

GENETICALLY ENGINEERED T CELLS HAVING IMPROVED PERSISTENCE IN CULTURE

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of priority to U.S. Provisional Patent Application No. 62/897,016, filed September 06, 2019, U.S. Provisional Patent Application No. 62/927,764, filed October 30, 2019, and U.S. Provisional Patent Application No. 63/034,646, filed June 04, 2020. Each of the prior applications is hereby incorporated by reference in its entirety.

10 BACKGROUND OF THE INVENTION

Chimeric antigen receptor (CAR) T-cell therapy uses genetically-modified T cells to more specifically and efficiently target and kill cancer cells. After T cells have been collected from the blood, the cells are engineered to include CARs on their surface. The CARs may be introduced into the T cells using CRISPR/Cas9 gene editing technology.

15 When these allogeneic CAR T cells are injected into a patient, the receptors enable the T cells to kill cancer cells.

T cells having improved persistence in culture are desired in CAR-T therapy. Such T cells live longer in both *in vitro* and *in vivo*, thereby conferring benefits in CAR-T cell manufacturing and clinical applications. However, it remains challenging to improve

20 persistence of T cells in culture.

SUMMARY OF THE INVENTION

The present disclosure is based, at least in part, on the development of a T cell bank comprising T cells with genetic editing of certain genes (*e.g.*, genes involved in cell self-renewal such as TET2, apoptosis such as FAS, T cell exhaustion or replicative senescence

25 such as CD70, or a combination thereof) to improve T cell persistence in cell culture. The genetically edited T cells disclosed herein showed enhanced cell expansion and proliferation capacity in culture, reduced apoptosis (*e.g.*, induced by FAS ligand), enhanced frequency of activation (*e.g.*, enhanced cytotoxicity), and enhanced CAR-T efficacy in animal models (via, *e.g.*, longer persistence).

30 *e.g.*, longer persistence). For example, CAR-T cells having a disrupted *TET2* gene showed both growth advantage and CAR-T enrichment effects *in vitro* and allows CAR-T cells to persist longer in animal models having both liquid and solid tumors. Further, the genetically edited T cells having a disrupted *TET2* gene showed cytokine-dependent growth, indicating safety. Such a T cell bank (*e.g.*, having a disrupted *TET2* gene) can be used for making

35 therapeutic T cells, for example, CAR-T cells.

Accordingly, one aspect of the present disclosure provides a population of genetically engineered T cells (a T cell bank), comprising: (i) a mutated gene involved in cell self-renewal; (ii) a disrupted gene involved in apoptosis; (iii) a disrupted gene involved in regulation of T cell exhaustion or replicative senescence; or (iv) a combination of any one of (i)-(iii). The population of genetically engineered T cells, as compared to the non-engineered T counterparts, has one or more of the following superior features: (a) enhanced expansion capacity in culture, (b) enhanced proliferation capacity, (c) a reduced apoptosis level, and (d) an enhanced frequency of activation. The T cell bank as disclosed herein may be derived from primary T cells of one or more donors, for example, one or more human donors.

10 In some embodiments, genes involved in cell self-renewal may comprise the *TET2* gene. A mutated *TET2* gene may be a disrupted *TET2* gene, which does not express a functional TET2 protein. Alternatively, a mutated *TET2* gene may be a modulated *TET2* gene that expresses a truncated version of *TET2* (e.g., a truncated version having a molecular weight of around 170 kDa). Any of the mutated *TET2* genes may be genetically edited by
15 CRISPR/Cas mediated gene editing, for example, using a guide RNA (gRNA) targeting a desired site in the *TET2* gene (either coding region or non-coding region). In some examples, the mutated *TET2* gene may have one or more genetic editing events in one or more of exon 1, exon 3, exon 4, exon 5 and exon 6. In particular examples, the mutated *TET2* gene is genetically edited using a gRNA comprising SEQ ID NO: 14, 18, 22, 26, 112, 116, or 120.

20 In some embodiments, genes involved in apoptosis may comprise *FAS*, and/or genes involved in T cell exhaustion may comprise *CD70*. Such genes may be disrupted, for example, via CRISPR/Cas-mediated gene editing. Exemplary gRNAs targeting *FAS* may comprise SEQ ID NO: 69, 73, 77, 81, or 85. Exemplary gRNAs targeting *CD70* may comprise SEQ ID NO: 34, 38, 42, 46, 50, 54, or 58.

25 In some embodiments, the population of genetically engineered T cells described herein may comprise a combination at least one mutated gene involved in cell self-renewal (e.g., *TET2*), at least one gene involved in apoptosis (e.g., *FAS*), and/or at least one gene involved in T cell exhaustion (e.g., *CD70*).

Any of the population of genetically engineered T cells as disclosed herein may
30 further comprise a disrupted beta-2-microglobulin (*$\beta 2M$*) gene, a disrupted T cell receptor alpha chain constant region (*TRAC*) gene, or a combination thereof. In some embodiments, the T cells may be further engineered to express a chimeric antigen receptor (CAR), for example, comprising a nucleic acid encoding the CAR. In some examples, the nucleic acid is inserted in the genome of the T cells. In specific examples, the genetically engineered T cells

may have a disrupted *TRAC* gene, in which a nucleotide acid encoding a chimeric antigen receptor may be inserted. In some embodiments, the CAR may target a tumor antigen. Examples include CD19, B cell maturation antigen (BCMA), or CD70.

5 In another aspect, the present disclosure provides a method for preparing T cell bank as disclosed herein. Such a method may comprise (a) providing a plurality of cells, which are T cells or precursor cells thereof; (b) genetically editing at least one gene involved in cell self-renewal (*e.g.*, *TET2*), at least one gene involved in apoptosis (*e.g.*, *FAS*), and/or at least one gene involved in regulation of T cell exhaustion (*e.g.*, *CD70*); and (c) producing the population of genetically engineered T cells.

10 In some embodiments, the T cells of step (a) may be derived from one or more suitable donors, for example, one or more human donors. In some embodiments, the T cells show cytokine-dependent growth.

In some embodiments, step (b) can be performed by delivering to the cells of (a) one or more RNA-guided nucleases, and one or more gRNAs specific to the one or more target genes disclosed herein. In some examples, the RNA-guided nuclease can be a Cas9 nuclease, for example, a *S. pyogenes* Cas9 nuclease. In some examples, the RNA-guided nuclease and the one or more gRNAs can be complexed in a ribonucleoprotein particle (RNP). Step (b) may be performed by a single electroporation event. Alternatively, step (b) may be performed by two sequential electroporation events.

20 In some embodiments, the target gene comprises *TET2*. Exemplary gRNA targeting *TET2* may be specific to exon 1, exon 3, exon 4, exon 5, and/or exon 6 of the *TET2* gene. Such gRNAs may comprise the nucleotide sequence of SEQ ID NO: 14, 18, 22, or 26. Alternatively or in addition, the target genes may comprise *FAS* and/or *CD70*. Exemplary gRNAs targeting *FAS* may comprise the nucleotide sequence of SEQ ID NO: 69, 73, 77, 81, or 85. Exemplary gRNAs targeting *CD70* may comprise the nucleotide sequence of SEQ ID NO: 34, 38, 42, 46, 50, 54, or 58.

30 In yet another aspect, the present disclosure provides a method for preparing genetically engineered T cells expressing a chimeric antigen receptor (CAR) using genetically engineered T cells from any of the T cell banks disclosed herein. Such a method may comprise: (a) providing a plurality of T cells from a T cell bank, which may comprise genetically engineered T cells having at least one gene involved in cell self-renewal (*e.g.*, *TET2*), at least one gene involved in apoptosis (*e.g.*, *FAS*), and/or at least one gene involved in regulation of T cell exhaustion (*e.g.*, *CD70*); (b) delivering to the plurality of the T cells a

nucleic acid encoding a CAR; and (c) producing genetically engineered T cells expressing the CAR.

In some embodiments, the plurality of T cells from the T cell bank further comprises a disrupted $\beta 2M$ gene. In other embodiments, the method may further comprise genetically editing a $\beta 2M$ gene, for example, delivering to the plurality of the T cells a gRNA targeting a $\beta 2M$ gene.

In some embodiments, the plurality of T cells from the T cell bank further comprises a disrupted *TRAC* gene. In other embodiments, the method may further comprise genetically editing a *TRAC* gene, for example, delivering to the plurality of the T cells a gRNA targeting a *TRAC* gene.

In any of the methods disclosed herein, a RNA-guided nuclease may be delivered to the plurality of the T cells from the T cell bank. In some examples, the RNA-guided nuclease can be a Cas9 nuclease, for example, a *S. pyogenes* Cas9 nuclease. In some examples, the RNA-guided nuclease and the gRNA(s) can be complexed in a ribonucleoprotein particle (RNP).

The genetically edited T cells prepared by any of the preparation methods disclosed herein are also within the scope of the present disclosure.

Any of the CAR-T cells disclosed herein may be used for therapeutic purposes (*e.g.*, eliminating disease cells targeted by the CAR polypeptide on the CAR-T cells) at a dose lower than a standard dose of CAR-T therapy, which refers to the dose of CAR-T cells expressing the same CAR polypeptide and lacking the generic edits for enhancing T cell persistence as disclosed herein (*e.g.*, with no genetic edits to *TET2*, *FAS*, and/or *CD70*).

Also within the scope of the present disclosures is a guide RNA (gRNA) targeting a *TET2* gene. In some embodiments, the gRNA comprises a nucleotide sequence specific to exon exon 5 of the *TET2* gene. In some examples, a *TET2* gene edited by the gRNA may express a truncated version of TET2. Such a gRNA may target the site of GGGATGTCCTATTGCTAAGT (SEQ ID NO: 125) in exon 5 of the *TET2* gene. In one example, the gRNA may comprise the nucleotide sequence of SEQ ID NO: 18. Alternatively, the gRNA may target the site of AGGGATGTCCTATTGCTAAG (SEQ ID NO: 126) in exon 5 of the *TET2* gene. In one example, the gRNA may comprise the nucleotide sequence of SEQ ID NO: 22.

In other embodiments, the gRNA may comprise a nucleotide sequence targeting exon 3 of the *TET2* gene. For example, the gRNA may comprise a nucleotide sequence targeting GATTCCGCTTGGTGAAAACG (SEQ ID NO: 129) in exon 3 of the *TET2* gene.

Alternatively, the gRNA may comprise a nucleotide sequence targeting CAGGACTCACACGACTATTC (SEQ ID NO: 131) in exon 3 of the *TET2* gene. In another example, the gRNA may comprise a nucleotide sequence targeting TTCCGCTTGGTGAAAACGAG (SEQ ID NO: 133) in exon 3 of the *TET2* gene.

5 Exemplary gRNAs may comprise the nucleotide sequence of SEQ ID NO: 112, 116, or 120.

In some embodiments, the gRNA may comprise a nucleotide sequence targeting exon 4 of the *TET2* gene. For example, the gRNA may comprise a nucleotide sequence targeting CATTAGGACCTGCTCCTAGA (SEQ ID NO: 124) in exon 4 of the *TET2* gene. In one example, the gRNA may comprise the nucleotide sequence of SEQ ID NO: 14.

10 In some embodiments, the gRNA may comprise a nucleotide sequence targeting exon 6 of the *TET2* gene. For example, the gRNA may comprise a nucleotide sequence targeting ACGGCACGCTCACCAATCGC (SEQ ID NO: 127) in exon 6 of the *TET2* gene. In one example, the gRNA may comprise the nucleotide sequence of SEQ ID NO: 26.

Any of the gRNAs disclosed herein may further comprise a scaffold sequence. In some instances, the gRNA may comprise one or more modified nucleotides, for example, one or more 2'-O-methyl phosphorothioate residues at the 5' and/or 3' terminus of the gRNA. Exemplary gRNAs targeting *TET2* are provided in **Table 3** (including unmodified sequences and modified sequences), all of which are within the scope of the present disclosure and can be used in any of the methods disclosed herein for genetic editing of the *TET2* genes in a host cell such as a T cell.

The details of one or more embodiments of the invention are set forth in the description below. Other features or advantages of the present invention will be apparent from the following drawings and detailed description of several embodiments, and also from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGs. 1A-1D include diagrams showing that mutations leading to either *TET2* gene knockout (KO) or protein truncation increased proliferation and expansion, in primary human T cells. **FIG. 1A:** a picture showing presence of TET2 proteins in primary human T cells edited by gRNAs target exon 5 of the *TET2* gene as determined by a Western Blot assay. No protein was detected in cells transfected with one of the gRNAs including TET2 exon 5_T1, and TET2 exon 5_T2. TET2 exon 5_T1 gRNA produced a truncated form of the TET2 protein. **FIG. 1B:** a picture showing presence of TET2 proteins in primary human T cells edited by gRNAs target exon 4 or exon 6 of the *TET2* gene as determined by a Western Blot

assay. No full-length protein was detected in cells transfected with TET2 exon 4_BG4, and TET2 exon 6_BG5. TET2 exon 6_BG5 treatment also produces truncated TET2 protein species. **FIG. 1C:** a graph showing that modulation of TET2 significantly enhanced T cell proliferation and expansion in culture. Deletion of TET2 by TET2 exon 5_T1 and TET2 exon 5_T2 increased T cell proliferation compared to the mock group. T cells with truncated TET2 (TET2 exon 5_T1) had greater proliferation compared to all other groups. **FIG. 1D:** a graph showing that modulation of TET2 significantly enhanced T cell proliferation in culture. Deletion of TET2 by either TET2 exon4_BG4 (BG4) or TET2 exon 6_BG5 (BG5) increased T cell proliferation and expansion compared to cells not receiving Cas9:sgRNA RNP.

FIGs. 2A-2C include diagrams showing that FAS knockout (KO) increased cytokine driven proliferation and rescued apoptosis in anti-BCMA CAR T cells *in vitro*. **FIG. 2A:** a graph showing highly efficient FAS gene editing in primary human T cells. FMO-FAS group represents fluorescence minus one group, a negative control for FAS signal. **FIG. 2B:** a graph showing that knockout of FAS improved IL-2/IL-7 driven proliferation of anti-BCMA CAR T cell *in vitro*. **FIG. 2C:** a graph showing that knockout of FAS rescued anti-BCMA CAR+ T cells from apoptosis induced by anti-FAS antibody.

FIGs. 3A and 3B include diagrams showing increased cell killing and cell proliferation in triple knockout (FAS/TET2/CD70 knockouts) T cells. **FIG. 3A:** a graph showing that FAS/TET2/CD70 triple knockout increased cell killing function (48 hour) of anti-CD19 CAR T cells. **FIG. 3B:** a graph showing that cell proliferation continued in triple knockout T cells after four weeks in culture.

FIG. 4 includes a graph showing expansion of CAR-T cells in mice with acute lymphoblastic leukemia. The mice were treated with anti-CD19 CAR-T cells having disrupted *TRAC* and *B2M* genes, and optionally in combination with disrupted TET2, CD70, and/or FAS as indicated.

FIGs. 5A-5B include graphs showing increase survival and tumor inhibition by CAR-T cells in mice with acute lymphoblastic leukemia. **FIG. 5A:** a plot showing increase survival with the addition of TET2 KO in CAR T cells. **FIG. 5B:** a graph showing the addition of a TET2 KO further reduces tumor burden in mice treated with CAR T cells.

FIGs. 6A-6E include graphs showing a protective effect of CAR T cells containing a TET2 KO when exposed to a tumor re-challenge. **FIG. 6A:** a graph showing the protective effect of TET2 KO in anti-CD19 CAR T cells with tumor re-challenge. **FIG. 6B:** a graph showing the protective effect of TET2 KO in anti-BCMA CAR T cells. **FIG. 6C:** a graph showing the protective effect of TET2 KO in anti-BCMA CAR T cells with tumor re-

challenge. **FIG. 6D**: a graph showing the protective effect of TET2 KO in anti-CD70 CAR T cells. **FIG. 6E**: a graph showing the protective effect of TET2 KO in anti-CD70 CAR T cells with tumor re-challenge.

FIG. 7 includes a graph showing cytokine independent growth of TRAC-/B2M-
5 /TET2- CAR T cells.

FIGs. 8A-8D include graphs showing advantageous features conferred by TET KO in anti-BCMA CAR-T cells as compared with TET WT anti-BCMA CAR-T cells. **FIG. 8A**: a diagram showing expansion growth curves of anti-BCMA CAR-T cells +/- TET2 KO following 3 rounds of MM.1S target cell stimulation. **FIG. 8B**: a diagram showing viability
10 curves of anti-BCMA CAR-T cells +/- TET2 KO following 3 rounds of MM.1S target cell stimulation. **FIG. 8C**: a diagram showing FACS surface expression curves of anti-BCMA CAR-T cells +/- TET2 KO following 3 rounds of MM.1S target cell stimulation. **FIG. 8D**: a chart showing cytokine secretion (IFN γ) response following MM1S stimulation of anti-BCMA CAR-T cells +/- TET2 KO.

FIGs. 9A-9E include diagrams showing persistence of allogenic human anti-CD19
15 CAR-T cells having disrupted *TET2*, *FAS*, and *CD70* genes. **FIG. 9A**: a chart showing continued cell proliferation, which is cytokine dependent. **FIG. 9B**: a chart showing cell killing activity of the allogenic human anti-CD19 CAR-T cells one month post spleen isolation. **FIG. 9C**: a graph showing interferon gamma secretion by the allogenic human
20 anti-CD19 CAR-T cells. **FIG. 9D**: a graph showing interleukin 2 (IL-2) secretion by the allogenic human anti-CD19 CAR-T cells. **FIG. 9E**: a chart showing survival of the allogenic human anti-CD19 CAR-T cells in the Nalm6/NOG mouse model.

DETAILED DESCRIPTION OF THE INVENTION

25 The present disclosure aims at establishing a T cell bank comprising T cells having improved persistence, a long-felt need in CAR-T therapy. Such a T cell bank may use *bona fide* T cells as the starting material, for example, non-transformed T cells, terminally differentiated T cells, T cells having stable genome, and/or T cells that depend on cytokines and growth factors for proliferation and expansion. Alternatively, such a T cell bank may use
30 T cells generated from precursor cells such as hematopoietic stem cells (*e.g.*, iPSCs), *e.g.*, *in vitro* culture. The T cell bank disclosed herein may confer one or more benefits in both CAR-T cell manufacturing and clinical applications.

Conventional allogenic CAR T cells are produced wherein a single donor leukopak is edited in most cases so that the cells can avoid components of the patient immune system and

thus do not cause GvHD. The process of expanding these CAR T cells can yield 10s to 100s of vial drug product. Patients may receive a single dose or multiple doses. During the manufacturing process, these CAR T cells lose potential due to various mechanisms, for example, apoptosis, exhaustion, replicative senescence, and other processes where the cells become less fit.

The edited T cell bank disclosed herein may provide a process whereby a single leukopak can create a cell bank of 10s to 100s of "vials" of cells, each of which can be used to create a multiple-vial drug product of allogeneic CAR T cells. Both the banked cells and the CAR T cells produced from them are expected to retain more potential than CAR T cells produced by the standard (non-cell bank) process (without one or more of the genetic editing events disclosed herein).

Other unlimited advantageous features of the T cell bank provided herein include:

- (a) Improved quality and consistency of drug products such as CAR-T products.
- (b) Greater potency and longer-lived potency of CAR-T cells produced from the T cell bank cells in human patients.
- (c) Reduced dosage requirement. Because the T cells disclosed herein have enhanced proliferation and expansion capacities, they can live longer *in vivo*. As such, a lower dose relative to standard CAR-T therapy could achieve substantially similar therapeutic effects as conventional CAR-T cells not having the gene edits disclosed herein (e.g., disruptions in *TET2*).
- (d) Increase safety. The growth of the genetically engineered T cells disclosed herein were found to depend on cytokine, indicating no transformation. Further, since lower doses may be sufficient, use of the CAR-T cells disclosed herein would be expected to reduce side effects commonly associated with CART-T therapy, for example, cytokine release syndrome (CRS), macrophage activation syndrome (MAS), tumor lysis syndrome (TLS), and/or neurotoxic effects.
- (e) Increased efficacy resulting from enhanced proliferation and expansion of the CAR-T cells disclosed herein, enhanced cytotoxicity, and prolonged persistence *in vivo*. Further, the T cell bank would provide the benefit of titratable dosing in patients to optimize safety and efficacy as noted above.
- (f) Extended therapeutic effects due to reduced exhaustion and/or replicative senescence and prolonged persistence of the T cells in the T cell bank both *in vitro* and *in vivo*.
- (g) Increasing the number of vial drug product, such as allogeneic CAR T cell

products, that can be created from a suitable natural source, such as a single leukopak.

Accordingly, provided herein are T cell banks that comprise T cells having improved persistence in culture, methods of producing such T cell banks, and methods of using such T cell banks for producing therapeutic T cells such as CAR-T cells. Components and processes (e.g., the CRISPR/Cas-mediated approach for gene editing and components used therein) for making the T cell banks disclosed herein are also within the scope of the present disclosure.

I. T Cell Bank Having Enhanced Persistence

The T cell bank disclosed herein comprises genetically engineered T cells having enhanced persistence in culture. Such genetically engineered T cells may have genetic editing of one or more genes involved in cell self-renewal, one or more genes involved in apoptosis, and/or one or more genes involved in T cell exhaustion. As shown by the studies disclosed herein, such genetically engineered T cells show one or more of the following superior features as relative to the non-edited T counterpart: enhanced expansion capacity in culture (e.g., expandable in culture for at least 4 weeks, e.g., at least 6 weeks; and/or at least 10-fold expandable, for example, at least 15-fold expandable, relative to the non-edited counterpart), enhanced proliferation capacity, greater T cell activation, and reduced apoptosis levels.

(i) Genetically Engineered T Cells in T Cell Banks

The genetically engineered T cells in the T cell bank disclosed herein comprise gene editing in one or more genes associated with T cell persistence in culture. "T cell persistence" as used herein refers to the tendency of T cells to continue to grow, proliferate, self-renew, expand, and maintain healthy activity in culture. In some instances, T cell persistence can be represented by the longevity that T cells can grow and expand *in vitro*, which can be measured by conventional methods and/or assays described herein. In other instances, T cell persistence can be represented by the reduction of cell death (e.g., apoptosis) or reduction in cell states characterized by exhaustion or replicative senescence. In yet other instances, T cell persistence can be presented by the maintenance of T cell activation capacity in culture.

T cell persistence of the genetically engineered T cells may be achieved by genetically editing one or more genes that function in regulating cell persistence *via* various pathways, for example, modulating cell self-renewal, apoptosis, and/or cell exhaustion. In some embodiments, the genetically engineered T cells may comprise gene editing of multiple genes involved in multiple pathways that regulate cell persistence. Such genetically

engineered T cells may have increased longevity of growth in culture. Further, CAR-T cells derived from such genetically engineered T cells may also have enhanced *in vivo* therapeutic efficacy.

The genetically engineered T cells may be derived from parent T cells (*e.g.*, non-edited wild-type T cells) obtained from a suitable source, for example, one or more mammal
5 donors. In some examples, the parent T cells are primary T cells (*e.g.*, non-transformed and terminally differentiated T cells) obtained from one or more human donors. Alternatively, the parent T cells may be differentiated from precursor T cells obtained from one or more suitable donor or stem cells such as hematopoietic stem cells or inducible pluripotent stem
10 cells (iPSC), which may be cultured *in vitro*.

In some embodiments, the genetically engineered T cells comprise one or more mutated genes involved in cell self-renewal, one or more disrupted genes involved in apoptosis, and/or one or more disrupted genes involved in cell exhaustion. Such T cells may be generated *via* gene editing (including genomic editing), a type of genetic engineering in
15 which nucleotide(s)/nucleic acid(s) is/are inserted, deleted, and/or substituted in a DNA sequence, such as in the genome of a targeted cell. Targeted gene editing enables insertion, deletion, and/or substitution at pre-selected sites in the genome of a targeted cell (*e.g.*, in a targeted gene or targeted DNA sequence). When a sequence of an endogenous gene is edited, for example by deletion, insertion or substitution of nucleotide(s)/nucleic acid(s), the
20 endogenous gene comprising the affected sequence may be knocked-out or knocked-down due to the sequence alteration. Therefore, targeted editing may be used to disrupt endogenous gene expression. "Targeted integration" refers to a process involving insertion of one or more exogenous sequences, with or without deletion of an endogenous sequence at the insertion site. Targeted integration can result from targeted gene editing when a donor template
25 containing an exogenous sequence is present.

As used herein, a "mutated gene" encompasses any type of genetic mutations introduced into a target gene. In some instances, a "mutated gene" can include a genetic mutation that leads to expression of a mutated gene product (for example, a truncated gene product). In other instances, a "mutated gene" may be a disrupted gene, which may contain a
30 genetic mutation that substantially or completely abolishes expression of the gene product.

As used herein, a "disrupted gene" refers to a gene comprising an insertion, deletion or substitution relative to an endogenous gene such that expression of a functional protein from the endogenous gene is reduced or inhibited. As used herein, "disrupting a gene" refers to a method of inserting, deleting or substituting at least one nucleotide/nucleic acid in an

endogenous gene such that expression of a functional protein from the endogenous gene is reduced or inhibited. Methods of disrupting a gene are known to those of skill in the art and described herein.

In some embodiments, a cell that comprises a disrupted gene does not express (*e.g.*, at the cell surface) a detectable level (*e.g.*, in an immune assay using an antibody binding to the encoded protein or by flow cytometry) of the protein encoded by the gene. A cell that does not express a detectable level of the protein may be referred to as a knockout cell.

In some embodiments, the genetically engineered T cells of the T cell bank disclosed herein may comprise a mutated *TET2* gene, a disrupted *FAS* gene, a disrupted *CD70* gene, or a combination thereof. In some instances, genetic editing of additional genes (*e.g.*, *β 2M* and/or *TRAC*) may also be included.

Tet Methylcytosine Dioxygenase 2 Gene (TET2) Editing

Self-renewal is the process by which cells (*e.g.*, T cells) divide and maintain the same cell state/identity. Genes involved in cell self-renewal refer to those that either positively regulate or negatively regulate cell self-renewal. The genetically engineered T cells disclosed herein may comprise genetic editing of a gene that positively regulates cell self-renewal to enhance its expression and/or bioactivity of the encoded protein product. Alternatively or in addition, the genetically engineered T cells may comprise genetic editing of a gene that negatively regulates cell self-renewal to disrupt its expression.

