

GENETICALLY ENGINEERED T CELLS WITH REGNASE-1 AND/OR TGFBR2 DISRUPTION HAVE IMPROVED FUNCTIONALITY AND PERSISTENCE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the filing dates of U.S. Provisional Application No. 63/082,357, filed September 23, 2020, U.S. Provisional Application No. 63/124,429, filed December 11, 2020, and U.S. Provisional Application No. 63/225,673, filed July 26, 2021. The entire contents of each of the prior applications are incorporated by reference herein.

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. 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 persistence of T cells in culture.

SUMMARY OF THE INVENTION

The present disclosure is based, at least in part, on the development of genetically edited T cells carrying a disrupted *Regnase 1* (*Reg1*) gene (e.g., “Reg1 Knockout T cells”), a disrupted *TGFBR2* gene (e.g., “TGFBR2 Knockout T cells”, or genetically edited T cells carrying both a disrupted *Reg1* gene and a disrupted *TGFBR2* gene, and effective methods of producing such genetically edited T cells *via* CRISPR/Cas-mediated gene editing using guide RNAs, for example, those targeting specific sites within the *Reg1* gene with high on-target editing efficiency and low off-target editing efficiency, and/or those targeting specific sites within the *TGFBR2* gene with high on-target editing efficiency and low off-target editing efficiency.

Such genetically engineered T cells exhibits at least one of the following advantageous features: (a) improved cell growth activity; (b) enhanced persistence; (c) reduced T cell exhaustion; (d) resistant to inhibitory effects induced by TGF- β ; (e) enhanced cell killing capacity; and (f) resistant to inhibitory effects by fibroblasts and/or inhibitory factors secreted

thereby.

The *Reg1* disrupted T cells, the *TGFBR2* disrupted T cells, or the *Reg1/TGFBR2* double disrupted T cells disclosed herein can further be genetically engineered to express a chimeric antigen receptor (CAR) targeting an antigen of interest, *e.g.*, an antigen associated with an undesired cell such as a cancer cell, and to comprise one or more additional disrupted genes, for example, *TRAC*, *β 2M*, *CD70*, or a combination thereof. The resultant CAR-expressing, *Reg1* disrupted T cells exhibit enhanced cytotoxic activity against target cells and anti-tumor activity as compared with CAR-T cells having a wild-type *Reg1* gene.

In one aspect, the present disclosure relates to a population of genetically engineered T cells, comprising: (i) a disrupted *Regnase-1* (*Reg1*) gene; and (ii) a disrupted Transforming Growth Factor Beta Receptor II (*TGFBR2*) gene; wherein the T cells are further engineered to express a chimeric antigen receptor (CAR). In another aspect, the present disclosure relates to a use of the population of genetically engineered T cells as described herein in the manufacture of a medicament for treating a cancer in a subject.

In another aspect, the present disclosure relates to a method for preparing the population of genetically engineered T cells having a disrupted *Reg1* gene and a disrupted *TGFBR2* gene, the method comprising: (a) providing a plurality of cells, which are T cells or precursor cells thereof; optionally wherein the plurality of T cells is a population of primary T cells of one or more human donors; (b) genetically editing the *Reg1* gene and the *TGFBR2* gene; and (c) producing the population of genetically engineered T cells having disrupted *Reg1* gene and the *TGFBR2* gene. In another aspect, the present disclosure relates to a guide RNA (gRNA) targeting a *Reg1* gene or a *TGFBR2* gene, which is set forth as described herein.

In some ~~aspect~~examples, the current disclosure is related to the development of genetically engineered CAR T cells that comprise a disrupted *Reg1* gene. The genetically engineered CAR T cells, in certain ~~aspect~~examples, are further genetically engineered to comprise a disrupted cluster of differentiation 70 (CD70) gene. In some ~~aspect~~examples, the CAR T cells described herein may express anti-CD70 CAR, anti-cluster of differentiation 19 (CD19) CAR or anti-B-cell maturation antigen (anti-BCMA) CAR.

The genetically edited T cells disclosed herein showed enhanced cell expansion, longevity and proliferation capacity in culture, enhanced potency (*e.g.*, enhanced cytotoxicity), and enhanced *CAR-T* efficacy in animal models (via, *e.g.*, longer persistence). Further, the genetically edited T cells showed cytokine-dependent growth, indicating safety. In addition,

disrupting both the *RegI* and *TGFBRII* genes exhibited synergistic effects in anti-tumor activity and CAR-T cell expansion and persistence as observed in animal models.

