For

example, the overall positive charge can be about +5, +6, +7, +8, +9, +10, +11, +12, +13, +14, +15, +16, +17, +18, +19, +20, +21, +22, +23, +24, +25, +26, +27, +28, +29, +30, +31, +32, +33, +34, +35, +36, +37, +38, +39 or +40.

The supercharged protein can be operably linked to the amino-terminus or the carboxy-terminus of the nuclease. It is also contemplated that the supercharged protein can be associated with the nuclease, without necessarily being covalently linked to the nuclease. An example of a supercharged protein is a superpositively charged GFP, for example, +36 GFP. +36 GFP can be operably linked to the amino or carboxy- terminus of Cas9 or a functional fragment thereof. See, for example, McNaughton et al., "Mammalian cell penetration, siRNA transfection, and DNA transfection by supercharged proteins," *PNAS* 106(15): 6111-6116. An example of a polypeptide comprising +36 GFP operably linked to the carboxy-terminus of Cas9 is provided herein as SEQ ID NO: 3.

The nuclease can also be operably linked to a supercharged protein and one or more positively charged peptides, for example, one or more transactivating transcriptional activator (TAT) peptide can be linked to the amino-terminus or the carboxy terminus of the nuclease. For example, and not to be limiting, a superpositively charged protein can be operably linked to the carboxy-terminus of the nuclease and one or more TAT peptides (for example, 1X TAT, 2X TAT, 3X TAT, 4X TAT, etc.) can be operably linked to the amino-terminus of the nuclease. An example of polypeptide comprising a TAT peptide operably linked to the amino-terminus of the nuclease and a superpositively charged GFP operably linked to the carboxy-terminus of the nuclease is provided herein as SEQ ID NO: 4. Polypeptide sequences that are at least about 75% identical to SEQ ID NO: 3 or SEQ ID NO: 4 are also provided. For example, polypeptide sequences that are at least about 75%, 80%, 85%, 90%, 95%, 99% or any percentage in between are also provided.

The nuclease can also be operably linked to a supercharged protein and one or more negatively charged peptides, for example, a negatively charged peptide of about 10 to about 25 amino acids in length, for example, SEQ ID NO: 50, can be operably linked to the carboxy-terminus of the site-directed nuclease. For example,and not to be limiting, a superpositively charged protein can be operably linked to the carboxy-terminus of the nuclease and a negatively charged peptide can be operably linked to the carboxy-terminus of the superpositively charged protein.

As used throughout, recombination is a process of exchange of genetic information between two polynucleotides. Homology-directed repair (HDR) refers to DNA repair that takes place, for example, during repair of double-strand breaks in cells. This process requires

nucleotide sequence homology and uses a donor molecule, for example, a single stranded or a double stranded nucleotide sequence as a template for repair of a target genomic sequence, i.e., the genomic sequence with the double-strand break, and leads to the transfer of genetic information from the donor to the target genomic sequence. Homology-directed repair can

5   result in a modification of the sequence of the target genomic sequence. For example, HDR can result in an insertion, a deletion or a mutation in the target genomic sequence. Part or all of the sequence of the donor polynucleotide can be incorporated into the target DNA. It is also contemplated that the donor polynucleotide, a portion of the donor polynucleotide, a copy of the donor polynucleotide, or a portion of a copy of the donor polynucleotide

10   integrates into the target DNA.

As used throughout, by non-homologous end joining (NHEJ) is meant the repair of double-strand breaks in DNA by direct ligation of the break ends to one another without the need for a homologous template (in contrast to homology-directed repair, which requires a homologous sequence to guide repair).

15   The complexes and methods provided herein can be used to correct any mutation in a target DNA by HDR. For example, and not to be limiting, the complexes can be used to replace an incorrect nucleotide sequence with a correct nucleotide sequence (e.g., to restore function to a target polynucleotide sequence that is impaired due to a loss of function mutation, i.e., a SNP) at a specific site in the genome. These mutations can be associated

20   with an autoimmune disorder, a genetic disease, a blood disorder, a T cell disorder, a monogenic disorder, cancer, a neurodegenerative disease, a cardiovascular disease or an infectious disease, to name a few. For example, and not to be limiting, the complexes and methods provided herein can be used to correct a mutation associated with sickle cell disease (i.e., a mutation in a hemoglobin gene, for example, a GAG to GTG mutation at codon 6 of

25   the beta-globin gene that results in a glutamic acid to valine substitution), severe combined immunodeficiency (SCID) (for example, a mutation in JAK3), beta thalassemia or Wiskott-Aldrich Syndrome.

Correction of single mutations or multiple mutations can be performed with one or more complexes. The complexes and methods provided herein can also be used to insert

30   sequences into a specific site in the genome to correct a deletion, as opposed to making a correction or a substitution. The complexes and methods provided herein can also be used to insert a nucleotide sequence that encodes an a functional polypeptide into a specific site in the genome of the cell, in order to express the functional polypeptide in the cell. The functional polypeptide can be a polypeptide that is endogenous (i.e., normally expressed by

8

the cell) or exogenous to the cell (i.e. not normally expressed by the cell). For example, chimeric antigen receptor (CAR) sequences can be inserted into the genome of a T cell precursor in order to generate cancer specific T cells for the treatment of cancer. In another example, the complexes and methods provided herein can be used to inhibit the activity of a gene at a specific site in the genome of the cell. For example, the complexes and methods provided herein can be used to insert sequences into the CXCR4 or CCR5 receptor to treat or prevent HIV infection.

The complexes provided herein can modify or alter target DNA with surprisingly high efficiency as compared to conventional CRISPR/Cas systems. The efficiency of alteration in a population of cells can be at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% or 80% or higher or any percentage in between these percentages. The efficiency of alteration can also be greater than or equal to about 80%. Therefore, also provided herein are populations of cells, wherein at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% or 80% or higher or any percentage in between are altered. For example, a mutation associated with sickle cell disease or another disorder has been corrected. If a population of cells comprising a mutation associated with sickle cell disease is contacted with a CRISPR/Cas complex described herein and the mutation is corrected in about 5% of the cells, the efficiency of modification or alteration is about 5%. Optionally, a population of cells wherein the mutation associated with sickle cell disease is corrected in about 30% of the cells, including, for example, 27%, 28% and 29% is sufficient to treat sickle cell disease, upon transplantation in a subject with sickle cell disease. Optionally, a mutation associated with sickle cell disease is corrected in about 40%, 50%, 60%, 70%, 80%, 90% or higher or any percentage in between, of the cells in the population.

In addition to altering the target DNA with high efficiency, the complexes provided herein can also increase the ratio of HDR to NHEJ in a population of cells contacted with the complex. The HDR/NHEJ ratio can be from about 10 to about 0.5. For example, the HDR/NHEJ ratio can be about 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5 or less or any ratio in between these ratios. In addition to high efficiency of correction and high rate of HDR to NHEJ, the cell survival rate for corrected cells can be at least about 50%, 60%, 70%, 80%, 90% or higher and any percentage in between.

Any cell(s) can be modified or derived using the complexes described herein. Introduction of the complex into the cells can be cell cycle dependent or cell cycle independent. Methods of synchronizing cells to increase the proportion of cells in a particular

phase, for example, the S-phase , are known in the art. See, for example, Takahashi et al. "Efficient introduction of a gene into hematopoietic cells in S-phase by electroporation," *Exp. Hematol.* 19(5):343-346 (1991). Depending on the type of cell to be modified, one of skill in the art can readily determine if cell cycle synchronization is necessary.

5   The cell(s) can be a eukaryotic cell, for example, a mammalian cell. The cell can also be prokaryotic or a plant cell. The cell can be a human cell. The cell can be a germ cell, a somatic cell, a stem cell, a precursor cell or a progenitor cell. The precursor cell can be, for example, a pluripotent stem cell or a multipotent stem cell, like a hematopoietic stem cell. As used throughout, pluripotent cells include induced pluripotent stem cells. Methods of making

10   induced pluripotent stem cells and known in the art and described in the Examples. The cell can also be CD34+ cell, optionally derived from an induced pluripotent stem cell. The CD34+ cell can be selected from the group consisting of a primary CD34+ hematopoietic progenitor cell, a CD34+ peripheral blood cell, a CD34+ cord blood cell and a CD34+ bone marrow cell. The cell can also be a primary cell, for example, a primary CD34+

15   hematopoietic progenitor cell. The cells are cells that are not cancer cells, cells that are not tumor cells or cells that are not transformed cells. Cells can be screened before or after correction for evidence of undesirable genetic characteristics. Further provided is a cell comprising any of the complexes described herein. The cell can be *in vitro, ex vivo* or *in vivo.*

20   Further provided is a method of site-specific modification of a target DNA in a population of cells comprising introducing into the cells any of the complexes described herein, wherein the complex is introduced into the cells under conditions that allow homology-directed repair (HDR) and integration of a donor nucleotide, for example, a ssODN or double stranded nucleotide sequence into the target DNA. The complex can be

25   introduced into the cell via nucleoporation. Methods for nucleoporation are known in the art. See, for example, Maasho et al. "Efficient gene transfer into the human natural killer cell line, NKL, using the amaxa nucleofection system," *Journal of Immunological Methods* 284(1-2): 133-140 (2004); and Aluigi et al. "Nucleofection is an efficient non-viral transduction technique for human bone marrow derived mesenchymal stem cells," *Stem Cells* 24(2): 454-

30   461 (2006)), both of which are incorporated herein in their entireties by this reference.

In some of the methods provided herein, the donor nucleotide, for example, a ssODN or a double stranded nucleotide sequence integrates into a target DNA and corrects a mutation in the target DNA. In the methods provided herein the ratio of HDR to NHEJ in a population of cells is increased relative to other CRISPR-Cas9 delivery methods. The

HDR/NHEJ ratio can be from about 10 to about 0.5. For example, the HDR/NHEJ ratio can be about 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5 or less or any ratio in between these ratios. In the methods provided herein, the efficiency of alteration by HDR can be at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80% or greater or

5   any percentage in between these percentages. The efficiency of alteration by HDR can also be greater than or equal to about 80%. For example, if a population of cells comprising a mutation associated with sickle cell anemia is contacted with a CRISPR/Cas complex described herein and the mutation is corrected in about 5% of the cells, the efficiency of alteration by HDR is about 5%. The population of cells can be obtained from the subject

10   having a disorder such that at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% or 80% or greater or any percentage in between these percentages, of the cells undergo HDR to correct a mutation associated with the disorder. In some cases greater than 80% of the cells from the subject will undergo HDR to correct a mutation associated with the disorder. In the methods described herein, between about 50%

15   and 99% of the cells survive after introduction of the complex. For example, great than about 50%, 60%, 70%, 80%, 90%, 95%, 99% or any percentage in between these percentages, of corrected cells survive after introduction of the complex.

Further provided is a method of treating a disease associated with a mutation in the genomic sequence encoding hemoglobin in a subject comprising: (a) introducing into a

20   population of cells obtained from the subject a complex comprising (1) a guide RNA (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of a cell, wherein the target DNA is a hemoglobin gene that comprises a mutation, and a second nucleotide sequence that interacts with a site-directed nuclease; (2) a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed

25   nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the guide RNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA to create a double stranded break; and (3) a single-stranded donor oligonucleotide (ssODN) that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and integrates into the target DNA to correct the mutation in

30   hemoglobin gene; and (b) transplanting the corrected cells into the subject.

In the methods for treating a disease associated with a mutation in the genomic sequence encoding hemoglobin in a subject, for example, sickle cell anemia, the subject with sickle cell anemia can optionally be a transfusion dependent subject or a subject with at least one silent infarction. The subject can also be less than about twelve months, eleven months,

ten months, nine months, eight months, seven months, six months, five months, four months, three months, two months, or one month in age. As infants are routinely screen for sickle cell disease, infants can be treated before symptoms of the disease manifest. The methods provided herein can further comprise diagnosing a subject with a disorder, for example,

5   sickle cell disease.

As set forth above, cells can be obtained from the subject with the disease or from a related donor. For example, bone marrow cells can be obtained or harvested from the subject. Bone marrow harvesting involves collecting stem cells with a needle placed into the soft center of the bone, the marrow. Bone marrow can be harvested for example, from the hip

10   bones or sternum of the subject. From about 500 ml to about 1 liter of bone marrow can be obtained from the subject.

In any of the methods provided herein the cell(s) can be a eukaryotic cell, for example, a human cell. The cell can be a germ cell, a stem cell, a precursor cell. The precursor cell can be, for example, a pluripotent stem cell or a hematopoietic stem cell. As

15   used throughout, pluripotent cells include induced pluripotent stem cells. Methods of making induced pluripotent stem cells and known in the art and described in the Examples. The cell can also be CD34+ cell. The CD34+ cell can be selected from the group consisting of a primary CD34+ hematopoietic progenitor cell, a CD34+ peripheral blood cell, a CD34+ cord blood cell and a CD34+ bone marrow cell. The cell can also be a primary cell, for example, a

20   primary CD34+ hematopoietic progenitor cell. The cells are that are not cancer cells, cells that are not tumor cells or cells that are not transformed cells. The cell can be *in vitro* or *ex vivo*. The cells can also be in a pharmaceutically acceptable composition.

The methods provided herein can further comprise culturing the cells corrected with HDR. For example, the cells can be cultured under conditions for expansion or under

25   conditions that promote differentiation of the corrected cells into T-cells. For example, and not to be limiting, using the methods provided herein, after a mutation has been corrected in induced pluripotent stem cells via HDR, the corrected cells can be co-cultured with human bone marrow stromal cells to generate CD34+ cells. The CD34+ cells can then be cultured under conditions that differentiate the CD34+ cells into T cells.

30   The methods provided herein can further comprise screening the corrected cells for the proper correction, other mutations, or NEJ prior to transplantation. Optionally cells can be screened to detect cells with one or more corrections.

In the methods provided herein, the cells can be transplanted into the subject after modification, for example, after correction of a mutation by HDR. The cells can be

transplanted into the subject with or without differentiation. For example, modified hematopoietic stem cells (HSCs) can be administered in a bone marrow transplant, wherein the HSCs are allowed to differentiate and mature *in vivo* in a subject Alternatively, the modified cells can be differentiated into a desired population of cells prior to transplantation.

5   As used herein, transplanting, introducing or administering cells to a subject refers to the placement of cells into a subject. For example, the cells described herein comprising a target DNA sequence corrected or modified according to the methods described herein can be transplanted into a subject, by an appropriate route which results in at least partial localization of the transplanted cells at a desired site. The cells can be implanted directly to

10   the desired site, or alternatively can be administered by any appropriate route which results in delivery to a desired location in the subject where at least a portion of the implanted cells remain viable. For example, the cells can be administered systemically, via intravenous infusion. The period of viability of the cells after administration to a subject can be as short as a few hours, e. g. twenty-four hours, to a few days, to as long as several years.