In some embodiments, the genetically engineered T cells may comprise a mutated gene involved in cell self-renewal. Such a gene may be *TET2* (Ten eleven translocation-2) a Methylcytosine Dioxygenase. *Tet2* is a dioxygenase that catalyzes the conversion of the modified genomic base methylcytosine to 5-hydroxymethylcytosine and to further intermediates leading to cytosine demethylation. This enzyme is involved in myelopoiesis, and defects in *TET2* have been reported to be associated with several myeloproliferative disorders. Structures of *TET2* genes are known in the art. For example, human *TET2* gene is located on chromosome 4q24. Additional information can be found in GenBank under Gene ID: 54790 or NCBI Reference Sequence: NM_001127208.2.

In some examples, the genetically engineered T cells may comprise a disrupted *TET2* gene such that the expression of *TET2* in the T cells is substantially reduced or eliminated completely. The disrupted *TET2* gene may comprise one or more genetic edits at one or more suitable target sites (*e.g.*, in coding regions or in non-coding regulatory regions such as promoter regions) that disrupt expression of the *TET2* gene. Such target sites may be identified based on the gene editing approach for use in making the genetically engineered T

cells. Exemplary target sites for the genetic edits may include exon 1, exon 3, exon 4, exon 5, exon 6, or a combination thereof. In some examples, one or more genetic edits may occur in exon 3, exon 4, exon 5, or exon 6. Such genetic editing may be induced by the CRISPR/Cas technology using a suitable guide RNA, for example, those listed in **Table 3**. The resultant edited *TET2* gene using a gRNA listed in **Table 3** may comprise one or more edited sequences provided in **Tables 15-21** below.

In other examples, the genetically engineered T cells may comprise a mutated *TET2* gene expressing a truncated version of TET2 protein, which may be a gain-of-function variant of TET2. Such a mutated *TET2* gene may have a genetic edit in exon 5 and produces a truncated TET2 variant having a molecular weight of about 170 kDa, which can be determined by a conventional method such as SDS-PAGE.

The term “about” as used herein means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, “about” can mean within an acceptable standard deviation, per the practice in the art. Alternatively, “about” can mean a range of up to $\pm 20\%$, preferably up to $\pm 10\%$, more preferably up to $\pm 5\%$, and more preferably still up to $\pm 1\%$ of a given value. Where particular values are described in the application and claims, unless otherwise stated, the term “about” is implicit and in this context means within an acceptable error range for the particular value.

In some examples, the T cell bank as disclosed herein may comprise genetically engineered T cells, at least 50% of which comprise a disrupted *TET2* gene (*e.g.*, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or above). In some examples, the T cell bank as disclosed herein may comprise genetically engineered T cells, at least 50% of which comprise a mutated *TET2* gene (*e.g.*, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or above), which may express a truncated version of TET2 such as that disclosed above.

FAS Gene Editing

Apoptosis is a process of programmed cell death that occurs in multicellular organisms. Genes involved in apoptosis refer to those that either positively regulate or negatively regulate this biological process. The genetically engineered T cells disclosed herein may comprise genetic editing of a gene that positively regulates cell apoptosis to disrupt its expression. Alternatively or in addition, the genetically engineered T cells may comprise genetic editing of a gene that negatively regulates cell apoptosis to enhance its

expression and/or biologic activity of the gene product.

In some embodiments, the genetically engineered T cells may comprise an edited gene involved in cell apoptosis, *e.g.*, disruption of a gene that positively regulates apoptosis. Such a gene may be a *FAS* gene, also known as FAS receptor, CD95, or apoptosis antigen 1 (APO-1). The *FAS* gene encodes a death receptor on cell surface that leads to apoptosis when triggered by FAS ligand. FASL-FAS induced apoptosis is one of the multiple apoptotic pathways in cells (another major pathway being the mitochondrial pathway). Structures of *FAS* genes are known in the art. For example, human *FAS* gene is located on chromosome 10q24.1. Additional information can be found in GenBank under Gene ID: 355.

In some examples, the genetically engineered T cells may comprise a disrupted *FAS* gene such that the expression of FAS in the T cells is substantially reduced or eliminated completely. The disrupted *FAS* gene may comprise one or more genetic edits at one or more suitable target sites (*e.g.*, in coding regions or in non-coding regulatory regions such as promoter regions) that disrupt expression of the *FAS* gene. Such target sites may be identified based on the gene editing approach for use in making the genetically engineered T cells. Exemplary target sites for the genetic edits may include exon 2, exon 3, or a combination thereof.

In some examples, the T cell bank as disclosed herein may comprise genetically engineered T cells, at least 50% of which comprise a disrupted *FAS* gene (*e.g.*, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or above).

CD70 Gene Editing

T cell exhaustion is a process of stepwise and progressive loss of T cell functions, which may be induced by prolonged antigen stimulation or other factors. Genes involved in T cell exhaustion refer to those that either positively regulate or negatively regulate this biological process. The genetically engineered T cells disclosed herein may comprise genetic editing of a gene that positively regulates T cell exhaustion to disrupt its expression. Alternatively or in addition, the genetically engineered T cells may comprise genetic editing of a gene that negatively regulates T cell exhaustion to enhance its expression and/or biologic activity of the gene product.

In some embodiments, the genetically engineered T cells may comprise an edited gene involved in T cell exhaustion, *e.g.*, disruption of a gene that positively regulates T cell exhaustion. Such a gene may be a Cluster of Differentiation 70 (*CD70*) gene. *CD70* is a member of the tumor necrosis factor superfamily and its expression is restricted to activated T and B lymphocytes and mature dendritic cells. *CD70* is implicated in tumor cell and

regulatory T cell survival through interaction with its ligand, CD27. CD70 and its receptor CD27 have multiple roles in immune function in multiple cell types including T cells (activated and T_{reg} cells), and B cells.

It was also found that disrupting the *CD70* gene in immune cells engineered to
5 express an antigen targeting moiety, enhanced anti-tumor efficacy against large tumors and induced a durable anti-cancer memory response. Specifically, the anti-cancer memory response prevented tumor growth upon re-challenge. Further, it has been demonstrated that disrupting the *CD70* gene results in enhanced cytotoxicity of immune cells engineered to
10 express an antigen targeting moiety at lower ratios of engineered immune cells to target cells, indicating the potential efficacy of low doses of engineered immune cells. See, *e.g.*, WO2019/215500, the relevant disclosures of which are incorporated by reference for the purpose and subject matter referenced herein.

Structures of *CD70* genes are known in the art. For example, human *CD70* gene is located on chromosome 19p13.3. The gene contains four protein encoding exons. Additional
15 information can be found in GenBank under Gene ID: 970.

In some examples, the genetically engineered T cells may comprise a disrupted *CD70* gene such that the expression of CD70 in the T cells is substantially reduced or eliminated completely. The disrupted *CD70* gene may comprise one or more genetic edits at one or more suitable target sites (*e.g.*, in coding regions or in non-coding regulatory regions such as
20 promoter regions) that disrupt expression of the *CD70* gene. Such target sites may be identified based on the gene editing approach for use in making the genetically engineered T cells. Exemplary target sites for the genetic edits may include exon 1, exon 2, exon 3, exon 4, or a combination thereof. See also WO2019/215500, the relevant disclosures of which are incorporated by reference for the purpose and subject matter referenced herein.

25 In some examples, the T cell bank as disclosed herein may comprise genetically engineered T cells, at least 50% of which comprise a disrupted *CD70* gene (*e.g.*, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or above).

β2M Gene Edit

In some embodiments, the genetically engineered T cells in the T cell bank as
30 disclosed herein may further comprise a disrupted *β2M* gene. *β2M* is a common (invariant) component of MHC I complexes. Disrupting its expression by gene editing will prevent host versus therapeutic allogeneic T cells responses leading to increased allogeneic T cell persistence. In some embodiments, expression of the endogenous *β2M* gene is eliminated to prevent a host-versus-graft response.

In some embodiments, at least 50% (*e.g.*, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or above) of the genetically engineered T cells in the T cell bank do not express a detectable level of $\beta 2M$ surface protein.

In some embodiments, an edited $\beta 2M$ gene may comprise a nucleotide sequence selected from the following sequences in **Table 1**. It is known to those skilled in the art that different nucleotide sequences in an edited gene such as an edited $\beta 2M$ gene (*e.g.*, those in **Table 1**) may be generated by a single gRNA. See also WO2019097305, the relevant disclosures of which are incorporated by reference for the subject matter and purpose referenced herein.

Table 1. Sequences of exemplary gRNAs targeting $\beta 2M$

Sequences	SEQ ID NO:
CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGCCTGGAGGCTATCCAGCGTGA GTCTCTCCTACCCCTCCCGCT	1
CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGCCTGGAGGCTATCCAGCGTGAG TCTCTCCTACCCCTCCCGCT	2
CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGAGGCTATCCAGCGTGAGTCT CTCCTACCCCTCCCGCT	3
CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGATAGCCTGGAGGCTATCCAG CGTGAGTCTCTCCTACCCCTCCCGCT	4
CGTGGCCTTAGCTGTGCTCGCGCTATCCAGCGTGAGTCTCTCCTACCCCTCCCGCT	5
CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGTGGCCTGGAGGCTATCCAGCG TGAGTCTCTCCTACCCCTCCCGCT	6

The genetically engineered T cells in the T cell bank disclosed herein may further comprise one or more additional gene edits (*e.g.*, gene knock-in or knock-out) to improve T cell function. Examples include knock-in or knock-out genes to improve target cell lysis, knock-in or knock-out genes to enhance performance of therapeutic T cells such as CAR-T cells prepared from cells of the T cell bank. Examples include knock-out of an immune checkpoint gene such as *PD-1*.

TRAC Gene Edit

In some embodiments, the genetically engineered T cells in the T cell bank as disclosed herein may further comprise a disrupted *TRAC* gene. This disruption leads to loss of function of the TCR and renders the engineered T cell non-alloreactive and suitable for allogeneic transplantation, minimizing the risk of graft versus host disease. In some embodiments, expression of the endogenous *TRAC* gene is eliminated to prevent a graft-versus-host response. See also WO2019097305, the relevant disclosures of which are incorporated by reference herein for the purpose and subject matter referenced herein.

It should be understood that more than one suitable target site/gRNA can be used for each target gene disclosed herein, for example, those known in the art or disclosed herein.

Additional examples can be found in, *e.g.*, WO2019097305, the relevant disclosures of which are incorporated by reference herein for the purpose and subject matter referenced herein.

Exemplary Genetically Engineered T cells in T cell Bank

In some embodiments, the T cell bank disclosed herein comprising a population of
5 genetically engineered T cells that comprise a disrupted *TET2* gene. Such genetically
engineered T cells may further comprise a disrupted *FAS* gene, a disrupted *CD70* gene, or
both. In some embodiments, the T cell bank disclosed herein may comprise a population of
genetically engineered T cells that comprise a combination of at least two of genetically
edited genes selected from a mutated *TET2* gene, a disrupted *FAS* gene, and a disrupted
10 *CD70* gene. Such genetically engineered T cells optionally may further comprise a disrupted
β2M gene, a disrupted *TRAC* gene, or both.

In some examples, the T cell bank disclosed herein comprising a population of
genetically engineered T cells that comprise a disrupted *TET2* gene as disclosed herein. In
some instances, at least 50% (*e.g.*, 60%, 70%, 80%, 90%, or 95%) of the genetically
15 engineered T cells in the T cell bank do not express surface TET2 at a detectable level as
measured by a conventional assay.

In some examples, the T cell bank disclosed herein comprising a population of
genetically engineered T cells that comprise a disrupted *TET2* gene and a disrupted *FAS*
gene. In some instances, at least 50% (*e.g.*, 60%, 70%, 80%, 90%, or 95%) of the genetically
20 engineered T cells in the T cell bank comprise the disrupted *TET2* gene and the disrupted
FAS gene.

In some examples, the T cell bank disclosed herein comprising a population of
genetically engineered T cells that comprise that comprise a disrupted *TET2* gene and a
disrupted *CD70* gene. In some instances, at least 50% (*e.g.*, 60%, 70%, 80%, 90%, or 95%)
25 of the genetically engineered T cells in the T cell bank comprise the mutated or disrupted
TET2 gene and the disrupted *CD70* gene.

In specific examples, the T cell bank disclosed herein comprising a population of
genetically engineered T cells that comprise a disrupted *TET2* gene, a disrupted *FAS* gene,
and a disrupted *CD70* gene. In some instances, at least 50% (*e.g.*, 60%, 70%, 80%, 90%, or
30 95%) of the genetically engineered T cells in the T cell bank comprise the mutated or
disrupted *TET2* gene, the disrupted *FAS* gene, and the disrupted *CD70* gene.

In some examples, the T cell bank disclosed herein comprising a population of
genetically engineered T cells that comprise a mutated *TET2* gene expressing any of the
truncated TET2 polypeptide as disclosed herein and a disrupted *FAS* gene. In some

instances, at least 50% (*e.g.*, 60%, 70%, 80%, 90%, or 95%) of the genetically engineered T cells in the T cell bank comprise the mutated *TET2* gene and the disrupted *FAS* gene.

In some examples, the T cell bank disclosed herein comprising a population of genetically engineered T cells that comprise a mutated *TET2* gene expressing a truncated
5 TET2 polypeptide as disclosed herein and a disrupted *CD70* gene. In some instances, at least 50% (*e.g.*, 60%, 70%, 80%, 90%, or 95%) of the genetically engineered T cells in the T cell bank comprise the mutated *TET2* gene and the disrupted *CD70* gene.

In specific examples, the T cell bank disclosed herein comprising a population of genetically engineered T cells that comprise a disrupted *TET2* gene, a disrupted *FAS* gene,
10 and a disrupted *CD70* gene. In some instances, at least 50% (*e.g.*, 60%, 70%, 80%, 90%, or 95%) of the genetically engineered T cells in the T cell bank comprise the disrupted *TET2* gene, the disrupted *FAS* gene, and the disrupted *CD70* gene.

In specific examples, the T cell bank disclosed herein comprising a population of genetically engineered T cells that comprise a mutated *TET2* gene expressing any of the
15 truncated TET2 polypeptide as disclosed herein, a disrupted *FAS* gene, and a disrupted *CD70* gene. In some instances, at least 50% (*e.g.*, 60%, 70%, 80%, 90%, or 95%) of the genetically engineered T cells in the T cell bank comprise the mutated *TET2* gene, the disrupted *FAS* gene, and the disrupted *CD70* gene.

In some embodiments, the genetically engineered T cells in the T cell bank, which
20 may comprise one or more of a mutated *TET2* gene (*e.g.*, disrupted or mutated to express a truncated version of TET2 as disclosed herein), a disrupted *FAS* gene, and a disrupted *CD70* gene may be expandable in culture for greater than 4 weeks, for example, greater than 5 weeks, greater than 6 weeks, greater than 8 weeks, and greater than 10 weeks. In some examples, the genetically engineered T cells in the T cell bank comprise a mutated *TET2* gene
25 expressing the truncated TET2 polypeptide as disclosed herein (optionally disruptions in *FAS* and/or *CD70*) and are expandable after 6 weeks (*e.g.*, after 7 weeks, after 8 weeks, after 9 weeks, or after 10 weeks) in culture. Such genetically engineered T cells may maintain the ability to be activated after 6 weeks (*e.g.*, after 7 weeks, after 8 weeks, after 9 weeks, or after 10 weeks) in culture. Further, such genetically engineered T cells have an increased
30 expansion capacity, which can be at least 10-fold (*e.g.*, at least 15-fold) higher than the non-engineered counterparts. Non-engineered counterparts refer to T cells having the same genetic background except for the gene involved in cell self-renewal (*e.g.*, *TET2*), apoptosis (*e.g.*, *FAS*), and/or T cell exhaustion (*e.g.*, *CD70*) as disclosed herein, *i.e.*, disrupted/mutated versus wild-type.

In some embodiments, the genetically engineered T cells in the T cell bank may comprise a disrupted *FAS* gene (optionally a mutated *TET2* gene, e.g., disrupted or expressing the truncated TET2 polypeptide as disclosed herein and/or a disrupted *CD70* gene) may have a reduced apoptosis tendency relative to the non-engineered counterparts.

5 For example, the level of FAS/FAS ligand-induced apoptosis of the genetically engineered T cells in culture may be less than 50% (e.g., less than 40%, less than 30%, less than 20% or lower) of the FAS/FAS ligand induced apoptosis in non-engineered counterparts.

In some embodiments, CAR-T cells generated using the genetically engineered T cells in the T cell bank comprise a disrupted *CD70* gene (optionally a mutated *TET2* gene, e.g., disrupted or expressing the truncated TET2 polypeptide as disclosed herein and/or a disrupted *FAS* gene) may have an improved potency (e.g., at least 50% higher, at least one-fold higher, at least 2-folds higher, at least 5-folds higher, or at least 10-folds higher) both *in vitro* and *in vivo* relative to the non-engineered counterparts.

10

In some embodiments, the T cells in the T cell bank may further engineered to express a chimeric antigen receptor (CAR), which are described in detail below.

15

(ii) Methods of Making T Cell Banks

The genetically engineered T cells of the T cell bank disclosed herein can be prepared by genetic editing of parent T cells or precursor cells thereof *via* a conventional gene editing method or those described herein.

20 (a) T cells

In some embodiments, T cells for use in making the T cell bank can be derived from one or more suitable mammals, for example, one or more human donors. T cells can be obtained from a number of sources, including, but not limited to, peripheral blood mononuclear cells, bone marrow, lymph nodes tissue, cord blood, thymus issue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In certain embodiments, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled person, such as sedimentation, e.g., FICOLL™ separation.

25

In some examples, T cells can be isolated from a mixture of immune cells (e.g., those described herein) to produce an isolated T cell population, which can be used for making the T cell bank disclosed herein. For example, after isolation of peripheral blood mononuclear cells (PBMC), both cytotoxic and helper T lymphocytes can be sorted into naive, memory, and effector T cell subpopulations either before or after activation, expansion, and/or genetic modification.

30

A specific subpopulation of T cells, expressing one or more of the following cell

surface markers: TCRab, CD3, CD4, CD8, CD27 CD28, CD38 CD45RA, CD45RO, CD62L, CD127, CD122, CD95, CD197, CCR7, KLRG1, MCH-I proteins and/or MCH-II proteins, can be further isolated by positive or negative selection techniques. In some embodiments, a specific subpopulation of T cells, expressing one or more of the markers selected from the group consisting of TCRab, CD4 and/or CD8, is further isolated by positive or negative selection techniques. In some embodiments, subpopulations of T cells may be isolated by positive or negative selection prior to genetic engineering and/or post genetic engineering.

An isolated population of T cells for use in making the T cell bank may express one or more of the T cell markers, including, but not limited to a CD3+, CD4+, CD8+, or a combination thereof. In some embodiments, the T cells are isolated from a donor, or subject, and first activated and stimulated to proliferate in vitro prior to undergoing gene editing.

In some instances, the T cell population for use in making the T cell bank comprises primary T cells isolated from one or more human donors. Such T cells are terminally differentiated, not transformed, depend on cytokines and/or growth factors for growth, and/or have stable genomes.

Alternatively, the T cells for use in making the T cell bank may be derived from stem cells (*e.g.*, HSCs or iPSCs) *via in vitro* differentiation.

To achieve sufficient amount of T cells for making the T cell bank, T cells from a suitable source can be subjected to one or more rounds of stimulation, activation and/or expansion. T cells can be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; and 6,867,041. In some embodiments, T cells can be activated and expanded for about 1 day to about 4 days, about 1 day to about 3 days, about 1 day to about 2 days, about 2 days to about 3 days, about 2 days to about 4 days, about 3 days to about 4 days, or about 1 day, about 2 days, about 3 days, or about 4 days prior to introduction of the genome editing compositions into the T cells.

In some embodiments, T cells are activated and expanded for about 4 hours, about 6 hours, about 12 hours, about 18 hours, about 24 hours, about 36 hours, about 48 hours, about 60 hours, or about 72 hours prior to introduction of the gene editing compositions into the T cells. In some embodiments, T cells are activated at the same time that genome editing compositions are introduced into the T cells. In some instances, the T cell population can be expanded and/or activated after the genetic editing as disclosed herein. T cell populations or isolated T cells generated by any of the gene editing methods described herein are also within

the scope of the present disclosure.

(b) Gene Editing Methods

Any of the genetically engineered T cells can be prepared using conventional gene editing methods or those described herein to edit one or more of the target genes disclosed herein (targeted editing). Targeted editing can be achieved either through a nuclease-independent approach, or through a nuclease-dependent approach. In the nuclease-independent targeted editing approach, homologous recombination is guided by homologous sequences flanking an exogenous polynucleotide to be introduced into an endogenous sequence through the enzymatic machinery of the host cell. The exogenous polynucleotide may introduce deletions, insertions or replacement of nucleotides in the endogenous sequence.

Alternatively, the nuclease-dependent approach can achieve targeted editing with higher frequency through the specific introduction of double strand breaks (DSBs) by specific rare-cutting nucleases (*e.g.*, endonucleases). Such nuclease-dependent targeted editing also utilizes DNA repair mechanisms, for example, non-homologous end joining (NHEJ), which occurs in response to DSBs. DNA repair by NHEJ often leads to random insertions or deletions (indels) of a small number of endogenous nucleotides. In contrast to NHEJ mediated repair, repair can also occur by a homology directed repair (HDR). When a donor template containing exogenous genetic material flanked by a pair of homology arms is present, the exogenous genetic material can be introduced into the genome by HDR, which results in targeted integration of the exogenous genetic material.

In some embodiments, gene disruption may occur by deletion of a genomic sequence using two guide RNAs. Methods of using CRISPR-Cas gene editing technology to create a genomic deletion in a cell (*e.g.*, to knock out a gene in a cell) are known (Bauer DE et al. Vis. Exp. 2015; 95:e52118).

Available endonucleases capable of introducing specific and targeted DSBs include, but not limited to, zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and RNA-guided nuclease such as CRISPR/Cas (*e.g.*, Clustered Regularly Interspaced Short Palindromic Repeats Associated protein 9 or CRISPR/Cas9). Additionally, DICE (dual integrase cassette exchange) system utilizing phiC31 and Bxb1 integrases may also be used for targeted integration. Some exemplary approaches are disclosed in detail below.

CRISPR-Cas9 Gene Editing System

The CRISPR-Cas9 system is a naturally-occurring defense mechanism in prokaryotes

that has been repurposed as an RNA-guided DNA-targeting platform used for gene editing. It relies on the DNA nuclease Cas9, and two noncoding RNAs, crisprRNA (crRNA) and trans-activating RNA (tracrRNA), to target the cleavage of DNA. CRISPR is an abbreviation for Clustered Regularly Interspaced Short Palindromic Repeats, a family of DNA sequences
5 found in the genomes of bacteria and archaea that contain fragments of DNA (spacer DNA) with similarity to foreign DNA previously exposed to the cell, for example, by viruses that have infected or attacked the prokaryote. These fragments of DNA are used by the prokaryote to detect and destroy similar foreign DNA upon re-introduction, for example, from similar viruses during subsequent attacks. Transcription of the CRISPR locus results in the formation
10 of an RNA molecule comprising the spacer sequence, which associates with and targets Cas (CRISPR-associated) proteins able to recognize and cut the foreign, exogenous DNA. Numerous types and classes of CRISPR/Cas systems have been described (see, *e.g.*, Koonin et al., (2017) Curr Opin Microbiol 37:67-78).

crRNA drives sequence recognition and specificity of the CRISPR-Cas9 complex
15 through Watson-Crick base pairing typically with a 20 nucleotide (nt) sequence in the target DNA. Changing the sequence of the 5' 20nt in the crRNA allows targeting of the CRISPR-Cas9 complex to specific loci. The CRISPR-Cas9 complex only binds DNA sequences that contain a sequence match to the first 20 nt of the crRNA, if the target sequence is followed by a specific short DNA motif (with the sequence NGG) referred to as a protospacer adjacent
20 motif (PAM).

TracrRNA hybridizes with the 3' end of crRNA to form an RNA-duplex structure that is bound by the Cas9 endonuclease to form the catalytically active CRISPR-Cas9 complex, which can then cleave the target DNA.

Once the CRISPR-Cas9 complex is bound to DNA at a target site, two independent
25 nuclease domains within the Cas9 enzyme each cleave one of the DNA strands upstream of the PAM site, leaving a double-strand break (DSB) where both strands of the DNA terminate in a base pair (a blunt end).

After binding of CRISPR-Cas9 complex to DNA at a specific target site and formation of the site-specific DSB, the next key step is repair of the DSB. Cells use two main
30 DNA repair pathways to repair the DSB: non-homologous end joining (NHEJ) and homology-directed repair (HDR).

NHEJ is a robust repair mechanism that appears highly active in the majority of cell types, including non-dividing cells. NHEJ is error-prone and can often result in the removal or addition of between one and several hundred nucleotides at the site of the DSB, though

such modifications are typically < 20 nt. The resulting insertions and deletions (indels) can disrupt coding or noncoding regions of genes. Alternatively, HDR uses a long stretch of homologous donor DNA, provided endogenously or exogenously, to repair the DSB with high fidelity. HDR is active only in dividing cells, and occurs at a relatively low frequency in most cell types. In many embodiments of the present disclosure, NHEJ is utilized as the repair operant.

Endonuclease for use in CRISPR

In some embodiments, the Cas9 (CRISPR associated protein 9) endonuclease is used in a CRISPR method for making the genetically engineered T cells as disclosed herein. The Cas9 enzyme may be one from *Streptococcus pyogenes*, although other Cas9 homologs may also be used. It should be understood, that wild-type Cas9 may be used or modified versions of Cas9 may be used (*e.g.*, evolved versions of Cas9, or Cas9 orthologues or variants), as provided herein. In some embodiments, Cas9 may be substituted with another RNA-guided endonuclease, such as Cpf1 (of a class II CRISPR/Cas system).

In some embodiments, the CRISPR/Cas system comprises components derived from a Type-I, Type-II, or Type-III system. Updated classification schemes for CRISPR/Cas loci define Class 1 and Class 2 CRISPR/Cas systems, having Types I to V or VI (Makarova et al., (2015) Nat Rev Microbiol, 13(11):722-36; Shmakov et al., (2015) Mol Cell, 60:385-397). Class 2 CRISPR/Cas systems have single protein effectors. Cas proteins of Types II, V, and VI are single-protein, RNA-guided endonucleases, herein called "Class 2 Cas nucleases." Class 2 Cas nucleases include, for example, Cas9, Cpf1, C2c1, C2c2, and C2c3 proteins. The Cpf1 nuclease (Zetsche et al., (2015) Cell 163:1-13) is homologous to Cas9, and contains a RuvC-like nuclease domain.