Accordingly, the present disclosure provides, in some aspectexamples, a population of genetically engineered T cells, comprising: (i) a disrupted *Regnase-1* (*RegI*) gene; and/or (ii) a disrupted Transforming Growth Factor Beta Receptor II (*TGFBRII*) gene. In some embodimentexamples, the population of genetically engineered T cells comprises (i). In some embodimentexamples, population of genetically engineered T cells comprises (ii). In other embodimentexamples, the population of genetically engineered T cells comprises both (i) and (ii). Any of the genetically engineered T cells may be further engineered to express a chimeric antigen receptor (CAR).

The population of genetically engineered T cells disclosed herein, as compared to non-engineered T cell counterparts, has one or more of the following features: (a) improved cell growth activity; (b) enhanced persistence; (c) reduced T cell exhaustion; (d) resistant to inhibitory effects induced by TGF- β ; (e) enhanced cell killing capacity; and (f) resistant to inhibitory effects by fibroblasts and/or inhibitory factors secreted thereby.

In some **embodimentexamples**, the disrupted *RegI* gene is genetically edited in exon 1, exon 2, exon 3, or exon 4. In some examples, the disrupted *RegI* gene is genetically edited in exon 2 and/or exon 4. Alternatively or in addition, the disrupted *TGFBR2* gene is genetically edited in exon 1, exon 2, exon 3, exon 4, or exon 5. In some examples, the disrupted *TGFBR2* gene is genetically edited in exon 4. In other examples, the disrupted *TGFBR2* gene is genetically edited in exon 5. The disrupted *RegI* gene, the disrupted *TGFBR2* gene, or both can be genetically edited by a CRISPR/Cas-mediated gene editing system.

In some instances, the CRISPR/Cas-mediated gene editing comprises a guide RNA (gRNA) targeting a site in the *RegI* gene that comprises a nucleotide sequence listed in **Table 22** (with or without PAM) (e.g., SEQ ID NO: 320, 322, 323, or 327, or the corresponding ones with PAM). For example, the gRNA targeting the *RegI* gene comprises a spacer that comprises the nucleotide sequence of listed in **Table 22** (e.g., SEQ ID NO: 24, 32, 36, or 52). In some examples, the disrupted *RegI* gene comprises a nucleotide sequence selected from those listed in **Tables 29-38** (e.g., **Table 31, 33, 34, or 38**).

In some instances, the CRISPR/Cas-mediated gene editing system comprises a guide RNA (gRNA) targeting a site in the *TGFBR2* gene that comprises a nucleotide sequence listed in **Table 39** (with or without PAM). For example, the gRNA targeting the *TGFBR2* gene comprises a spacer listed in **Table 39**, for example, having a nucleotide sequence of any one of SEQ ID NOs: 270, 302, 308, and 312. In some examples, the disrupted *TGFBR2* gene may comprise a nucleotide sequence selected from those listed in **Tables 40-48** (e.g., **Table 43**).

Any of the gRNAs disclosed herein may further comprise a scaffold sequence. For example, the gRNA targeting the *RegI* gene comprises any of the nucleotide sequences listed in **Table 22**. Examples include 22, 23, 30, 31, 34, 35, 50, and 51. Alternatively or in addition, the gRNA targeting the *TGFBR2* gene may comprise any of the nucleotide sequences provided in **Table 39**. Examples include SEQ ID NOs: 270, 271, 300, 301, 306, 307, 312, and 313.

Any of the populations of genetically engineered T cells disclosed herein may further comprise: (iii) a disrupted T cell receptor alpha chain constant region (*TRAC*) gene, (iv) a disrupted beta-2-microglobulin ($\beta 2M$) gene, (v) a disrupted *CD70* gene, or (vi) a combination of any of (iii)-(v). In some **embodimentexamples**, the T cells comprise a disrupted T cell receptor alpha chain constant region (*TRAC*) gene. Alternatively or in addition, the T cells comprise a disrupted beta-2-microglobulin ($\beta 2M$) gene. Any of the T cells disclosed herein may also comprise a disrupted *CD70* gene. In some examples, the disrupted *TRAC* gene, the disrupted $\beta 2M$ gene, and/or the disrupted *CD70* gene is genetically edited by one or more CRISPR/Cas-