15   For *ex vivo* methods, cells can be autologous cells, i.e., a cell or cells taken from a subject who is in need of modification of a target DNA in the cell or cells (i.e., the donor and recipient are the same individual). As described herein, the modification can be, for example correction of a mutation, insertion of a sequence that inhibits activity of a protein or insertion of a sequence that increases expression of a protein, for example, insertion of a sequence

20   encoding a chimeric antigen receptor that can be used to target cancer cells. Autologous cells can be used to avoid immunological reactions that can result in rejection of the cells. In other words, when using autologous cells, the donor and recipient are the same subject. Alternatively, the cells can be heterologous, e.g., taken from a donor, preferably a related donor. The second subject can be of the same or different species. Typically, when the cells

25   come from a donor, they will be from a donor who is sufficiently immunologically compatible with the recipient to reduce the chances of transplant rejection, and/or to reduce the need for immunosuppressive therapy. The cells can also be obtained from a xenogeneic source, i.e., a non-human mammal that has been genetically engineered to be sufficiently immunologically compatible with the recipient, or the recipient's species. Any of the

30   methods of treating a disorder described herein can further comprise administering one or more immunosuppressants to the subject.

In the methods involving transplantation, a subject optionally undergoes myeloablative therapy prior to transplantation of any of the cells described herein. The myeloablative therapy can include administering one or more doses of chemotherapy,

radiation therapy, or both, that results in severe or complete depletion of healthy bone marrow cells. In another example, the subject can undergo submyeloablative therapy that includes administering one or more doses of chemotherapy, radiation therapy, or both, that depletes a portion of the healthy bone marrow cells. The cells can also be transplanted into subjects that have undergone nonablative chemotherapy. For example, the cells can be transplanted into a subject that has been treated with Busulfan, Fludarabine and/or Treosulfan.

In the methods involving transplantation, an effective dose or amount of corrected cells is administered to the subject. The terms effective amount and effective dosage are used interchangeably. The term effective amount is defined as any amount necessary to produce a desired physiologic response. In some methods, about 1×10^6 to about 7×10^6 corrected cells/kg can be administered, but this amount can vary depending on the associated disorder. The percentage of corrected cells that Effective amounts and schedules for administering the cells may be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for administration are those large enough to produce the desired effect (e.g., treatment of a disease, for example, sickle cell anemia). The dosage should not be so large as to cause substantial adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, type of disease, the extent of the disease or disorder, route of administration, or whether other drugs are included in the regimen, and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any contraindications. Dosages can vary, and the agent can be administered in one or more dose administrations daily, for one or multiple days as needed.

As used throughout, a subject can be a vertebrate, more specifically a mammal (e.g., a human, horse, cat, dog, cow, pig, sheep, goat, mouse, rabbit, rat, and guinea pig). The term does not denote a particular age or sex. Thus, adult and newborn subjects, whether male or female, are intended to be covered. As used herein, patient or subject may be used interchangeably and can refer to a subject with or at risk of developing a disorder. The term patient or subject includes human and veterinary subjects.

As used herein the terms treatment, treat, or treating refers to a method of reducing one or more of the effects of the disorder or one or more symptoms of the disorder, for example, sickle cell disease, by eliciting an immune response in the subject. Thus in the disclosed method, treatment can refer to a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% reduction in the severity of sickle cell disease and other disorders. For

example, a method for treating sickle cell disease is considered to be a treatment if there is a 10% reduction in one or more symptoms of the infection in a subject as compared to a control. Thus the reduction can be a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or any percent reduction in between 10% and 100% as compared to native or control levels. It is understood that treatment does not necessarily refer to a cure or complete ablation of the disorder or symptoms of the disorder.

Also provided is a method of correcting a mutation associated with a T-cell disorder comprising introducing into a population of cells obtained from a subject with the T-cell disorder a complex comprising: (a) a guide RNA (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of a cell, wherein the target DNA comprises the mutation associated with the T-cell disorder, and a second nucleotide sequence that interacts with a site-directed nuclease; (b) a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the gRNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA that comprises the mutation associated with the T-cell disorder to create a double stranded break in the target DNA; and (c) a single stranded donor oligonucleotide (ssODN) comprising a third nucleotide sequence that hybridizes to a genomic sequence flanking the double stranded break in the target DNA and that integrates into the target DNA to correct the mutation associated with the T-cell disorder, wherein the complex is introduced into the cell under conditions that allow homology-directed repair (HDR) to correct the mutation associated with the T-cell disorder.

In the methods provided herein, the target DNA comprising a mutation associated with a T-cell disorder can be a target DNA that encodes a protein associated with T-lymphocyte development. For example, the target DNA can encode JAK3. Such corrected cells can be used, for example, in the treatment of SCID.

In addition to correcting mutations in the genome of a cell, the complexes and methods provided herein can also be used to insert functional polypeptides at specific sites in the genome of a cell, such that the polypeptide is expressed by the cell. The polypeptide can be expressed in the cell or on the cell surface.

Also provided is a method of making tumor-specific T-cell precursor cells comprising introducing into a population of T-cell precursor cells a complex comprising: (a) a guide (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of the T cell precursor cells and a second nucleotide sequence that interacts with a

site-directed nuclease; (b) a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the gRNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA to create a double stranded break; and

5   (c) donor nucleotide sequence comprising a third nucleotide sequence that encodes a chimeric antigen receptor (CAR) and a fourth nucleotide sequence that hybridizes to a genomic sequence flanking the double stranded break in the target DNA, wherein the complex is introduced into the T-cell precursor cells under conditions that allow homology-directed repair (HDR) and integration of the third nucleotide sequence into the target DNA to form

10   modified T-cell precursor cells that express the CAR.

The T cell precursor cells can be obtained from a subject with cancer. As set forth above, the HDR/NHEJ ratio can be from about 10 to about 0.5. For example, the HDR/NHEJ ratio can be about 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0.5 or any ratio in between these ratios. In the methods provided herein, the efficiency of alteration by HDR can be at least about 5%, 10%,

15   15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80% or any percentage in between these percentages. The efficiency of alteration by HDR can also be greater than or equal to about 80%. For example, when using the methods described herein, if a nucleotide sequence encoding an functional polypeptide, for example, a nucleotide sequence that encodes a CAR, is inserted in about 5% of the cells, the efficiency of alteration

20   by HDR is about 5%. The population of cells can be obtained from the subject that has cancer such that at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75% or 80% or any percentage in between these percentages, of the cells undergo HDR to insert a nucleotide sequence that encodes a chimeric antigen receptor (CAR) and form cells that express the CAR. In some cases greater than 80% of the cells from the

25   subject will undergo HDR to correct a mutation associated with the disorder.

The modified T-cell precursor cells that express the CAR can be transplanted into a subject with cancer. As used herein, cancer is a disease characterized by the rapid and uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of cancers include

30   but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer and the like. The modified T-cell precursor cells that express the CAR exhibit anti-tumor immunity when the antigen binding domain binds to its corresponding antigen.

Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are

5   disclosed that while specific reference of each various individual and collective combinations and permutations of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a method is disclosed and discussed and a number of modifications that can be made to a number of molecules including the method are discussed, each and every combination and permutation of the method, and the

10   modifications that are possible are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed. This concept applies to all aspects of this disclosure including, but not limited to, steps in methods using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed, it is understood that each of these additional steps can

15   be performed with any specific method steps or combination of method steps of the disclosed methods, and that each such combination or subset of combinations is specifically contemplated and should be considered disclosed.

Publications cited herein and the material for which they are cited are hereby specifically incorporated by reference in their entireties.

20

EXAMPLES
Example 1

Correction of SCID by CRISPR/Cas9 enhanced gene replacement

25   Mutations of the Janus family kinase JAK3 gene cause severe combined immunodeficiency (SCID). JAK3 deficiency in humans is characterized by the absence of circulating T cells and natural killer (NK) cells with normal numbers of poorly functioning B cells (T–B+NK–). As shown herein, using SCID patient-specific induced pluripotent stem cells (iPSCs) and a T cell *in vitro* differentiation system, a complete block in early T cell

30   development of JAK3-deficient cells was demonstrated. Correction of the novel JAK3 mutation by CRISPR/Cas9 enhanced gene replacement restores normal T cell development, including the production of mature T-cell populations with a broad T Cell Receptor (TCR) repertoire. Whole genome sequencing of corrected cells demonstrated no CRISPR/Cas9 off-target modifications. Thus, provided herein is a novel approach for the study of human

lymphopoiesis and a method for gene replacement therapy in humans with immunodeficiencies.

Allogeneic hematopoietic stem cell (HSC) transplantation is currently the only established therapy for SCID; however, delayed immune recovery and risk of graft-vs-host disease present significant risks. Treatment by retroviral-based gene therapy has been successfully demonstrated for X-linked SCID. However, severe adverse effects of insertional mutagenesis have been observed with retroviral gene therapy. Self-inactivating lentiviral vectors have been used effectively in recent clinical trials, but long-term follow-up is needed to thoroughly address safety concerns.

Provided herein is an alternative therapeutic strategy in which patient-specific induced pluripotent stem cells (iPSCs) are derived, and disease-causing mutations are corrected by gene replacement using a CRISPR-Cas9 complex. These corrected iPSCs could optionally be differentiated into hematopoietic progenitors for transplantation into patients to treat the disease (Hanna et al., "Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin," *Science* 318: 1920-1923 (2007)). As shown herein, differentiation of JAK3-deficient human T cells is blocked at an early developmental stage. Also demonstrated is that correction of the human JAK3 mutation by CRISPR/Cas9 enhanced gene replacement restores the differentiation potential of early T cell progenitors. These corrected progenitors are capable of producing NK cells and mature T cell populations expressing a broad repertoire of T-cell antigen receptors (TCR). These studies establish a powerful system for determining the mechanism of immunodeficiency in human SCID patients and for testing pharmacological and genetic therapies for the disorder.

Patient Information

The male patient was enrolled in an Institutional Review Board-approved study in accordance with the Declaration of Helsinki. The family history was negative for immune deficiencies. For the first 8 months of age he had poor weight gain, diarrhea, and recurrent bronchiolitis requiring frequent hospitalization. He was admitted to the hospital at 8 months of age with severe respiratory distress and oral thrush. Bronchoscopy with bronchial alveolar lavage demonstrated bacterial (pseudomonas, H flu, S. pneumonia) and viral organisms (respiratory syncytial virus). Immunologic evaluations demonstrated severe hypogammaglobulinemia, with an IgE<3, IgA<4, IgG=29, IgM=26. Immune phenotyping of peripheral blood demonstrated complete absence of CD3+ T cells and NK cells, though B cells were present (absolute B cell count=875). Mitogen studies demonstrated a complete lack of response to concanavalin A, poke weed mitogen and phytohemagglutinin A. The

diagnosis of SCID was confirmed by genetic testing, with a homozygous C>T nucleotide substitution in exon 14 of the JAK3 gene, resulting in the replacement of an arginine codon (CGA) with a stop codon (TGA) at amino acid position 613. This is the first report linking this JAK3 variant (rs149316157) to a clinical case of SCID. The patient underwent a reduced intensity conditioning matched unrelated bone marrow transplant, and is doing well now two years off therapy with complete immune reconstitution.

Human iPSC reprogramming and characterization

For iPSC induction, 5×10^4 primary keratinocytes were seeded into one well of a 6-well plate. On the following day, keratinocytes were transduced with 1 mL of virus supernatant and 1 mL of human keratinocyte medium containing polybrene at a final concentration of 4 μg/mL. The keratinocytes were spinfected at 800 x g for 45 minutes (day 1). The transduction procedure was repeated again the next day. On day 3, cells were changed to fresh human keratinocyte medium and cultured for two more days. On day 5, the keratinocytes were trypsinized and transferred to a 10 cm dish pre-seeded with mitomycin C-treated murine embryonic fibroblasts (MEFs) and cultured in human keratinocyte medium. On day 7, cells were changed to human ES medium and continuously cultured in the same dish for 3-4 weeks. ES medium was changed daily. Potential iPSC colonies were visible after 2-3 weeks. These colonies were individually picked and expanded on MEFs for analysis. To remove the integrated lentiviral and polycistronic sequences, iPSCs were infected with a Cre-expressing adenovirus (rAd-Cre-IE). Individual colonies were picked and Cre-mediated removal of floxed sequences was verified by PCR using the primers gctaattcactcccaaagaagacaag (SEQ ID NO: 5) and cttcagcaagccgagtcctg (SEQ ID NO: 6).

Generation of CD34+ cells and T cells with OP9 co-culture

The procedure was described previously (Chang et al., "Broad T-cell receptor repertoire in T-lymphocytes derived from human induced pluripotent stem cells," PloS one 9, e97335 (2014)). This method was used with the following modifications. Cultures of hiPSCs in one well of a 6 well plate were treated as described by Ohnuki et al (Ohnuki M, "Generation and characterization of human induced pluripotent stem cells. *Curr Protoc Stem Cell Biol* Chapter 4: Unit 4A 2 (2009)) with CTK solution to make small cell clumps. Cell clumps were then transferred to a 10 cm plate that was pre-seeded with 2-day old OP9 cells in α-MEM-based medium containing 10% FBS, 1X penicillin/streptomycin and 100 μM mono-thioglycerol. The medium was changed every other day, and cells were cultured for 18 days without splitting. After 18 days of co-culture, cells were harvested by treating with dissociation solution (0.15% collagenase IV and 0.015% hyaluronidase in α-MEM medium)

for about 30 minutes and followed by 0.25% trypsin for another 30 minutes. CD34+ cells were then purified on anti- CD34+ magnetic beads (MicroBead Kit; Miltenyi Biotec, Bergisch Gladbach, Germany). For T cell differentiation, these CD34+ cells were plated onto OP9-DL4 cells and cultured with α-MEM medium containing 20% FBS, 5 ng/mL hFlt3-L, 5 ng/mL hIL-7, and 10 ng/mL hSCF. The medium was changed every other day, and cells were transferred to new OP9-DL4 plates every 4 days.

T cell stimulation

In vitro derived T cells from hiPSCs were stimulated by incubation with CD3/28 beads (Invitrogen, Carlsbad, CA) according to the manufacturers' protocol for 3 days prior to analysis by flow cytometry, as previously described (Chang et al., 2014).