In some embodiments, the Cas nuclease is from a Type-II CRISPR/Cas system (*e.g.*, a Cas9 protein from a CRISPR/Cas9 system). In some embodiments, the Cas nuclease is from a Class 2 CRISPR/Cas system (a single-protein Cas nuclease such as a Cas9 protein or a Cpf1 protein). The Cas9 and Cpf1 family of proteins are enzymes with DNA endonuclease activity, and they can be directed to cleave a desired nucleic acid target by designing an appropriate guide RNA, as described further herein.

In some embodiments, a Cas nuclease may comprise more than one nuclease domain. For example, a Cas9 nuclease may comprise at least one RuvC-like nuclease domain (*e.g.*, Cpf1) and at least one HNH-like nuclease domain (*e.g.*, Cas9). In some embodiments, the Cas9 nuclease introduces a DSB in the target sequence. In some embodiments, the Cas9 nuclease is modified to contain only one functional nuclease domain. For example, the Cas9

nuclease is modified such that one of the nuclease domains is mutated or fully or partially deleted to reduce its nucleic acid cleavage activity. In some embodiments, the Cas9 nuclease is modified to contain no functional RuvC-like nuclease domain. In other embodiments, the Cas9 nuclease is modified to contain no functional HNH-like nuclease domain. In some

5 embodiments in which only one of the nuclease domains is functional, the Cas9 nuclease is a nickase that is capable of introducing a single-stranded break (a “nick”) into the target sequence. In some embodiments, a conserved amino acid within a Cas9 nuclease domain is substituted to reduce or alter a nuclease activity. In some embodiments, the Cas nuclease nickase comprises an amino acid substitution in the RuvC-like nuclease domain.

10 Exemplary amino acid substitutions in the RuvC-like nuclease domain include D10A (based on the *S. pyogenes* Cas9 nuclease). In some embodiments, the nickase comprises an amino acid substitution in the HNH-like nuclease domain. Exemplary amino acid substitutions in the HNH-like nuclease domain include E762A, H840A, N863A, H983A, and D986A (based on the *S. pyogenes* Cas9 nuclease).

15 In some embodiments, the Cas nuclease is from a Type-I CRISPR/Cas system. In some embodiments, the Cas nuclease is a component of the Cascade complex of a Type-I CRISPR/Cas system. For example, the Cas nuclease is a Cas3 nuclease. In some

embodiments, the Cas nuclease is derived from a Type-III CRISPR/Cas system. In some

embodiments, the Cas nuclease is derived from Type-IV CRISPR/Cas system. In some

20 embodiments, the Cas nuclease is derived from a Type-V CRISPR/Cas system. In some

embodiments, the Cas nuclease is derived from a Type-VI CRISPR/Cas system.

Guide RNAs (gRNAs)

The CRISPR technology involves the use of a genome-targeting nucleic acid that can

25 direct the endonuclease to a specific target sequence within a target gene for gene editing at the specific target sequence. The genome-targeting nucleic acid can be an RNA. A genome-targeting RNA is referred to as a “guide RNA” or “gRNA” herein. A guide RNA comprises at least a spacer sequence that hybridizes to a target nucleic acid sequence within a target gene for editing, and a CRISPR repeat sequence.

30 In Type II systems, the gRNA also comprises a second RNA called the tracrRNA sequence. In the Type II gRNA, the CRISPR repeat sequence and tracrRNA sequence hybridize to each other to form a duplex. In the Type V gRNA, the crRNA forms a duplex. In both systems, the duplex binds a site-directed polypeptide, such that the guide RNA and site-directed polypeptide form a complex. In some embodiments, the genome-targeting nucleic

35 acid provides target specificity to the complex by virtue of its association with the site-

directed polypeptide. The genome-targeting nucleic acid thus directs the activity of the site-directed polypeptide.

As is understood by the person of ordinary skill in the art, each guide RNA is designed to include a spacer sequence complementary to its genomic target sequence. See
 5 Jinek et al., *Science*, 337, 816-821 (2012) and Deltcheva et al., *Nature*, 471, 602-607 (2011).

In some embodiments, the genome-targeting nucleic acid (*e.g.*, gRNA) is a double-molecule guide RNA. In some embodiments, the genome-targeting nucleic acid (*e.g.*, gRNA) is a single-molecule guide RNA.

A double-molecule guide RNA comprises two strands of RNA molecules. The first
 10 strand comprises in the 5' to 3' direction, an optional spacer extension sequence, a spacer sequence and a minimum CRISPR repeat sequence. The second strand comprises a minimum tracrRNA sequence (complementary to the minimum CRISPR repeat sequence), a 3' tracrRNA sequence and an optional tracrRNA extension sequence.

A single-molecule guide RNA (referred to as a "sgRNA") in a Type II system
 15 comprises, in the 5' to 3' direction, an optional spacer extension sequence, a spacer sequence, a minimum CRISPR repeat sequence, a single-molecule guide linker, a minimum tracrRNA sequence, a 3' tracrRNA sequence and an optional tracrRNA extension sequence. The optional tracrRNA extension may comprise elements that contribute additional functionality (*e.g.*, stability) to the guide RNA. The single-molecule guide linker links the minimum
 20 CRISPR repeat and the minimum tracrRNA sequence to form a hairpin structure. The optional tracrRNA extension comprises one or more hairpins. A single-molecule guide RNA in a Type V system comprises, in the 5' to 3' direction, a minimum CRISPR repeat sequence and a spacer sequence.

A spacer sequence in a gRNA is a sequence (*e.g.*, a 20 nucleotide sequence) that
 25 defines the target sequence (*e.g.*, a DNA target sequences, such as a genomic target sequence) of a target gene of interest. In some embodiments, the spacer sequence range from 15 to 30 nucleotides. For example, the spacer sequence may contain 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides. In some embodiments, a spacer sequence contains 20 nucleotides.

30 The "target sequence" is in a target gene that is adjacent to a PAM sequence and is the sequence to be modified by an RNA-guided nuclease (*e.g.*, Cas9). The "target sequence" is on the so-called PAM-strand in a "target nucleic acid," which is a double-stranded molecule containing the PAM-strand and a complementary non-PAM strand. One of skill in the art recognizes that the gRNA spacer sequence hybridizes to the complementary sequence located

in the non-PAM strand of the target nucleic acid of interest. Thus, the gRNA spacer sequence is the RNA equivalent of the target sequence. For example, if the target sequence is 5'-AGAGCAACAGTGCTGTGGCC-3' (SEQ ID NO: 7), then the gRNA spacer sequence is 5'-AGAGCAACAGUGCUGUGGCC-3' (SEQ ID NO: 8). The spacer of a gRNA interacts with
5 a target nucleic acid of interest in a sequence-specific manner *via* hybridization (*i.e.*, base pairing). The nucleotide sequence of the spacer thus varies depending on the target sequence of the target nucleic acid of interest.

In a CRISPR/Cas system herein, the spacer sequence is designed to hybridize to a region of the target nucleic acid that is located 5' of a PAM recognizable by a Cas9 enzyme
10 used in the system. The spacer may perfectly match the target sequence or may have mismatches. Each Cas9 enzyme has a particular PAM sequence that it recognizes in a target DNA. For example, *S. pyogenes* recognizes in a target nucleic acid a PAM that comprises the sequence 5'-NRG-3', where R comprises either A or G, where N is any nucleotide and N is immediately 3' of the target nucleic acid sequence targeted by the spacer sequence.

15 In some embodiments, the target nucleic acid sequence has 20 nucleotides in length. In some embodiments, the target nucleic acid has less than 20 nucleotides in length. In some embodiments, the target nucleic acid has more than 20 nucleotides in length. In some embodiments, the target nucleic acid has at least: 5, 10, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30 or more nucleotides in length. In some embodiments, the target nucleic acid has at
20 most: 5, 10, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30 or more nucleotides in length. In some embodiments, the target nucleic acid sequence has 20 bases immediately 5' of the first nucleotide of the PAM. For example, in a sequence comprising 5'-NNNNNNNNNNNNNNNNNNNNNRG-3', the target nucleic acid can be the sequence that corresponds to the Ns, wherein N can be any nucleotide, and the underlined NRG sequence is
25 the *S. pyogenes* PAM.

The guide RNA disclosed herein may target any sequence of interest *via* the spacer sequence in the crRNA. In some embodiments, the degree of complementarity between the spacer sequence of the guide RNA and the target sequence in the target gene can be about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100%. In some
30 embodiments, the spacer sequence of the guide RNA and the target sequence in the target gene is 100% complementary. In other embodiments, the spacer sequence of the guide RNA and the target sequence in the target gene may contain up to 10 mismatches, *e.g.*, up to 9, up to 8, up to 7, up to 6, up to 5, up to 4, up to 3, up to 2, or up to 1 mismatch.

Non-limiting examples of gRNAs that may be used as provided herein are provided in

WO2019097305, the relevant disclosures of which are herein incorporated by reference for the purposes and subject matter referenced herein. For any of the gRNA sequences provided herein, those that do not explicitly indicate modifications are meant to encompass both unmodified sequences and sequences having any suitable modifications.

The length of the spacer sequence in any of the gRNAs disclosed herein may depend on the CRISPR/Cas9 system and components used for editing any of the target genes also disclosed herein. For example, different Cas9 proteins from different bacterial species have varying optimal spacer sequence lengths. Accordingly, the spacer sequence may have 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, 35, 40, 45, 50, or more than 50 nucleotides in length. In some embodiments, the spacer sequence may have 18-24 nucleotides in length. In some embodiments, the targeting sequence may have 19-21 nucleotides in length. In some embodiments, the spacer sequence may comprise 20 nucleotides in length.

In some embodiments, the gRNA can be an sgRNA, which may comprise a 20 nucleotide spacer sequence at the 5' end of the sgRNA sequence. In some embodiments, the sgRNA may comprise a less than 20 nucleotide spacer sequence at the 5' end of the sgRNA sequence. In some embodiments, the sgRNA may comprise a more than 20 nucleotide spacer sequence at the 5' end of the sgRNA sequence. In some embodiments, the sgRNA comprises a variable length spacer sequence with 17-30 nucleotides at the 5' end of the sgRNA sequence. Examples are provided in **Table 2** below. In these exemplary sequences, the fragment of “n” refers to the spacer sequence at the 5' end.

Table 2. Exemplary sgRNA Formulas

SEQ ID NO.	sgRNA sequence
9	nnnnnnnnnnnnnnnnnnnguuuuagagcuagaaauagcaaguuaaaaauagg cuaguccguuaucaacuugaaaaaguggcaccgagucggugcuuuu
10	nnnnnnnnnnnnnnnnnnnguuuuagagcuagaaauagcaaguuaaaaauagg cuaguccguuaucaacuugaaaaaguggcaccgagucggugc
11	n ₍₁₋₇₎ ₃₀₎ guuuuagagcuagaaauagcaaguuaaaaauaggcuaguccguuaucaacu qaaa aaqugcacccgaucgugcu ₍₁₋₈₎

In some embodiments, the sgRNA comprises comprise no uracil at the 3' end of the
25 sgRNA sequence. In other embodiments, the sgRNA may comprise one or more uracil at the
3' end of the sgRNA sequence. For example, the sgRNA can comprise 1-8 uracil residues, at
the 3' end of the sgRNA sequence, *e.g.*, 1, 2, 3, 4, 5, 6, 7, or 8 uracil residues at the 3' end of
the sgRNA sequence.

Any of the gRNAs disclosed herein, including any of the sgRNAs, may be

unmodified. Alternatively, it may contain one or more modified nucleotides and/or modified backbones. For example, a modified gRNA such as an sgRNA can comprise one or more 2'-O-methyl phosphorothioate nucleotides, which may be located at either the 5' end, the 3' end, or both.

5 In certain embodiments, more than one guide RNAs can be used with a CRISPR/Cas nuclease system. Each guide RNA may contain a different targeting sequence, such that the CRISPR/Cas system cleaves more than one target nucleic acid. In some embodiments, one or more guide RNAs may have the same or differing properties such as activity or stability within the Cas9 RNP complex. Where more than one guide RNA is used, each guide RNA
10 can be encoded on the same or on different vectors. The promoters used to drive expression of the more than one guide RNA is the same or different.

In some embodiments, the gRNAs disclosed herein target a *TET2* gene, for example, target a site within exon 1, exon 2, exon 3, exon 4, exon 5, or exon 6 of the *TET2* gene. Such a gRNA may comprise a spacer sequence complementary (complete or partially) to the target
15 sequences in exon 3 or exon 5 of a *TET2* gene, or a fragment thereof. Exemplary target sequences of *TET2* and exemplary gRNA sequences are provided in **Table 3** below:

Table 3. Exemplary *TET2* gRNA Sequences/Target Sequences

gRNA Sequences		
Name	Unmodified Sequence	Modified Sequence
TET2 exon 4_BG4 (a.k.a., TET2-4)	CAUUAGGACCUGCUCCUAGAguu uuagagcuagaaauagcaaguua aaauaaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 12)	C*A*U*UAGGACCUGCUCCUAGA guuuuagagcuagaaauagcaag uuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 13)
TET2 exon 4_BG4 spacer	CAUUAGGACCUGCUCCUAGA (SEQ ID NO: 14)	C*A*U*UAGGACCUGCUCCUAGA (SEQ ID NO: 15)
TET2 exon 5_T1 (a.k.a., TET2-m5)	GGAUGUCCUAUUGCUAAGUguu uuagagcuagaaauagcaaguua aaauaaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 16)	G*G*G*AUGUCCUAUUGCUAAGU guuuuagagcuagaaauagcaag uuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 17)
TET2 exon 5_T1spacer	GGAUGUCCUAUUGCUAAGU (SEQ ID NO: 18)	G*G*G*AUGUCCUAUUGCUAAGU (SEQ ID NO: 19)
TET2 exon5 _T2	AGGGAUGUCCUAUUGCUAAGguu uuagagcuagaaauagcaaguua aaauaaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 20)	A*G*G*GAUGUCCUAUUGCUAAG guuuuagagcuagaaauagcaag uuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 21)
TET2 exon 5_T2spacer	AGGGAUGUCCUAUUGCUAAG (SEQ ID NO: 22)	A*G*G*GAUGUCCUAUUGCUAAG (SEQ ID NO: 23)

TET2 exon 6_BG5 (<i>a.k.a.</i> , TET2-5)	ACGGCACGCUCACCAAUCGCguu uugagcuagaaauagcaaguuaa aauaaggcuaguccguuaucaac uugaaaaagu ggcaccgagucggugcUUUU (SEQ ID NO: 24)	A*C*G*GCACGCUCACCAAUCGC guuuuagagcuagaaauagcaag uuaaaauaaggcuaguccguuau caacuugaaaaagu ggcaccgagucggugcU*U*U*U (SEQ ID NO: 25)
TET2 exon 6_BG5 spacer	ACGGCACGCUCACCAAUCGC (SEQ ID NO: 26)	A*C*G*GCACGCUCACCAAUCGC (SEQ ID NO: 27)
TET2 exon 3_T1 spacer (<i>a.k.a.</i> , TET2-1)	GAUUCGCUUGGUGAAAACG (SEQ ID NO: 112)	G*A*U*UCCGCUUGGUGAAAACG (SEQ ID NO: 113)
TET2 exon 3_T1	GAUUCGCUUGGUGAAAACGguu uuagagcuagaaauagcaaguua aaauaaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 114)	G*A*U*UCCGCUUGGUGAAAACG guuuuagagcuagaaauagcaag uuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 115)
TET2 exon 3_T2 spacer	CAGGACUCACACGACUAUUC (SEQ ID NO: 116)	C*A*G*GACUCACACGACUAUUC (SEQ ID NO: 117)
TET2 exon 3_T2	CAGGACUCACACGACUAUUCguu uuagagcuagaaauagcaaguua aaauaaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 118)	C*A*G*GACUCACACGACUAUUC guuuuagagcuagaaauagcaag uuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 119)
TET2 exon 3_T3 spacer	UUCGCUUGGUGAAAACGAG (SEQ ID NO: 120)	U*U*C*CGCUUGGUGAAAACGAG (SEQ ID NO: 121)
TET2 exon 3_T3	UUCGCUUGGUGAAAACGAGguu uuagagcuagaaauagcaaguua aaauaaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 122)	U*U*C*CGCUUGGUGAAAACGAG guuuuagagcuagaaauagcaag uuaaaauaaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 123)
Target Sequences		
Name	Target Sequence (PAM)	
TET2 exon 4_BG4 (<i>a.k.a.</i> , TET2-4)	CATTAGGACCTGCTCCTAGA (TGG) (SEQ ID NO: 28)	
	CATTAGGACCTGCTCCTAGA (SEQ ID NO: 124)	
TET2 exon 5_T1	GGGATGTCCTATTGCTAAGT (GGG) (SEQ ID NO: 29)	
	GGGATGTCCTATTGCTAAGT (SEQ ID NO: 125)	
TET2 exon 5_T2	AGGGATGTCCTATTGCTAAG (TGG) (SEQ ID NO: 30)	
	AGGGATGTCCTATTGCTAAG (SEQ ID NO: 126)	
TET2 exon 6_BG5	ACGGCACGCTCACCAATCGC (CGG) (SEQ ID NO: 31)	
	ACGGCACGCTCACCAATCGC (SEQ ID NO: 127)	
TET2 exon 3_T1	GATTCCGCTTGGTGAAAACG (AGG) (SEQ ID NO: 128)	
	GATTCCGCTTGGTGAAAACG (SEQ ID NO: 129)	
TET2 exon 3_T2	CAGGACTCACACGACTATTC (TGG) (SEQ ID NO: 130)	
	CAGGACTCACACGACTATTC (SEQ ID NO: 131)	
TET2 exon 3_T3	TTCCGCTTGGTGAAAACGAG (GGG) (SEQ ID NO: 132)	
	TTCCGCTTGGTGAAAACGAG (SEQ ID NO: 133)	

*: 2'-O-methyl phosphorothioate residue

In some embodiments, a gRNA targeting a site in exon 3 of *TET2* (*e.g.*, those listed in

Table 3 above) may be used for editing the *TET2* gene. For example, a gRNA targeting the site of GATTCCGCTTGGTGAAAACG (SEQ ID NO: 129) may be used, *e.g.*, a gRNA comprising a spacer of SEQ ID NO: 112 (unmodified) or SEQ ID NO: 113 (modified). Such a gRNA may comprise (*e.g.*, consists of) the nucleotide sequence of SEQ ID NO: 114 (unmodified) or SEQ ID NO: 115 (modified). T cells edited by a gRNA targeting the site of SEQ ID NO: 129 may comprise a modified *TET2* gene having at least one of the modifications listed in **Table 15** below.

In another specific example, a gRNA targeting the site of CAGGACTCACACGACTATTC (SEQ ID NO: 131) may be used, *e.g.*, a gRNA comprising a spacer of SEQ ID NO: 116 (unmodified) or SEQ ID NO: 117 (modified). Such a gRNA may comprise (*e.g.*, consists of) the nucleotide sequence of SEQ ID NO: 118 (unmodified) or SEQ ID NO: 119 (modified). T cells edited by a gRNA targeting the site of SEQ ID NO: 131 may comprise a modified *TET2* gene having at least one of the modifications listed in **Table 16** below.

In another specific example, a gRNA targeting the site of TTCCGCTTGGTGAAAACGAG (SEQ ID NO: 133) may be used, *e.g.*, a gRNA comprising a spacer of SEQ ID NO: 120 (unmodified) or SEQ ID NO: 121 (modified). Such a gRNA may comprise (*e.g.*, consists of) the nucleotide sequence of SEQ ID NO: 122 (unmodified) or SEQ ID NO: 123 (modified). T cells edited by a gRNA targeting the site of SEQ ID NO: 133 may comprise a modified *TET2* gene having at least one of the modifications listed in **Table 17** below.

In other embodiments, a gRNA specific to the *TET2* gene may target a site within exon 5. For example, a gRNA targeting the site of GGGATGTCCTATTGCTAAGT (SEQ ID NO: 125) in exon 5 may be used, *e.g.*, a gRNA comprising a spacer of SEQ ID NO: 18 (unmodified) or SEQ ID NO: 19 (modified). Such a gRNA may comprise (*e.g.*, consisting of) the nucleotide sequence of SEQ ID NO: 16 (unmodified) or SEQ ID NO: 17 (modified). T cells edited by a gRNA targeting the site of SEQ ID NO: 125 may comprise a modified *TET2* gene having at least one of the modifications listed in **Table 19** below. In some instances, the genetic editing of the *TET2* gene using such a gRNA may lead to expression of a truncated version of a TET2 protein, which may have a molecular weight about 170 kDa.

In other examples, a gRNA targeting the site of AGGGATGTCCTATTGCTAAG (SEQ ID NO: 126) in exon 5 may be used, *e.g.*, a gRNA comprising a spacer of SEQ ID NO: 22 (unmodified) or SEQ ID NO: 23 (modified). Such a gRNA may comprise (*e.g.*, consisting of) the nucleotide sequence of SEQ ID NO: 20 (unmodified) or SEQ ID NO: 21 (modified).

T cells edited by a gRNA targeting the site of SEQ ID NO: 126 may comprise a modified *TET2* gene having at least one of the modifications listed in **Table 20** below.

In other embodiments, a gRNA specific to the *TET2* gene may target a site within exon 4. For example, a gRNA targeting the site of CATTAGGACCTGCTCCTAGA (SEQ ID NO: 124) in exon 4 may be used, *e.g.*, a gRNA comprising a spacer of SEQ ID NO: 14 (unmodified) or SEQ ID NO: 15 (modified). Such a gRNA may comprise (*e.g.*, consisting of) the nucleotide sequence of SEQ ID NO:12 (unmodified) or SEQ ID NO: 13 (modified). T cells edited by a gRNA targeting the site of SEQ ID NO: 124 may comprise a modified *TET2* gene having at least one of the modifications listed in **Table 21** below.

In other embodiments, a gRNA specific to the *TET2* gene may target a site within exon 6. For example, a gRNA targeting the site of ACGGCACGCTCACCAATCGC (SEQ ID NO: 127) in exon 6 may be used, *e.g.*, a gRNA comprising a spacer of SEQ ID NO: 26 (unmodified) or SEQ ID NO: 27 (modified). Such a gRNA may comprise (*e.g.*, consisting of) the nucleotide sequence of SEQ ID NO:24 (unmodified) or SEQ ID NO:25 (modified). T cells edited by a gRNA targeting the site of SEQ ID NO: 127 may comprise a modified *TET2* gene having at least one of the modifications listed in **Table 18** below.

In some embodiments, the gRNAs disclosed herein target a *CD70* gene, for example, target a site within exon 1 or exon 3 of a *CD70* gene. Such a gRNA may comprise a spacer sequence complementary (complete or partially) to the target sequences in exon 1 or exon 3 of a *CD70* gene, or a fragment thereof. Exemplary target sequences in a *CD70* gene and exemplary gRNAs specific to the *CD70* gene are provided in **Table 4** below.

Table 4. Exemplary *CD70* gRNA Sequences/Target Sequences

gRNA Sequences		
Name	Unmodified Sequence	Modified Sequence
CD70 sgRNA (E1_T1)	UCACCAAGCCCGCGACCA AUguuuuagagcuagaaauagcaagu uaaaauaaggcuaguccguuaucaacu ugaaaaaaggcaccgagucggugcU UUU (SEQ ID NO: 32)	U*C*A*CCAAGCCCGCGAC CAAUguuuuagagcuagaaauagca aguuaaaauaaggcuaguccguuauca acuugaaaaaaggcaccgagucggug cU*U*U*U (SEQ ID NO: 33)
CD70 sgRNA (E1_T1) spacer	UCACCAAGCCCGCGACCA AU (SEQ ID NO: 34)	U*C*A*CCAAGCCCGCGAC CAAU (SEQ ID NO: 35)
CD70 sgRNA (E1_T3)	AUCACCAAGCCCGCGACC AAguuuuagagcuagaaauagcaagu uaaaauaaggcuaguccguuaucaacu ugaaaaaaggcaccgagucggugcU UUU (SEQ ID NO: 36)	A*U*C*ACCAAGCCCGCGA CCAAguuuuagagcuagaaauagca aguuaaaauaaggcuaguccguuauca acuugaaaaaaggcaccgagucggug cU*U*U*U (SEQ ID NO: 37)
CD70 sgRNA (E1_T3) spacer	AUCACCAAGCCCGCGACC AA (SEQ ID NO: 38)	A*U*C*ACCAAGCCCGCGA CCAA (SEQ ID NO: 39)

CD70 sgRNA (E1_T4)	CGGUGCGGCGCAGGCCCU AUguuuuagagcuagaaauagcaagu uaaaauaaggcuaguccguuaucaacu ugaaaaaaggcaccgagucggugcU UUU (SEQ ID NO: 40)	C*G*G*UGCGGCGCAGGCC CUAUguuuuagagcuagaaauagca aguuaaaauaaggcuaguccguuauca acuugaaaaaaggcaccgagucggug cU*U*U*U (SEQ ID NO: 41)
CD70 sgRNA (E1_T4) spacer	CGGUGCGGCGCAGGCCCU AU (SEQ ID NO: 42)	C*G*G*UGCGGCGCAGGCC CUAU (SEQ ID NO: 43)
CD70 sgRNA (E1_T7)); also referred to as: T7	GCUUUGGUCCCAUUGGUC GCguuuuagagcuagaaauagcaagu uaaaauaaggcuaguccguuaucaacu ugaaaaaaggcaccgagucggugcU UUU (SEQ ID NO: 44)	G*C*U*UUGGUCCCAUUGG UCGCguuuuagagcuagaaauagca aguuaaaauaaggcuaguccguuauca acuugaaaaaaggcaccgagucggug cU*U*U*U (SEQ ID NO: 45)
CD70 sgRNA (E1_T7) spacer	GCUUUGGUCCCAUUGGUC GC (SEQ ID NO: 46)	G*C*U*UUGGUCCCAUUGG UCGC (SEQ ID NO: 47)
CD70 sgRNA (E1_T8); also referred to as: T8	GCCCGCAGGACGCACCCA UAGuuuuagagcuagaaauagcaagu uaaaauaaggcuaguccguuaucaacu ugaaaaaaggcaccgagucggugcU UUU (SEQ ID NO: 48)	G*C*C*CGCAGGACGCACC CAUAGuuuuagagcuagaaauagca aguuaaaauaaggcuaguccguuauca acuugaaaaaaggcaccgagucggug cU*U*U*U (SEQ ID NO: 49)
CD70 sgRNA (E1_T8) spacer	GCCCGCAGGACGCACCCA UA (SEQ ID NO: 50)	G*C*C*CGCAGGACGCACC CAUA (SEQ ID NO: 51)
CD70 sgRNA (E1_T10)	GUGCAUCCAGCGCUUCGC ACguuuuagagcuagaaauagcaagu uaaaauaaggcuaguccguuaucaacu ugaaaaaaggcaccgagucggugcU UUU (SEQ ID NO: 52)	G*U*G*CAUCCAGCGCUUC GCACguuuuagagcuagaaauagca aguuaaaauaaggcuaguccguuauca acuugaaaaaaggcaccgagucggug cU*U*U*U (SEQ ID NO: 53)
CD70 sgRNA (E1_T10) spacer	GUGCAUCCAGCGCUUCGC A C (SEQ ID NO: 54)	G*U*G*CAUCCAGCGCUUC GCAC (SEQ ID NO: 55)
CD70 sgRNA (E3_T1)	CAGCUACGUAUCCAUCGU GAGuuuuagagcuagaaauagcaagu uaaaauaaggcuaguccguuaucaacu ugaaaaaaggcaccgagucggugcU UUU (SEQ ID NO: 56)	C*A*G*CUACGUAUCCAUC GUGAGuuuuagagcuagaaauagca aguuaaaauaaggcuaguccguuauca acuugaaaaaaggcaccgagucggug cU*U*U*U (SEQ ID NO: 57)
CD70 sgRNA (E3_T1) spacer	CAGCUACGUAUCCAUCGU GA (SEQ ID NO: 58)	C*A*G*CUACGUAUCCAUC GUGA (SEQ ID NO: 59)
Target Sequences		
Name	Target Sequence (PAM)	
CD70 target sequence with and without (PAM) (E1_T1)	TCACCAAGCCCGCGACCAAT (GGG) (SEQ ID NO: 60)	
	TCACCAAGCCCGCGACCAAT (SEQ ID NO:134)	
CD70 target sequence with and without (PAM) (E1_T3)	ATCACCAAGCCCGCGACCAA (TGG) (SEQ ID NO: 61)	
	ATCACCAAGCCCGCGACCAA (SEQ ID NO:135)	
CD70 target sequence with and without (PAM) (E1_T4)	CGGTGCGGCGCAGGCCCTAT (GGG) (SEQ ID NO: 62)	
	CGGTGCGGCGCAGGCCCTAT (SEQ ID NO:136)	
CD70 target sequence with and without (PAM) (E1_T7)	GCTTTGGTCCCATTGGTCGC (GGG) (SEQ ID NO: 63)	
	GCTTTGGTCCCATTGGTCGC (SEQ ID NO:137)	

CD70 target sequence with and without (PAM) (E1_T8)	GCCCGCAGGACGCACCCATA (GGG) (SEQ ID NO: 64)
CD70 target sequence with and without (PAM) (E1_T10)	GCCCGCAGGACGCACCCATA (SEQ ID NO: 138)
CD70 target sequence with and without (PAM) (E1_T10)	GTGCATCCAGCGCTTCGCAC (AGG) (SEQ ID NO: 65)
CD70 target sequence with and without (PAM) (E1_T10)	GTGCATCCAGCGCTTCGCAC (SEQ ID NO: 139)
CD70 target sequence with and without (PAM) (E3_T1)	CAGCTACGTATCCATCGTGA (TGG) (SEQ ID NO: 66)
CD70 target sequence with and without (PAM) (E3_T1)	CAGCTACGTATCCATCGTGA (SEQ ID NO: 140)

*: 2'-O-methyl phosphorothioate residue

In some embodiments, the gRNAs disclosed herein target a *FAS* gene, for example, target a site within exon 1, exon 2, or exon 3 of a *FAS* gene. Such a gRNA may comprise a spacer sequence complementary (complete or partially) to the target sequences in exon 1, exon 2, or exon 3 of a *FAS* gene, or a fragment thereof. Exemplary target sequences in a *FAS* gene and exemplary gRNAs specific to the *FAS* gene are provided in **Table 5** below.