mediated gene editing system

In some [embodimentexamples](#), the genetically engineered T cells may comprise a nucleic acid encoding the CAR, and wherein the nucleic acid is inserted in the genome of the T cells. In some instances, the nucleic acid encoding the CAR is inserted in the disrupted *RegI* gene, the disrupted *TGFBR2* gene, the disrupted *TRAC* gene, the disrupted $\beta 2M$, or the disrupted *CD70* gene. In some examples, the nucleic acid encoding the CAR is inserted in the disrupted *TRAC* gene. In specific examples, the nucleic acid encoding the CAR may replace the deleted fragment comprising SEQ ID NO: 69 in the *TRAC* gene. In some examples, the disrupted *RegI* gene may comprise a nucleotide sequence listed in **Tables 29-38** (e.g., **Table 31, 33, 34, or 38**). In some examples, the disrupted *TGFBR2* gene may comprise a nucleotide sequence selected from those listed in **Tables 40-48** (e.g., **Table 43**). In some examples, the disrupted *TRAC* gene may comprise a nucleotide sequence of any one of SEQ ID NOs: 75-82 (see **Table 24**). In some examples, the disrupted $\beta 2M$ may comprise a nucleotide sequence of any one of SEQ ID NOs: 83-88 (see **Table 25**). In some examples, the disrupted *CD70* gene may comprise a nucleotide sequence of any one of SEQ ID NOs: 89-94 (see **Table 26**).

Any of the CAR constructs disclosed herein may comprise an extracellular antigen binding domain specific to a tumor antigen, a co-stimulatory signaling domain of 4-1BB or CD28, and a cytoplasmic signaling domain of CD3 ζ . In some examples, the tumor antigen is CD19. In some examples, the tumor antigen is BCMA. In some examples, the tumor antigen is CD70. In some examples, the tumor antigen is CD33. In some examples, the tumor antigen is PTK7.

In some [embodimentexamples](#), the CAR binds CD19 (anti-CD19 CAR). The extracellular antigen binding domain in the anti-CD19 CAR can be a single chain variable fragment (scFv) that binds CD19 (anti-CD19 scFv). In some instances, the anti-CD19 scFv may comprise (i) a heavy chain variable region (V_H) that comprises the same heavy chain complementary determining regions (CDRs) as those in SEQ ID NO: 124; and (ii) a light chain variable region (V_L) that comprises the same light chain CDRs as those in SEQ ID NO: 125. In some examples, the V_H comprises the amino acid sequence of SEQ ID NO: 124 and the V_L comprises the amino acid sequence of SEQ ID NO: 125. In one example, the anti-CD19 scFv comprises the amino acid sequence of SEQ ID NO: 120. In one specific example, the anti-CD19 CAR comprises the amino acid sequence of SEQ ID NO: 118 (with an N-terminal signal peptide) or SEQ ID NO: 353 (without N-terminal signal peptide).

In some [embodimentexamples](#), the CAR binds CD70 (anti-CD70 CAR). The extracellular

antigen binding domain in the anti-CD70 CAR can be a single chain variable fragment (scFv) that binds CD70 (anti-CD70 scFv). In some instances, the anti-CD70 scFv comprises (i) a heavy chain variable region (V_H) that comprises the same heavy chain complementary determining regions (CDRs) as those in SEQ ID NO: 143; and (ii) a light chain variable region (V_L) that comprises the same light chain CDRs as those in SEQ ID NO: 144. In some examples, the V_H comprises the amino acid sequence of SEQ ID NO: 143 and the V_L comprises the amino acid sequence of SEQ ID NO: 144. In one example, the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 140 or 142. In one specific example, the anti-CD70 CAR comprises the amino acid sequence of SEQ ID NO: 138 (with an N-terminal signal peptide) or SEQ ID NO:354 (without N-terminal signal peptide)..

In some **embodimentexamples**, the CAR binds BCMA (anti-BCMA CAR). The extracellular antigen binding domain in the anti-BCMA CAR can be a single chain variable fragment (scFv) that binds BCMA (anti-BCMA CAR). In some instances, the anti-BCMA scFv comprises (i) a heavy chain variable region (V_H) that comprises the same heavy chain complementary determining regions (CDRs) as those in SEQ ID NO: 149; and (ii) a light chain variable region (V_L) that comprises the same light chain CDRs as those in SEQ ID NO: 150. In some examples, the V_H comprises the amino acid sequence of SEQ ID NO: 149 and the V_L comprises the amino acid sequence of SEQ ID NO: 150. In one example, the anti-BCMA scFv comprises the amino acid sequence of SEQ ID NO: 148. In one specific example, the anti-BCMA CAR comprises the amino acid sequence of SEQ ID NO: 146 (with an N-terminal signal peptide) or SEQ ID NO:355 (without N-terminal signal peptide).

The genetically engineered T cells disclosed herein may be derived from primary T cells of one or more human donors. In some instances, the genetically engineered T cells show cytokine-dependent growth.

In other **aspectexamples**, the present disclosure provides a method for preparing any of the populations of genetically engineered T cells disclosed herein. In some instances, the method may comprise: (a) providing a plurality of cells, which are T cells or precursor cells thereof; (b) genetically editing the *RegI* gene and/or the *TGFBR2* gene; and (c) producing the population of genetically engineered T cells having disrupted *RegI* gene and/or the *TGFBR2* gene. In some examples, the T cells of step (a) are derived from primary T cells of one or more human donors. In some examples, step (b) comprises genetically editing the *RegI* gene. In some examples, step (b) comprises genetically editing the *TGFBR2* gene. In some examples, step (b) comprises genetically editing both the *RegI* gene and the *TGFBR2* gene.