Flow Cytometry

Cells were harvested and washed before analysis with an LSRFortessa cell analyzer (BD Bioscience, San Jose, CA). For cell surface staining, propidium iodide (PI, Sigma-Aldrich, St. Louis, MO) was used to exclude dead cells. For apoptosis assay, harvested cells were first stained with cell surface antibodies for 30 min. After washing once with 1X PBS, the cells were resuspended in 100 µL of Annexin Binding Buffer (Invitrogen, Carlsbad, CA) containing Annexin V-647 (Invitrogen, Carlsbad, CA) and PI and incubated for 15 min before adding 400 µL of Annexin Binding Buffer with PI. Antibodies were obtained from BD Biosciences unless otherwise indicated: CD3 (Percp-Cy5-5, clone UCHT1), CD4 (PE-Cy7, clone SK3), CD7 (APC, BV510, clone M-T701), CD8 (APC-Cy7, clone SK1), CD16 (PE, clone B73.1), CD25 (FITC, clone 2A3), CD34 (PE-Cy7, clone WM59), CD43 (PE, clone 1G10), CD56- PE (clone MY31), CD69 (FITC, clone L78), NKG2D-PE (clone 1D11), TCR-αβ (FITC, PE, clone T10B9.1A-31), TCR-Vδ1-FITC (Fisher Scientific, Pittsburgh, PA, Clone TS8.2), TCR-Vδ2-PE (clone B6), TCRVγ9- FITC (clone B3), TNF-α-PE-Cy7 (clone MAB11), Beta Mark TCR Repertoire Kit (Beckman Coulter, Atlanta, GA).

Vector Construction

The polycistronic OSKM vector was previously described (Chang et al.,"Polycistronic lentiviral vector for "hit and run" reprogramming of adult skin fibroblasts to induced pluripotent stem cells," *Stem cells* 27: 1042-1049 (2009)). The Lenti-hDL4-mCherry plasmid was constructed by cloning a PCR-amplified human DL4 cDNA (Open Biosystems, LaFayette, CO), an IRES fragment (Open Biosystems) and mCherry cDNA into a lentiviral vector (pDL171) which contains the EF1α promoter. PCR reactions were performed using PrimeStar polymerase (Takara, Mountain View).

To construct CRISPR plasmids, gRNA oligos were designed and introduced into pX330 and pX335 plasmids following the Zhang lab protocol (Addgene, Cambridge, MA). To construct the JAK3 repair plasmid, wild type human genomic DNA was PCR amplified using JAK3 primer sets (5' arm:

5   gtcgacgtcgacgctcagtgaagctgaagtattccttctgcttcacagggcgaccactac (SEQ ID NO: 7) and atttaaatcctcccctcgaacccttaccaaactcctatgcatactacag (SEQ ID NO:8); 3' arm: ttaattaattaattagcattttaggttcaggttgtgagaacactagaagagaacaagtca (SEQ ID NO: 9) and gtatacgtatacgcatacctggagaggggacaaggtcttgagatgcgagggt (SEQ ID NO: 10). After digesting with enzymes (5' arm: SalI and SwaI; 3' arm: PacI and BstZ17I), the PCR products were

10   cloned into a plasmid containing a LoxP-PGK-Neo-LoxP fragment. All of the oligos used in this study were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). To construct the BCL2 lentiviral plasmid, a primer set (forward: agccaccttaattaagccaccatggcgcacgctgggagaacggggtacgata (SEQ ID NO: 11) and reverse: taacagagagaagttcgtggctccggatcccttgtggcccagataggcacccagggtgat (SEQ ID NO: 12)) was used

15   to amplify the human BCL2 cDNA (Open Biosystems) fragment. The product was linked with GFP through a 2A sequence by PCR and cloned into the pDL171 vector. gRNA-F1 caccGTG AGA TAC AGA TAC AGA CA (SEQ ID NO: 13) gRNA-R1 aaacTGT CTG TAT CTG TAT CTC AC (SEQ ID NO: 14) gRNA-F2 caccgAAT GAT TTG CCT GGA ATG CC (SEQ ID NO: 14) gRNA-R2 aaacGGC ATT CCA GGC AAA TCA TTc (SEQ ID NO: 15)

20   gRNA-F3 caccgCAG CCT AGG CAA AGG CCT GC (SEQ ID NO: 16) gRNA-R3 aaacGCA GGC CTT TGC CTA GGC TGc (SEQ ID NO: 17) gRNA-F4 caccgTGC CAA CAG AAC TGC CTG AT (SEQ ID NO: 18) gRNA-R4 aaacATC AGG CAG TTC TGT TGG Cac (SEQ ID NO: 19) gRNA-F5 caccGAC CAG GGT GCA AGT GTG GA (SEQ ID NO: 20) gRNA-R5 aaacTCC ACA CTT GCA CCC TGG TC (SEQ ID NO: 21) gRNA-F6

25   caccGCT CCT CAG CCT GGC ATT CA (SEQ ID NO: 22) gRNA-R6 aaacTGA ATG CCA GGC TGA GGA GC (SEQ ID NO: 23)

Cell culture

IPSCs were cultured on mitomycin C-treated MEFs derived from E14.5 CF-1 embryos in ES cell media consisting of DMEM F-12 supplemented with 1X non-essential

30   amino acids, 1X penicillin-streptomycin, 1X L-glutamine (all from Mediatech, Corning, NY), 20% KnockOut Serum Replacement (Invitrogen), 2-βME (Sigma) and 5-10 ng/mL bFGF (Invitrogen). Human primary keratinocytes were cultured in DermaLife K Medium Complete Kit (LifeLine Cell Technology, Frederick, MD). OP9 cells were purchased from ATCC and

grown in α-MEM medium with 20% FBS and penicillin-streptomycin. OP9-DL4 cells were established by transducing OP9 cells with a lentivirus containing hDL4 and mCherry.

Virus Production

For preparation of lentivirus, 10 μg of the lentiviral vector, 2.5 μg of the envelope plasmid (pMDG), and 7.5 μg of the packaging plasmid (pCMBVdR8.9.1) were co-transfected into 5x106 293T cells by Fugene 6 (Roche, Nutley, NJ or Promega, Madison, WI). Virus-containing supernatant was collected 2 days after transfection and passed through a 0.45 μm filter.

Gene targeting

IPSCs were treated with 0.25% trypsin for 5 minutes to generate single cell suspensions. After washing twice with 1X PBS, 1 to 2 million cells were mixed with 5 μg of JAK3 repair plasmid and 5 μg of pX330-JAK3 or pX335-JAK3 plasmids for Nucleofection (Human Stem Cell Nucleofector Kit, program A-023, Lonza, Alpharetta, GA) and plating onto MEFs. Two to four days later, hES medium containing 30 μg/mL of G418 was added to the plates to select for drug resistant colonies. The colonies were picked 3 to 4 weeks later and expanded for genomic DNA extraction. For PCR genotyping, a 5' primer set (tgctaaagcgcatgctccagact (SEQ ID NO: 24) and gtcttcatctcagggtcggct (SEQ ID NO: 25) and a 3' primer set (cctctctgtgcattatggcag (SEQ ID NO: 26) and gccttctatcgccttcttg (SEQ ID NO: 27)) were used. To remove the Neo selection marker, hiPSCs were infected with a Cre-expressing adenovirus (rAd-Cre-IE).

RT-PCR

Total RNA was isolated from *in-vitro* derived cells with Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized with 0.5 to 2 μg of total RNA using Superscript First-strand Synthesis System (Invitrogen) according to the manufacturer's instructions. SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA) was used for qPCR according to the manufacturer's instructions. Primer sets used for qPCR are GAPDH (F: actcctccacctttgacgct (SEQ ID NO: 28), R: tcccctcttcaagggtctacatg (SEQ ID NO: 29)); PU.1 (F: gtgcaaaatggaagggtttc (SEQ ID NO: 30), R: ggagctccgtgaagttgttc (SEQ ID NO: 31)); GATA3 (F: tgtttcctttcactggccaca (SEQ ID NO: 32), R: aacggcaactggtgaacggta (SEQ ID NO: 33)); BCL11B (F: ggcgatgccagaatagatgccg (SEQ ID NO: 34), R: ccaggccacttggctcctctatctccaga (SEQ ID NO: 35)); RAG1 (F: ccttactgttgagactgcaatatcc (SEQ ID NO: 36), R: ctgaagtcccagtatatacttcacac (SEQ ID NO: 37)); RAG2 (F: cccagaagcagtaataatcatcgag (SEQ ID NO: 38), R: atgtgggatgtagtagatcttgc (SEQ ID NO: 39)); pTa (F: gggtcttacctcagcagttac (SEQ ID NO: 40), R: cctcacacagtgtgacgcag (SEQ ID NO: 41));

BCL2 (F: gactgagtacctgaaccggc (SEQ ID NO: 42), R: gggccaaactgagcagagtc (SEQ ID NO: 43)); BAX (F: aagaccagggtggttgggac (SEQ ID NO: 44), R: gtaagaaaaatgcccacgtc (SEQ ID NO: 45)); and JAK3 (F: agtcagacgtctggagcttc (SEQ ID NO: 46), R: gtgagcagtgaaggcatgagtc (SEQ ID NO: 47)). All values were normalized relative to GAPDH expression.

Whole Genome Sequencing and Analysis

DNA from iPSCs was sheared using a Covaris S2 Focused-ultrasonicator: 130 μL samples in microTUBEs were subjected to two 40-second cycles of 10% Duty Cycle, Intensity of 4, and 200 Cycles per Burst in Frequency Sweeping Mode. DNA Chip (DNA 1000 Kit; Agilent Technologies, Santa Clara, CA) analysis using an Agilent 2100 Bioanalyzer indicated an average fragment size of 400 bp. Library preparation was performed using an NEBNext Ultra DNA Library Prep Kit for Illumina (NEB #E7370), and the final library concentration was determined by qPCR using a KAPA Illumina Library Quantification Kit (KK4835; KAPA Biosystems, Wilmington, MA) and an Applied Biosystems ViiA 7 Real-Time PCR System (Life Technologies). Sequencing clusters were produced on the flow cell using an Illumina TruSeq PE Cluster Kit v3 – cBot – HS (PE-401-3001) and an Illumina cBot. WGS was performed using an Illumina TruSeq SBS Kit v3 – HS – 200 cycles (FC-401-3001) and an Illumina HiSeq 2500 upgrade to generate 2x100 single-index paired-end reads for bioinformatic analysis. Probable off-target sites were identified by aligning the CRISPR/Cas9 guide sequences to the hg19 reference genome using EMBOSS fuzznuc software (v6.6.0.0) (Rice et al., "EMBOSS: the European Molecular Biology Open Software Suite," *Trends in Genetics* : TIG 16: 276-277 (2000)) and allowing for a maximum of three mismatches; 1193 sites were predicted for the first guide sequence (GTGAGATACAGATACAGACA) (SEQ ID NO: 48) and 257 sites for the second guide sequence (AATGATTTGCCTGGAATGCC) (SEQ ID NO: 49). All of the reads from the WGS for each sample were mapped to the hg19 reference genome using the BWA (v0.7.5a) mem algorithm (Li and Durbin, "Fast and accurate long-read alignment with Burrows-Wheeler transform," *Bioinformatics* 26: 589-595 (2010)) and duplicate reads were removed using Picard-tools (v1.100) (http://picard.sourceforge.net). Local realignment and base quality re-calibration were performed using GATK (v2.7-2) (McKenna et al., "The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data," *Genome research* 20: 1297-1303 (2010)). Both SNVs and indels were called using the GATK HaplotypeCaller. Additionally, SNVs and indels were separately re-calibrated as described in GATK Best Practices and quality filters were applied. The variants from the reference genome that were common to all four iPSC samples were excluded from

CRISPR/Cas9 off-target analysis. The non-excluded variants were screened using Bedtools (v2.17.0) (Quinlan and Hall, "BEDTools: a flexible suite of utilities for comparing genomic features," Bioinformatics 26: 841-842 (2010)) to determine if they fell within the probable off-target sites. The analysis shows that none of these variants reside in the off-target sites

5   and suggests these mutations were randomly accumulated. All of the functional variants (excluded and non-excluded) with a low allele frequency (< 1%, dbSNP 138) were then annotated using the ANNOVAR software package and screened for known associations with diseases in HGMD and ClinVar (v20140902); additionally, all of the hits with a high CADD score (CADD >= 20) were also screened for associations with complex diseases using the

10   GWAS Catalog and COSMIC (v70). No validated disease-associated variants were identified in the databases queried. Of particular interest, the JAK3 C1837T (p.R613X) mutation was also not validated to associate with a disease, though the SNP (rs149316157) is predicted to be significantly deleterius, with a GERP score of 3.85 and a CADD score (CADD phred-like score) of 38. Therefore, the JAK3 C1837T variant was associated for the

15   first time with a clinical case of SCID.

Accession codes

The WGS data can be accessed at the NCBI SRA database with the accession number SRP056149.