Table 5. Exemplary *FAS* gRNA Sequences/Target Sequences

gRNA Sequences		
Name	Unmodified Sequence	Modified Sequence
FAS sgRNA (FAS_Ex2_T1)	GUGACUGACAUAACUCCAAguu uuagagcuagaaaauagcaaguua aaaauaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 67)	G*U*G*ACUGACAUAACUCCAA guuuuagagcuagaaaauagcaag uuaaaaauaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 68)
FAS sgRNA (FAS_Ex2_T1) spacer	GUGACUGACAUAACUCCAA (SEQ ID NO: 69)	G*U*G*ACUGACAUAACUCCAA (SEQ ID NO: 70)
FAS sgRNA (FAS_Ex2_T2)	CACUUGGGCAUUAACACUUUguu uuagagcuagaaaauagcaaguua aaaauaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 71)	C*A*C*UUGGGCAUUAACACUUU guuuuagagcuagaaaauagcaag uuaaaaauaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 72)
FAS sgRNA (FAS_Ex2_T2) spacer	CACUUGGGCAUUAACACUUU (SEQ ID NO: 73)	C*A*C*UUGGGCAUUAACACUUU (SEQ ID NO: 74)
FAS sgRNA (FAS_Ex2_T3)	UUGGAAGGCCUGCAUCAUGAguu uuagagcuagaaaauagcaaguua aaaauaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 75)	U*U*G*GAAGGCCUGCAUCAUGA guuuuagagcuagaaaauagcaag uuaaaaauaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 76)
FAS sgRNA (FAS_Ex2_T3) spacer	UUGGAAGGCCUGCAUCAUGA (SEQ ID NO: 77)	U*U*G*GAAGGCCUGCAUCAUGA (SEQ ID NO: 78)
FAS sgRNA (FAS_Ex3_T1)	CUAGGGACUGCACAGUCAAUguu uuagagcuagaaaauagcaaguua aaaauaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 79)	C*U*A*GGGACUGCACAGUCAAU guuuuagagcuagaaaauagcaag uuaaaaauaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 80)
FAS sgRNA (FAS_Ex3_T1) spacer	CUAGGGACUGCACAGUCAAU (SEQ ID NO: 81)	C*U*A*GGGACUGCACAGUCAAU (SEQ ID NO: 82)

FAS sgRNA (FAS_Ex3_T2)	ACUGCGUGCCCUGCCAAGAAguu uuagagcuagaaauagcaaguua aaaauaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 83)	A*C*U*GCGUGCCCUGCCAAGAA guuuuagagcuagaaauagcaag uuaaaaauaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 84)
FAS sgRNA (FAS_Ex3_T2) spacer	ACUGCGUGCCCUGCCAAGAA (SEQ ID NO: 85)	A*C*U*GCGUGCCCUGCCAAGAA (SEQ ID NO: 86)
Target sequences		
Name	Target Sequence (PAM)	
FAS_Ex2_T1 target sequence with and without (PAM)	GTGACTGACATCAACTCCAA (GGG) (SEQ ID NO: 87) GTGACTGACATCAACTCCAA (SEQ ID NO: 141)	
FAS_Ex2_T2 target sequence with and without (PAM)	CACTTGGGCATTAACACTTT (TGG) (SEQ ID NO: 88) CACTTGGGCATTAACACTTT (SEQ ID NO: 142)	
FAS_Ex2_T3 target sequence with and without (PAM)	TTGGAAGGCCTGCATCATGA (TGG) (SEQ ID NO: 89) TTGGAAGGCCTGCATCATGA (SEQ ID NO: 143)	
FAS_Ex3_T1 target sequence with and without (PAM)	CTAGGGACTGCACAGTCAAT (GGG) (SEQ ID NO: 90) CTAGGGACTGCACAGTCAAT (SEQ ID NO: 144)	
FAS_Ex3_T2 target sequence with and without (PAM)	ACTGCGTGCCCTGCCAAGAA (GGG) (SEQ ID NO: 91) ACTGCGTGCCCTGCCAAGAA (SEQ ID NO: 145)	

*: 2'-O-methyl phosphorothioate residue

In other embodiments, a gRNA specific to the *FAS* gene may target a site within exon 2. For example, a gRNA targeting the site of SEQ ID NO: 142 in exon 2 of the *FAS* gene may be used, *e.g.*, a gRNA comprising a spacer of SEQ ID NO: 73 (unmodified) or SEQ ID NO: 74 (modified). Such a gRNA may comprise (*e.g.*, consisting of) the nucleotide sequence of SEQ ID NO: 71 (unmodified) or SEQ ID NO: 72 (modified) (*e.g.*, FAS-Exon2-T2 gRNA). T cells edited by a gRNA targeting the site of SEQ ID NO: 142 may comprise a modified *FAS* gene having at least one of the modifications listed in **Table 26** below.

In other embodiments, a gRNA specific to the *FAS* gene may target a site within exon 3. For example, a gRNA targeting the site of SEQ ID NO: 144 in exon 3 of the *FAS* gene may be used, *e.g.*, a gRNA comprising a spacer of SEQ ID NO: 81 (unmodified) or SEQ ID NO: 82 (modified). Such a gRNA may comprise (*e.g.*, consisting of) the nucleotide sequence of SEQ ID NO: 79 (unmodified) or SEQ ID NO: 80 (modified) (*e.g.*, FAS-Exon3-T1 gRNA). T cells edited by a gRNA targeting the site of SEQ ID NO: 144 may comprise a modified *FAS* gene having at least one of the modifications listed in **Table 27** below.

In other embodiments, a gRNA specific to the *FAS* gene may target a site within exon 3. For example, a gRNA targeting the site of SEQ ID NO: 145 in exon 3 of the *FAS* gene

- may be used, *e.g.*, a gRNA comprising a spacer of SEQ ID NO: 85 (unmodified) or SEQ ID NO: 86 (modified). Such a gRNA may comprise (*e.g.*, consisting of) the nucleotide sequence of SEQ ID NO: 83 (unmodified) or SEQ ID NO: 84 (modified) (*e.g.*, FAS-Exon3-T2 gRNA). T cells edited by a gRNA targeting the site of SEQ ID NO: 145 may comprise a modified
- 5 FAS gene having at least one of the modifications listed in **Table 28** below.

In some embodiments, the gRNAs disclosed herein target a $\beta 2M$ gene, for example, target a suitable site within a $\beta 2M$ gene. See also International Application No. PCT/US2018/032334, filed May 11, 2018, the relevant disclosures of which are incorporated by reference herein for the purpose and subject matter referenced herein. Other gRNA

10 sequences may be designed using the $\beta 2M$ gene sequence located on Chromosome 15 (GRCh38 coordinates: Chromosome 15: 44,711,477-44,718,877; Ensembl: ENSG00000166710). In some embodiments, gRNAs targeting the $\beta 2M$ genomic region and RNA-guided nuclease create breaks in the $\beta 2M$ genomic region resulting in Indels in the $\beta 2M$ gene disrupting expression of the mRNA or protein.

15 In some embodiments, the gRNAs disclosed herein target a TRAC gene. See also International Application No. PCT/US2018/032334, filed May 11, 2018, the relevant disclosures of which are incorporated by reference herein for the subject matter and purpose referenced herein. Other gRNA sequences may be designed using the TRAC gene sequence located on chromosome 14 (GRCh38: chromosome 14: 22,547,506-22,552,154; Ensembl;

20 ENSG00000277734). In some embodiments, gRNAs targeting the TRAC genomic region and RNA-guided nuclease create breaks in the TRAC genomic region resulting Indels in the TRAC gene disrupting expression of the mRNA or protein.

Exemplary spacer sequences and gRNAs targeting a $\beta 2M$ gene or TRAC gene are provided in **Table 6** below.

25 **Table 6. gRNA Sequences/Target Sequences**

Name	Unmodified Sequence	Modified Sequence
TRAC sgRNA	AGAGCAACAGUGCUGUGGCCguu uuagagcuagaaauagcaaguua aaauaaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 92)	A*G*A*GCAACAGUGCUGUGGCC guuuuagagcuagaaauagcaag uuaaaaaaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 93)
TRAC sgRNA spacer	AGAGCAACAGUGCUGUGGCC (SEQ ID NO: 94)	A*G*A*GCAACAGUGCUGUGGCC (SEQ ID NO: 95)
$\beta 2M$ sgRNA	GCUACUCUCUCUUUCUGGCCguu uuagagcuagaaauagcaaguua aaauaaggcuaguccguuaucaa cuugaaaaaguggcaccgagucg gugcUUUU (SEQ ID NO: 96)	G*C*U*ACUCUCUCUUUCUGGCC guuuuagagcuagaaauagcaag uuaaaaaaggcuaguccguuau caacuugaaaaaguggcaccgag ucggugcU*U*U*U (SEQ ID NO: 97)

β2M sgRNA spacer	GCUACUCUCUCUUUCUGGCC (SEQ ID NO: 98)	G*C*U*ACUCUCUCUUUCUGGCC (SEQ ID NO: 99)
Target Sequences		
Name	Target Sequence (PAM)	
TRAC target sequence with and without (PAM)	AGAGCAACAGTGCTGTGGCC (TGG) (SEQ ID NO: 100)	
	AGAGCAACAGTGCTGTGGCC (SEQ ID NO: 146)	
β2M target sequence with and without (PAM)	GCTACTCTCTCTTTCTGGCC (TGG) (SEQ ID NO: 101)	
	GCTACTCTCTCTTTCTGGCC (SEQ ID NO: 147)	

*: 2'-O-methyl phosphorothioate residue

By way of illustration, guide RNAs used in the CRISPR/Cas/Cpf1 system, or other smaller RNAs can be readily synthesized by chemical means, as illustrated below and described in the art. While chemical synthetic procedures are continually expanding, purifications of such RNAs by procedures such as high performance liquid chromatography (HPLC, which avoids the use of gels such as PAGE) tends to become more challenging as polynucleotide lengths increase significantly beyond a hundred or so nucleotides. One approach used for generating RNAs of greater length is to produce two or more molecules that are ligated together. Much longer RNAs, such as those encoding a Cas9 or Cpf1 endonuclease, are more readily generated enzymatically. Various types of RNA modifications can be introduced during or after chemical synthesis and/or enzymatic generation of RNAs, *e.g.*, modifications that enhance stability, reduce the likelihood or degree of innate immune response, and/or enhance other attributes, as described in the art.

In some examples, the gRNAs of the present disclosure can be are produced *in vitro* transcription (IVT), synthetic and/or chemical synthesis methods, or a combination thereof. Enzymatic (IVT), solid-phase, liquid-phase, combined synthetic methods, small region synthesis, and ligation methods are utilized. In one embodiment, the gRNAs are made using IVT enzymatic synthesis methods. Methods of making polynucleotides by IVT are known in the art and are described in WO2013/151666. Accordingly, the present disclosure also includes polynucleotides, *e.g.*, DNA, constructs and vectors are used to *in vitro* transcribe a gRNA described herein.

Various types of RNA modifications can be introduced during or after chemical synthesis and/or enzymatic generation of RNAs, *e.g.*, modifications that enhance stability, reduce the likelihood or degree of innate immune response, and/or enhance other attributes, as described in the art. In some embodiments, non-natural modified nucleobases can be introduced into any of the gRNAs disclosed herein during synthesis or post-synthesis. In certain embodiments, modifications are on internucleoside linkages, purine or pyrimidine

bases, or sugar. In some embodiments, a modification is introduced at the terminal of a gRNA with chemical synthesis or with a polymerase enzyme. Examples of modified nucleic acids and their synthesis are disclosed in WO2013/052523. Synthesis of modified polynucleotides is also described in Verma and Eckstein, Annual Review of Biochemistry, 5 vol. 76, 99-134 (1998).

In some embodiments, enzymatic or chemical ligation methods can be used to conjugate polynucleotides or their regions with different functional moieties, such as targeting or delivery agents, fluorescent labels, liquids, nanoparticles, etc. Conjugates of polynucleotides and modified polynucleotides are reviewed in Goodchild, Bioconjugate 10 Chemistry, vol. 1(3), 165-187 (1990).

In some embodiments of the present disclosure, a CRISPR/Cas nuclease system for use in genetically editing any of the target genes disclosed here may include at least one guide RNA. In some examples, the CRISPR/Cas nuclease system may contain multiple gRNAs, for example, 2, 3, or 4 gRNAs. Such multiple gRNAs may target different sites in a 15 same target gene. Alternatively, the multiple gRNAs may target different genes. In some embodiments, the guide RNA(s) and the Cas protein may form a ribonucleoprotein (RNP), *e.g.*, a CRISPR/Cas complex. The guide RNA(s) may guide the Cas protein to a target sequence(s) on one or more target genes as those disclosed herein, where the Cas protein cleaves the target gene at the target site. In some embodiments, the CRISPR/Cas complex is 20 a Cpf1/guide RNA complex. In some embodiments, the CRISPR complex is a Type-II CRISPR/Cas9 complex. In some embodiments, the Cas protein is a Cas9 protein. In some embodiments, the CRISPR/Cas9 complex is a Cas9/guide RNA complex.

In some embodiments, the indel frequency (editing frequency) of a particular CRISPR/Cas nuclease system, comprising one or more specific gRNAs, may be determined 25 using a TIDE analysis, which can be used to identify highly efficient gRNA molecules for editing a target gene. In some embodiments, a highly efficient gRNA yields a gene editing frequency of higher than 80%. For example, a gRNA is considered to be highly efficient if it yields a gene editing frequency of at least 80%, at least 85%, at least 90%, at least 95%, or 100%.

30 *Delivery of Guide RNAs and Nucleases to T Cells*

The CRISPR/Cas nuclease system disclosed herein, comprising one or more gRNAs and at least one RNA-guided nuclease, optionally a donor template as disclosed below, can be delivered to a target cell (*e.g.*, a T cell) for genetic editing of a target gene, via a conventional method. In some embodiments, components of a CRISPR/Cas nuclease system

as disclosed herein may be delivered to a target cell separately, either simultaneously or sequentially. In other embodiments, the components of the CRISPR/Cas nuclease system may be delivered into a target together, for example, as a complex. In some instances, gRNA and an RNA-guided nuclease can be pre-complexed together to form a ribonucleoprotein (RNP), which can be delivered into a target cell.

RNPs are useful for gene editing, at least because they minimize the risk of promiscuous interactions in a nucleic acid-rich cellular environment and protect the RNA from degradation. Methods for forming RNPs are known in the art. In some embodiments, an RNP containing an RNA-guided nuclease (*e.g.*, a Cas nuclease, such as a Cas9 nuclease) and one or more gRNAs targeting one or more genes of interest can be delivered to a cell (*e.g.*, a T cell). In some embodiments, an RNP can be delivered to a T cell by electroporation.

In some embodiments, an RNA-guided nuclease can be delivered to a cell in a DNA vector that expresses the RNA-guided nuclease in the cell. In other examples, an RNA-guided nuclease can be delivered to a cell in an RNA that encodes the RNA-guided nuclease and expresses the nuclease in the cell. Alternatively or in addition, a gRNA targeting a gene can be delivered to a cell as a RNA, or a DNA vector that expresses the gRNA in the cell.

Delivery of an RNA-guided nuclease, gRNA, and/or an RNP may be through direct injection or cell transfection using known methods, for example, electroporation or chemical transfection. Other cell transfection methods may be used.

Other Gene Editing Methods

Besides the CRISPR method disclosed herein, additional gene editing methods as known in the art can also be used in making the genetically engineered T cells disclosed herein. Some examples include gene editing approaches involving zinc finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), restriction endonucleases, meganucleases, homing endonucleases, and the like.

ZFNs are targeted nucleases comprising a nuclease fused to a zinc finger DNA binding domain (ZFBD), which is a polypeptide domain that binds DNA in a sequence-specific manner through one or more zinc fingers. A zinc finger is a domain of about 30 amino acids within the zinc finger binding domain whose structure is stabilized through coordination of a zinc ion. Examples of zinc fingers include, but not limited to, C2H2 zinc fingers, C3H zinc fingers, and C4 zinc fingers. A designed zinc finger domain is a domain not occurring in nature whose design/composition results principally from rational criteria, *e.g.*, application of substitution rules and computerized algorithms for processing information

in a database storing information of existing ZFP designs and binding data. See, for example, U.S. Pat. Nos. 6,140,081; 6,453,242; and 6,534,261; see also WO 98/53058; WO 98/53059; WO 98/53060; WO 02/016536 and WO 03/016496. A selected zinc finger domain is a domain not found in nature whose production results primarily from an empirical process
5 such as phage display, interaction trap or hybrid selection. ZFNs are described in greater detail in U.S. Pat. No. 7,888,121 and U.S. Pat. No. 7,972,854. The most recognized example of a ZFN is a fusion of the FokI nuclease with a zinc finger DNA binding domain.

A TALEN is a targeted nuclease comprising a nuclease fused to a TAL effector DNA binding domain. A “transcription activator-like effector DNA binding domain”, “TAL
10 effector DNA binding domain”, or “TALE DNA binding domain” is a polypeptide domain of TAL effector proteins that is responsible for binding of the TAL effector protein to DNA. TAL effector proteins are secreted by plant pathogens of the genus *Xanthomonas* during infection. These proteins enter the nucleus of the plant cell, bind effector-specific DNA sequences via their DNA binding domain, and activate gene transcription at these sequences
15 via their transactivation domains. TAL effector DNA binding domain specificity depends on an effector-variable number of imperfect 34 amino acid repeats, which comprise polymorphisms at select repeat positions called repeat variable-diresidues (RVD). TALENs are described in greater detail in US Patent Application No. 2011/0145940. The most recognized example of a TALEN in the art is a fusion polypeptide of the FokI nuclease to a
20 TAL effector DNA binding domain.

Additional examples of targeted nucleases suitable for use as provided herein include, but are not limited to, Bxb1, phiC31, R4, PhiBT1, and Wβ/SPBc/TP901-1, whether used individually or in combination.

Any of the nucleases disclosed herein may be delivered using a vector system,
25 including, but not limited to, plasmid vectors, DNA minicircles, retroviral vectors, lentiviral vectors, adenovirus vectors, poxvirus vectors; herpesvirus vectors and adeno-associated virus vectors, and combinations thereof.

Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding nucleases and donor templates in cells (*e.g.*, T cells). Non-
30 viral vector delivery systems include DNA plasmids, DNA minicircles, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell.

Methods of non-viral delivery of nucleic acids include electroporation, lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, naked RNA, capped RNA, artificial virions, and agent-enhanced uptake of DNA. Sonoporation using, *e.g.*, the Sonitron 2000 system (Rich-
5 Mar) can also be used for delivery of nucleic acids. Some specific examples are provided below.

II. Use of T Cell Banks for Generating Therapeutic T Cells

The genetically engineered T cells from the T cell bank disclosed herein can be used
10 to produce therapeutic T cells such as CAR-T cells. In some embodiments, a nucleic acid encoding a therapeutic agent such as a CAR may be introduced into cells from the T cell bank to generate the therapeutic T cells. In other embodiments, the T cells in the T cell bank have been engineered to express a therapeutic agent such as a CAR. Such T cells may be obtained from the T cell bank, and optionally expanded *in vitro*, to produce the therapeutic T
15 cells for use in subjects in need of the treatment. Therapeutic T cells generated from the T cell bank are expected to live longer and be more potent *in vivo*. As such, a lower dose of such therapeutic T cells would be needed in therapy, which would result in lower side effects. In addition, a higher number of CAR-T cells can be generated from T cells isolated from a suitable natural source such as a single leukopak, as compared with conventional approaches
20 using T cells (*e.g.*, with a wild-type *TET2* gene, a wild-type *FAS* gene, and/or a wild-type *CD70* gene).

Chimeric Antigen Receptor (CAR)

A chimeric antigen receptor (CAR) refers to an artificial immune cell receptor that is engineered to recognize and bind to an antigen expressed by undesired cells, for example,
25 disease cells such as cancer cells. A T cell that expresses a CAR polypeptide is referred to as a CAR T cell. CARs have the ability to redirect T-cell specificity and reactivity toward a selected target in a non-MHC-restricted manner. The non-MHC-restricted antigen recognition gives CAR T cells the ability to recognize an antigen independent of antigen processing, thus bypassing a major mechanism of tumor escape. Moreover, when expressed on T cells, CARs
30 advantageously do not dimerize with endogenous T Cell Receptor (TCR) alpha and beta chains.

There are various generations of CARs, each of which contains different components. First generation CARs join an antibody-derived scFv to the CD3zeta (ζ or ζ) intracellular signaling domain of the T cell receptor through hinge and transmembrane domains. Second

generation CARs incorporate an additional co-stimulatory domain, *e.g.*, CD28, 4-1BB (41BB), or ICOS, to supply a costimulatory signal. Third-generation CARs contain two costimulatory domains (*e.g.*, a combination of CD27, CD28, 4-1BB, ICOS, or OX40) fused with the TCR CD3 ζ chain. Maude et al., *Blood*. 2015; 125(26):4017-4023; Kakarla and Gottschalk, *Cancer J.* 2014; 20(2):151-155). Any of the various generations of CAR constructs is within the scope of the present disclosure.

Generally, a CAR is a fusion polypeptide comprising an extracellular domain that recognizes a target antigen (*e.g.*, a single chain fragment (scFv) of an antibody or other antibody fragment) and an intracellular domain comprising a signaling domain of the T Cell Receptor (TCR) complex (*e.g.*, CD3 ζ) and, in most cases, a co-stimulatory domain. (Enblad et al., *Human Gene Therapy*. 2015; 26(8):498-505). A CAR construct may further comprise a hinge and transmembrane domain between the extracellular domain and the intracellular domain, as well as a signal peptide at the N-terminus for surface expression. Examples of signal peptides include MLLLVTSLLLCELPHPAFLIP (SEQ ID NO: 102) and MALPVTALLPLALLLHAARP (SEQ ID NO: 103). Other signal peptides may be used.

(i) Antigen Binding Extracellular Domain

The antigen-binding extracellular domain is the region of a CAR polypeptide that is exposed to the extracellular fluid when the CAR is expressed on cell surface. In some instances, a signal peptide may be located at the N-terminus to facilitate cell surface expression. In some embodiments, the antigen binding domain can be a single-chain variable fragment (scFv, which may include an antibody heavy chain variable region (V_H) and an antibody light chain variable region (V_L) (in either orientation). In some instances, the V_H and V_L fragment may be linked via a peptide linker. The linker, in some embodiments, includes hydrophilic residues with stretches of glycine and serine for flexibility as well as stretches of glutamate and lysine for added solubility. The scFv fragment retains the antigen-binding specificity of the parent antibody, from which the scFv fragment is derived. In some embodiments, the scFv may comprise humanized V_H and/or V_L domains. In other embodiments, the V_H and/or V_L domains of the scFv are fully human.