In some **embodimentexamples**, step (b) is performed by one or more CRISPR/Cas-mediated gene editing systems. For example, step (b) can be performed by delivering to the plurality of cells an RNA-guided nuclease and a gRNA targeting the *RegI* gene. In some instances, the gRNA targeting the *RegI* gene may be specific to an exon of the *RegI* gene, e.g., exon 2 or exon 4. In some examples, the gRNA targeting the *RegI* gene comprises a spacer that comprises a nucleotide sequence listed in **Table 22** (e.g., SEQ ID NO: 24, 32, 36, or 52).

Alternatively or in addition, step (b) can be performed, *inter alia*, by delivering to the plurality of cells an RNA-guided nuclease and a gRNA targeting the *TGFBR2* gene. For example, the gRNA targeting the *TGFBR2* gene may be specific to an exon of the *TGFBR2* gene, e.g., exon 1, exon 2, exon 3, exon 4, and exon 5. In one example, the gRNA targeting the *TGFBR2* gene is specific to exon 4. In another example, the gRNA targeting the *TGFBR2* gene is specific to exon 5. In some instances, the gRNA targeting the *TGFBR2* gene comprises a spacer listed in **Table 39**. Examples include SEQ ID NOs: 272, 302, 308, and 314.

Any of the gRNAs disclosed herein may further comprise a scaffold sequence. For example, the gRNA targeting the *RegI* gene may comprise any of the nucleotide sequences listed in **Table 22**. Examples include SEQ ID NO: 22, 23, 30, 31, 34, 35, 50, and 51. Alternatively or in addition, the gRNA targeting the *TGFBR2* gene may comprise any of the nucleotide sequences provided in **Table 39**. Examples include SEQ ID NOs: 270, 271, 300, 301, 306, 307, 312, and 313.

In any of the methods disclosed herein, the plurality of T cells in step (a) comprises one or more of the following genetic modifications: (i) engineered to express a chimeric antigen receptor (CAR); (ii) has a disrupted T cell receptor alpha chain constant region (*TRAC*) gene; (iii) has a disrupted $\beta 2M$ gene; and (iv) has a disrupted *CD70* gene.

Alternatively, any of the methods disclosed herein may further comprise:

- (i) delivering to the T cells a nucleic acid encoding a chimeric antigen receptor (CAR);
- (ii) genetically editing a *TRAC* gene to disrupt its expression;
- (iii) genetically editing a $\beta 2M$ gene to disrupt its expression;
- (iv) genetically editing a *CD70* gene to disrupt its expression; or
- (v) a combination thereof.

In some **embodimentexamples**, one or more of (i)-(iv) are performed by one or more CRISPR/Cas-mediated gene editing system comprising one or more RNA-guided nucleases and one or more gRNAs targeting the *TRAC* gene, the $\beta 2M$ gene, and/or the *CD70* gene. In

some examples, the gRNA targeting the *TRAC* gene comprises a spacer that comprises the nucleotide sequence of SEQ ID NO: 61. In some examples, the gRNA targeting the *β2M* gene comprises a spacer that comprises the nucleotide sequence of SEQ ID NO: 65. In some examples, the gRNA targeting the *CD70* gene comprises a spacer that comprises the nucleotide sequence of SEQ ID NO: 57. See **Table 23**.

In some [embodimentexamples](#), the method disclosed herein may comprise delivering to the T cells one or more ribonucleoprotein particles (RNP), which may comprise the RNA-guided nuclease, one or more of the gRNAs, and the nucleic acid encoding the CAR. In some examples, the RNA-guided nuclease is a Cas9 nuclease, for example, a *S. pyogenes* Cas9 nuclease.

In some [embodimentexamples](#), the nucleic acid encoding the CAR is in an AAV vector. In some instances, the nucleic acid encoding the CAR comprises a left homology arm and a right homology arm flanking the nucleotide sequence encoding the CAR. The left homology arm and the right homology arm are homologous to a genomic locus in the T cells, allowing for insertion of the nucleic acid into the genomic locus. In some examples, the genomic locus is in the *RegI* gene. In some examples, the genomic locus is in the *TGFBR2* gene. In some examples, the genomic locus is in the *TRAC* gene. In some examples, the genomic locus is in the *β2M* gene. In some examples, the genomic locus is in the *CD70* gene.