JAK3-deficient human T cells express low levels of BCL2 and die at an early

20   **developmental stage**

IPSCs were generated from skin keratinocytes (Chang et al., 2009) of a SCID patient homozygous for a C>T nucleotide substitution in exon 14 of the JAK3 gene. This mutation replaces a CGA codon (arginine at 613) with a TGA stop codon (p.R613X). As described above, the four-month-old patient presented with a T-B+NK- clinical phenotype. To

25   determine whether this SCID phenotype can be recapitulated *in vitro*, differentiation of patient-specific iPSCs to T lymphocytes using a two-step OP9 and OP9-DL4 system (Chang et al., 2014) was attempted. JAK3-deficient iPSCs grew at a rate comparable to control iPSCs derived from healthy donors, and these iPSCs efficiently differentiated into CD34+ hematopoietic progenitors (HPs) on OP9 stromal cell monolayers. However, when the

30   JAK3-deficient, iPSC-derived CD34+ HPs were plated on OP9-DL4 stromal monolayers, T-cell differentiation was absent compared to controls (Fig. 1). No CD3+ T cells or CD3-CD16+CD56+ NK cells were observed (Fig.1A), and no CD4+CD8+ double positive (DP), CD4+ single positive (SP), or CD8+ single positive (SP) T cells were detected (Fig.1B). Jak3 knockout (KO) mice have a small thymus due to a block in thymocyte differentiation at

the CD4-CD8- double negative (DN) stage prior to productive TCR rearrangement. To further understand the developmental defects resulting from a JAK3 mutation in humans, T lineage commitment and maturation of JAK3-deficient cells compared to normal JAK3 WT controls was assayed. IPSC-derived CD34+ cells were plated on OP9-DL4 monolayers, and

5   cells were harvested and analyzed for lymphocyte markers at T-cell induction day (TD) 14, 21, 28 and 35 (Fig. 4A). In normal controls, 1.2 X 107 CD7+ cells (84% of cells counted in the lymphoid gate) were generated at TD14 from 1-2 X 106 CD34+ cells. T cell markers CD4, CD8, CD3 and TCR αβ were sequentially detected upon T cell maturation. At TD35, more than 50% of the population was CD8 SP cells. In JAK3-deficient cells, only 4.5 X 104

10   CD7+ cells (38.9% of cells counted in lymphoid gate) were generated at TD14 from 1-2 X 10^6 CD34+ cells. The number of CD7+ cells decreased during extended culture and T cell markers CD3, CD4, CD8 and TCR αβ were not significantly expressed. During the transition through early T cell progenitors (ETPs), the CD4-CD8- (DN) to CD4+CD8+ (DP) stages are directed by precise activation and repression of specific transcription factors. In control cells,

15   the silencing of PU.1 and induction of GATA3 and BCL11B (Fig. 1C) suggest that these cells proceed to the onset of T lineage commitment (DN2 to DN3) followed by TCR rearrangement. In contrast, in JAK3-deficient cells PU.1 accumulates and GATA3 and BCL11B levels are reduced (Fig. 1C). These data suggest that human JAK3-deficient cells arrest before or at the DN2 stage, which is similar to the stage at which T cells die in Jak3

20   KO mice. Interestingly, human JAK3-deficient cells may express sufficient RAG1, RAG2 and PTCRA (Fig. 1C) to perform TCR rearrangement, but the cells do not survive long enough to proceed to this important developmental stage. These profound defects in lymphocyte development of JAK3-deficient cells can be explained by the absence of IL-7 signaling which plays an important role in lymphoid progenitor survival and differentiation.

25   IL-7/JAK3 signaling maintains thymocyte homeostasis by regulating the BCL2 family of apoptotic regulators. Thymocytes and peripheral T cells from Jak3 KO mice have a high apoptotic index in part through selectively elevating BAX, a pro-apoptotic factor, and by reducing expression of BCL2, an anti-apoptotic factor. Similarly, in these studies, an increase in apoptosis of *in vitro*-derived human JAK3-deficient cells compared to controls at

30   TD10 (9% to 2.2%) and TD17 (7% to 1.9%) (Fig. 2A). Consistent with this phenotype, BAX levels were increased and BCL2 levels were reduced in JAK3-deficient cells compared to controls (Fig. 2B). Forced expression of Bcl2 rescues T, but not B or NK cell development in γc–deficient mice (Kondo et al., *Immunity* 7: 155-162 (1997)). Transplantation of Jak3 KO mice with Bcl2-expressing Jak3 KO bone marrow cells also improves peripheral T cell

numbers (Wen et al., *Molecular and cellular biology* 21: 678-689 (2001)). To determine whether overexpression of BCL2 will rescue T cell developmental defects of human JAK3-deficient cells, in vitro-derived, JAK3-deficient CD34+ cells were transduced with a lentivirus containing a BCL2- 2A-GFP polycistron driven by EF1a promoter. After transduction, CD34+ cells were plated on OP9-DL4 monolayers and assayed for NK and T cell markers at TD 28. No CD3-CD16+CD56+ NK cells were found in GFP- (JAK3-; BCL2 low) or GFP+ cells (JAK3-; BCL2+) (Fig 2C). These findings suggest that BCL2 released the blockage at the DN stage in JAK3-deficient cells. Interestingly, a second developmental arrest was evident at the DP stage; no further differentiation of CD8+CD4+ DP positive cells was observed in GFP+ cells (Fig 2C). In summary, the studies described above demonstrate that human SCID phenotypes can be recapitulated *in vitro* with patient-derived iPSCs. JAK3 deficiency results in proliferative defects in DN thymocytes. Forced expression of BCL2 enhances survival of DN cells, which further differentiate into DP thymocytes. Nevertheless, DP thymocytes fail to mature to SP T cells, and this defect may result from the absence of IL7/JAK3 signaling.

Correction of the JAK3 deficiency in SCID hiPSCs by CRISPR/Cas9 enhanced gene replacement

To determine whether normal T cell development can be restored in JAK3-deficient SCID patient cells, the JAK3 mutation was corrected in iPSCs by CRISPR/Cas9 enhanced gene replacement. Six guide RNAs within introns upstream and downstream of exon 14 were designed to target wtCas9 or nCas9 near the C1837T mutation, and a correction template was used for gene replacement (Fig. 3A). IPSCs were nucleofected with two plasmids expressing the D10A Cas9 nickase and paired guide RNAs or a single plasmid expressing wild-type Cas9 and a single guide RNA. Cells were grown in medium containing G418 for 2 weeks post nucleofection. Individual colonies were picked, expanded, and genotyped by PCR (Fig. 3B Top). The efficiency of CRISPR/Cas9–mediated JAK3 gene correction is shown in Fig. 3C. Three clones from WT Cas9 + gRNA #1, 3 clones from WT Cas9 + gRNA #2 and 6 clones from Cas9 nickase + paired gRNAs #1 and #2 were further verified by Sanger sequencing. In 12 sequenced clones, 2 homozygous corrected clones (1 clone from Cas9 nickase + paired gRNA #1 and #2, and 1 clone from WT Cas9 + gRNA #1) and 10 heterozygous corrected clones were identified (Fig. 3D). Restoration of JAK3 gene expression was demonstrated by RT-PCR (JAK3 mRNA) (Fig. 3B; lower left panel) and western blot (JAK3 protein) (Fig. 3B; lower right).

Specificity of CRISPR/Cas9 directed JAK3 correction

The potential for off-target, CRISPR/Cas9 directed genome modifications raises some concerns about the use of this approach for therapy in humans. In cancer cell lines, relatively high levels of off-target mutagenesis by Cas9-gRNAs have been described. To determine the specificity of CRISPR/Cas9 directed JAK3 correction in human SCID iPSCs, Whole genome

5   sequencing was performed before and after gene replacement. The genomes of two heterozygous and one homozygous corrected clones were sequenced. The two heterozygous clones were corrected with gRNA #2 + wild type Cas9, and the homozygous clone was corrected with gRNA #1 + gRNA #2 + nickase Cas9 (D10A). The 20-base CRISPR guide sequences were mapped to the human reference genome, allowing up to 3 mismatches in

10   order to identify possible off-target sites. These sites were then analyzed for variations in the iPSC samples following CRISPR/Cas9 directed gene replacement. WGS analysis of one homozygous and two heterozygous corrected iPSC lines demonstrated that no mutations (SNVs nor indels) were introduced into the predicted off-target sites, suggesting a strong specificity for the CRISPR/Cas9 directed gene replacement.

15   **Restoration of T cell development after CRISPR/Cas9 directed JAK3 correction**

To determine whether T cell development is restored after JAK3 gene correction, T cell lineage commitment and maturation were assayed. T cell differentiation sequentially passes through intermediates observed *in vivo*: CD34+CD7+ T/NK committed stage; CD7+CD4+CD8− immature, SP stage; CD4+CD8+ DP stage; and finally, CD3+CD8+

20   TCRαβ mature stage. Mature T cells are polyclonal, proliferate, and secrete cytokines in response to mitogens. Therefore, JAK3 corrected hiPSCs were differentiated into hematopoietic progenitors on OP9 monolayers, and CD34+ cells were positively selected on anti-CD34 magnetic beads. These cells were plated on OP9-DL4 monolayers, and nonadherent cells were analyzed for lymphocyte markers at TD14, 21, 28 and 35 (Fig. 4).

25   Similar to control cells, 1-2 X 106 CD34+ JAK3 corrected cells differentiated into 4.7 X 106 CD7+ cells (91% of cells counted in lymphoid gate) at TD14. After further differentiation to TD21, TD28 and TD35, T cell maturation markers CD3, CD4, CD8 and TCR αβ were abundantly observed (Fig. 4A). To determine whether TCR rearrangement is reestablished in JAK3-corrected T cells, TCR Vβ typing was performed by flow cytometry

30   and summarized in Figure 4B. JAK3-corrected T cells expressed all the Vβ segments that we tested (19 of 25); therefore, a broad TCR repertoire was restored. Finally, the integrity of the TCR signaling pathway, a surrogate of T cell function, in JAK3-corrected T cells, was examined by measuring cell surface activation markers following anti-CD3/CD28 stimulation. On Day 3 post-stimulation, the percentage of CD3+CD25+CD69+ T cells

increased from 0.68% to 59.7% in JAK3-corrected T cells similar to the increase observed in control cells (0.01% to 37.6%) (Fig. 4C). These data and results described above demonstrate that correction of the JAK3 C1837T (p.R613X) mutation by CRISPR/Cas9 enhanced gene replacement in an *in vitro* iPSC model system restores normal T cell development with the

5   capacity to produce functional, mature T cell populations with a broad TCR repertoire.

In humans, the phenotype of lymphocytes in the peripheral blood of SCID patients has been well described, but studies on critical steps of lymphoid commitment and thymocyte development have been difficult to perform. Access to bone marrow and thymocyte samples from untreated patients with SCID is challenging since these conditions

10   are rare and infants typically present with life-threatening infections requiring urgent HSC transplantation to survive. The strategy described herein for studying human SCID bypasses these restrictions; large numbers of hematopoietic progenitors can be produced from patient specific iPSCs *in vitro*, and the mechanisms responsible for immunodeficiency can be precisely determined. Demonstrated herein is that T cell development in human JAK3-

15   deficient SCID is completely blocked before or at the CD4-CD8- (DN2) stage. Interestingly, forced expression of BCL2 enhances survival of DN cells, which further differentiate into DP thymocytes. However, DP thymocytes fail to mature to SP T cells, and this defect may result from the absence of IL7/JAK3 signaling. It is also demonstrated that correction of the human JAK3 mutation by CRISPR/Cas9 enhanced gene replacement restores the

20   differentiation potential of early T cell progenitors. Corrected progenitors are capable of producing NK cells and mature T cell populations expressing a broad TCR repertoire. Whole-genome sequencing analysis of one homozygous and two heterozygous corrected iPSC lines demonstrates that no mutations (SNVs nor indels) are introduced into the predicted off-target sites, suggesting a strong specificity for the CRISPR/Cas9 directed gene

25   replacement.

In the methods described herein, CD34+ HSCs can be generated from hiPSCs by co-culturing with human bone marrow stromal stem (hMSC) cells (See Figure 5). The HSCs produced by this method from patient-specific iPSC after gene correction/modification could be transplanted back into the patient to treat diseases such as sickle cell disease (SCD), SCID

30   or cancer. In the methods described herein, T cells can be generated by culturing hiPSC derived CD34+ cells by co-culturing the hiPSC derived CD34+ cells with hMSC-DL4 (See Figure 6). HSCs produced by this method from patient-specific iPSC after correction/modification could be transplanted back into the patient to treat diseases. The T cells can comprise γδ T cells. As shown in Figure 7, γδ T cells expressing recombinant T cell

receptor (TCR) can be efficiently produced from genetically modified iPSC. Production of γδ T cells expressing TCR specific for tumor antigens provide a cellular therapy for cancer.

<u>Example 2</u>

5   **Correction of a mutation associated with Sickle Cell Anemia
by CRISPR/Cas9 enhanced gene replacement**

Vector Construction

          The human codon optimized *S. pyogenes* Cas9 with both N-terminal and C-terminal

10   nuclear localization sequences (nls-Cas9-nls) were PCR cloned from px330 vector (Addgene ID: 42230) into a modified pET-28b (EMD Biosciences) vector with a His$_6$-SUMO tag at the N-terminus. A gene block cassette containing a short linker peptide followed by a supercharged GFP with a net charge of +36 and a 23 amino acid influenza virus hemagglutinin HA-2 variant peptide INF7 (GLFEAIEGFIENGWEGMIDGWYG)(SEQ ID

15   NO: 50) was codon optimized for *E. coli* and synthesized (IDT DNA) and cloned to fuse with the C-terminus of the nls-Cas9-nls. An HIV-TAT peptide (YGRKKRRQRRRPPQ))(SEQ ID NO: 51) coding sequence was also synthesized (IDT DNA) and cloned to fuse with the N-terminus of the nls-Cas9-nls.

Protein Overexpression and Purification

20   The pET-SUMO-scCas9 plasmid was transformed into *E. coli* strain Rosetta™ 2(DE3) cells (EMD Millipore, Billerica, MA) in LB medium. The cells were grown at 37°C until the optical density reached 0.6 at 600nm. Induction of protein overexpression was achieved by adding 0.5 mM isopropyl-1-thio-ß-D-galactopyranoside (IPTG) and culturing overnight at 18°C in a shaker. The harvested cells were re-suspended in Ni-binding buffer

25   (20mM Tris-HCl pH 8.0, 1.5 M NaCl, 25 mM imidazole and 0.2 mM TCEP) and lysed by Emulsiflex C3 high pressure homogenizer (Avestin). Polyethyleneimine (PEI) with final concentration of 0.4% was added into the cleared lysate to precipitate the nucleic acids. The proteins in the supernatant after centrifugation was then precipitated by ammonium sulfate to remove the PEI and re-dissolved in the Ni-binding buffer. The proteins were first purified by

30   a HisTrap nickel affinity column (GE Healthcare) followed by overnight digestion with SUMO protease Ulp1 at 4°C. The cleaved His-SUMO tag was then removed via a second HisTrap column. The flow though containing the scCas9 protein was diluted to reach the final NaCl concentration of 0.5 M and purified on a HiTrap Heparin column (GE Healthcare) by gradient elution with buffer containing 20mM Tris-HCl pH 8.0, 2.0 M NaCl, and 0.2 mM

35   TCEP. The eluted scCas9 protein was further purified by a size exclusion column Superdex

200 16/600 (GE Healthcare) in gel filtration buffer (20mM Tris-HCl pH 8.0, 0.5 M NaCl, and

0.2 mM TCEP), sterilized by passing through a 0.22 µm filter and concentrated by an

Amicon Centrifugal Unit (EMD Millipore) with 100 kDa cutoff. The concentrated protein

was quantified by UV spectrophotometer and flash frozen in liquid nitrogen.