The antigen-binding extracellular domain may be specific to a target antigen of interest, for example, a pathologic antigen such as a tumor antigen. In some embodiments, a tumor antigen is a "tumor associated antigen," referring to an immunogenic molecule, such as a protein, that is generally expressed at a higher level in tumor cells than in non-tumor cells, in which it may not be expressed at all, or only at low levels. In some embodiments, tumor-associated structures, which are recognized by the immune system of the tumor-harboring

host, are referred to as tumor-associated antigens. In some embodiments, a tumor-associated antigen is a universal tumor antigen, if it is broadly expressed by most types of tumors. In some embodiments, tumor-associated antigens are differentiation antigens, mutational antigens, overexpressed cellular antigens or viral antigens. In some embodiments, a tumor antigen is a “tumor specific antigen” or “TSA,” referring to an immunogenic molecule, such as a protein, that is unique to a tumor cell. Tumor specific antigens are exclusively expressed in tumor cells, for example, in a specific type of tumor cells.

Exemplary tumor antigens include, but are not limited to, CD19, CD33, BCMA, and CD70. Any known antibodies specific to such tumor antigens, for example, those approved for marketing and those in clinical trials, can be used for making the CAR constructs disclosed herein.

(ii) Transmembrane Domain

The CAR polypeptide disclosed herein may contain a transmembrane domain, which can be a hydrophobic alpha helix that spans the membrane. As used herein, a “transmembrane domain” refers to any protein structure that is thermodynamically stable in a cell membrane, preferably a eukaryotic cell membrane. The transmembrane domain can provide stability of the CAR containing such.

In some embodiments, the transmembrane domain of a CAR as provided herein can be a CD8 transmembrane domain. In other embodiments, the transmembrane domain can be a CD28 transmembrane domain. In yet other embodiments, the transmembrane domain is a chimera of a CD8 and CD28 transmembrane domain. Other transmembrane domains may be used as provided herein. In some embodiments, the transmembrane domain is a CD8a transmembrane domain containing the sequence of

FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG
 AVHTRGLDFACDIYIWA PLAGTCGVLLLSLVITLYCNHRNR (SEQ ID NO: 104) or
 IYIWAPLAGTCGVLLLSLVITLY (SEQ ID NO: 105). Other transmembrane domains may be used.

(iii) Hinge Domain

In some embodiments, a hinge domain may be located between an extracellular domain (comprising the antigen binding domain) and a transmembrane domain of a CAR, or between a cytoplasmic domain and a transmembrane domain of the CAR. A hinge domain can be any oligopeptide or polypeptide that functions to link the transmembrane domain to the extracellular domain and/or the cytoplasmic domain in the polypeptide chain. A hinge

domain may function to provide flexibility to the CAR, or domains thereof, or to prevent steric hindrance of the CAR, or domains thereof.

In some embodiments, a hinge domain may comprise up to 300 amino acids (*e.g.*, 10 to 100 amino acids, or 5 to 20 amino acids). In some embodiments, one or more hinge domain(s) may be included in other regions of a CAR. In some embodiments, the hinge domain may be a CD8 hinge domain. Other hinge domains may be used.

(iv) Intracellular Signaling Domains

Any of the CAR constructs contain one or more intracellular signaling domains (*e.g.*, CD3 ζ , and optionally one or more co-stimulatory domains), which are the functional end of the receptor. Following antigen recognition, receptors cluster and a signal is transmitted to the cell.

CD3 ζ is the cytoplasmic signaling domain of the T cell receptor complex. CD3 ζ contains three (3) immunoreceptor tyrosine-based activation motif (ITAM)s, which transmit an activation signal to the T cell after the T cell is engaged with a cognate antigen. In many cases, CD3 ζ provides a primary T cell activation signal but not a fully competent activation signal, which requires a co-stimulatory signaling.

In some embodiments, the CAR polypeptides disclosed herein may further comprise one or more co-stimulatory signaling domains. For example, the co-stimulatory domains of CD28 and/or 4-1BB may be used to transmit a full proliferative/survival signal, together with the primary signaling mediated by CD3 ζ . In some examples, the CAR disclosed herein comprises a CD28 co-stimulatory molecule. In other examples, the CAR disclosed herein comprises a 4-1BB co-stimulatory molecule. In some embodiments, a CAR includes a CD3 ζ signaling domain and a CD28 co-stimulatory domain. In other embodiments, a CAR includes a CD3 ζ signaling domain and 4-1BB co-stimulatory domain. In still other embodiments, a CAR includes a CD3 ζ signaling domain, a CD28 co-stimulatory domain, and a 4-1BB co-stimulatory domain.

Table 7 provides examples of signaling domains derived from 4-1BB, CD28 and CD3-zeta that may be used herein.

Table 7. Exemplary Intracellular Signaling Domain Sequences

Name	Sequence	SEQ ID NO:
4-1BB	AAACGGGGCAGAAAGAACTCCTGTATATATTCAAACAACCATTATGAGACAGTACAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAA GAAGAAGAAGGAGGATGTGAAGT	106
	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	107

Name	Sequence	SEQ ID NO:
CD28	TCAAAGCGGAGTAGGTTGTTGCATTCCGATTACATGAATATGACTCCTCGC CGGCCTGGGCCGACAAGAAAACATTACCAACCCCTATGCCCCCACCAGAGAC TTCGCTGCGTACAGGTCC	108
	SKRSRLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRS	109
CD3 ζ	CGAGTGAAGTTTTCCCGAAGCGCAGACGCTCCGGCATATCAGCAAGGACAG AATCAGCTGTATAACGAAGTGAATTGGGACGCCGCGAGGAGTATGACGTG CTTGATAAACGCCGGGGGAGAGACCCGAAATGGGGGTAACCCCGAAGA AAGAATCCCAAGAAGGACTCTACAATGAATCCAGAAGGATAAGATGGCG GAGGCTACTCAGAAATAGGTATGAAGGGCGAACGACGACGGGAAAAGGT CACGATGGCCTCTACCAAGGGTTGAGTACGGCAACCAAAGATACGTACGAT GCACTGCATATGCAGGCCCTGCCTCCAGA	110
	RVKFSRSADAPAYQQGQNLNLYNELNLGRREEYDVLDRRGRDPEMGGKPRR KNPQEGLYNELQKDKMAEAYSEIGMKGERRRKGHDGLYQGLSTATKDTYD ALHMQLPFR	111

Non-limiting examples of CAR constructs are provided in WO2019097305 and WO2019215500, and WO2020/095107, the relevant disclosures of which are herein incorporated by reference for the purposes and subject matter referenced herein. Some examples are provided below:

Anti-CD19 CAR (SEQ ID NO: 148):

MLLLVTSLLLCELPHPAFLIPDIQMTQTSSLSASLGDRVTISCRASQDISKYLWYQQKPDGTVKL
LIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEITGSTSGSG
KPGSGEGSTKGEVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSETT
YYNSALKSRLTIKDNSKSQVFLKMNSLQTDITAIYCAKHYYYGGSYAMDYWGQTSVTVSSAAAFV
PVFLPAKPTTTPAPRPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLL
LSLVITLYCNHRNRKRRLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSRVKFSRSADAPAYQ
QQGQNLNLYNELNLGRREEYDVLDRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERR
RKGHDGLYQGLSTATKDTYDALHMQLPFR

Anti-BCMA CAR (SEQ ID NO: 149):

MALPVTALLPLALLHAARPQVQLVQSGAELKKPGASVKVSKASGNTLTNYVIHWVRQAPGQRLW
MGYILPYNDLTQYSQKFQGRVTITRDKSASTAYMELSSLRSEDTAVYYCTRWDWDGFFDPWGQGTIVT
VSSGGGSGGGGSGGGGSEIVMTQSPATLSVSPGERASISCRASQSLVHSNGNTHLHWYQQRPGQAPR
LLIYSVSNRFSEVPARFSGSGSGTDFTLTISVSEDFAVYYCSQTSHPYTFGGGKLEIKSAAAFV
PVFLPAKPTTTPAPRPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLL
LSLVITLYCNHRNRKRGRKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCELRVKFSRSADAPA
YQQGQNLNLYNELNLGRREEYDVLDRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGER
RRKGHDGLYQGLSTATKDTYDALHMQLPFR

Anti-CD70 CAR (SEQ ID NO: 150):

MALPVTALLPLALLHAARPQVQLVQSGAEVKKPGASVKVSKASGYFTNYGMNWVRQAPGQGLKW
MGWINTYTGEPYADAFKGRVTMTSDTISSTAYMELSLRSDDTAVYYCARDYGDYGMWYWGQGTIVT
VSSGGGSGGGGSGGGGSDIVMTQSPDSLAVSLGERATINCRASKSVSTSGYSFMHWYQQKPGQPPK
LLIYLASNLESQVDFRSGSGSGTDFTLTISLQAEADVAVYYCQHSREVPWTFGQGTKEIKSAAAFV
PVFLPAKPTTTPAPRPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLL
LSLVITLYCNHRNRKRGRKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCELRVKFSRSADAPA

YQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGER
RRGKGHDGLYQGLSTATKDTYDALHMQALPPR

Exemplary anti-CD33 CAR constructs can be found in WO2020095107, the relevant
5 disclosures of which are incorporated by reference for the subject matter and purpose
referenced herein.

Delivery of CAR Construct to T Cells

In some embodiments, a nucleic acid encoding a CAR can be introduced into any of
10 the genetically engineered T cells from the T cell bank disclosed herein by methods known to
those of skill in the art. For example, a coding sequence of the CAR may be cloned into a
vector, which may be introduced into the genetically engineered T cells for expression of the
CAR. A variety of different methods known in the art can be used to introduce any of the
nucleic acids or expression vectors disclosed herein into an immune effector cell. Non-
15 limiting examples of methods for introducing nucleic acid into a cell include: lipofection,
transfection (e.g., calcium phosphate transfection, transfection using highly branched organic
compounds, transfection using cationic polymers, dendrimer-based transfection, optical
transfection, particle-based transfection (e.g., nanoparticle transfection), or transfection using
liposomes (e.g., cationic liposomes)), microinjection, electroporation, cell squeezing,
20 sonoporation, protoplast fusion, impalefection, hydrodynamic delivery, gene gun,
magnetofection, viral transfection, and nucleofection.

In specific examples, a nucleic acid encoding a CAR construct can be delivered to a
cell using an adeno-associated virus (AAV). AAVs are small viruses which integrate site-
specifically into the host genome and can therefore deliver a transgene, such as CAR.
25 Inverted terminal repeats (ITRs) are present flanking the AAV genome and/or the transgene
of interest and serve as origins of replication. Also present in the AAV genome are rep and
cap proteins which, when transcribed, form capsids which encapsulate the AAV genome for
delivery into target cells. Surface receptors on these capsids which confer AAV serotype,
which determines which target organs the capsids will primarily bind and thus what cells the
30 AAV will most efficiently infect. There are twelve currently known human AAV serotypes.
In some embodiments, the AAV for use in delivering the CAR-coding nucleic acid is AAV
serotype 6 (AAV6).

Adeno-associated viruses are among the most frequently used viruses for gene
therapy for several reasons. First, AAVs do not provoke an immune response upon
35 administration to mammals, including humans. Second, AAVs are effectively delivered to

target cells, particularly when consideration is given to selecting the appropriate AAV serotype. Finally, AAVs have the ability to infect both dividing and non-dividing cells because the genome can persist in the host cell without integration. This trait makes them an ideal candidate for gene therapy.

5 A nucleic acid encoding a CAR can be designed to insert into a genomic site of interest in the host T cells. In some embodiments, the target genomic site can be in a safe harbor locus.

 In some embodiments, a nucleic acid encoding a CAR (*e.g.*, via a donor template, which can be carried by a viral vector such as an adeno-associated viral (AAV) vector) can be
10 designed such that it can insert into a location within a *TRAC* gene to disrupt the *TRAC* gene in the genetically engineered T cells and express the CAR polypeptide. Disruption of *TRAC* leads to loss of function of the endogenous TCR. For example, a disruption in the *TRAC* gene can be created with an endonuclease such as those described herein and one or more gRNAs targeting one or more *TRAC* genomic regions. Any of the gRNAs specific to a *TRAC*
15 gene and the target regions disclosed herein can be used for this purpose.

 In some examples, a genomic deletion in the *TRAC* gene and replacement by a CAR coding segment can be created by homology directed repair or HDR (*e.g.*, using a donor template, which may be part of a viral vector such as an adeno-associated viral (AAV) vector). In some embodiments, a disruption in the *TRAC* gene can be created with an
20 endonuclease as those disclosed herein and one or more gRNAs targeting one or more *TRAC* genomic regions, and inserting a CAR coding segment into the *TRAC* gene.

 A donor template as disclosed herein can contain a coding sequence for a CAR. In some examples, the CAR-coding sequence may be flanked by two regions of homology to allow for efficient HDR at a genomic location of interest, for example, at a *TRAC* gene using
25 a gene editing method known in the art. In some examples, a CRISPR-based method can be used. In this case, both strands of the DNA at the target locus can be cut by a CRISPR Cas9 enzyme guided by gRNAs specific to the target locus. HDR then occurs to repair the double-strand break (DSB) and insert the donor DNA coding for the CAR. For this to occur correctly, the donor sequence is designed with flanking residues which are complementary to
30 the sequence surrounding the DSB site in the target gene (hereinafter “homology arms”), such as the *TRAC* gene. These homology arms serve as the template for DSB repair and allow HDR to be an essentially error-free mechanism. The rate of homology directed repair (HDR) is a function of the distance between the mutation and the cut site so choosing overlapping or nearby target sites is important. Templates can include extra sequences

flanked by the homologous regions or can contain a sequence that differs from the genomic sequence, thus allowing sequence editing.

Alternatively, a donor template may have no regions of homology to the targeted location in the DNA and may be integrated by NHEJ-dependent end joining following
5 cleavage at the target site.

A donor template can be DNA or RNA, single-stranded and/or double-stranded, and can be introduced into a cell in linear or circular form. If introduced in linear form, the ends of the donor sequence can be protected (*e.g.*, from exonucleolytic degradation) by methods known to those of skill in the art. For example, one or more dideoxynucleotide residues are
10 added to the 3' terminus of a linear molecule and/or self-complementary oligonucleotides are ligated to one or both ends. See, for example, Chang et al., (1987) Proc. Natl. Acad. Sci. USA 84:4959-4963; Nehls et al., (1996) Science 272:886-889. Additional methods for protecting exogenous polynucleotides from degradation include, but are not limited to, addition of terminal amino group(s) and the use of modified internucleotide linkages such as, for
15 example, phosphorothioates, phosphoramidates, and O-methyl ribose or deoxyribose residues.

A donor template can be introduced into a cell as part of a vector molecule having additional sequences such as, for example, replication origins, promoters and genes encoding antibiotic resistance. Moreover, a donor template can be introduced into a cell as naked
20 nucleic acid, as nucleic acid complexed with an agent such as a liposome or poloxamer, or can be delivered by viruses (*e.g.*, adenovirus, AAV, herpesvirus, retrovirus, lentivirus and integrase defective lentivirus (IDLV)).

A donor template, in some embodiments, can be inserted at a site nearby an endogenous promoter (*e.g.*, downstream or upstream) so that its expression can be driven by
25 the endogenous promoter. In other embodiments, the donor template may comprise an exogenous promoter and/or enhancer, for example, a constitutive promoter, an inducible promoter, or tissue-specific promoter to control the expression of the CAR gene. In some embodiments, the exogenous promoter is an EF1 α promoter. Other promoters may be used.

Furthermore, exogenous sequences may also include transcriptional or translational
30 regulatory sequences, for example, promoters, enhancers, insulators, internal ribosome entry sites, sequences encoding 2A peptides and/or polyadenylation signals.

When needed, additional gene editing (*e.g.*, gene knock-in or knock-out) can be introduced into therapeutic T cells produced from the T cell bank as disclosed herein to improve T cell function and therapeutic efficacy. For example, if $\beta 2M$ knockout can be

performed to reduce the risk of or prevent a host-versus-graft response. Other examples include knock-in or knock-out genes to improve target cell lysis, knock-in or knock-out genes to enhance performance of therapeutic T cells such as CAR-T cells prepared from cells of the T cell bank. Examples include knock-out of an immune checkpoint gene such as *PD-1*.

5

III. Therapeutic Applications

The therapeutic T cells generated using the genetically engineered T cells of the T cell bank would be expected to maintain T cell health enabled by the mutation in the *TET2* gene, the disruption of the *FAS* gene, the disruption of the *CD70* gene, or a combination thereof.

10 For example, maintaining T cell health may extend expansion during manufacturing, thereby increasing yield and consistency. In another example, maintaining T cell health may rescue exhausted/unhealthy T cells, thereby enabling potentially lower doses in patients and more robust responses.

The therapeutic T cells generated using the T cell bank disclosed herein can be
15 administered to a subject for therapeutic purposes, for example, treatment of a solid tumor targeted by the CAR construct expressed by the therapeutic T cells.

The step of administering may include the placement (*e.g.*, transplantation) of the therapeutic T cells into a subject by a method or route that results in at least partial localization of the therapeutic T cells at a desired site, such as a tumor site, such that a desired
20 effect(s) can be produced. Therapeutic T cells can be administered by any appropriate route that results in delivery to a desired location in the subject where at least a portion of the implanted cells or components of the cells remain viable. 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, or even the life time of the subject, *i.e.*, long-term
25 engraftment. For example, in some aspects described herein, an effective amount of the therapeutic T cells can be administered *via* a systemic route of administration, such as an intraperitoneal or intravenous route.

In some embodiments, the therapeutic T cells are administered systemically, which refers to the administration of a population of cells other than directly into a target site, tissue,
30 or organ, such that it enters, instead, the subject's circulatory system and, thus, is subject to metabolism and other like processes. Suitable modes of administration include injection, infusion, instillation, or ingestion. Injection includes, without limitation, intravenous, intramuscular, intra-arterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular,

intraarticular, sub capsular, subarachnoid, intraspinal, intracerebro spinal, and intrasternal injection and infusion. In some embodiments, the route is intravenous.

A subject may be any subject for whom diagnosis, treatment, or therapy is desired. In some embodiments, the subject is a mammal. In some embodiments, the subject is a human.

5 In some instances, the therapeutic T cells may be autologous ("self") to the subject, i.e., the cells are from the same subject. Alternatively, the therapeutic T cells can be non-autologous ("non-self," *e.g.*, allogeneic, syngeneic or xenogeneic) to the subject. "Allogeneic" means that the therapeutic T cells are not derived from the subject who receives the treatment but from different individuals (donors) of the same species as the subject. A
10 donor is an individual who is not the subject being treated. A donor is an individual who is not the patient. In some embodiments, a donor is an individual who does not have or is not suspected of having the cancer being treated. In some embodiments, multiple donors, *e.g.*, two or more donors, are used.

In some embodiments, an engineered T cell population being administered according
15 to the methods described herein comprises allogeneic T cells obtained from one or more donors. Allogeneic refers to a cell, cell population, or biological samples comprising cells, obtained from one or more different donors of the same species, where the genes at one or more loci are not identical to the recipient (*e.g.*, subject). For example, an engineered T cell population, being administered to a subject can be derived from one or more unrelated
20 donors, or from one or more non-identical siblings. In some embodiments, syngeneic cell populations may be used, such as those obtained from genetically identical donors, (*e.g.*, identical twins). In some embodiments, the cells are autologous cells; that is, the engineered T cells are obtained or isolated from a subject and administered to the same subject, *i.e.*, the donor and recipient are the same.

25 An effective amount refers to the amount of a population of engineered T cells needed to prevent or alleviate at least one or more signs or symptoms of a medical condition (*e.g.*, cancer), and relates to a sufficient amount of a composition to provide the desired effect, *e.g.*, to treat a subject having a medical condition. An effective amount also includes an amount sufficient to prevent or delay the development of a symptom of the disease, alter the course of
30 a symptom of the disease (for example but not limited to, slow the progression of a symptom of the disease), or reverse a symptom of the disease. It is understood that for any given case, an appropriate effective amount can be determined by one of ordinary skill in the art using routine experimentation.

Because of the enhanced persistence and efficacy of the therapeutic T cells produced from the T cell bank disclosed herein, the dose of the therapeutic T cells provided herein may be lower than the standard dose of CAR-T cells prepared by conventional approaches (*e.g.*, using T cells that do not have one or more of the genetic editing events disclosed herein, including a mutated *TET2* gene, a disrupted *FAS* gene, and/or a disrupted *CD70* gene). In some examples, the effective amount of the therapeutic T cells disclosed herein may be at least 2-fold lower, at least 5-fold lower, at least 10-fold lower, at least 20-fold lower, at least 50-fold lower, or at least 100-fold lower than a standard dose of a CAR-T therapy. In some examples, an effective amount of the therapeutic T cells disclosed herein may be less than 10^6 cells, *e.g.*, 10^5 cells, 5×10^4 cells, 10^4 cells, 5×10^3 cells, or 10^3 cells. In some examples described herein, the cells are expanded in culture prior to administration to a subject in need thereof.

The efficacy of a treatment using the therapeutic T cells disclosed herein can be determined by the skilled clinician. A treatment is considered “effective”, if any one or all of the signs or symptoms of, as but one example, levels of functional target are altered in a beneficial manner (*e.g.*, increased by at least 10%), or other clinically accepted symptoms or markers of disease (*e.g.*, cancer) are improved or ameliorated. Efficacy can also be measured by failure of a subject to worsen as assessed by hospitalization or need for medical interventions (*e.g.*, progression of the disease is halted or at least slowed). Methods of measuring these indicators are known to those of skill in the art and/or described herein. Treatment includes any treatment of a disease in subject and includes: (1) inhibiting the disease, *e.g.*, arresting, or slowing the progression of symptoms; or (2) relieving the disease, *e.g.*, causing regression of symptoms; and (3) preventing or reducing the likelihood of the development of symptoms.

Combination therapies are also encompassed by the present disclosure. For example, the therapeutic T cells disclosed herein may be co-used with other therapeutic agents, for treating the same indication, or for enhancing efficacy of the therapeutic T cells and/or reducing side effects of the therapeutic T cells.

KITS

The present disclosure also provides kits for use in producing the T cell bank, the therapeutic T cells, and for therapeutic uses,

In some embodiments, a kit provided herein may comprise components for performing genetic edit of one or more of *TET2* gene, *FAS* gene, and *CD70* gene, and optionally a population of immune cells to which the genetic editing will be performed

(*e.g.*, a leukopak). A leukopak sample may be an enriched leukapheresis product collected from peripheral blood. It typically contains a variety of blood cells including monocytes, lymphocytes, platelets, plasma, and red cells. The components for genetically editing one or more of the target genes may comprise a suitable endonuclease such as an RNA-guided
5 endonuclease and one or more nucleic acid guides, which direct cleavage of one or more suitable genomic sites by the endonuclease. For example, the kit may comprise a Cas enzyme such as Cas 9 and one or more gRNAs targeting a *TET2* gene, a *FAS* gene, and/or a *CD70* gene. Any of the gRNAs specific to these target genes can be included in the kit. Such a kit may further comprise components for further gene editing, for example, gRNAs
10 and optionally additional endonucleases for editing other target genes such as *β2M* and/or *TRAC*.

In some embodiments, a kit provided herein may comprise a population of genetically engineered T cells of the T cell bank as disclosed herein, and one or more components for producing the therapeutic T cells as also disclosed herein. Such
15 components may comprise an endonuclease suitable for gene editing and a nucleic acid coding for a CAR construct of interest. The CAR-coding nucleic acid may be part of a donor template as disclosed herein, which may contain homologous arms flanking the CAR-coding sequence. In some instances, the donor template may be carried by a viral vector such as an AAV vector. The kit may further comprise gRNAs specific to a *TRAC*
20 gene for inserting the CAR-coding sequence into the *TRAC* gene.

In yet other embodiments, the kit disclosed herein may comprise a population of therapeutic T cells as disclosed for the intended therapeutic purposes.

Any of the kit disclosed herein may further comprise instructions for making the T cell bank, the therapeutic T cells, or therapeutic applications of the therapeutic T cells. In
25 some examples, the included instructions may comprise a description of using the gene editing components to genetically engineer one or more of the target genes (*e.g.*, *TET2*, *FAS*, *CD70*, or a combination thereof) for making the T cell bank from parent T cells of a suitable source (*e.g.*, those described herein). In other examples, the included instructions may comprise a description of how to introduce a nucleic acid encoding a CAR
30 construction into the T cells of the T cell bank for making therapeutic T cells.

Alternatively, the kit may further comprise instructions for administration of the therapeutic T cells as disclosed herein to achieve the intended activity, *e.g.*, eliminating disease cells targeted by the CAR expressed on the therapeutic T cells. The kit may further comprise a description of selecting a subject suitable for treatment based on identifying

whether the subject is in need of the treatment. The instructions relating to the use of the therapeutic T cells described herein generally include information as to dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (*e.g.*, multi-dose packages) or sub-unit doses. Instructions
 5 supplied in the kits of the disclosure are typically written instructions on a label or package insert. The label or package insert indicates that the therapeutic T cells are used for treating, delaying the onset, and/or alleviating a disease or disorder in a subject.

The kits provided herein are in suitable packaging. Suitable packaging includes, but is not limited to, vials, bottles, jars, flexible packaging, and the like. Also
 10 contemplated are packages for use in combination with a specific device, such as an infusion device for administration of the therapeutic T cells. A kit may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The container may also have a sterile access port.

15 Kits optionally may provide additional components such as buffers and interpretive information. Normally, the kit comprises a container and a label or package insert(s) on or associated with the container. In some embodiment, the disclosure provides articles of manufacture comprising contents of the kits described above.

20 ***General techniques***

The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as *Molecular Cloning: A*
 25 *Laboratory Manual*, second edition (Sambrook, et al., 1989) Cold Spring Harbor Press; *Oligonucleotide Synthesis* (M. J. Gait, ed. 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J. E. Cellis, ed., 1989) Academic Press; *Animal Cell Culture* (R. I. Freshney, ed. 1987); *Introduction to Cell and Tissue Culture* (J. P. Mather and P. E. Roberts, 1998) Plenum Press; *Cell and Tissue Culture: Laboratory*
 30 *Procedures* (A. Doyle, J. B. Griffiths, and D. G. Newell, eds. 1993-8) J. Wiley and Sons; *Methods in Enzymology* (Academic Press, Inc.); *Handbook of Experimental Immunology* (D. M. Weir and C. C. Blackwell, eds.): *Gene Transfer Vectors for Mammalian Cells* (J. M. Miller and M. P. Calos, eds., 1987); *Current Protocols in Molecular Biology* (F. M. Ausubel, et al. eds. 1987); *PCR: The Polymerase Chain Reaction*, (Mullis, et al., eds. 1994); *Current Protocols in Immunology* (J. E. Coligan et al., eds., 1991); *Short Protocols*
 35

in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C. A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: a practice approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal antibodies: a practical approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using antibodies: a laboratory manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J. D. Capra, eds. Harwood Academic Publishers, 1995); *DNA Cloning: A practical Approach*, Volumes I and II (D.N. Glover ed. 1985); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds.(1985»; *Transcription and Translation* (B.D. Hames & S.J. Higgins, eds. (1984»; *Animal Cell Culture* (R.I. Freshney, ed. (1986»; *Immobilized Cells and Enzymes* (IRL Press, (1986»; and B. Perbal, *A practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.).