In some examples, the method comprising disrupting the *TRAC* gene by a CRISPR/Cas-mediated gene editing system comprising a gRNA comprising the nucleotide sequence of SEQ ID NO: 59 and the nucleic acid encoding the CAR is inserted at the site targeted by the gRNA. Alternatively or in addition, the method may comprise delivering to the T cells a nucleic acid encoding a CAR, which is specific to CD70, and genetically editing the *CD70* gene to disrupt its expression.

Any population of the genetically engineered T cells prepared by a method disclosed herein is also within the scope of the present disclosure.

Further, the present disclosure provides a method for eliminating undesired cells in a subject, the method comprising administering to a subject in need thereof any of the populations of genetically engineered T cells disclosed herein. In some [embodimentexamples](#), the undesired cells are cancer cells, for example, hematopoietic cancer cells or solid tumor cells. In some [embodimentexamples](#), the undesired cells are CD19⁺. In some [embodimentexamples](#), the undesired cells are BCMA⁺. In some [embodimentexamples](#), the undesired cells are CD70⁺. In some [embodimentexamples](#), the undesired cells are CD33⁺. In some [embodimentexamples](#), the undesired cells are PTK7⁺.

In yet other [aspect examples](#), provided herein is a guide RNA (gRNA) targeting a *RegI* gene, comprising a nucleotide sequence specific to a fragment in exon 2 or exon 4 of the *RegI* gene. In some [embodiment examples](#), the gRNA comprises a spacer listed in **Table 22** (e.g., SEQ ID NO: 24, 32, 36 or 52). Such a gRNA may further comprise a scaffold sequence. Alternatively or in addition, the gRNA comprises one or more modified nucleotides. For example, the gRNA comprises one or more 2'-O-methyl phosphorothioate residues at the 5' and/or 3' terminus of the gRNA. Examples of gRNAs targeting *RegI* include any of those listed in **Table 22** (e.g., SEQ ID NO: 22, 23, 30, 31, 34, 35, 50, or 51; see also disclosures herein).

In still other [aspect examples](#), provided herein is a guide RNA (gRNA) targeting a *TGFBR2* gene, comprising a nucleotide sequence specific to a fragment in exon 1, exon 2, exon 3, exon 4, or exon 5 of the *TGFBR2* gene. In some examples, the gRNA comprises a nucleotide sequence specific to exon 4 of the *TGFBR2* gene. In other examples, the gRNA comprises a nucleotide sequence specific to exon 5 of the *TGFBR2* gene. In some instances, the gRNA comprises a spacer having the nucleotide sequence listed in **Table 39** (e.g., SEQ ID NOs: 272, 302, 308, and 314). Such a gRNA may further comprise a scaffold sequence. Alternatively or in addition, the gRNA comprises one or more modified nucleotides. For example, the gRNA comprises one or more 2'-O-methyl phosphorothioate residues at the 5' and/or 3' terminus of the gRNA. Examples of gRNAs targeting the *TGFBR2* gene include any of those listed in **Table 39** (e.g., SEQ ID NOs: 270, 271, 300, 301, 306, 307, 312, and 313).

Also within the scope of the present disclosure are any of the genetically engineered T cells, gRNAs targeting *RegI*, or gRNAs targeting *TGFBR2* for use in treating a target disease as disclosed herein (e.g., cancer such as those disclosed herein), or uses of such for manufacturing a medicament for the intended therapeutic purposes

The details of one or more [embodiment examples](#) of the [invention disclosure](#) are set forth in the description below. Other features or advantages of the present [invention disclosure](#) will be apparent from the following drawings and detailed description of several [embodiment examples](#), and also from the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGs. 1A and 1B include diagrams showing that exemplary CAR T cells (anti-CD70 CAR T cells) with *RegI* KO exhibit superior **in vitro** expansion **FIG. 1A**: Proliferation of anti-CD70 CAR T cells (CAR T) with *RegI* KO using one of the 10 guides (Z01-Z10) targeting *RegI* as indicated. CAR T indicates anti-CD70 CAR T cells with an unedited (wild-type) *RegI* gene. **FIG. 1B**: Proliferation of anti-CD70 CAR T cells with *RegI* KO using guide REG1-Z10

What Is Claimed Is:

1. A population of genetically engineered T cells, comprising:
 - (i) a disrupted *Regnase-1* (*Reg1*) gene; and~~or~~
 - (ii) a disrupted Transforming Growth Factor Beta Receptor II (*TGFBR2*) gene;

wherein the T cells are further engineered to express a chimeric antigen receptor (CAR).