5   **Guide RNA Preparation**

Template DNA for sgRNA transcription was generated by PCR with primer set

adding a T7 promoter and a polyA sequences. sgRNA was *in vitro* transcribed by T7 RNA

polymerase using T7 Ribomax Express System (Promega, Madison, WI) according to the

manufacturer's manual. The transcribed RNA was purified by phenol: chloroform extraction,

10   ethanol precipitation and followed by column purification with MEGAclear™ Transcription

Clean-Up Kit (Ambion, Austin, TX). The purified gRNA was quantified by UV

spectrophotometer and stored in -80°C freezer.

Single-stranded DNA Donors

Single-stranded DNA (ssODN) donors were synthesized by IDT DNA as shown

15   below.

Single-stranded Donor DNAs for HBB sickle correction	
HBB-T2-ssODN	ATCCACGTTCACCTTGCCCCACAGGGCAGTAACGGCAGACTTCT CCtCAGGAGTCAGGTGCACCATGGTGTCTGTTTGAGGTTGCTAGT GA (SEQ ID NO: 52)
HBB-T2-ssODN-wobble	CTTCATCCACGTTCACCTTGCCCCACAGGGCAGTAACGGCAGAtT TtTCCtCAGGAGTCAGGTGCACCATGGTGTCTGTTTGAGGTTGCT AGTGA (SEQ ID NO: 53)

Cell Culture

Human sickle patient iPSC were derived from skin fibroblasts and were maintained

on Matrigel (BD) in mTeSR™1 medium (Stem Cell Technologies, Vancouver, CA) with

20   penicillin/streptomycin.

scCas9-sgRNA-ssODN complex preparation and nucleofection

1/10 volume of 10x PBS was added into sgRNA to reach 1x final concentration. The

sgRNA was annealed on PCR thermo cycler with slow decreasing of temperature from 95°C

to 4°C. After annealing, scCas9 protein was added into the sgRNA with a 1:1.5 protein-to-

25   RNA molar ratio and mixed quickly by tapping the tube until all the transient precipitation

was gone. The mixture was incubated in room temperature for 10 minutes in dark. Then, 1

molar ratio amount of ssODN was added into the mixture and incubated for additional 10
minutes in dark to form the scCas9-sgRNA-ssODN complex.

One day before nucleofection, cells were detached by Accutase (Stem Cell
Technologies) and 1×10^6 cells /well cells were seeded on a 6-well plate with 10 μM Rock
5   inhibitor (Y-27632) (EMD Millipore). For each experiment, 5×10^5 hsIPSCs were
resuspended as single cells in 100μl supplemented Human Stem Cell Nucleofector Solution
1(Lonza) and scCas9-sgRNA-ssODN complex was then mixed with the cell solution. The
cells were nucleofected with program A-023 using a Nucleofector II device (Lonza, Basel,
Switzerland). The efficiency of HBB genome correction was analyzed by ddPCR two days
10   post nucleofection.

Detection of sickle correction by ddPCR

The cells nucleofected with the scCas9-sgRNA-ssODN complex were lysed by
prepGEM Tissue DNA extraction reagent (ZyGEM, Hamilton, NZ) following manufacturer's
manual and 1:3 diluted with water. In a 22 μl ddPCR reaction, 11 μl 2 x ddPCR mix (Bio-
15   rad) was mixed with 1ul each of 5μM allele-specific FAM or VIC Taqman probes set forth
below, 0.2 μl each of a 100 μM forward and reverse primer, and 8.6 μl diluted genomic
DNA. Droplets were generated by QX200 Droplet Generator (Bio-rad, Hercules, CA)
according to the manufacturer's manual. The reaction mix was then transferred into a 96-well
PCR plate and the PCR was performed on a standard thermal cycler (Bio-rad). The program
20   for PCR was : Step 1: 95°C 10 min; Step 2: 95°C 30s; Step 3: 55°C 1 min; repeat steps 2-3
for 39 times; Step 4: 98°C 10 min; Step 5: 8°C hold. After PCR was done, the plate was then
analyzed by QX200 Droplet Reader (Bio-rad).

T7-sgRNA transcription template primers	
T7-T2-F	TAATACGACTCACTATAGGGTAACGGCAGACTTCTCCAC (SEQ ID NO: 54)
T7-polyA-R	AAAAAGCACCGACTCGGTGCC (SEQ ID NO: 55)

25   **Taqman Probes:**

HBB-wb-FAM-TM	FAM-TCCTGaGGAaAAaT-MGB (SEQ ID NO: 56)
HBB-wt-FAM-TM	FAM-TGACTCCTGAGGAGAA-MGB (SEQ ID NO: 57)
HBB-sk-VIC-TM	VIC-ACTCCTGTGGAGAAG-MGB (SEQ ID NO: 58)

ddPCR Primers:

R196	HBB-TaqM-f2	CAGAGCCATCTATTGCTTACATTTG (SEQ ID NO: 59)
R197	HBB-TaqM-r1	GGCCTCACCACCAACTTCAT (SEQ ID NO: 60)

As set forth above, a complex that includes a guide RNA (gRNA), modified recombinant Cas9 protein (mrCas9) and a single-stranded oligodeoxyribonucleotide (ssODN) can be introduced into human stem cells or derivatives thereof to correct a single base mutation that causes disease. Table 1 and Figure 8 illustrate results from the introduction of a sickle cell correction complex (gRNA-mrCas9-ssODN) into induced Pluripotent Stem Cells (iPSC) derived from skin cells of a sickle cell patient. IPSCs were derived as described in Example 1. The correction complex was introduced into sickle iPSC by nucleoporation and 2 days later genomic DNA was analyzed by digital PCR, using the primers set forth above, and sequenced. Over 65% of the cells contained at least one corrected gene. One corrected gene is sufficient to cure the disease. The results were confirmed as follows. Two days after introduction of the correction complex, the cells were plated in culture dishes, and 43 individual iPSC colonies were isolated. Genomic DNA was isolated from these colonies and the beta-globin gene was sequenced. Sixty-five percent of the colonies contained at least one corrected beta-globin gene (S corrected to A).

Table 1

	gRNA-mrCas9-ssODN	
Pooled ddPCR result (2-day)	68.6%	
Total colonies picked after 2 weeks	48	
Mixed colonies	5	
Total single colonies	43	
A/A	14	32.6%
A/S	4	9.3%
S/S	3	7.0%
A/indel	10	23.3%
S/indel	6	14.0%
Indel/indel	6	14.0%
Clones with at least 1 allele corrected	28	65.1%
Clones with indels	22	51.2%
Clones with genome modification	40	93.0%
Total number of alleles	86	
Total "A" alleles (corrected)	42	48.8%
Total "S" alleles (uncorrected)	16	18.6%
Total "indel" alleles	28	32.6%
A:(A+S) *comparable to ddPCR result	42/58 = 72.4%	
HR:NHEJ (A:indel) ratio	1.50	

Similar studies were performed with patient primary bone marrow CD34+ cells. The protocol was as follows. Bone marrow was obtained from a sickle patient by an IRB approved protocol. CD34+ cells were purified on a Miltenyi anti-CD34+ beads (Miltenyi, Bergisch Gladbach, Germany). The cells were nucleoporated with the complex prepared as described above. After nucleoporation, the cells plated in methycult and BFU-E, CFU-E and CFU-GEMM colonies were picked after two weeks and analyzed for corrected alleles. Table 2 and Figure 9 illustrate results from the introduction of a sickle cell correction complex (gRNA-mrCas9-ssODN) into patient primary bone marrow CD34+ cells. After twelve days of *in vitro* differentiation, DNA was analyzed by digital PCR (ddPCR) and sequenced. Approximately equal amounts of betaA and betaS mRNA were observed (See Figure 9). Immediately after nucleoporation, some of the cells were culture in erythroid differentiation medium for up to eightenn days and enucleated red blood cells were analyzed for HbA. An isoelectric focusing (IEF) gel of *in vitro* differentiated red blood cells from the corrected sickle patient CD34+ cells showed an HbA (normal hemoglobin) to HbS (hemoglobin with sickle cell mutation) ratio of about 1:3, which is sufficient to inhibit sickling and treat the disease (See Figure 10).

Table 2

Complex for nucleofection	Cas9wt-36GFP-T2-ssODN	
Nucleofection Program	P4 DN-100	
BFU-E/CFU-E/GEMM colonies picked on D10 and D15	21/23/7	
Total colonies*	**51**	
A/A	2	4%
A/S	4	8%
S/S	19	37%
A/indel	5	10%
S/indel	15	29%
Indel/indel	6	12%
Clones with at least 1 allele corrected	11	22%
Clones with indels	24	47%
Clones with genome modification	29	57%
Total number of alleles	**102**	
Total "A" alleles (corrected)	13	13%
Total "S" alleles (uncorrected)	57	56%
Total "indel" alleles	32	31%
A:(A+S) *comparable to ddPCR result	13/70 = 18.6%	
HR:NHEJ (A: indel) ratio	0.41	

Sequences

SEQ ID NO: 1

5   TAACGGCAGACTTCTCCAC

SEQ ID NO: 2
GTAACGGCAGACTTCTCCACGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG
CTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTT

SEQ ID NO:3
10   Cas9-supercharged GFP construct
mdykdhdgdykdhdidykddddkmapkkkrkvgihgvpaadkkysigldigtnsvgwavitdeykvpskkfkvlgntdrh
sikknligallfdsgetaeatrlkrtarrrytrrknricylqeifsnemakvddsffhrleesflveedkkherhpifgnivdevayhek
yptiyhlrkklvdstdkadlrliylalahmikfrghfliegdlnpdnsdvdklfiqlvqtynqlfeenpinasgvdakailsarlsksrrl
enliaqlpgekknglfgnlialslgltpnfksnfdlaedaklqlskdtydddldnllaqigdqyadlflaaknlsdaillsdilrvnteitk
15   aplsasmikrydehhqdltllkalvrqqlpekykeiffdqskngyagyidggasqeefykfikpilekmdgteellvklnredllrk
qrtfdngsiphqihlgelhailrrqedfypflkdnrekiekiltfripyyvgplargnsrfawmtrkseetitpwnfeevvdkgasaqs
fiermtnfdknlpnekvlpkhsllyeyftvyneltkvkyvtegmrkpaflsgeqkkaivdllfktnrkvtvkqlkedyfkkiecfds
veisgvedrfnaslgtyhdllkiikdkdfldneenediledivltltlfedremieerlktyahlfddkvmkqlkrrrytgwgrlsrkli
ngirdkqsgktildflksdgfanrnfmqlihddsltfkediqkaqvsgqgdslhehianlagspaikkgilqtvkvvdelvkvmgr
20   hkpeniviemarenqttqkgqknsrermkrieegikelgsqilkehpventqlqneklylyylqngrdmyvdqeldinrlsdydv
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qitkhvaqildsrmntkydendklirevkvitlksklvsdfrkdfqfykvreinnyhhahdaylnavvgtalikkypklesefvyg
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tevqtggfskesilpkrnsdkliarkkdwdpkkyggfdsptvaysvlvvakvekgkskklksvkellgitimerssfeknpidfle

akgykevkkdliiklpkyslfelengrkrmlasagelqkgnelalpskyvnflylashyeklkgspedneqkqlfveqhkhyldei
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qlggdkrpaatkkagqakkkkgsgsngssgsaskgerlfrgkvpilvelkgdvnghkfsvrgkgkgdatrgkltlkficttgklpv
pwptlvttltygvqcfsrypkhmkrhdffksampkgyvqertisfkkdgkyktraevkfegrtlvnriklkgrdfkekgnilghkl

5   rynfnshkvyitadkrkngikakfkirhnvkdgsvqladhyqqntpigrgpvllprnhylstrsklskdpkekrdhmvllefvtaa
gikhgrderyk

SEQ ID NO: 4
TAT-Cas9-supercharged GFP construct
ygrkkrrqrrrppqaggsmdykdhdgdykdhdidykddddkmapkkkrkvgihgvpaadkkysigldigtnsvgwavitd

10   eykvpskkfkvlgntdrhsikknligallfdsgetaeatrlkrtarrrytrrknricylqeifsnemakvddsffhrleesflveedkkh
erhpifgnivdevayhekyptiyhlrkklvdstdkadlrliylalahmikfrghfliegdlnpdnsdvdklfiqlvqtynqlfeenpin
asgvdakailsarlsksrrlenliaqlpgekknglfgnlialslgltpnfksnfdlaedaklqlskdtydddldnllaqigdqyadlflaa
knlsdaillsdilrvnteitkaplsasmikrydehhqdltllkalvrqqlpekykeiffdqskngyagyidggasqeefykfikpilek
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15   itpwnfeevvdkgasaqsfiermtnfdknlpnekvlpkhsllyeyftvyneltkvkyvtegmrkpaflsgeqkkaivdllfktnrk
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gilqtvkvvdelvkvmgrhkpeniviemarenqttqkgqknsrermkrieegikelgsqilkehpventqlqneklylyylqngr
dmyvdqeldinrlsdydvdhivpqsflkddsidnkvltrsdknrgksdnvpseevvkkmknywrqllnaklitqrkfdnltkaer

20   gglseldkagfikrqlvetrqitkhvaqildsrmntkydendklirevkvitlksklvsdfrkdfqfykvreinnyhhahdaylnavv
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gitimerssfeknpidfleakgykevkkdliiklpkyslfelengrkrmlasagelqkgnelalpskyvnflylashyeklkgsped
neqkqlfveqhkhyldeiieqisefskrviladanldkvlsaynkhrdkpireqaeniihlftltnlgapaafkyfdttidrkrytstke

25   vldatlihqsitglyetridlsqlggdkrpaatkkagqakkkkgsgsngssgsaskgerlfrgkvpilvelkgdvnghkfsvrgkgk
gdatrgkltlkficttgklpvpwptlvttltygvqcfsrypkhmkrhdffksampkgyvqertisfkkdgkyktraevkfegrtlvnr
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dpkekrdhmvllefvtaagikhgrderykggsggsvdglfeaiegfiengwegmidgwyg

What is claimed is:

1. A method of making tumor-specific T-cell precursor cells comprising introducing into a population of T-cell precursor cells a complex comprising:

   a. a guide (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of the T cell precursor cells and a second nucleotide sequence that interacts with a site-directed nuclease;

   b. a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the gRNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA to create a double stranded break; and

   c. a donor nucleic acid sequence comprising a third nucleotide sequence that encodes a chimeric antigen receptor (CAR) and a fourth nucleotide sequence that hybridizes to a genomic sequence flanking the double stranded break in the target DNA,

   wherein the complex is introduced into the T-cell precursor cells under conditions that allow homology-directed repair (HDR) and integration of the third nucleotide sequence into the target DNA to form modified T-cell precursor cells that express the CAR.

2. The method of claim 1, wherein the cells are selected from the group consisting of hematopoietic stem cells or pluripotent stem cells.