Without further elaboration, it is believed that one skilled in the art can, based on the above description, utilize the present invention to its fullest extent. The following specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All publications cited herein are incorporated by reference for the purposes or subject matter referenced herein.

EXAMPLES

Example 1: Effects of TET2 Knockout (KO) or Mutation on Primary Human T Cells

(i) Efficient Knockout or Mutation of TET2 by Cas9:sgRNA RNPs in T cells

This example describes efficient editing of the *TET2* gene in T cells derived from primary human PBMC cells *ex vivo* using CRISPR/Cas9 gene editing. Genomic segments of the *TET2* gene containing the fourth, fifth and sixth protein coding exons were used as input in gRNA design software. Desired gRNAs were those that would lead to deletions in the coding sequence, disrupting the amino acid sequence of TET2, and leading to out of frame/loss of function allele (referred to as “TET2 knockout” alleles or truncation of the TET2 protein). Four (4) *in silico*-identified gRNA spacer sequences targeting the *TET2* gene were synthesized and the gRNAs were specifically modified, as indicated in **Table 1**. While the gRNAs in **Table 1** were modified with 2'-O-methyl phosphorothioate modifications, unmodified gRNAs, or gRNAs with other modifications, may be used. See also WO2019097305, the relevant disclosures of which are incorporated by reference for the purpose and subject matter referenced herein.

Activated T cells derived from primary human PBMC cells were transfected (electroporated) with a ribonucleoprotein particle (RNP) containing Cas9 nuclease and a

synthetic modified sgRNA targeting the *TET2* gene (sequences in **Table 3** above) or controls (no Cas9, no gRNA). Six (6) days post transfection, cells were immunoblotted to assess indel efficiency.

As show in **FIGs. 1A-1B**, no TET2 protein was detected determined by a capillary-based size-separation immunoassay termed Simple Western (protein simple, San Jose, CA) in cells treated with gRNAs TET2 exon 4_BG4, TET2 exon 5_T1, TET2 exon 5_T2, or TET2 exon 6_BG5, and a truncated form(s) of the TET2 protein (having a lower apparent molecular weight than wild-type TET2) was detected in cells treated with either the TET2 exon 5_T1 gRNA or Exon 6_BG5 gRNA.

(ii) *TET2 KO or Mutation Increased T cell Proliferation and Expansion*

To assess the effect of TET2 modulation (including disruption or truncated mutation) on the ability of T cells to expand in cytokine containing media (IL-2+IL-7), cells with disrupted or mutated *TET2* gene were generated as described above. Equal numbers of cells were plated at 1.5×10^6 cells/ml of cytokine containing media (IL-2+IL-7), cell counts were recorded every 2-3 days, and cells density was adjusted at 1.5×10^6 cells/ml in fresh cytokine containing media (IL-2+IL-7). T cells containing a disruption in the *TET2* gene induced by TET2 exon5_T1 or TET2 exon5_T2 expanded to greater levels as compared to control cells without a *TET2* gene disruption (**FIG. 1B**). More than 6 weeks post-transfection, TET2 edited cells with TET2 exon5_T1 or TET2 exon5_T2 gRNAs kept proliferating and the modulation of TET2 by TET2 exon5_T1 gRNA (resulting in a truncated form of TET2) results in greater cell yields in culture compared to other groups and caused an increase of more than 250 fold in the cell yield compared to non-transfected cells (WT). **FIG. 1B**.

To assess if disruptions of other TET2 coding exons leads to greater T cell expansion T cells were generated with disruptions in the TET2 gene after treatment with TET2 exon 4_BG4 and TET2 exon 6_BG5 gRNAs. After the T cells were edited they were grown for 4 weeks under standard T-cell culture conditions (X-vivo medium (04-744, Lonza), supplemented with 5% human AB serum (HP1022, Valley Biomedical), 50 ng/ml IL-2 (rhIL-2; 130-097-745, Miltenyi Biotech) and 10 ng/ml IL-7 (rhIL-7; Cellgenix 001410-050). After four weeks, 3×10^6 cells were plated into a G-Rex (Gas Permeable Rapid Expansion, Wilson Wolf P/N80660M) 6 well plate. Cell expansion was assessed as by counting viable cells once a week for a ten-week period. The expansion was recorded as a fold change of the initial plating of 3×10^6 cells to the most current cell count (*i.e.* current cell count/ 3×10^6 cells). Cells were maintained at 1×10^6 cells per mL in complete media. As shown in **FIG. 1D**, TET2 KO produced by disrupting either exon 4 or exon6 of the TET2 gene led to 15-19 fold expansion

over the No RNP treated control T-Cells during a 10 week enumeration period (14 weeks total in culture).

In sum, TET2 modified T cells were found to be expandable in culture for greater than 4 weeks, much longer than non-modified T cells.

5

(iii) TET2 KO or Truncated Mutation Reduces Apoptotic Cell numbers in extended cell cultures

The number of TET2 deficient cells undergoing apoptosis in extended cell culture was evaluated at day 36 post-electroporation. Briefly, an aliquot of cells was washed and stained with FITC-conjugated annexin V along with 7-AAD in annexin V binding buffer (BioLegend) for 15 minutes at room temperature. Cells were then washed and resuspend in annexin V binding buffer for analysis by flow cytometry. As shown in **Table 8** below, T cells edited with TET2 exon5_T1 or TET2 exon5_T2 gRNAs showed a reduction in apoptotic cells and an increase in the percentage of healthy cells by 7-8 folds compared to unedited cells.

Table 8. Percentage of Healthy Cells at Day 36 Post-Electroporation

	Healthy Cells	Annexin-V ⁺ Cells	7-AAD ⁺ Cells	Annexin-V ⁺ /7-AAD ⁺ Cells
Mock	8.48%	60.3%	0.48%	30.7%
TET2 exon5_T1	61.3%	21.1%	0.21%	17.4%
TET2 exon5_T2	64.3%	20.5%	0.13%	16.1%

(iv) TET2 KO and Truncated Mutation Increased the number of activatable T cells in extended cell cultures

To assess the ability of TET2-modified T cells to be activated in extended cell cultures, cells with disrupted *TET2* gene were generated as described above. At day forty-five (45) post-transfection, 0.5×10^6 T cells were treated with BD Golgiplug for one (1) hour followed by activation with PMA/Ionomycin for four (4) hours in the presence of Golgiplug. Cells were collected and surface-stained for activation markers and intracellularly-stained for IFN- γ production. T cells containing a disruption and truncated mutation in the *TET2* gene caused by TET2 exon3_T3 and TET2 exon5_T1, respectively, showed a greater frequency of activated and IFN- γ -positive cells (CD25⁺, IFN- γ ⁺ cells), as compared to control cells without a *TET2* gene disruption or T cells transfected with other gRNAs. See the results in **Table 9** below.

Table 9. Percentage of IFN- γ /CD25⁺ T cells in Genetically Edited T cells

	Mock	TET2 exon5_T1	TET2 exon5_T2
CD25 ⁺ /IFN- γ ⁺ cell%	0	13.1	4.75

Example 2: FAS Knockout (KO) Increased Proliferation and Reduced Apoptosis*(i) Knockout of FAS by Cas9:sgRNA RNPs in T cells*

5 This example describes efficient editing of the FAS gene in primary human T cells *ex vivo* using the CRISPR/Cas9 gene editing approach. Desired gRNAs were those that would lead to insertions or deletions in the coding sequence, disrupting the amino acid sequence of FAS, leading to out of frame/loss of function allele(s) (referred to as “FAS knockout” alleles). All five (5) in silico-identified gRNA spacer sequences targeting the FAS gene were
 10 synthesized.

Primary human T cells from two healthy donors were transfected (electroporated) with a ribonucleoprotein particle (RNP) containing a Cas9 nuclease and a synthetic modified sgRNA (with 2'-O-methyl phosphorothioate modifications) targeting the FAS gene (sequences in **Table 5** above) or controls (no Cas9, no gRNA). Six (6) days post
 15 transfection, cells were processed by flow cytometry (primary antibody: PE Dazzle 594 anti-human FAS antibody, clone DX2, Biolegend) to assess FAS expression levels at the cell surface.

As shown in **FIG. 2A**, the gRNAs listed in **Table 5** above all led to certain level of FAS knockout with gRNA FAS Ex3_T2 having the highest editing efficiency.

20

(ii) FAS KO Increased Cytokine Driven Proliferation of Anti-BCMA CAR T cells

To evaluate the effect of FAS and/or CD70 knockout on cell proliferation, anti-BCMA CAR T cells were utilized. The following groups of edited T BCMA-CAR T cells were generated:

- 25 TRAC-/B2M-/anti-BCMA CAR+ (Control; 2KO, BCMA CAR+)
 TRAC-/B2M-/FAS-/anti-BCMA CAR+ (3KO (FAS), BCMA CAR+)
 TRAC-/B2M-/CD70-/anti-BCMA CAR+ (3KO (CD70), BCMA CAR+)
 TRAC-/B2M-/FAS-/CD70-/anti-BCMA CAR+ (4KO, BCMA CAR+)

Edited cells were enriched for TRAC-/B2M- cells by magnetic depletion of
 30 CD3+B2M+ cells. Briefly, cells were labeled with anti-CD3 Biotin (Biolegend Cat# 300404)

anti- β 2M Biotin (Biolegend Cat# 316308) antibodies, each at 0.5 μ g per 1×10^6 cells in 100 μ l volume at 4 °C for 15 min, washed and incubated with Streptavidin labeled magnetic microbeads (Miltenyi Biotec, 130-048-101) for 15 min at 4 °C. Cells were resuspended in buffer and passed through LS columns (Miltenyi Biotec, 130-042-401) according to the manufacturer's protocol.

To determine the effect of FAS or CD70 on IL-2/IL-7 driven T cell proliferation, the edited T cells (1×10^6 cells/ml) were cultured in growth medium (X-vivo medium (04-744, Lonza), supplemented with 5% human AB serum (HP1022, Valley Biomedical), 50 ng/ml IL-2 (rhIL-2; 130-097-745, Miltenyi Biotec) and 10 ng/ml IL-7 (rhIL-7; Cellgenix 001410-050) for up to four weeks. At indicated days, the cells were counted and re-seeded in fresh medium at 1.5×10^6 cells/ml in appropriate culture dishes.

As shown in **FIG. 2B**, knockout of FAS or CD70 improved IL-2/IL-7 driven proliferation of anti-BCMA CAR T cells *in vitro* as compared to controls (*i.e.*, anti-BCMA CAR T cells comprising endogenous FAS and CD70). Knockout of both FAS and CD70 showed a synergistic effect on proliferation competence of anti-BCMA CAR T cells as relative to knockout of only FAS or CD70. **FIG. 2B**.

(iii) FAS KO Rescued Anti-BCMA CAR T cells From Anti-FAS Antibody Induced Apoptosis

The effect of FAS KO on apoptotic cell death of anti-BCMA CAR+ T cells following exposure to anti-FAS antibody was evaluated. Briefly, to activate FAS-FASL signaling pathway, anti-BCMA CAR+ T cells were exposed to anti-FAS antibody (1 μ g/ml, BioLegend, Cat No. 305704) for 48 hrs. At the end of the treatment, an aliquot of cells was washed and stained with fluorochrome-conjugated annexin V along with 7-AAD in annexin V binding buffer (BioLegend) for 15 minutes at room temperature. Cells were then washed and resuspended in annexin V binding buffer for analysis by flow cytometry.

As shown in **FIG. 2C**, deletion of FAS (FAS KO) rescued anti-BCMA CAR+ T cells from apoptosis induced by anti-FAS antibody, as demonstrated by the decrease in the percentage of apoptotic cells both in the 3KO (TRAC-/B2M-/FAS-) and 4KO (TRAC-/B2M-/FAS-/CD70-) cells.

Example 3: Triple Knockout of CD70, TET2, and FAS Enhanced Cytolytic Activity of Anti-CD19 CAR T Cells

Following preparation of edited anti-CD19 CAR T cells, the functional activity of the CAR T cells was verified using a flow cytometry-based cytotoxicity assay. See

WO2019/097305 for disclosures of anti-CD19 CAR, the relevant disclosures of which are incorporated by reference for the purpose and subject matter referenced herein. The anti-CD19 CAR T cells (TRAC-/β2M-/CD19 CAR+ and TRAC-/β2M-/CD70-/TET2-/FAS-/CD19 CAR+) were co-cultured with a CD19-expressing cancer cell line (target cells): Raji (ATCC ccl-86). The target cells were labeled with 5 μM efluor670 (eBiosciences), washed and incubated in co-cultures with the TRAC-/β2M-/anti-CD19 CAR+, or TRAC-/β2M-/CD70-/TET2-/FAS-/ anti-CD19 CAR+ at varying ratios (0.01, 0.05, 0.1, 0.5, 1:1 T cells:target cells). The target cells were seeded at 100,000 cells per well in a 96-well, U-bottom plate. The co-culture was incubated for 48 hours. After incubation, wells were washed and media was replaced with 200 μL of media containing a 1:500 dilution of 5 mg/mL DAPI (Molecular Probes). Twenty five (25) μL of CountBright beads (Life Technologies) were then added to each well and the cell cultures were analyzed for cell viability by flow cytometry (*i.e.*, viable cells being negative for DAPI staining).

Percent cell lysis of the target cells (*e.g.*, Nalm6 or Raji cells) was then determined using the following formula:

$$\text{Percent cell lysis} = (1 - ((\text{total number of target cells in a test sample}) \div (\text{total number of target cells in a control sample}))) \times 100;$$

In this formula, a test sample refers to target cells (*e.g.*, Raji cells) co-cultured with (1) TRAC-/β2M-/CD19 CAR+ T cells, or (2) TRAC-/β2M-/CD70-/TET2-/FAS-/ anti-CD19 CAR+ cells; and a control sample refers to target cells alone that had not been co-cultured with CAR-T cells.

Disruption of the CD70, TET2 and FAS genes led to enhanced cytolytic activity of the anti-CD19 CAR-T cells against the Raji cell line at low CAR-T to target ratios. **FIG. 3A.** See WO2019097305 for descriptions of anti-CD19 CAR-T cells (*e.g.*, TC1 cells). The relevant disclosures of this PCT publication is hereby incorporated by reference for the purpose and subject matter referenced herein. The increased activity conferred by CD70, TET2 and FAS loss against the Raji cell line indicates that in challenging tumor environments, particularly when CAR-T to tumor ratios are low, CD70, TET2, and FAS loss may have substantial benefit to the CAR-T cells in eradicating tumor cells.

Generation of T cell Bank and Serial Edits After Cell Bank Expansion

Further, the use of CRISPR/Cas9 gene editing technology is explored for producing a human T cell bank, where the TET2 gene is initially knocked-out, and then is further edited after four weeks in culture. Specifically, the FAS Cell Surface Death Receptor (FAS) gene, and the Cluster of Differentiation 70 (CD70) gene were edited at four weeks by

CRISPR/Cas9 gene editing to produce T cells deficient in an additional one or two more genes. Initial editing was conducted in activated primary human T cells and included the electroporation of control T Cells (no RNP) and T cells with a TET2 Cas9/gRNA RNP complex. The nucleofection mix contained the Nucleofector™ Solution, 5×10^6 cells, 1 μ M Cas9, and 5 μ M gRNA (as described in Hendel *et al.*, *Nat Biotechnol.* 2015; 33(9):985-989, PMID: 26121415).

Editing conducted after four weeks included re-electroporating the TET2 knockout T cells and the control T cells without RNP. For the generation of double knockout T cells, the TET2 knockout cells were electroporated with an RNP complex, containing Cas9 protein and one of the following sgRNAs: FAS or CD70. For the generation of triple knockout T cells, the TET2 knockout cells were electroporated with two different RNP complexes, each RNA complex containing Cas protein and one of the following sgRNAs: FAS and CD70. The exemplary gRNAs for use in gene editing of the TET2, FAS, and CD70 genes are provided in Table 10 below:

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Table 10. TET2 FAS CD70 gRNA Sequences

gRNA Sequences		
Name	Unmodified Sequence	Modified Sequence
TET2 sgRNA (TET2 exon 4_BG4)	cauuaggaccugcuccuag aguuuuagagcuagaaa gcaaguuaaaauaaggcua guccguuaucaacuugaaa aaguggcaccgagucggug cuuuu (SEQ ID NO:12)	c*a*u*uaggaccugcuccu agaguuuuagagcuagaaa agcaaguuaaaauaaggcua guccguuaucaacuugaaa aaguggcaccgagucggugcu *u*u*u (SEQ ID NO: 13)
FAS sgRNA	acugcgugcccugccaaga aguuuuagagcuagaaa gcaaguuaaaauaaggcua guccguuaucaacuugaaa aaguggcaccgagucggug cuuuu (SEQ ID NO: 83)	a*c*u*gcugcccugcca gaaguuuuagagcuagaaa agcaaguuaaaauaaggcua guccguuaucaacuugaaa aaguggcaccgagucggugcu *u*u*u (SEQ ID NO: 84)
CD70 sgRNA	GCUUUGGUCCAUUGGUCGCG uuuagagcuagaaaagcaagu uaaaauaaggcuaguccguuau caacuugaaaaaguggcaccga gucggugcUUUU (SEQ ID NO: 44)	G*C*U*UUGGUCCAUUGGUCGCG uuuagagcuagaaaagcaagu aaaaauaaggcuaguccguuau cuugaaaaaguggcaccgagucgg ugcU*U*U*U (SEQ ID NO: 45)

*: 2'-O-methyl phosphorothioate residue

To assess whether further editing the cell bank affects cell expansion, cell numbers were enumerated among, single, double, triple gene edited T cells (unedited T cells were used as a control) over a seven-week period of post editing. 1×10^6 cells were generated and

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plated for each genotype of T cells. After electroporation, cell expansion was assessed as by counting viable cells once a week for a seven-week period. Cells were maintained at 1×10^6 cells per mL in complete media.

As shown in **FIG. 3B**, cell proliferation (expansion) continued after the post second electroporation in the edited cell bank conditions. Cell proliferation was observed among the single TET2 (cell bank), double (TET2-/CD70-) or (TET2-/FAS-), or triple (TET2-/CD70-/FAS), knockout T cells, as indicated by the number of viable cells. These data suggest that serial gene editing to a TET2- T cell bank does not impact T cell health as measured by T cell proliferation after a period of recovery.

Example 4: *In vivo* effect of TET2, FAS and CD70 KO on allogeneic CAR T Cells in the Intravenous Disseminated Nalm-6 Human Acute Lymphoblastic Leukemia Tumor Xenograft Model

A disseminated mouse model was utilized to further assess the *in vivo* efficacy of allogeneic CAR T cells lacking B2M and TRAC, as well as TET2, FAS and/or CD70.

The intravenous disseminated model (disseminated model) using a CD19+ B-ALL derived Nalm-6 Human Acute Lymphoblastic Leukemia tumor cell line in NOG mice was used to demonstrate the efficacy of anti-CD19/TRAC⁻/B2M⁻ CAR T cells (anti-CD19 CAR T cells) with or without the additional gene edits disclosed herein (*e.g.*, TET2, FAS and/or CD70). The anti-CD19 CAR T cells were made as described in Example 3. See also WO2019/097305.

Efficacy of the anti-CD19 CAR T cells was evaluated in the disseminated model using methods employed by Translations Drug Development, LLC (Scottsdale, AZ) and described herein. In brief, 40, 5-8 week old female CIEA NOG (NOD.Cg-Prkdc^{scid}I12rg^{tm1Sug}/JicTac) mice were individually housed in ventilated microisolator cages, maintained under pathogen-free conditions, 5-7 days prior to the start of the study. At the start of the study, the mice were divided into 8 treatment groups as shown in **Table 11**. The mice were inoculated with Nalm6-Fluc-GFP (Nalm6-Fluc-Neo/eGFP--Puro) cells intravenously to model disseminated disease. On Day 1, all mice received an intravenous injection of 0.5×10^6 Nalm6 cells/mouse. On Day 4, Groups 2-8 received an intravenous injection of CAR T cells (4×10^6 CAR+ cells/mouse) as indicated in **Table 11**.

Table 11. Treatment groups

Group	Nalm6 tumor cells 0.5×10^6 cells/mouse	CAR T cells (i.v.) 4×10^6 cells/mouse	N
1	X	NA	5
2	X	anti-CD19 CAR/TRAC ⁻ /B2M ⁻	5
3	X	anti-CD19 CAR/TRAC ⁻ /B2M ⁻ /CD70 ⁻	5

Group	Nalm6 tumor cells 0.5x10 ⁶ cells/mouse	CAR T cells (i.v.) 4x10 ⁶ cells/mouse	N
4	X	anti-CD19 CAR/TRAC-/B2M-/TET2-	5
5	X	anti-CD19 CAR/TRAC-/B2M-/FAS-	5
6	X	anti-CD19 CAR/TRAC-/B2M-/TET2-/CD70-	5
7	X	anti-CD19 CAR/TRAC-/B2M-/FAS-/CD70-	5
8	X	anti-CD19 CAR/TRAC-/B2M-/FAS-/TET2-/CD70-	5

During the course of the study, the mice were monitored daily and body weight was measured two times weekly. Blood samples (0.1ml/mouse) were collected from all mice by submandibular bleed at days 2, 7, 14, 21, 42 and 50 to assess CAR T cell expansion over the course of the study. Bioluminescence (BLI; total ROI, photon/s) was measured twice weekly beginning on Day 4 of the study. A significant endpoint was the time to peri-morbidity and the effect of T-cell engraftment was also assessed. The percentage of animal mortality and time to death were recorded for every group in the study. Mice were euthanized prior to reaching a moribund state. Mice may be defined as moribund and sacrificed if one or more of the following criteria were met:

- Loss of body weight of 20% or greater sustained for a period of greater than 1 week;
- Tumors that inhibit normal physiological function such as eating, drinking, mobility and ability to urinate and or defecate;
- Prolonged, excessive diarrhea leading to excessive weight loss (>20%); or
- Persistent wheezing and respiratory distress.

Animals were also considered moribund if there was prolonged or excessive pain or distress as defined by clinical observations such as: prostration, hunched posture, paralysis/paresis, distended abdomen, ulcerations, abscesses, seizures and/or hemorrhages.

(i) In vivo Survival Rate

Mice in groups receiving TRAC/B2M⁻CAR T cells with an additional TET2 knockout, either alone or in combination with CD70 and/or FAS (Groups 4, 6 and 8), exhibited an increase in survival relative to mice in both the untreated (Group 1) and those treated with TRAC/B2M⁻ allogeneic anti-CD19 CAR T cells with a wild-type TET2 allele (Group 2) and did not succumb to Nalm6 leukemia even out to 153 days after treatment. While CD70 loss (Group 3; anti-CD19 CAR/TRAC⁻/B2M⁻/CD70⁻) did not increase survival relative to Group 2 mice, the addition of the TET2 knock-out prolonged survival of mice in Group 6 (anti-CD19 CAR/TRAC⁻/B2M⁻/TET2⁻/CD70⁻) and group 8 (anti-CD19 CAR/TRAC⁻/B2M⁻/FAS⁻/TET2⁻/CD70⁻) as compared to the Group 2 mice. See **Table 12** below.

Table 12. Survival Days of Mice Treated by CAR-T Cells

Group	CAR T cells (i.v.) 4×10^6 cells/mouse	Median Survival (Days)
1	NA	24
2	anti-CD19 CAR/TRAC-/B2M-	102
3	anti-CD19 CAR/TRAC-/B2M-/CD70-	46
4	anti-CD19 CAR/TRAC-/B2M-/TET2-	>153
5	anti-CD19 CAR/TRAC-/B2M-/FAS-	>153
6	anti-CD19 CAR/TRAC-/B2M-/TET2-/CD70-	>153
7	anti-CD19 CAR/TRAC-/B2M-/FAS-/CD70-	55
8	anti-CD19 CAR/TRAC-/B2M-/FAS-/TET2-/CD70-	137

To assess if the effects of the TET2 KO observed in allogeneic anti-CD19 CAR+/TRAC-/B2M- T cells were specific to sgRNA TET2 exon4 BG4, a separate lot of cells was prepared from a different healthy donor and tested in the Nalm6/*in vivo* model as above. Mice treated with 4×10^6 CAR+ cells that had disruption of the TET2 locus with either of the following sgRNAs (TET2 exon6_BG5, TET2 exon4_BG4 or TET2 exon3_T1) all prolonged survival of mice longer than mice receiving TRAC-/B2M-/CAR+ T cells (**FIG. 5A**; All TET2- groups showed statistically significant survival vs TRAC-/B2M-/CAR+ T cells ($p=.0021$); Log-Rank test) and showed reduced Nalm6-luciferase activity over time (**FIG 5B**). Thus, Tet2 improved CAR T efficacy in the Nalm6 disseminated mouse model.

Taken together, TET2 knockout leads to marked cell expansion, leading to enhancement of the function of CAR T cells in an *in vivo* model of CD19+ malignancy. Furthermore, TET2 KO allows CAR-T cells to carry edits in other genes that may have additional benefits to the cells (*e.g.*, anti-apoptosis/anti-senescence) but do not prolong survival in this leukemia model on their own.

(ii) CAR T cell expansion in vivo

CAR T cell expansion was assessed by measuring the CAR copy number by ddPCR of DNA isolated from blood samples collected throughout the study as described above.

DNA was isolated from mouse tissue using the Qiagen Dneasy blood and tissue kit (Qiagen, Venlo, Netherlands). Total mass of nucleic acid from RBC-lysed samples was quantitated using either Nanodrop (Thermo Fisher Scientific) or DropSense96 (trinean, Gentbrugge, Belgium) machines. Primers and 6-carboxyfluorescein (FAM)-labeled probe sets (provided in **Table 12** below) were designed to quantitate the levels of the integrated

CAR construct into the human TRAC locus by droplet digital PCR (ddPCR). ddPCR was performed using the Bio-Rad Automated Droplet Generator, Bio-Rad T100 Thermal Cycler, and Bio-Rad QX200 Droplet Reader machine(s) (Bio-rad Laboratories, Hercules, CA). QuantaSoft Version 1.7.4.0917 (Bio-rad Laboratories) software was used to calculate the absolute number of integrated CAR copies per sample. Finally, the number of detected CAR alleles was divided by the input total DNA amount to compute the absolute number of CAR copies per mass of input sample. The ddPCR assay detects the number of copies of integrated CAR transgene per mass of genomic DNA (gDNA) by amplifying an 866 bp amplicon spanning endogenous TRAC sequence and the CAR expression cassette promoter (EF-1 α). In brief, qualification of the assay yielded linear data ($R^2 > 0.95$) within the range tested (2 to 300,000 copies per μ g of gDNA) as well as generated a % relative error (%RE) and % coefficient of variation (% CV) within normal ranges (%RE $\leq 100\%$ and %CV $\leq 20\%$) for conditions $\geq$ LLOQ. The LLOD and LLOQ were calculated based on the available data and the LLOD was set to 5 copies per 0.2 μ g of gDNA and the LLOQ was set to 40 copies per 0.2 μ g.