~~2. The population of genetically engineered T cells of claim 1, which comprises both (i) and (ii).~~

3. The population of genetically engineered T cells of claim 1 ~~or claim 2~~, wherein the population of genetically engineered T cells, as compared to non-engineered T cell counterparts, has one or more of the following features:

- (a) improved cell growth activity;
- (b) enhanced persistence;
- (c) reduced T cell exhaustion;
- (d) resistant to inhibitory effects induced by TGF- β ;
- (e) enhanced cell killing capacity; and
- (f) resistant to inhibitory effects by fibroblasts and/or inhibitory factors secreted thereby.

~~4. The population of genetically engineered T cells of any one of claims 1-3, wherein the T cells are further engineered to express a chimeric antigen receptor (CAR).~~

5. The population of genetically engineered T cells of ~~any one of claims 1-4~~ or 2, wherein the disrupted *Reg1* gene is genetically edited in exon 2 ~~and/or~~ exon 4, and/or, wherein the disrupted *TGFBR2* gene is genetically edited in exon 1, exon 2, exon 3, exon 4, or exon 5.

~~6. The population of genetically engineered T cells of any one of claims 1-5, wherein the disrupted *TGFBR2* gene is genetically edited in exon 1, exon 2, exon 3, exon 4,~~

~~or exon 5, optionally wherein the disrupted *TGFBRII* gene is genetically edited in exon 4 or exon 5.~~

~~74.~~ The population of genetically engineered T cells of ~~any one of claims 1-63,~~ wherein the disrupted *Reg1* gene, ~~the disrupted *TGFBRII* gene, or both are~~ is genetically edited by a CRISPR/Cas-mediated gene editing system, which

~~8.~~ ~~The population of genetically engineered T cells of claim 7, wherein the CRISPR/Cas-mediated gene editing~~ comprises a guide RNA (gRNA) targeting a site in the *Reg1* gene that comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 327, 318, 319, 320, 321, 322, 323, 324, 325, and 326, which optionally is selected from the group consisting of SEQ ID NOs: 327, 320, 322, and 323, and/or listed in Table 22, which optionally is selected from the group consisting of SEQ ID NO: 320, 322, 323, and 327.

~~9.~~ ~~The population of genetically engineered T cells of claim 8, wherein the gRNA targeting the *Reg1* gene comprises a nucleotide sequence listed in Table 22, which optionally is selected from the group consisting of SEQ ID NO: 24, 32, 36, or 52.~~

~~10.~~ ~~The population of genetically engineered T cells of claim 9, wherein the disrupted *Reg1* gene comprises a nucleotide sequence selected from those listed in Table 31, 33, 34, or 38.~~

~~11.~~ ~~The population of genetically engineered T cells of any one of claims 7-10,~~ wherein the disrupted *TGFBRII* gene is genetically edited by a CRISPR/Cas-mediated gene editing ~~system~~system, which comprises a guide RNA (gRNA) targeting a site in the *TGFBRII* gene that comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 269, 275, 281, 287, 293, 299, 305, 311, and 317, which optionally is selected from the group consisting of SEQ ID NOs: 317, 275, 305, and 311.

5. The population of genetically engineered T cells of claim 4, wherein the gRNA targeting the *Reg1* gene comprises a spacer sequence set forth as SEQ ID NO: 52, 16, 20, 24, 28, 32, 36, 40, 44, or 48, which optionally is selected from the group consisting of SEQ ID NOs: 52, 24, 32, and 36, and/or

wherein the gRNA targeting the *TGFBRII* gene comprises a spacer sequence selected from the group consisting of SEQ ID NOs: 314, 266, 272, 278, 284, 290, 296, 302, and 308, which optionally is selected from the group consisting of SEQ ID NOs: 314, 272, 302, and 308.

optionally wherein the gRNA targeting the *Reg1* gene comprises the nucleotide sequence of SEQ ID NO: 50, 22, 30, or 34,

optionally wherein the gRNA targeting the *TGFBRII* gene comprises the nucleotide sequence of SEQ ID NO: 312, 270, 300, or 306.

~~12. The population of genetically engineered T cells of claim 11, wherein the gRNA targeting the *TGFBRII* gene comprises a spacer having a nucleotide sequence selected from the group consisting of SEQ ID NOs: 266, 272, 278, 284, 290, 296, 302, 308, and 314.~~

~~13. The population of genetically engineered T cells of any one of claims 7-12, wherein the gRNA further comprises a scaffold sequence.~~

~~14. The population of genetically engineered T cells of claim 13, wherein:~~

~~the gRNA targeting the *Reg1* gene comprises the nucleotide sequence of any of SEQ ID NO: 22, 30, 34, or 50; and/or~~

~~the gRNA targeting the *TGFBRII* gene comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 270, 300, 306, or 312.~~