3. The method of claim 2, wherein the pluripotent stem cells are induced pluripotent stem cells.

4. The method of claim 1-3, wherein the supercharged protein is operably linked to the amino-terminus or the carboxy-terminus of the nuclease.

5. The method of any of claims 1-4, wherein the recombinant site-directed nuclease operably linked to a supercharged protein further comprises a trans-activating transcriptional activator (TAT) peptide operably linked to the amino-terminus of the site-directed nuclease.

6. The method of any of claims 1-5, wherein the supercharged protein has an overall positive charge that is greater than its corresponding unmodified protein.

7. The method of claim 6, wherein the overall positive charge is from about +5 to about about +40.

8. The method of any of claims 1-7, wherein the supercharged protein is superpositively charged green fluorescent protein (GFP).

9. The method of claim 8, wherein the supercharged protein is a superpositively charged +36 GFP.

10. The method of any of claims 1-9, wherein the nuclease is Cas9.

11. The method of any of claims 1-10, wherein the molar ratio of gRNA to site-directed nuclease operably linked to a supercharged protein to ssODN is from about 1:1:1 to about 1.5:1:1.

12. The method of any of claims 1-11, wherein the ratio of homology-directed repair to nonhomologous end joining in the population of T-cell precursor cells is at leat about 0.5.

13. The method of any of claims 1-12, wherein the complex is introduced into the cells by nucleoporation.

14. The method of any of claims 1-13, wherein at least 5% of the population of T-cell precursor cells are modified by HDR to form modified T-cell precursor cells that express the CAR.

15. The method of any of claims 1-14, further comprising isolating the modified T-cell precursor cells.

16. The method of claim 15, further comprising culturing the modified T-cell precursor cells.

17. The method of claim 16, wherein the modified T-cell precursor cells are cultured under conditions for expansion.

18. The method of claim 16 or 17, further comprising culturing the modified T-cell precursor cells under conditions that promote differentiation of the modified T-cell precursor cells into T cells that express the CAR.

19. A method of treating cancer in a subject comprising transplanting into the subject cells obtained by the method of any of claims 1-18.

20. The method of claim 19, wherein the transplantation is autologous.

21. A complex for making tumor-specific T-cell precursor cells comprising:
    a. a guide (gRNA) comprising a first nucleotide sequence that hybridizes to a target DNA in the genome of the T cell precursor cells and a second nucleotide sequence that interacts with a site-directed nuclease;
    b. a recombinant site-directed nuclease operably linked to a supercharged protein, wherein the site-directed nuclease comprises an RNA-binding portion that interacts with the second nucleotide sequence of the gRNA and wherein the site-directed nuclease specifically binds and cleaves the target DNA to create a double stranded break; and
    c. a donor nucleic acid sequence comprising a third nucleotide sequence that encodes a chimeric antigen receptor (CAR) and a fourth nucleotide sequence that hybridizes to a genomic sequence flanking the double stranded break in the target DNA.

22. The complex of claim 21 wherein the supercharged protein is operably linked to the amino-terminus or the carboxy-terminus of the nuclease.

23. The complex of any of claims 21 or 22 wherein the recombinant site-directed nuclease operably linked to a supercharged protein further comprises a trans-activating

transcriptional activator (TAT) peptide operably linked to the amino-terminus of the site-directed nuclease.

24. The complex of any of claims 21-23, wherein the supercharged protein has an overall positive charge that is greater than its corresponding unmodified protein.

25. The complex of claim 24, wherein the overall positive charge is from about +5 to about about +40.

26. The complex of any of claims 21-25, wherein the supercharged protein is superpositively charged green fluorescent protein (GFP).

27. The complex of claim 26, wherein the supercharged protein is a superpositively charged +36 GFP.

28. The complex of any of claims 21-27, wherein the nuclease is Cas9.

29. The complex of any of claims 21-28, wherein the molar ratio of gRNA to site-directed nuclease operably linked to a supercharged protein to ssODN is from about 1:1:1 to about 1.5:1:1.

FIGURE 1A
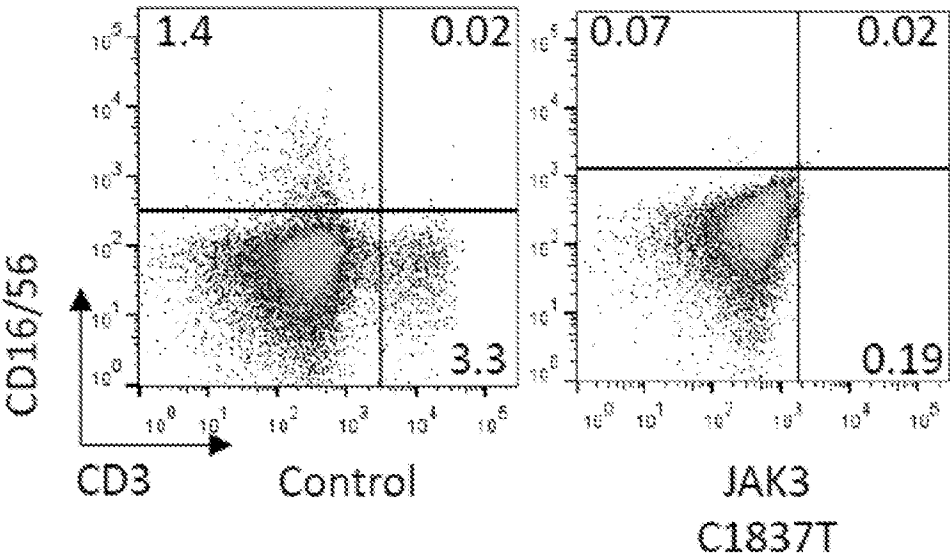
FIGURE 1B
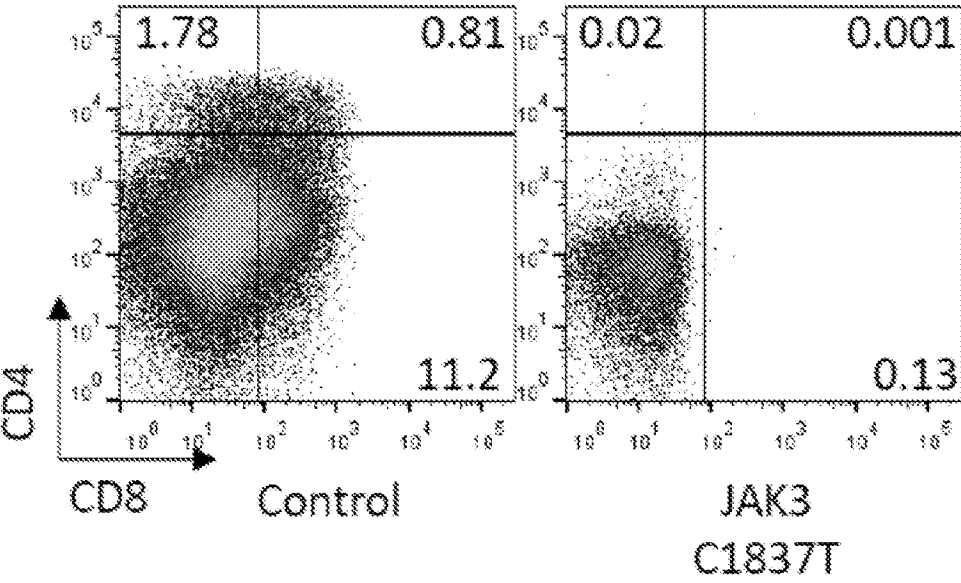
FIGURE 1C
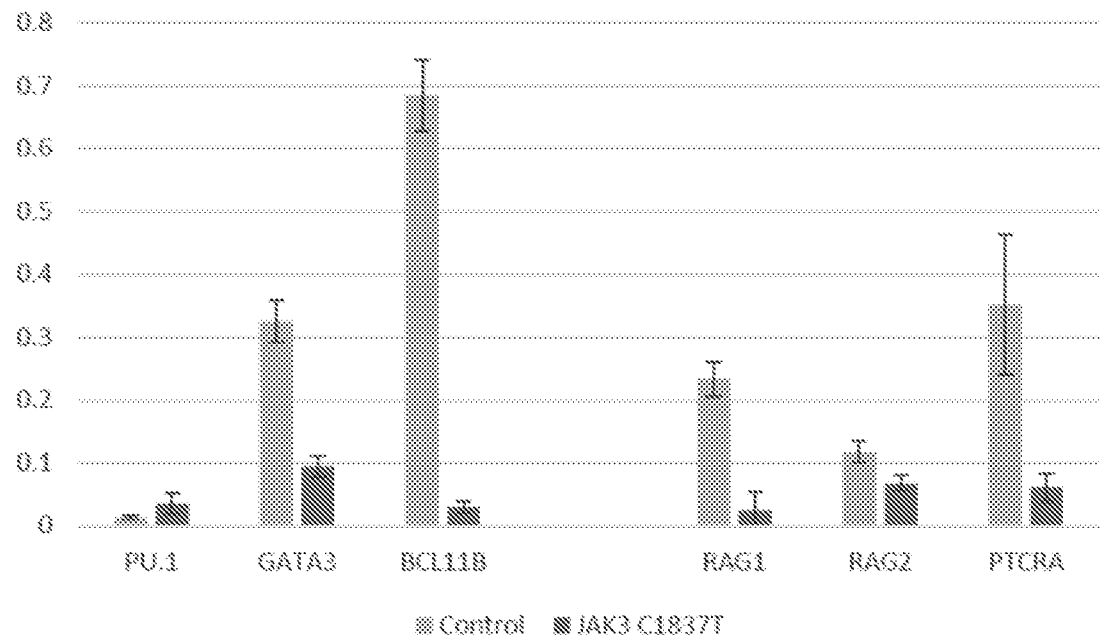
FIGURE 2A
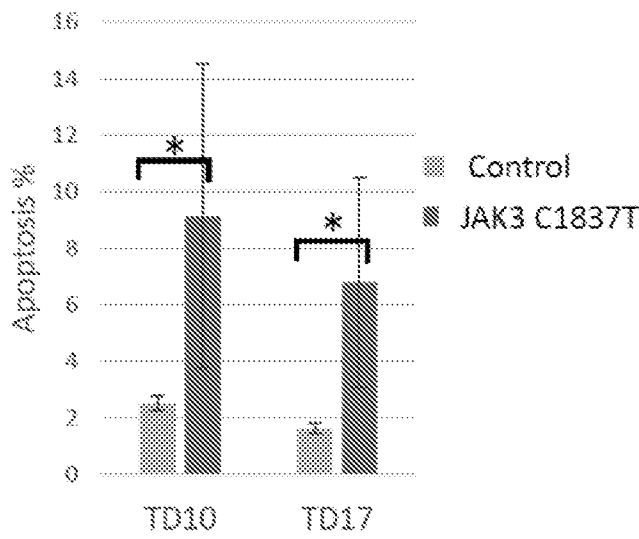
FIGURE 2B
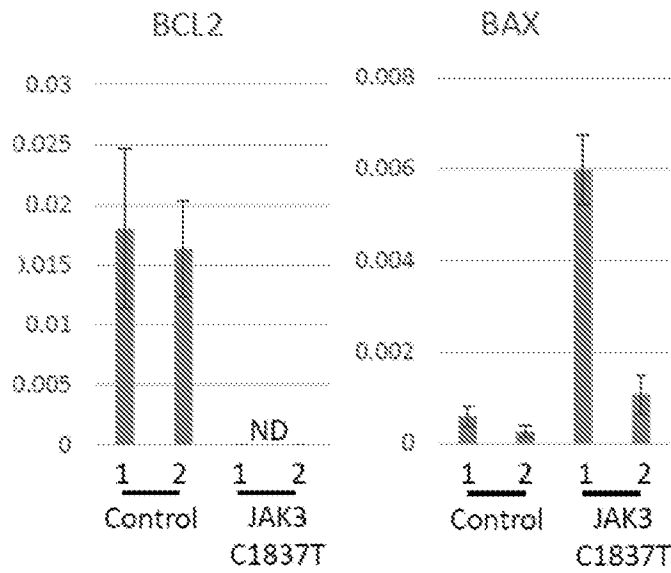
FIGURE 2C

FIGURE 3A

A.