Table 13. Primers and probes used for ddPCR

CAR primers and probe	
Anti-CD19 CAR-20-30_dd_1 Forward	GGCACCATATTCATTTGCAGGTGAA (SEQ ID NO: 151)
Anti-CD19 CAR-20-30_dd_1 Reverse	ATGTGCGCTCTGCCCACTGACGGGC (SEQ ID NO: 152)
Anti-CD19 CAR-20-30_dd_1 Probe (FAM)	AGACATGAGGTCTATGGACTTCAGGCTCC (SEQ ID NO: 153)

These analysis demonstrate that the addition of a TET2 KO to allogeneic CAR T cells (TRAC⁻/B2M⁻) allowed the T cells to expand to larger levels in the blood of treated mice (*e.g.*, Groups 4, 6, and 8) compared to groups treated with the allogeneic CAR T cells without a TET2 KO (*e.g.*, Groups 2, 3, 5, and 7) (**FIG. 4**). This expansion was apparent at day 21 of the study and passed day 100. FAS KO also appeared to give a more modest expansion to CAR T cells apparent at day 50 (*e.g.*, Group 5 or 7 vs. Group 2) (**FIG. 4**).

In sum, all groups with loss of TET2 had expanded CAR-T cells in the peripheral blood and groups with loss of FAS displayed increased levels at relatively later time points.

Example 5: Knock-Out of TET2 Increases the Persistence of Functional CAR-T Cells in Liquid and Solid Tumor Models

To assess the mechanism of how TET2 KO increases CAR-T potency, the effects of TET2 disruption on CAR T cells persistence was examined in several re challenge models. In a re-challenge model of CD19+ malignancy NOG mice first received Nalm6-luciferase cells and were dosed with CAR-T cells as described above. Mice were dosed with 8×10^6 CAR+ CD19 CAR-T cells that were all TRAC-/B2M- along with groups that had an additional disruption of TET2 induced using one of three gRNAs: TET2 exon4_BG4, TET2 exon3_T1, of TET2 exon6_BG5. While untreated mice had quickly succumb to leukemia all CAR-T cell groups cleared the initial Nalm6-luciferase leukemia within a 30 day period (**FIG. 6A**). At Day 35 of the study all mice received a second dose of cancer cells, using a more aggressive cancer cell line. The CD19+ Burkitt lymphoma derived Raji-luciferase cell line was injected into mice at 0.5×10^6 cells per mouse intravenously (i.v.). Naïve mice (mice that had not received Nalm-luc cells) quickly succumb to Raji lymphomas. However, mice that had previously cleared Nalm6 tumors after receiving the TRAC-/B2M-/TET2- cells better controlled the second tumor than mice that had received just TRAC-/B2M- CAR T cells (**FIG. 6A**). These data provide evidence that TET2 disruption allows for the continued persistence of functional CAR-T cells. This effect is independent of the TET2 genomic segment disrupted as cells edited with several distinct sgRNAs gave similar results.

To assess the ability of TET2 KO to increase the persistence of CAR-T cells in a different liquid tumor model (multiple myeloma) in the context of a different CAR construct (anti-BCMA CAR) BCMA targeting CAR- T cells were made from a healthy donor. Anti-BCMA CAR T Cells were made as previously described. Groups of CAR-T cells were made that were TRAC-/B2M- and TRAC-/B2M-/TET2- (using either TET2 exon4_BG4, TET2 exon6_BG5 or TET2 exon5_T1 sgRNA). NOG mice were initially inoculated subcutaneously in their right flanks with 1×10^7 multiple myeloma derived RPMI-8226 cells. After 9 days, when tumors were palpable, 1×10^6 CAR+ cells were injected i.v. While untreated mice quickly had succumb to tumor endpoint (2000 mm³) both TRAC-/B2M- and TRAC-/B2M-/TET2- groups efficiently cleared the primary RPMI-8226 tumors (**FIG. 6B**). On day 32 of the study mice previously treated with CAR+ T cells were subjected to a tumor re-challenge by injecting 1×10^7 fresh RPMI-8226 cells into the left flank of mice. A new group of naïve mice were injecting 1×10^7 fresh RPMI-8226 cells into the left flank (untreated group). While TRAC-/B2M- BCMA-CAR-T cells could control of the re-challenge to some extent, TRAC-/B2M-/TET2- BCMA-CAR-T cells quickly eliminated these new tumor cells (**FIG. 6C**). Taken together TET2 disruption

allows CAR-T cells to persist longer in aggressive re-challenge models in multiple liquid tumor models. This effect multiple CAR T cells targeting different cancer antigens.

To assess the ability of TET2 loss to increase the persistence of CAR-T cells in solid tumors CD70 targeting CAR T cells (anti-CD70 CAR T cells) were made from a healthy donor. Groups of CAR-T cells were made that were TRAC-/B2M-/CD70- and TRAC-/B2M-/CD70-/TET2- (*e.g.*: TET2 exon4) BG4 sgRNA). NOG mice were initially inoculated subcutaneously in their right flanks with 5×10^6 renal cell carcinoma (RCC) derived A498 cells. When tumors reached 500 mm^3 1×10^7 CAR-T cells were injected i.v. While untreated mice quickly had succumb to tumor endpoint (2000 mm^3) both TRAC-/B2M-/CD70- and TRAC-/B2M-/CD70-/TET2- efficiently cleared the primary A498 tumors (**FIG. 6D**). On day 54 of the study mice were subjected to a tumor re-challenge by injecting 5×10^6 RCC-derived Caki-1 cells into the left flank of mice. A new group of naïve mice were injecting 1×10^7 fresh RPMI-8226 cells into the left flank (untreated group). While TRAC-/B2M-/CD70-/CD70-/CAR-T cells showed modest control of the Caki-1 tumors TRAC-/B2M-/CD70-/TET2-/CD70-/CAR-T cells quickly eliminated these tumor cells (**FIG. 6E**). Taken together TET2 disruption allows CAR-T cells to persist longer in aggressive re-challenge models of both liquid and solid tumors.

Example 6. TET2 Deficient CAR-T Cells Depend on Cytokines for Growth.

To assess the tumorigenic potential of CAR-T cells that lack TET2, anti-CD19 CAR T cells were produced that were TRAC-/B2M- and either wild type for TET2 or had a TET2 disruption (TET2 disruption was generating using either TET2 exon4_BG4, TET2 exon6_BG5, TET2-exon5_T1). Cells were then used to assess for the ability of genome edited cells to display growth factor and/or cytokine independent growth in culture. 2×10^6 cells were placed in either full T cell media (containing 5% human serum + IL2 and IL7) or in media containing serum but lacking IL-2 and IL-7. Cells were then counted weekly using trypan blue and a Countess automated cell counter (Thermo Fisher Scientific). While all groups of cells could grow in media containing serum and cytokines, no outgrowths were detected over a 6 week period from any of the groups. **FIG. 7**. These data demonstrate that TET2 disruption does not lead to direct oncogenic transformation of human peripheral blood derived T cells as these cells continue to depend on cytokines for their growth.

Example 7. On-Target and Off-Target Editing of TET-2 Guide RNAs

On-target and off-target editing efficiencies of various TET2-targeting gRNAs were examined following the method disclosed in **Example 1** above. Briefly, activated T cells

derived from primary human PBMC cells were transfected (electroporated) with a ribonucleoprotein particle (RNP) containing Cas9 nuclease and a synthetic modified sgRNA targeting the TET2 gene (sequences in **Table 3** above) or controls (no Cas9, no gRNA). Six (6) days post transfection, cells were immunoblotted to assess the effect of on-target TET2 editing on protein expression.

For genomic on- and off-target assessment, the same electroporation methods were used to generate two cell populations of edited cells from two different donor T cells (termed 1 and 2). Cells were gene edited with each of the seven guides listed in Tables 3 and 14, and then collected six (6) days post transfection. These samples were analyzed with hybrid capture, a homology-dependent method to enrich on- and off-target sites, combined with next-generation sequencing. Briefly, on- and off-target sites with homology to each gRNA target site were identified computationally, single-stranded RNA probes were used to enrich these sites from bulk genomic DNA, these enriched sites were sequenced with next-generation sequencing, and the data were analyzed for insertions and deletions indicating repair following CRISPR/Cas9-mediated gene editing.

The results are provided in **Table 14** below.

Table 14. On and Off Target Results by Hybrid Capture

Guide	gRNA target sequence + (PAM)	Number of predicted off target sites tested	On-target mean editing hyb cap ^a	Effect of editing on Tet2 protein expression	Detected off-targets
TET2-exon3-T1	GATTCGCTTGGTGAAAACG (AGG) (SEQ ID NO: 128)	36	89.0%	KO	NO
TET2-exon3-T2	CAGGACTCACACGACTATTC (TGG) (SEQ ID NO: 130)	57	87.7%	Reduced	NO
TET2-exon3-T3	TTCCGCTTGGTGAAAACGAG (GGG) (SEQ ID NO: 132)	54	80.0%	KO/truncation	NO
TET2-exon6-BG5	ACGGCAGCTCACCAATCGC (CGG) (SEQ ID NO: 31)	6	93.8%	KO/truncation	Yes, one: intronic to PRKAR1B, a regulatory subunit of PKA
TET2-exon5-T1	GGGATGTCCTATTGCTAAGT (GGG) (SEQ ID NO: 29)	59	93.0%	KO/truncation	Yes, one: exonic to TET1 with 3 mismatches relative to guide
TET2-exon5-T2	AGGGATGTCCTATTGCTAAG (TGG) (SEQ ID NO: 30)	103	96.0%	KO	Yes, one
TET2-exon4-BG4	CATTAGGACCTGCTCCTAGA (TGG) (SEQ ID NO: 28)	90	94.9%	KO	Yes, two

^a Average across donors 1 and 2.

Analysis of on-target indel profiles in T cells

The data used to quantify off-target editing were also used to quantify and summarize the most frequent on-target indels for all TET2 guides listed in **Table 14**. This data was generated from hybrid capture of the TET2 locus combined with next-generation sequencing

5 in two donors (termed Donor 1 and Donor 2).

Following gene editing, hybrid capture analysis of the TET2 locus in a population of T cells following CRISPR/Cas9 gene editing to produce TET2 - T cells results in specific indel frequencies and edited gene sequences at the TET2 locus (**Tables 15-21**; deletions as dashes and insertions in bold).

10 For the purposes of individual sequence quantification from hybrid capture data, sequence reads aligning across the TET2 on-target site, 20 bp upstream and downstream of the cut site, were selected and considered for indel sequence quantification. From the selected reads, the sequence within 10 bp upstream and downstream of each putative cut site (~3bp upstream of the PAM (Jinek, et al., Science 2012) was quantified as a representative

15 region of on-target non-homologous end joining (NHEJ) editing. The data on these on-target gene edited sequences is presented in the tables below, with the frequencies of these sequences representing the percent of all sequences spanning the on-target site within 20 bp upstream and downstream of each cut site. The indels for each guide are shown relative to an on-target reference sequence in Tables 15-21. The reference sequence is centered on the

20 cleavage site with 10 bp in either direction, ending 4 bp 3' of the PAM.

Table 15. On-Target Gene Edited Sequences > 1% Frequency in At Least One Gene Edited T Cell Donor for the TET2-Exon3-T1 gRNA.

Reference on-target sequence ^a : CTTGGTGAAAACG (AGG) GGCC (SEQ ID NO: 154)						
SEQ ID NO:	Gene Edited Sequence ^b :	Reference Sequence ^c (SEQ ID NO: 154)	Donor 1 (%)	Donor 2 (%)	Mean (%)	Std. Dev. (%)
155	CTTGGTGAAA-CGAGGGGCC	CTTGGTGAAAACGAGGGGCC	27.7	25.6	26.7	1.5
156	CTTGGTGAAAA A CGAGGGGCC	CTTGGTGAAAA-CGAGGGGCC	13.7	15.5	14.6	1.3
157	CTTGGTGAA--CGAGGGGCC	CTTGGTGAAAACGAGGGGCC	4.8	4.2	4.5	0.4
158	CTTGGTGA-----GGGGCC	CTTGGTGAAAACGAGGGGCC	4.8	3.7	4.2	0.8
	-----	CTTGGTGAAAACGAGGGGCC	2.9	3.2	3.0	0.2
	CTTGG-----GGCC	CTTGGTGAAAACGAGGGGCC	2.9	2.1	2.5	0.6
	-----AGGGGCC	CTTGGTGAAAACGAGGGGCC	2.4	2.4	2.4	0.0
	CTTGG-----CC	CTTGGTGAAAACGAGGGGCC	1.8	1.6	1.7	0.1
	C-----C	CTTGGTGAAAACGAGGGGCC	1.2	1.4	1.3	0.1
	CTTGG-----GCC	CTTGGTGAAAACGAGGGGCC	1.2	1.6	1.4	0.3
	CTT-----	CTTGGTGAAAACGAGGGGCC	1.2	1.7	1.4	0.3

	-----GCC	CTTGGTGAAAACGAGGGGCC	0.9	1.2	1.0	0.2
	-----CC	CTTGGTGAAAACGAGGGGCC	0.6	1.2	0.9	0.5

^a On-target sequence centered on cleavage site, with 10 bp in either direction. For comparison, the portion of the gRNA target sequence aligning with the Reference on-target sequence is underlined and the PAM is indicated by parenthesis.

^b Deletions indicated by dashes (-); insertions indicated by bold

5 ^c Positions of inserted bases in the gene edited sequence indicated by dashes (-) in the Reference Sequence

Table 16. On-Target Gene Edited Sequences > 1% Frequency in At Least One Gene Edited T Cell Donor for the TET2-exon3-T2 gRNA.

Reference on-target sequence ^a : CACACGACTATTC(TGG)CTTC (SEQ ID NO: 159)						
SEQ ID NO:	Gene Edited Sequence ^b	Reference Sequence ^c (SEQ ID NO: 159)	Donor 1 (%)	Donor 2 (%)	Mean (%)	Std. Dev. (%)
160	CACACGACT-----GGCTTC	CACACGACTATTCCTGGCTTC	14.1	14.7	14.4	0.4
161	CACACGACT-----TC	CACACGACTATTCCTGGCTTC	11.5	11.8	11.6	0.2
162	CACACGACT--TCTGGCTTC	CACACGACTATTCCTGGCTTC	5.1	3.4	4.2	1.2
163	CACACGACTAT-CTGGCTTC	CACACGACTATTCCTGGCTTC	2.9	3.7	3.3	0.6
164	CACACGACTAATTCTGGCTTC	CACACGACTA-TTCTGGCTTC	4.1	2.5	3.3	1.1
165	CACACGACTATTCTGGCTTC	CACACGACTATT-CTGGCTTC	3	3.4	3.2	0.3
166	CACACGACT-TTCTGGCTTC	CACACGACTATTCCTGGCTTC	3.4	2.2	2.8	0.8
	CACACGACT-----	CACACGACTATTCCTGGCTTC	2.9	2.2	2.6	0.5
	-----	CACACGACTATTCCTGGCTTC	2.4	2.7	2.6	0.2
	-----CTTC	CACACGACTATTCCTGGCTTC	1.5	1.6	1.6	0.1
	CACAC-----	CACACGACTATTCCTGGCTTC	1.6	1.5	1.6	0.1
167	CACACG-----TTCTGGCTTC	CACACGACTATTCCTGGCTTC	1.8	1	1.4	0.6
	CAC-----	CACACGACTATTCCTGGCTTC	1.3	1.4	1.4	0.1
	-----TTCTGGCTTC	CACACGACTATTCCTGGCTTC	1.5	1.4	1.4	0.1
	C-----	CACACGACTATTCCTGGCTTC	1.4	1	1.2	0.3
168	CACAC-----TGGCTTC	CACACGACTATTCCTGGCTTC	1.1	1.2	1.2	0.1
	CACACG-----GCTTC	CACACGACTATTCCTGGCTTC	1.2	1	1.1	0.1
	-----TC	CACACGACTATTCCTGGCTTC	1.1	1.1	1.1	0
169	CACACGACTA--CTGGCTTC	CACACGACTATTCCTGGCTTC	1.1	1	1	0.1
	-----GGCTTC	CACACGACTATTCCTGGCTTC	1.2	0.7	1	0.4
	CACACGAC-----	CACACGACTATTCCTGGCTTC	0.7	1.1	0.9	0.3
170	CACACGACT--CTGGCTTC	CACACGACTATTCCTGGCTTC	1.2	0.6	0.9	0.4

10 ^a On-target sequence centered on cleavage site, with 10 bp in either direction. For comparison, the portion of the gRNA target sequence aligning with the Reference on-target sequence is underlined and the PAM is indicated by parenthesis.

^b Deletions indicated by dashes (-); insertions indicated by bold

^c Positions of inserted bases in the gene edited sequence indicated by dashes (-) in the Reference Sequence

15

Table 17. On-Target Gene Edited Sequences > 1% Frequency in At Least One Gene Edited T Cell Donor for the TET2-exon3-T3 gRNA.

Reference on-target sequence ^a : TGGTGAAAACGAG(GGG)CCTT (SEQ ID NO: 171)						
SEQ ID NO:	Gene Edited Sequence ^b	Reference Sequence ^c (SEQ ID NO: 171)	Donor 1 (%)	Donor 2 (%)	Mean (%)	Std. Dev. (%)

172	TGGTGAAC CC GAGGGGCCTT	TGGTGAAC-GAGGGGCCTT	17.3	19.9	18.6	1.8
	TGG-----CCTT	TGGTGAACGAGGGGCCTT	3.9	4	4	0.1
	-----	TGGTGAACGAGGGGCCTT	3.8	3.7	3.8	0.1
	-----CTT	TGGTGAACGAGGGGCCTT	3.2	2.7	3	0.4
	TGG-----GGCCTT	TGGTGAACGAGGGGCCTT	3.3	2.7	3	0.4
173	TGGTGA-----GGGGCCTT	TGGTGAACGAGGGGCCTT	3.6	2.3	3	0.9
174	TGGTGAAC-CGAGGGGCCTT	TGGTGAACGAGGGGCCTT	3.5	2.3	2.9	0.8
	T-----	TGGTGAACGAGGGGCCTT	2.2	2.3	2.2	0.1
	TGG-----GCCTT	TGGTGAACGAGGGGCCTT	1.7	1.6	1.6	0.1
	TGGTGAA-----	TGGTGAACGAGGGGCCTT	1	1.2	1.1	0.1
	TGGTGAA-----	TGGTGAACGAGGGGCCTT	1.3	0.9	1.1	0.3
175	TGGTGAA--CGAGGGGCCTT	TGGTGAACGAGGGGCCTT	1.1	0.9	1	0.1
	-----CCTT	TGGTGAACGAGGGGCCTT	1.2	0.8	1	0.3
	-----TT	TGGTGAACGAGGGGCCTT	0.7	1.1	0.9	0.3

^a On-target sequence centered on cleavage site, with 10 bp in either direction. For comparison, the portion of the gRNA target sequence aligning with the Reference on-target sequence is underlined and the PAM is indicated by parenthesis.

^b Deletions indicated by dashes (-); insertions indicated by bold

5 ^c Positions of inserted bases in the gene edited sequence indicated by dashes (-) in the Reference Sequence

Table 18. On-Target Gene Edited Sequences > 1% Frequency in At Least One Gene Edited T Cell donor for the TET2-exon6-BG5 gRNA.

10

Reference on-target sequence ^a : <u>GCTCACC</u> AAATCGC(CGG)TGTG (SEQ ID NO: 176)						
SEQ ID NO:	Gene Edited Sequence ^b	Reference Sequence ^c (SEQ ID NO: 176)	Donor 1 (%)	Donor 2 (%)	Mean (%)	Std. Dev. (%)
	-----CGCCGGTGTG	GCTCACC ^a AAATCGCCGGTGTG	20.8	22	21.4	0.8
177	GCTCACC ^a AA-CGCCGGTGTG	GCTCACC ^a AAATCGCCGGTGTG	7.3	8.2	7.8	0.6
178	GCTCACC ^a AA T CGCCGGTGTG	GCTCACC ^a AA-TCGCCGGTGTG	6.1	7.4	6.8	0.9
179	GCTCACC ^a AA-CGCCGGTGTG	GCTCACC ^a AAATCGCCGGTGTG	4.3	5.6	4.9	0.9
	-----CGGTGTG	GCTCACC ^a AAATCGCCGGTGTG	5.8	3.5	4.6	1.6
180	GCTCA-----CCGGTGTG	GCTCACC ^a AAATCGCCGGTGTG	3.6	3.8	3.7	0.1
181	GCTCACC---CGCCGGTGTG	GCTCACC ^a AAATCGCCGGTGTG	2.3	2.2	2.2	0.1
	-----GCCGGTGTG	GCTCACC ^a AAATCGCCGGTGTG	2.2	1.7	2	0.4
182	GCTCAC----CGCCGGTGTG	GCTCACC ^a AAATCGCCGGTGTG	2.3	1.5	1.9	0.6
	-----G	GCTCACC ^a AAATCGCCGGTGTG	2.1	1.4	1.8	0.5
183	GCTCACC ^a AA T CGCCGGTGTG	GCTCACC ^a AAAT-CGCCGGTGTG	2	1.4	1.7	0.4
184	GCTCA-----CGCCGGTGTG	GCTCACC ^a AAATCGCCGGTGTG	1.3	1.8	1.6	0.4
185	GCTCACC ^a AA T CGCCGGTGTG	GCTCACC ^a AAAT-CGCCGGTGTG	1.1	1.9	1.5	0.6
	-----	GCTCACC ^a AAATCGCCGGTGTG	2	0.9	1.4	0.8
	GCTCA-----	GCTCACC ^a AAATCGCCGGTGTG	1.2	0.8	1	0.3
186	GCTCACC ^a AA T CGCCGGTGTG	GCTCACC ^a AAAT-CGCCGGTGTG	1.2	0.5	0.8	0.5
187	GCTCACC ^a AA TCAAGGC ACGC CGGTGTG	GCTCACC ^a AAAT-----CGCCGGTGTG	1.1	0	0.6	0.8

^a On-target sequence centered on cleavage site, with 10 bp in either direction. For comparison, the portion of the gRNA target sequence aligning with the Reference on-target sequence is underlined and the PAM is indicated by parenthesis.

^b Deletions indicated by dashes (-); insertions indicated by bold

^c Positions of inserted bases in the gene edited sequence indicated by dashes (-) in the Reference Sequence

Table 19. On-Target Gene Edited Sequences > 1% Frequency in At Least One Gene Edited T Cell Donor for the TET2-exon5-T1 gRNA.

5

Reference on-target sequence ^a : CCTATTGCTAAGT(GGG)TAAG (SEQ ID NO: 188)						
SEQ ID NO:	Gene Edited Sequence ^b	Reference Sequence ^c (SEQ ID NO: 188)	Donor 1 (%)	Donor 2 (%)	Mean (%)	Std. Dev. (%)
189	CCTATTGCT-AGTGGGTAAG	CCTATTGCTAAGTGGGTAAG	10.1	9.6	9.8	0.4
190	CCTATTGCT AA AGTGGGTAAG	CCTATTGCT-AAGTGGGTAAG	7.4	9.3	8.4	1.3
191	CCTAT-----TGGGTAAG	CCTATTGCTAAGTGGGTAAG	6.9	6	6.4	0.6
	-----	CCTATTGCTAAGTGGGTAAG	4.1	5.8	4.9	1.2
	-----AGTGGGTAAG	CCTATTGCTAAGTGGGTAAG	4	4.8	4.4	0.6
	-----GGGTAAG	CCTATTGCTAAGTGGGTAAG	3.7	2.2	3	1.1
192	CCTATTGC--AGTGGGTAAG	CCTATTGCTAAGTGGGTAAG	2.3	1.8	2	0.4
	-----GTAAG	CCTATTGCTAAGTGGGTAAG	1.9	1.9	1.9	0
	CCTATTGCTA-----	CCTATTGCTAAGTGGGTAAG	1.1	2	1.6	0.6
193	C-----AGTGGGTAAG	CCTATTGCTAAGTGGGTAAG	1.3	1.8	1.6	0.4
	-----GGTAAG	CCTATTGCTAAGTGGGTAAG	1.4	1.5	1.4	0.1
194	CCTATTGC-----TAAG	CCTATTGCTAAGTGGGTAAG	1.5	1.4	1.4	0.1
195	CCTATTGCTA T AGTGGGTAAG	CCTATTGCTA-AGTGGGTAAG	1.4	1.3	1.4	0.1
	CCTAT-----	CCTATTGCTAAGTGGGTAAG	1.1	1.5	1.3	0.3
196	CCTATTG---AGTGGGTAAG	CCTATTGCTAAGTGGGTAAG	1.2	1.2	1.2	0
	-----TGGGTAAG	CCTATTGCTAAGTGGGTAAG	0.9	1.4	1.2	0.4
197	CC-----AGTGGGTAAG	CCTATTGCTAAGTGGGTAAG	1.2	1.2	1.2	0
	-----GTGGGTAAG	CCTATTGCTAAGTGGGTAAG	1.8	0.7	1.2	0.8
198	CCTATT-----GTGGGTAAG	CCTATTGCTAAGTGGGTAAG	1.5	0.8	1.2	0.5
199	CCT-----AGTGGGTAAG	CCTATTGCTAAGTGGGTAAG	1	1.3	1.2	0.2
200	CCTAT-----AGTGGGTAAG	CCTATTGCTAAGTGGGTAAG	1.2	1	1.1	0.1
	CCTA-----	CCTATTGCTAAGTGGGTAAG	0.7	1.2	1	0.4
201	CCTATTGCTA--TGGGTAAG	CCTATTGCTAAGTGGGTAAG	1.3	0.7	1	0.4
	CC-----TAAG	CCTATTGCTAAGTGGGTAAG	0.9	1.2	1	0.2
202	CCTATTGCTA C AGTGGGTAAG	CCTATTGCTA-AGTGGGTAAG	0.9	1.1	1	0.1
	-----AAGTGGGTAAG	CCTATTGCTAAGTGGGTAAG	1.1	0.8	1	0.2
203	CCTATTGCTA-----TAAG	CCTATTGCTAAGTGGGTAAG	1.2	0.5	0.8	0.5

^a On-target sequence centered on cleavage site, with 10 bp in either direction. For comparison, the portion of the gRNA target sequence aligning with the Reference on-target sequence is underlined and the PAM is indicated by parenthesis.