156. The population of genetically engineered T cells of any one of claims 1-~~145~~, which further comprises:

(iii) a disrupted T cell receptor alpha chain constant region (*TRAC*) gene,

(iv) a disrupted beta-2-microglobulin ($\beta 2M$) gene,

(v) a disrupted *CD70* gene, or

(vi) a combination of any of (iii)-(v);

~~16. The population of genetically engineered T cells of claim 15, wherein the T cells comprise a disrupted T cell receptor alpha chain constant region (*TRAC*) gene.~~

~~17. The population of genetically engineered T cells of claim 15 or claim 16,~~

~~wherein the T cells comprise a disrupted beta-2-microglobulin ($\beta 2M$) gene.~~

~~18. —The population of genetically engineered T cells of any one of claims 15-17, wherein the T cells comprise a disrupted *CD70* gene.~~

~~19. —The population of genetically engineered T cells of any one of claims 15-18, optionally wherein the disrupted *TRAC* gene, the disrupted $\beta 2M$ gene, and/or the disrupted *CD70* gene is genetically edited by one or more CRISPR/Cas-mediated gene editing system.~~

~~207. The population of genetically engineered T cells of any one of claims 41-196, wherein the T cells comprise a nucleic acid encoding the CAR, and wherein the nucleic acid is inserted in the genome of the T cells.~~

~~21. —The population of genetically engineered T cells of claim 20, optionally, wherein the nucleic acid encoding the CAR is inserted in the disrupted *Reg1* gene, the disrupted *TGFBRII* gene, the disrupted *TRAC* gene, the disrupted $\beta 2M$, or the disrupted *CD70* gene.~~

~~228. The population of genetically engineered T cells of claim 217, wherein the nucleic acid encoding the CAR is inserted in the disrupted *TRAC* gene, and optionally wherein the nucleic acid encoding the CAR replaces the deleted fragment comprising SEQ ID NO: 69 in the *TRAC* gene.~~

~~23. —The population of genetically engineered T cells of claim 15, wherein the disrupted *Reg1* gene comprises a nucleotide sequence listed in Table 31, 33-34, or 38, the disrupted *TRAC* gene comprises a nucleotide sequence listed in Table 24; the disrupted $\beta 2M$ comprises a nucleotide sequence listed in Table 25 and/or the disrupted *CD70* gene comprises a nucleotide sequence listed in Table 26.~~

~~249. The population of genetically engineered T cells of any one of claims 41-238, wherein the CAR comprises an extracellular antigen binding domain specific to a tumor antigen, a co-stimulatory signaling domain of 4-1BB or CD28, and a cytoplasmic signaling domain of CD3 ζ .~~

~~25. — The population of genetically engineered T cells of claim 24, optionally,~~
wherein the tumor antigen is CD19, BCMA, CD70, CD33, or PTK7.

~~26~~10. The population of genetically engineered T cells of claim ~~249~~, wherein the CAR binds CD19 (anti-CD19 CAR) and wherein the extracellular antigen binding domain in the anti-CD19 CAR is a single chain variable fragment (scFv) that binds CD19 (anti-CD19 scFv).~~1~~

~~27. — The population of genetically engineered T cells of claim 24, optionally,~~
wherein the anti-CD19 scFv comprises (i) a heavy chain variable region (V_H) that comprises the same heavy chain complementary determining regions (CDRs) as those in SEQ ID NO: 124; and (ii) a light chain variable region (V_L) that comprises the same light chain CDRs as those in SEQ ID NO: 125; optionally wherein the V_H comprises the amino acid sequence of SEQ ID NO: 124 and the V_L comprises the amino acid sequence of SEQ ID NO: 125.~~1~~

~~28. — The population of genetically engineered T cells of claim 27, optionally,~~
wherein the anti-CD19 scFv comprises the amino acid sequence of SEQ ID NO: 120.

~~29~~11. The population of genetically engineered T cells of claim ~~28~~10, wherein the anti-CD19 CAR comprises the amino acid sequence of SEQ ID NO: 117 or SEQ ID NO: 353.

~~30~~12. The population of genetically engineered T cells of claim ~~24~~11, wherein the CAR binds CD70 (anti-CD70 CAR) and wherein extracellular antigen binding domain in the anti-CD70 CAR is a single chain variable fragment (scFv) that binds CD70 (anti-CD70 scFv).~~1~~

~~31. — The population of genetically engineered T cells of claim 30, optionally,~~
wherein the anti-CD70 scFv comprises (i) a heavy chain variable region (V_H) that comprises the same heavy chain complementary determining regions (CDRs) as those in SEQ ID NO: 143; and (ii) a light chain variable region (V_L) that comprises the same light chain CDRs as those in SEQ ID NO: 144; optionally wherein the V_H comprises the amino acid sequence of SEQ ID NO: 143 and the V_L comprises the amino acid sequence of SEQ ID NO: 144.~~1~~

~~32. — The population of genetically engineered T cells of claim 31, optionally,~~

wherein the anti-CD70 scFv comprises the amino acid sequence of SEQ ID NO: 140 or 142.