JAK3 genomic locus
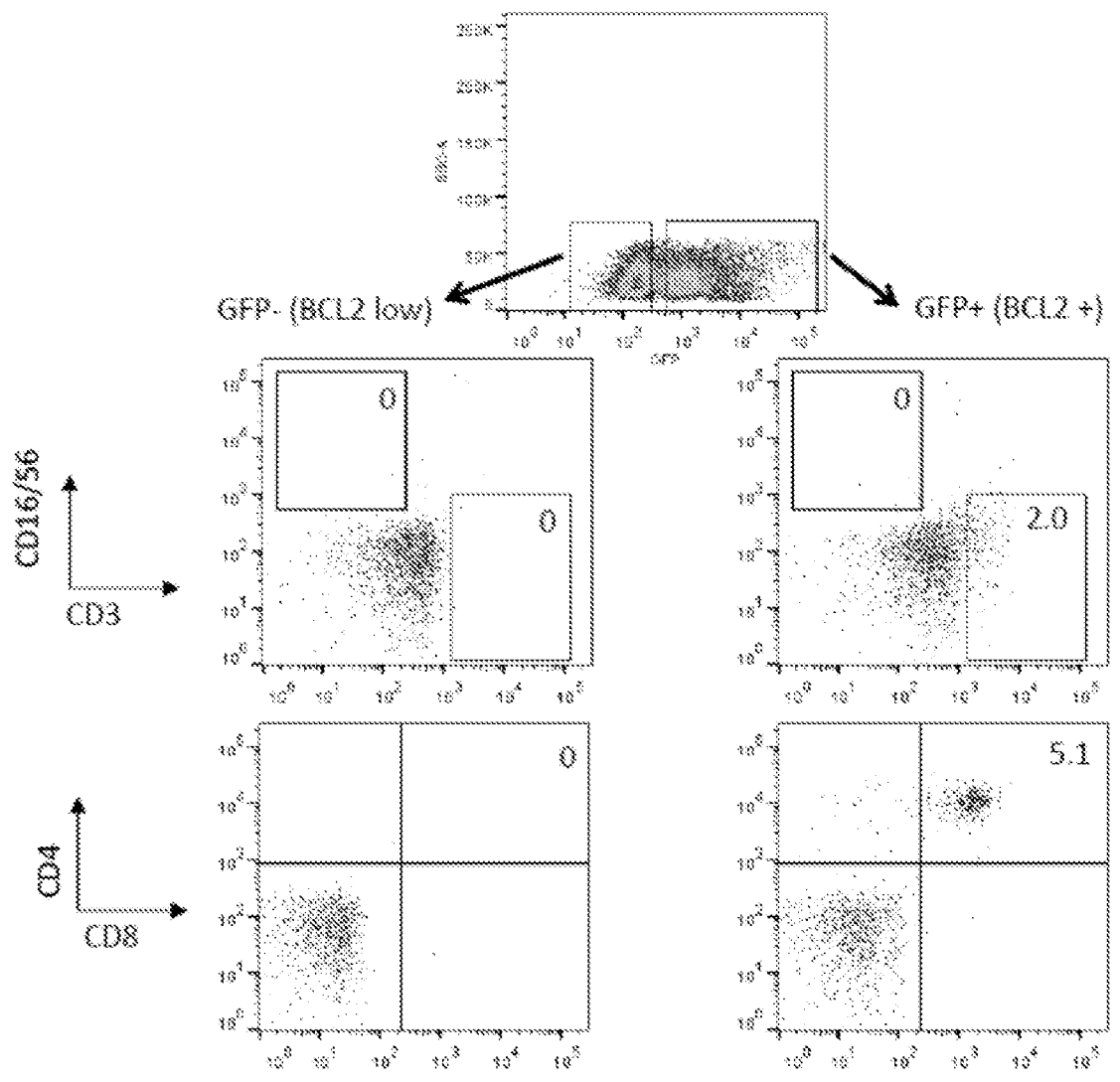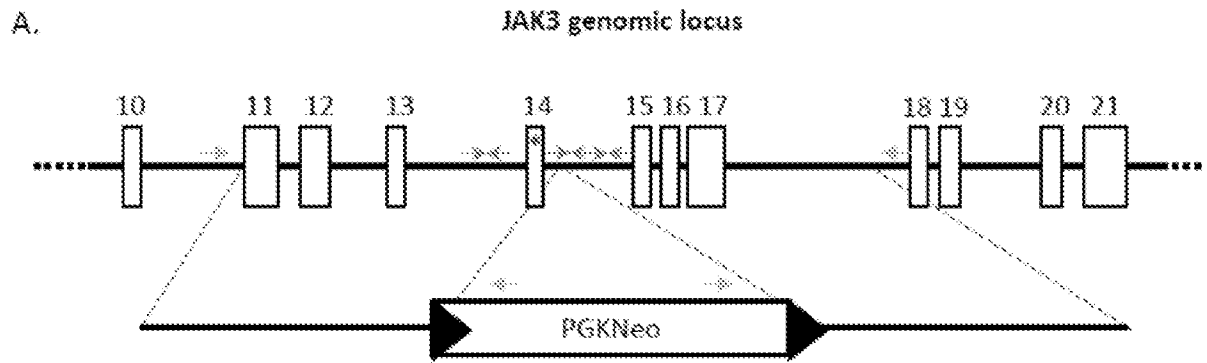
FIGURE 3B

B.
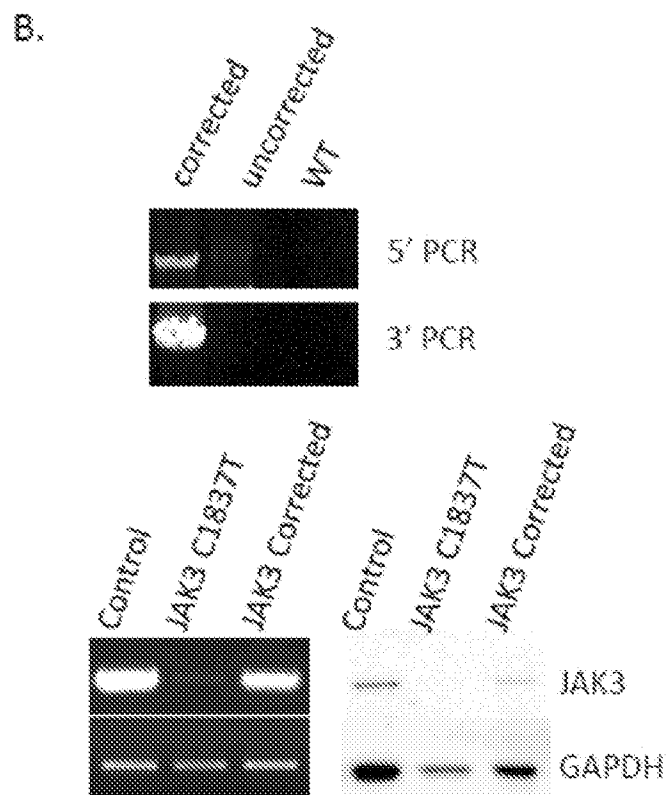
FIGURE 3C

	Colonies examined	PCR positive colonies	%
gRNA #1	39	9	23
gRNA #2	45	33	73.3
gRNA #3	16	1	6.25
gRNA #4	9	3	33.3
gRNA #5	3	0	0
gRNA #6	7	0	0
gRNA #1 + #2	14	14	100
gRNA #3 + #4	3	0	0
gRNA #5 + #6	4	0	0

FIGURE 3D

JAK3 patient
C1837T

Heterozygous
corrected

Homozygous
corrected

FIGURE 4A

—▲—JAK3 C1837T
—●—JAK3 Corrected
—■—Control

FIGURE 4B



TCR Vβ family
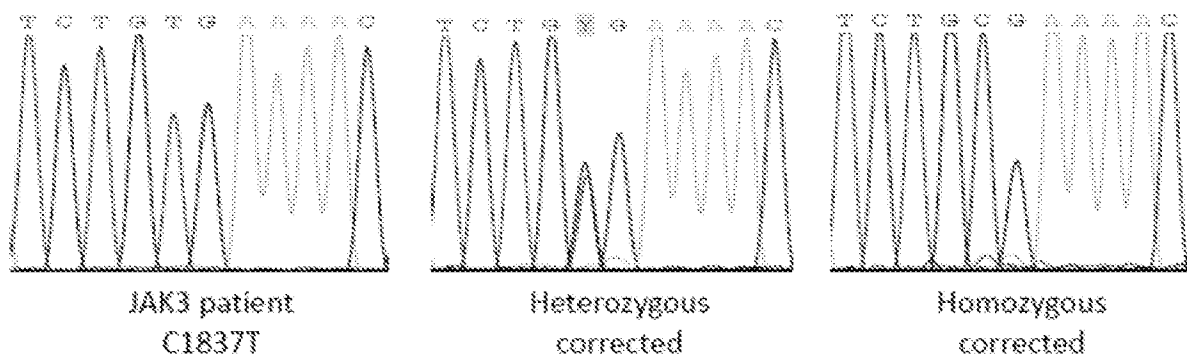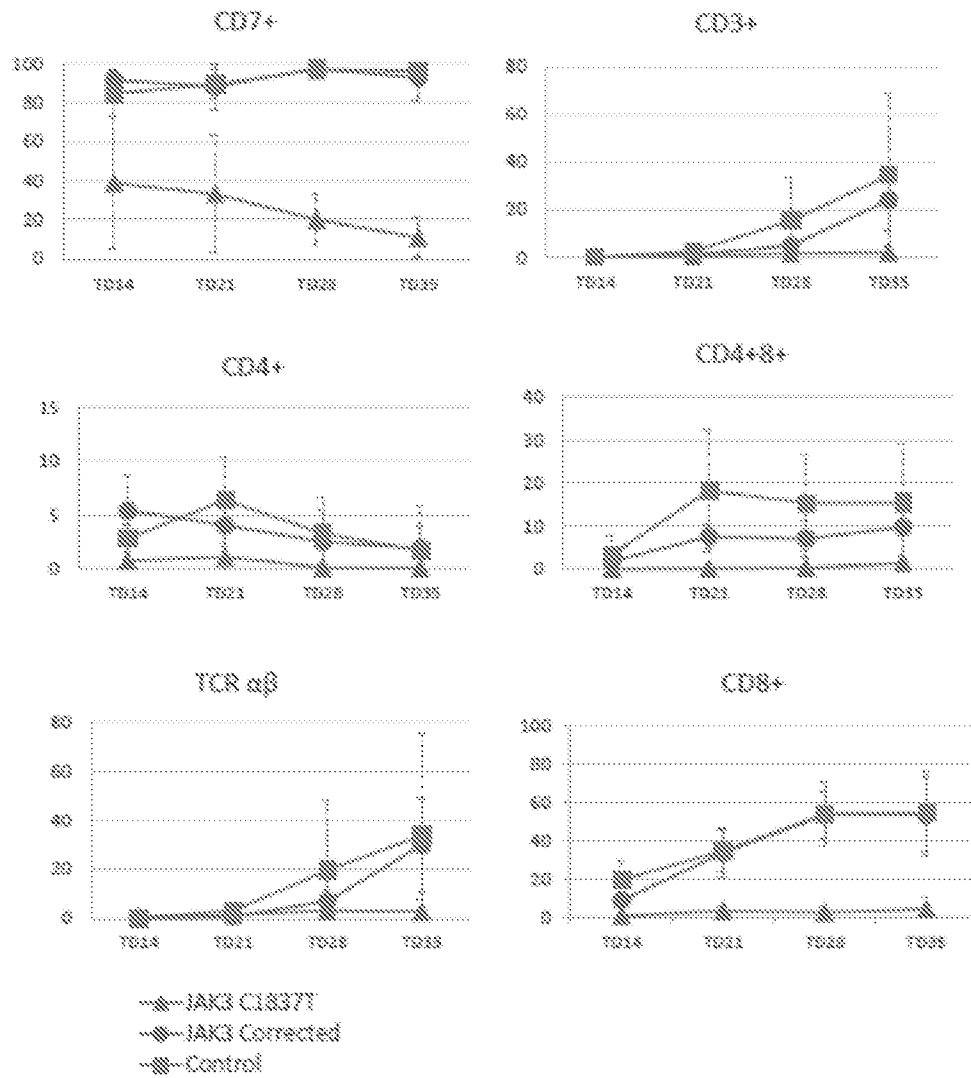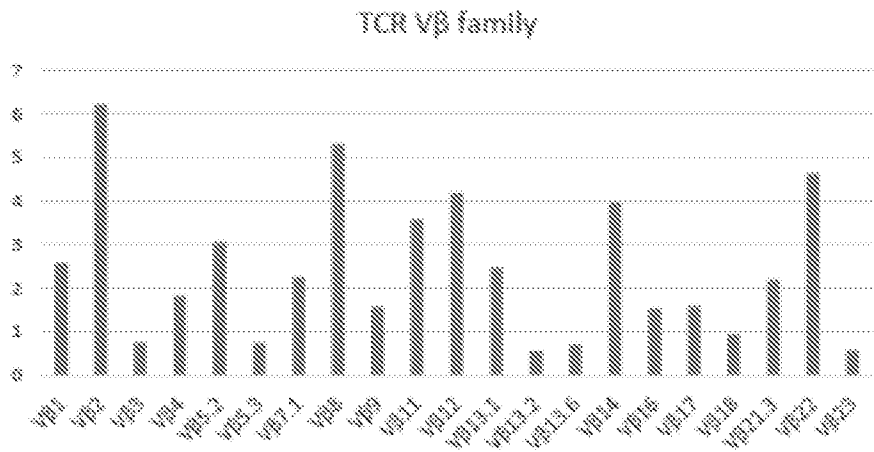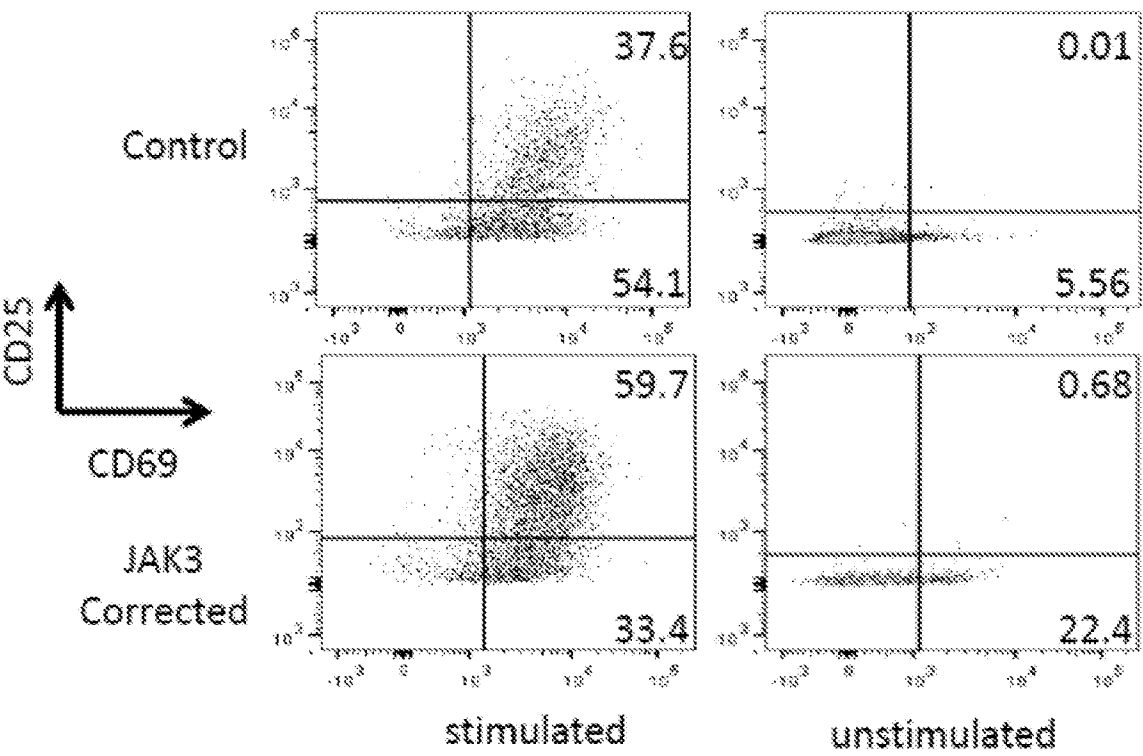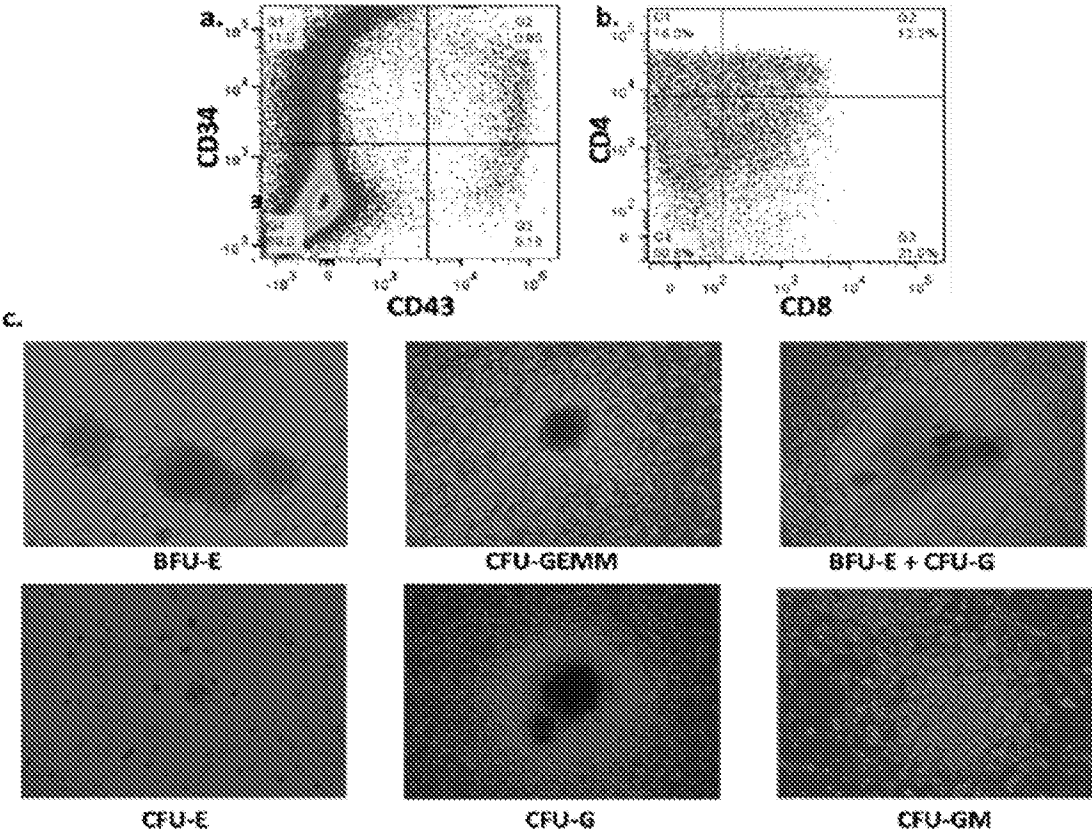
FIGURE 4C