^b Deletions indicated by dashes (-); insertions indicated by bold

10 ^c Positions of inserted bases in the gene edited sequence indicated by dashes (-) in the Reference Sequence

Table 20. On-Target Gene Edited Sequences > 1% Frequency in At Least One Gene Edited T Cell Donor for the TET2-exon5-T2 gRNA.

15

Reference on-target sequence ^a : TCCTATTGCTAAG(TGG)GTAA (SEQ ID NO: 204)						
SEQ ID NO:	Gene Edited Sequence ^b	Reference Sequence ^c (SEQ ID NO: 204)	Donor 1 (%)	Donor 2 (%)	Mean (%)	Std. Dev. (%)

205	TCCTATTGCTTAAGTGGGTAA	TCCTATTGC-TAAGTGGGTAA	60.7	67.1	63.9	4.5
206	TCCTAT---TAAGTGGGTAA	TCCTATTGCTAAGTGGGTAA	2.5	1.7	2.1	0.6
	-----GGGTAA	TCCTATTGCTAAGTGGGTAA	2.6	1.5	2	0.8
207	TCCTATTGC-AAGTGGGTAA	TCCTATTGCTAAGTGGGTAA	2.1	1.5	1.8	0.4
	-----	TCCTATTGCTAAGTGGGTAA	1.9	1.8	1.8	0.1
208	TCCTATTG-TAAGTGGGTAA	TCCTATTGCTAAGTGGGTAA	1.6	1.5	1.6	0.1
209	TCCTAT-----TGGGTAA	TCCTATTGCTAAGTGGGTAA	1.7	1	1.4	0.5

^a On-target sequence centered on cleavage site, with 10 bp in either direction. For comparison, the portion of the gRNA target sequence aligning with the Reference on-target sequence is underlined and the PAM is indicated by parenthesis.

^b Deletions indicated by dashes (-); insertions indicated by bold

5 ^c Positions of inserted bases in the gene edited sequence indicated by dashes (-) in the Reference Sequence

Table 21. On-Target Gene Edited Sequences > 1% Frequency in At Least One Gene Edited T Cell Donor for the TET2-exon4-BG4 gRNA.

Reference on-target sequence ^a : <u>ACCTGCTCCTAG</u> (TGG)GTAT (SEQ ID NO: 210)						
SEQ ID NO:	Gene Edited Sequence ^b	Reference Sequence ^c (SEQ ID NO: 210)	Donor 1 (%)	Donor 2 (%)	Mean (%)	Std. Dev. (%)
211	ACCTGCTCCTTAGATGGGTAT	ACCTGCTCCT-AGATGGGTAT	48.4	51.3	49.8	2.1
	ACCT-----	ACCTGCTCCTAGATGGGTAT	8.6	6.4	7.5	1.6
212	ACCTGCTCC-AGATGGGTAT	ACCTGCTCCTAGATGGGTAT	3.8	3.3	3.6	0.4
213	ACCTGCT---AGATGGGTAT	ACCTGCTCCTAGATGGGTAT	2.7	3.5	3.1	0.6
	ACCTG-----GGTAT	ACCTGCTCCTAGATGGGTAT	2.4	2.2	2.3	0.1
	-----	ACCTGCTCCTAGATGGGTAT	2.2	2	2.1	0.1
214	ACCTGCTC-TAGATGGGTAT	ACCTGCTCCTAGATGGGTAT	1.7	1	1.4	0.5
	A-----TGGGTAT	ACCTGCTCCTAGATGGGTAT	1.2	1.3	1.2	0.1
215	ACCTG----TAGATGGGTAT	ACCTGCTCCTAGATGGGTAT	1.5	0.7	1.1	0.6
216	ACCTGCTCCTA--TGGGTAT	ACCTGCTCCTAGATGGGTAT	1.1	0.7	0.9	0.3

10 ^a On-target sequence centered on cleavage site, with 10 bp in either direction. For comparison, the portion of the gRNA target sequence aligning with the Reference on-target sequence is underlined and the PAM is indicated by parenthesis.

^b Deletions indicated by dashes (-); insertions indicated by bold

^c Positions of inserted bases in the gene edited sequence indicated by dashes (-) in the Reference Sequence

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Example 8. TET2 Knock-Out in Anti-BCMA CAR-T Cells Confer Growth Advantage and CAR-T Enrichment

Chimeric antigen receptor (CAR) T-cell therapy uses genetically modified T cells to target and kill cancer cells more specifically and efficiently. After T cells have been collected from the blood, the cells are engineered to include CARs on their surface. The CARs may be introduced into the T cells using CRISPR/Cas9 gene editing technology. When these allogeneic CAR T cells are injected into a patient, the receptors enable the T cells to kill cancer cells.

This example further explores advantageous features arising from TET2 knock-out via gene editing in CAR-T cells, for example, with multiple rounds of target antigen

stimulation.

(I) Generating TET2 KO (TET2⁻) or TET2 WT (TET2⁺) anti-BCMA CAR-T cells

Activated primary human T cells were electroporated (EP1) with Cas9: TET2 sgRNA 1 RNP complex to generate a polyclonal TET2 KO population. 72 hours later these cells or wild type (WT) activated primary T cells were electroporated again (EP2) with Cas9: gRNA RNP complexes (RNPs) and adeno-associated adenoviral vectors (AAVs) to generate TET2-/TRAC- β 2M-/anti-BCMA CAR+ (TET2-/anti-BCMA CAR) or TRAC- β 2M-/anti-BCMA CAR+ T cells (TET2+/anti-BCMA CAR). Two control groups were generated where either AAV or RNP were excluded in EP2 (TET2-/AAV- and TET2-/RNP-, respectively).

Recombinant AAV serotype 6 (AAV6) comprising one of the nucleotide sequences encoding an anti-BCMA CAR (SEQ ID NO: 149) were delivered with Cas9:sgRNA RNPs (1 μ M Cas9, 5 μ M gRNA) to activated allogeneic human T cells. The following sgRNAs were used: TET2 (SEQ ID NO: 13), TRAC (SEQ ID NO: 93) and β 2M (SEQ ID NO: 97).

About one (1) week post electroporation (EP1), cells were assessed for TET2 protein knockdown by automated Western blot (WesTM, Proteinsimple) using anti-TET2 polyclonal antibody (Diagenode #C15410255-100) at 1:500 dilution. The results obtained from this assay confirm no detection of TET2 in TET2 KO (TET2⁻) anti-BCMA CAR-T cells, while TET2 expression was detected in control cells and in TET2 WT (TET2⁺) anti-BCMA CAR-T cells.

On Day 9 after EP2 the cells were also processed for flow cytometry to assess TRAC and β 2M knockout levels, and anti-BCMA CAR expression levels at the cell surface of the edited cell population. The cells were stained with a panel of antibodies indicated in **Table 22** below.

Table 22. Antibody Panel for Detecting CAR-T Cell Surface Proteins

Antibody	Source	Fluor	Dilution
IgG, F(ab') ₂ fragment specific	Jackson Immuno-research, #109-006-097	Biotinylated; detected with SA-APC	1:20
TCR $\alpha\beta$	Miltenyi, #130-099-661	PE	1:100
β 2M	Biolegend, #316318	PECy7	1:100
CD8	Biolegend, #344742	BV605	1:100
CD4	Biolegend, #300546	BV510	1:100
Streptavidin-APC (SA-APC)	ThermoFisher (eBioscience), #17-	APC	1:100

	4317-82		
7-AAD	BD Biosciences, 559925	PerCP range	1:500

The results are shown in **Table 23** below. For all anti-BCMA CAR-T cells and TRAC-/β2M- control cells (TET2-/AAV-), >85% of viable cells lacked expression of TCR and >74% lacked expression of β2M, with >72% of anti-BCMA CAR-T cell populations being both TRAC-/B2M-. Live CAR-T cells were gated by their forward scatter (FSC) and side scatter (SSC) profiles, and with 7-AAD dye.

Table 23. Percent positive populations of surface anti-BCMA CAR expression and TRAC/B2M knockdown 9 days after EP2

Sample	% BCMA CAR positive	TRAC -	B2M -	TRAC-B2M-
TET2-/anti-BCMA CAR-T	34.80%	89.50%	81.90%	74.70%
TET2+/anti-BCMA CAR-T	41.30%	85.00%	83.20%	72.30%
TET2-/AAV-	0.11%	84.90%	74.70%	63.80%
TET2-/RNP-	0.62%	11.27%	0.18%	0.07%

(II). Antigen stimulation of TET2 KO (TET2-) or TET2 WT (TET2+) anti-BCMA CAR-T cells

The different cell populations were expanded for a week after determining CAR expression. 1 million cells of each CAR-T population (TET2-/anti-BCMA CAR-T, TET2+/anti-BCMA CAR-T, TET2-/AAV- and TET2-/RNP-) were then co-cultured with target cell line MM.1S at a 1:1 E:T ratio, in triplicate, in a 12-well tissue culture plate in full T cell media, and incubated at 37 °C for 48 hours (stimulation round 1) or 72 hours (stimulation rounds 2 & 3), a sufficient time period previously established to ensure all target cells were killed by the cocultured CAR-T cells. A total of 3 rounds of stimulation were performed where, after each stimulation CAR-T cells were collected, washed, counted with Trypan Blue (for viability assessment) and replated at 1 million cells/well with fresh target cells at an E:T of 1:1, in triplicate. Additionally, a portion of the cells were evaluated for CAR-T expression by FACS using an antibody panel indicated in **Table 24** below. After 3 rounds of stimulation, CAR-T cells were collected and expanded to monitor for growth and % viability, as well as periodic FACS evaluation of surface CAR expression using the antibody panel indicated in **Table 24**.

Table 24. Antibody Panel for Detecting CAR-T Cell Surface Proteins

Antibody	Source	Fluor	Dilution
Anti-BCMA CAR	US63/069889	APC	1:100
CD8	BioLegend, #344742	BV605	1:100
CD4	BioLegend, #300546	BV510	1:100
CD57	BioLegend, #322306	FITC	1:100
Lag3	BioLegend, #369310	PE-Cy7	1:100
CD45RA	BioLegend, #304128	APC-Cy7	1:100
CCR7	BioLegend, #353210	PacBlue	1:100
PD1	BioLegend, #329906	PE	1:100
7-AAD	BD Biosciences, #559925	PerCP range	1:500

The results of extended culture of these anti-BCMA CAR-T populations indicate that knocking out TET2 in T cells confers a proliferative advantage to CAR-T cells that have been stimulated with a target cell line (**FIG. 8A**), without a significant difference in overall viability (**FIG. 8B**). Additionally, a preferential enrichment of BCMA CAR-T positive cells in the TET2 KO cells was observed as compared to TET2+/anti-BCMA CAR-T cells or non-stimulated cells (**FIG. 8C**).

(III). Comparison of functional features between TET2 KO anti-BCMA CAR-T cells (TET2-/anti-BCMA CAR T) and TET2 WT anti-BCMA CAR-T cells (TET2+/anti-BCMA CAR T)

Functional activity of anti-BCMA CAR T cells (evidenced by effector cytokine secretion) was assessed using cytokine release assays for Interferon gamma (IFN γ) after each round of target cell stimulation. After above-mentioned periods of coculture, supernatant media from the co-cultured cells were collected and the levels of IFN γ was measured using an ELISA (RD Systems) following the manufacturer's instructions. The MILLIPLEX kit (Millipore, catalog # HCYTOMAG-60K) using magnetic microspheres, HCYIFNG-MAG (Millipore, catalog # HCYIFNG-MAG) was used to quantify IFN- γ secretion in samples from the cocultured stimulation assay. The assay was conducted following manufacturer's protocol.

In short, MILLIPLEX[®] standard and quality control (QC) samples were reconstituted, and serial dilutions of the working standards from 10,000 pg/mL to 3.2 pg/mL were prepared. MILLIPLEX[®] standards, QCs and cell supernatants were added to each plate, and assay

media was used to dilute the supernatants. All samples were incubated with HCYIFNG-MAG beads for 2 hours. After incubation, the plate was washed using an automated magnetic plate washer. Human cytokine/chemokine detection antibody solution was added to each well and incubated for 1 hour followed by incubation with Streptavidin-Phycoerythrin for 30 minutes.

5 The plate was subsequently washed, samples were resuspended with 150 μ L Sheath Fluid, and agitated on a plate shaker for 5 minutes. The samples were read using the Luminex[®] 100/200[™] instrument with xPONENT[®] software and data acquisition and analysis was completed using MILLIPLEX[®] Analyst software. The Median Fluorescent Intensity (MFI) data is automatically analyzed using a 5-parameter logistic curve-fitting method for

10 calculating the cytokine concentration measured in the unknown samples.

Results showed that there was no significant difference in IFN γ secretion in anti-BCMA CAR-T cells with or without TET2 KO after multiple rounds of target cell line stimulation under these conditions (**FIG. 8D**). The control cells TCR- β 2M- (AAV negative) and non-CAR edited (RNP negative) showed no significant or specific IFN γ secretory

15 response in the presence of MM1S cells.

In summary, the studies described above show that: (a) TET2 KO anti-BCMA CAR-T cells (TET2-/anti-BCMA CAR T) showed a growth advantage over TET2 WT anti-BCMA CAR T cells (TET2+/anti-BCMA CAR T) after MM1S antigen stimulation; (b) TET2 KO anti-BCMA CAR-T cells showed an enrichment for anti-BCMA CAR positive cells over

20 TET2 WT anti-BCMA CAR T cells post antigen stimulation; and (c) TET2 KO did not alter anti-BCMA CAR-T functional activity (IFN γ secretion) compared to TET2 WT anti-BCMA CAR T cells with multiple antigen stimulation. These results have demonstrated that engineering a TET2 knockout in CAR-T cells (using anti-BCMA CAR-T cells as an example here) confers both a growth advantage and CAR-T enrichment effect. This provides proof of

25 concept evidence that boosting CAR-T cell proliferation *in vitro* by gene editing is feasible and can be used to generate a cell bank capable of higher CAR-T yield without compromising function.

Example 9. On-Target and Off-Target Editing of FAS Guide RNAs

30 On-target and off-target editing efficiencies of various FAS-targeting gRNAs were examined for the three guides exhibiting the highest gene editing efficiency FAS-ex2-T2, FAS-ex3_T1, FAS-ex3_T2 (see **FIG. 2A**). Briefly, activated T cells derived from primary human PBMC cells were transfected (electroporated) with a ribonucleoprotein particle (RNP) containing Cas9 nuclease and a synthetic modified sgRNA targeting the FAS gene

(sequences in **Table 5** above) or prepared as untreated controls (no electroporation, no Cas9, no gRNA).

For genomic on- and off-target assessment, the same electroporation methods were used to generate two cell populations of edited cells from two different donor T cells (termed 1 and 2). Cells were gene edited with three of the top guides listed in **Table 5**, and then collected ten (10) days post transfection. These samples were analyzed with hybrid capture, a homology-dependent method to enrich on- and off-target sites, combined with next-generation sequencing. Briefly, on- and off-target sites with homology to each gRNA target site were identified computationally, single-stranded RNA probes were used to enrich these sites from bulk genomic DNA, these enriched sites were sequenced with next-generation sequencing, and the data were analyzed for insertions and deletions indicating repair following CRISPR editing.

The results are provided in **Table 25** below.

Table 25. On and Off Target Results by Hybrid Capture

Guide	gRNA target sequence + (PAM)	Number of predicted off target sites tested	On-target mean editing hyb cap (%) ^a	Detected off-targets
FAS-ex2_T2	CACTTGGGCATTAACTTT (TGG) (SEQ ID NO: 88)	172	98.6	One intergenic 1.1%, one 0.6% off-target
FAS-ex3_T1	CTAGGGACTGCACAGTCAAT (GGG) (SEQ ID NO: 90)	146	95.3	None
FAS-ex3_T2	ACTGCGTGCCCTGCCAAGAA (GGG) (SEQ ID NO: 91)	111	99.2	None

^a Average across donors 1 and 2.

Analysis of on-target indel profiles in T cells

The data used to quantify off-target editing were also used to quantify and summarize the most frequent on-target indels for all FAS guides listed in **Table 25**. This data was generated from hybrid capture of the FAS locus combined with next-generation sequencing in two donors (termed Donor 1 and Donor 2).

Following gene editing, hybrid capture analysis of the FAS locus in a population of T cells following CRISPR/Cas9 gene editing to produce FAS - T cells results in specific indel frequencies and edited gene sequences at the FAS locus (**Tables 26-28**; deletions as dashes and insertions in bold).

For the purposes of individual sequence quantification from hybrid capture data, sequence reads aligning across the FAS on-target site, 20 bp upstream and downstream of the

cut site, were selected and considered for indel sequence quantification. From the selected reads, the sequence within 10 bp upstream and downstream of each putative cut site (~3bp upstream of the PAM (Jinek, et al., Science 2012) was quantified as a representative region of on-target non-homologous end joining (NHEJ) editing. The data on these on-target gene edited sequences is presented in the tables below, with the frequencies of these sequences representing the percent of all sequences spanning the on-target site within 20 bp upstream and downstream of each cut site. The indels for each guide are shown relative to an on-target reference sequence in Tables 22-24. The reference sequence is centered on the cleavage site with 10 bp in either direction, ending 4 bp 3' of the PAM.

10

Table 26. On-Target Gene Edited Sequences > 1% Frequency in At Least One Gene Edited T Cell Donor for the FAS-Exon2-T2 gRNA.

Reference on-target sequence ^a : GCATTAACACTTT (TGG)ACGA (SEQ ID NO:217)						
SEQ ID NO:	Gene Edited Sequence ^b	Reference Sequence ^c	Donor 1 (%)	Donor 2 (%)	Mean (%)	Std. Dev. (%)
218	GCATTAACACTTTT T GGACGA	GCATTAACACTTTT--GGACGA	43.3	40.4	41.8	2.1
219	GCATTAACACTTTT TT GGACGA	GCATTAACACTTTT--GGACGA	19.6	18.1	18.8	1.1
	-----ACGA	GCATTAACACTTTTGGACGA	3.4	5.4	4.4	1.4
220	GCATTAACA-TTTTGGACGA	GCATTAACACTTTTGGACGA	3.7	4.4	4.1	0.5
	-----	GCATTAACACTTTTGGACGA	2.1	2.3	2.2	0.1
	GCATTAA-----	GCATTAACACTTTTGGACGA	2.0	1.8	1.9	0.1
	GCA-----	GCATTAACACTTTTGGACGA	1.3	1.7	1.5	0.3
221	GCATTAACACTTT--GGACGA	GCATTAACACTTTTGGACGA	1.4	1.3	1.4	0.1
222	GCATTA----TTTGGACGA	GCATTAACACTTTTGGACGA	1.1	1.6	1.4	0.4
223	GCATTAACACTTTT TT GGACGA	GCATTAACACTTTT--GGACGA	1.4	1.1	1.2	0.2
224	GCATTAAC--TTTGGACGA	GCATTAACACTTTTGGACGA	1.0	1.2	1.1	0.1
	GCAT-----	GCATTAACACTTTTGGACGA	0.7	1.1	0.9	0.3

^a On-target sequence centered on cleavage site, with 10 bp in either direction. For comparison, the portion of the gRNA target sequence aligning with the Reference on-target sequence is underlined and the PAM is indicated by parenthesis.

^b Deletions indicated by dashes (-); insertions indicated by bold

^c Positions of inserted bases in the gene edited sequence indicated by dashes (-) in the Reference Sequence

Table 27. On-Target Gene Edited Sequences > 1% Frequency in At Least One Gene Edited T Cell Donor for the FAS-Exon3-T1 gRNA.

Reference on-target sequence ^a : CTGCACAGTCAAT (GGG)GATG (SEQ ID NO: 225)						
SEQ ID NO:	Gene Edited Sequence ^b	Reference Sequence ^c	Donor 1 (%)	Donor 2 (%)	Mean (%)	Std. Dev. (%)
	-----	CTGCACAGTCAATGGGGATG	24.9	19.1	22.0	4.1
226	CTGCACAGTCA A ATGGGGATG	CTGCACAGTC--AATGGGGATG	5.6	7.1	6.4	1.1
227	CTGCACAGT C CAATGGGGATG	CTGCACAGT--CAATGGGGATG	5.1	4.2	4.6	0.6

	-----GGGATG	CTGCACAGTCAATGGGGATG	4.1	4.5	4.3	0.3
228	CTGCA---CAATGGGGATG	CTGCACAGTCAATGGGGATG	3.8	4.0	3.9	0.1
229	CTGCACAGTC-ATGGGGATG	CTGCACAGTCAATGGGGATG	3.1	2.9	3.0	0.1
	CTGCA-----	CTGCACAGTCAATGGGGATG	2.8	3.2	3.0	0.3
230	CTGCACAGT-AATGGGGATG	CTGCACAGTCAATGGGGATG	2.5	3.3	2.9	0.6
231	CTGCACA---AATGGGGATG	CTGCACAGTCAATGGGGATG	2.6	2.9	2.8	0.2
232	CTG-----CAATGGGGATG	CTGCACAGTCAATGGGGATG	1.8	2.1	2.0	0.2
	C-----TG	CTGCACAGTCAATGGGGATG	1.9	2.0	2.0	0.1
	CTGC-----	CTGCACAGTCAATGGGGATG	1.8	1.7	1.8	0.1
233	CTGCACAG--AATGGGGATG	CTGCACAGTCAATGGGGATG	1.1	2.5	1.8	1.0
	-----G	CTGCACAGTCAATGGGGATG	1.5	1.8	1.6	0.2
	-----GGGGATG	CTGCACAGTCAATGGGGATG	1.2	1.8	1.5	0.4
234	CTGCACAGTC--TGGGGATG	CTGCACAGTCAATGGGGATG	1.4	1.3	1.4	0.1
	CTGCACAGT-----	CTGCACAGTCAATGGGGATG	1.0	1.5	1.2	0.4
235	CTGCACAGTC-----	CTGCACAGTCAATGGGGATG	1.0	1.4	1.2	0.3
236	-----AATGGGGATG	CTGCACAGTCAATGGGGATG	1.1	0.7	0.9	0.3

^a On-target sequence centered on cleavage site, with 10 bp in either direction. For comparison, the portion of the gRNA target sequence aligning with the Reference on-target sequence is underlined and the PAM is indicated by parenthesis.

^b Deletions indicated by dashes (-); insertions indicated by bold

5 ^c Positions of inserted bases in the gene edited sequence indicated by dashes (-) in the Reference Sequence

Table 28. On-Target Gene Edited Sequences > 1% Frequency in At Least One Gene Edited T Cell Donor for the FAS-Exon3-T2 gRNA.

Reference on-target sequence ^a : GCCCTGCCAAGAA (GGG) AAGG (SEQ ID NO: 237)						
SEQ ID NO:	Gene Edited Sequence ^b	Reference Sequence ^c	Donor 1 (%)	Donor 2 (%)	Mean (%)	Std. Dev. (%)
238	GCCCTGCC AA AAGGGAAGG	GCCCTGCC-AAGAAGGGAAGG	45.7	37.6	41.7	5.7
239	GCCCTGCC-AGAAGGGAAGG	GCCCTGCCAAGAAGGGAAGG	19.4	26.6	23.0	5.1
	-----	GCCCTGCCAAGAAGGGAAGG	9.6	9.2	9.4	0.3
	G-----	GCCCTGCCAAGAAGGGAAGG	1.1	1.4	1.2	0.2
240	GCCCTGCC AAA AAGGGAAGG	GCCCTGCC--AAGAAGGGAAGG	1.2	0.9	1.0	0.2
241	GCCCT----AGAAGGGAAGG	GCCCTGCCAAGAAGGGAAGG	0.5	1.1	0.8	0.4

10 ^a On-target sequence centered on cleavage site, with 10 bp in either direction. For comparison, the portion of the gRNA target sequence aligning with the Reference on-target sequence is underlined and the PAM is indicated by parenthesis.

^b Deletions indicated by dashes (-); insertions indicated by bold

^c Positions of inserted bases in the gene edited sequence indicated by dashes (-) in the Reference Sequence

15 Example 10. Derivation of an allogeneic human CD19 CAR-T cell line

Splenocytes were isolated from 3 mice that had been dosed with TRAC-/B2M-/FAS-/TET2-/CD70- anti CD19 CAR-expressing T cells (produced from a healthy human donor's peripheral blood T cells). These mice had controlled Nalm6 leukemia >3 months post infusion. The isolated splenocytes were cultured in human T cell media containing IL2/IL7 and human serum and outgrowths of cells were monitored. Cells from one of the mouse spleen isolates showed outgrowth over time (these cells are referred to as: isolated cells).

These isolated cells were >99% TRAC-/B2M-/CAR+ and had a CD4 phenotype along with high frequency indels at FAS, CD70, TET2 after 2 months of culture. More specifically, FACS analysis showed that 97.8% are live cells, 99.1% are TCR and B2M negative, 99.1% are CAR positive, and 99.8% are CD4 positive in the tested cell population.

5 The isolated cells maintained their dependency on human cytokines IL2 and IL7 (FIG. 9A). These cells also retain their ability to kill CD19+ cells (FIG. 9B) and further maintained cytotoxic and cytokine release functions *in vitro* (FIGs. 9C and 9D, respectively). The isolated cells were again reinjected into a Nalm6-leukemia model and showed efficacy comparable to a fresh lot of TRAC-/B2M-/anti-CD19 CAR T cells. (FIG. 9E).

10 Following this second round of *in vivo* testing with the isolated cells, cells were again isolated from 1 mouse and further cultured/expanded for several weeks in cytokine containing media. These re-isolated cells maintained the same phenotype as the cells from the initial *in vivo* isolation. The re-isolated cells are used in a second experiment to assess their ability to control Nalm6 leukemia.

15 This example suggests that persistent T cell lines can be produced from normal healthy donor (*e.g.*, human donor) derived peripheral blood T cells that maintain functionality *in vitro* and *in vivo* and have the potential to serve as a cell bank and/or therapeutic.

OTHER EMBODIMENTS

20 All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

25 From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the claims.

EQUIVALENTS

30 While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to

be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Inventive embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with “and/or” should be construed in the same FAShion, i.e., “one or more” of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally,
5 additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (i.e. “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or
10 “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase “at least one,” in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily
15 including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “at least one” refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least
20 one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one,
25 optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method
30 are recited.