FIGURE 5

FIGURE 6
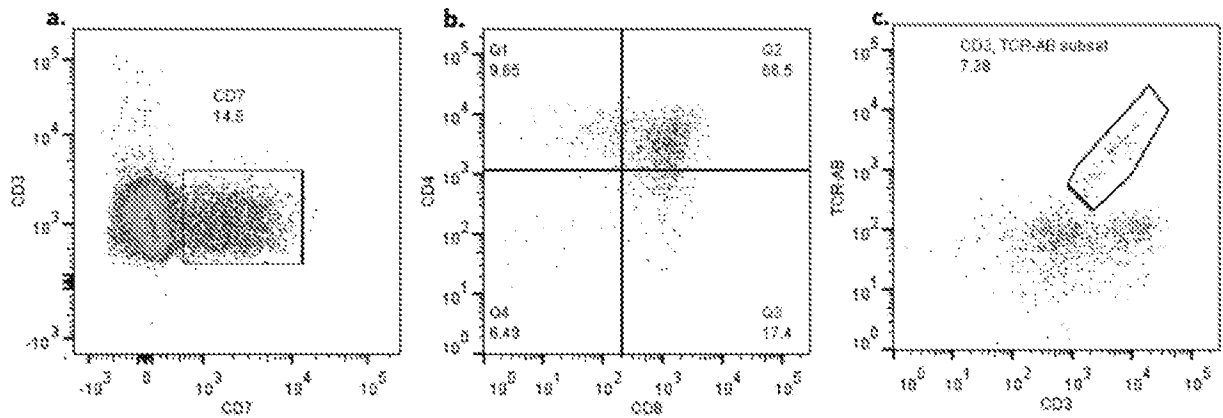
FIGURE 7
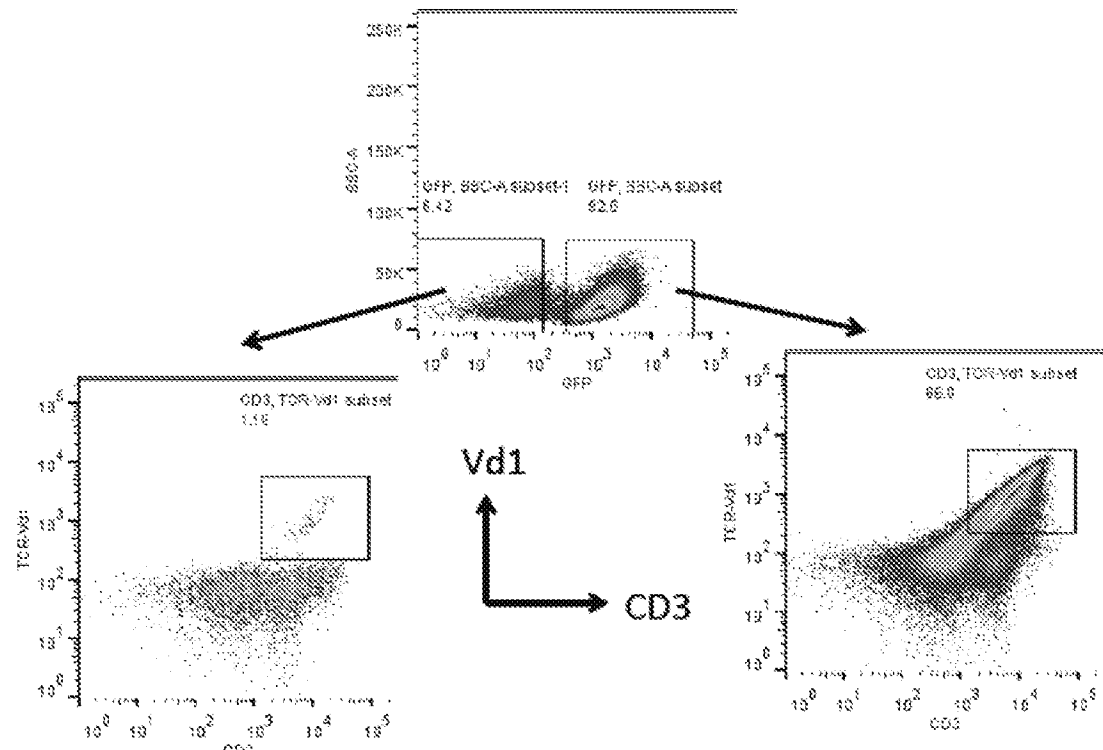
FIGURE 8

Sanger sequencing results
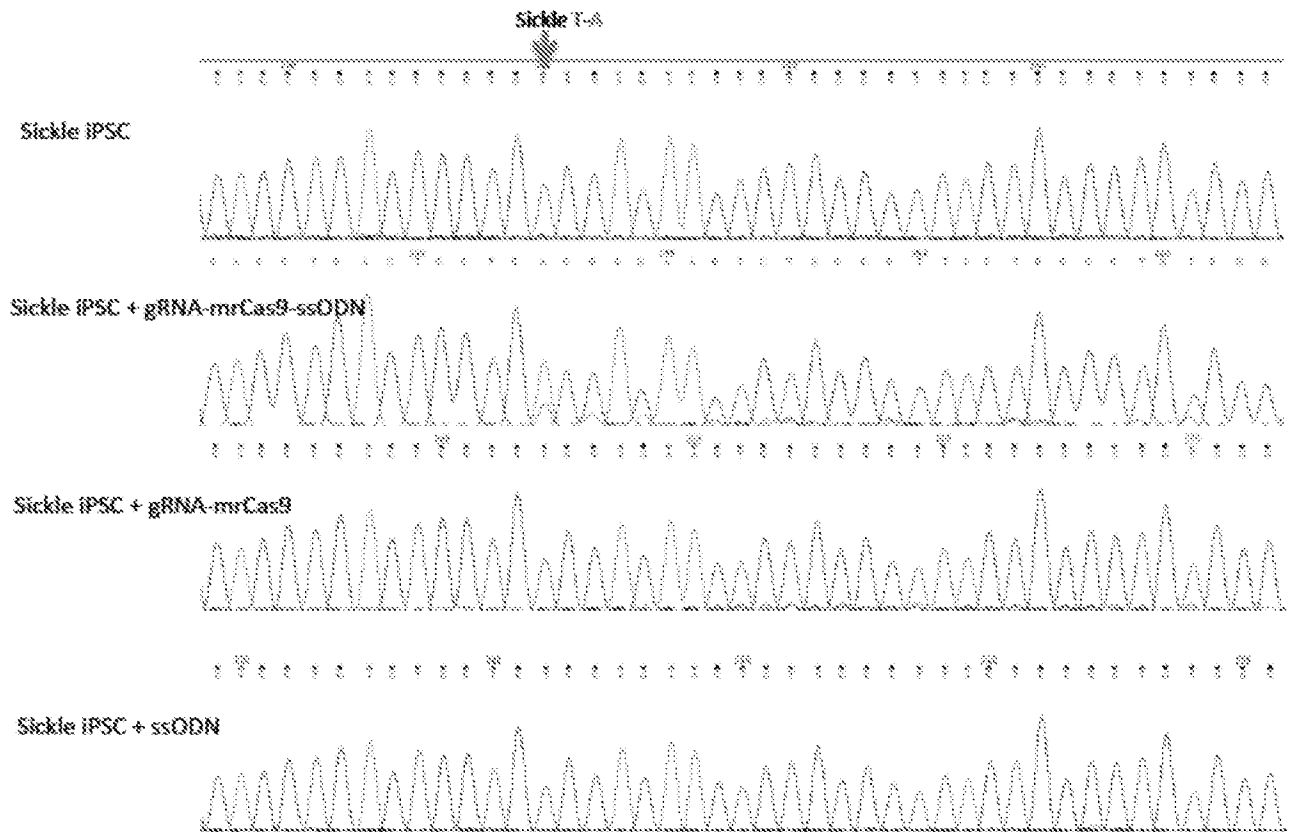
FIGURE 9
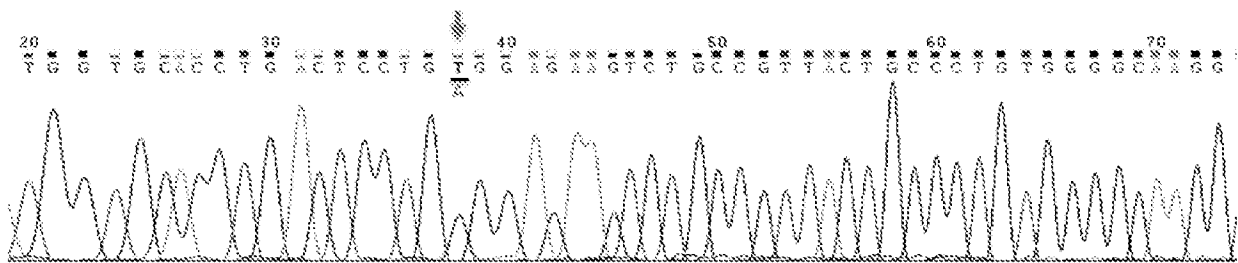
FIGURE 10
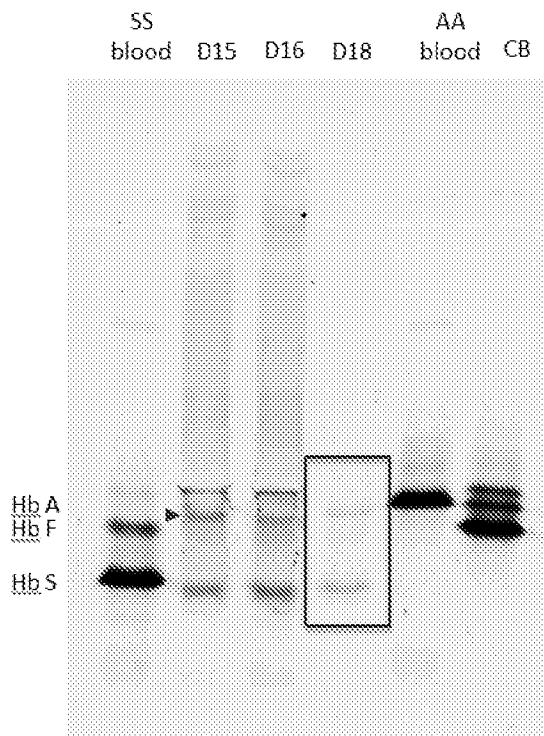
A. CLASSIFICATION OF SUBJECT MATTER

(see extra sheet)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/68, C12N 15/00, 15/113, 15/90, 9/22, A61K 48/00.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSearch, EMBL, NCBI, PAJ, Espacenet, DWPI, PCT Online, USPTO DP, CIPO (Canada PO), SIPO DB, Freepatentsonline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHANG CHIA-WEI et al. Broad T-Cell Receptor Repertoire in T-Lymphocytes Derived from Human Induced Pluripotent Stem Cells, PLOS ONE, 2014, Vol.9, Issue 5, e97335. doi: 10.1371/journal.pone.0097335, pp.1-10, especially abstract, pp.1-2	1-4, 19-20
X Y	BIALK PAWEL et al. Regulation of Gene Editing Activity Directed by Single-Stranded Oligonucleotides and CRISPR/Cas9 Systems, PLOS ONE, 08.06.2015, 10(6), e0129308. doi: 10.1371/journal.pone.0129308, pp.1-19, especially abstract, pp.3-5, 12-16	21-23 1-4, 19-20

☐ Further documents are listed in the continuation of Box C.　　　☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document but published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 October 2016 (14.10.2016)	27 October 2016 (27.10.2016)
Name and mailing address of the ISA/RU: Federal Institute of Industrial Property, Berezhkovskaya nab., 30-1, Moscow, G-59, GSP-3, Russia, 125993 Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37	Authorized officer U. Berezhnaya Telephone No. 495 531 65 15

Form PCT/ISA/210 (second sheet) (January 2015)

C12Q 1/68 (2006.01)
C12N 15/00 (2006.01)
C12N 15/113 (2010.01)
C12N 15/90 (2006.01)
C12N 9/22 (2006.01)
A61K 48/00 (2006.01)

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:

   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:

   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-18, 24-29

   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)