3313. The population of genetically engineered T cells of claim 3212, wherein the anti-CD70 CAR comprises the amino acid sequence of SEQ ID NO: 138 or SEQ ID NO:354.

~~34.—The population of genetically engineered T cells of claim 24, wherein the CAR binds BCMA (anti-BCMA CAR) and wherein the extracellular antigen binding domain in the anti-BCMA CAR is a single chain variable fragment (scFv) that binds BCMA (anti-BCMA CAR).~~

~~35.—The population of genetically engineered T cells of claim 34, wherein the anti-BCMA scFv comprises (i) a heavy chain variable region (V_H) that comprises the same heavy chain complementary determining regions (CDRs) as those in SEQ ID NO: 149; and (ii) a light chain variable region (V_L) that comprises the same light chain CDRs as those in SEQ ID NO: 150; optionally wherein the V_H comprises the amino acid sequence of SEQ ID NO: 149 and the V_L comprises the amino acid sequence of SEQ ID NO: 150.~~

~~36.—The population of genetically engineered T cells of claim 35, wherein the anti-BCMA scFv comprises the amino acid sequence of SEQ ID NO: 148.~~

~~37.—The population of genetically engineered T cells of claim 36, wherein the anti-BCMA CAR comprises the amino acid sequence of SEQ ID NO: 146 or SEQ ID NO:355.~~

3814. The population of genetically engineered T cells of any one of claims 1-3713, wherein the genetically engineered T cells are derived from primary T cells of one or more human donors;₂

~~39.—The population of genetically engineered T cells of any one of claims 1-38, optionally,~~ wherein the genetically engineered T cells show cytokine-dependent growth.

15. The population of genetically engineered T cells of any one of claims 1-14 for use as a medicament.

16. Use of the population of genetically engineered T cells of any one of claims 1-

14 in the manufacture of a medicament for treating a cancer in a subject.

17. The use of claim 16, wherein the cancer is a hematopoietic cancer or a solid tumor.

18. The use of claim 16 or claim 17, wherein the cancer is CD19⁺, BCMA⁺, CD70⁺, CD33⁺, or PTK7⁺.

4019. A method for preparing the population of genetically engineered T cells having a disrupted *Reg1* gene and a disrupted *TGFBR2* gene~~of claim 1~~, the method comprising:

- (a) providing a plurality of cells, which are T cells or precursor cells thereof; optionally wherein the plurality of T cells is a population of primary T cells of one or more human donors;
- (b) genetically editing the *Reg1* gene and/or the *TGFBR2* gene; and
- (c) producing the population of genetically engineered T cells having disrupted *Reg1* gene and~~or~~ the *TGFBR2* gene.

~~41. The method of claim 40, wherein step (b) comprises genetically editing both the *Reg1* gene and the *TGFBR2* gene.~~

4220. The method of claim ~~40-19~~or claim 41, wherein step (b) is performed by one or more CRISPR/Cas-mediated gene editing systems~~.~~, and

~~43. The method of any one of claims 40-42, wherein step (b) is performed by delivering to the plurality of cells an RNA-guided nuclease, and a gRNA targeting the *Reg1* gene, and a gRNA targeting the *TGFBR2* gene.~~

21. The method of claim 19 or claim 20, wherein the gRNA targeting the *Reg1* gene is set forth in claim 4 or claim 5, and/or wherein the gRNA targeting the *TGFBR2* gene is set forth in claim 4 or claim 5.

~~44. The method of claim 43, wherein the gRNA targeting the *Reg1* gene is specific to an exon of the *Reg1* gene selected from the group consisting of exon 2 and exon 4.~~

~~45. The method of claim 44, wherein the gRNA targeting the *Reg1* gene comprises a spacer having a nucleotide sequence of SEQ ID NO: 24, 32, 36, or 52.~~

~~46. The method of any one of claims 40-45, wherein step (b) is performed by delivering to the plurality of cells an RNA-guided nuclease and a gRNA targeting the *TGFBR2* gene.~~

~~47. The method of claim 46, wherein the gRNA targeting the *TGFBR2* gene is specific to an exon of the *TGFBR2* gene selected from the group consisting of exon 1, exon 2, exon 3, exon 4, and exon 5, preferably wherein the gRNA targeting the *TGFBR2* gene is specific to exon 4 or exon 5.~~

~~48. The method of claim 47, wherein the gRNA targeting the *TGFBR2* gene comprises a spacer having a nucleotide sequence selected from the group consisting of SEQ ID NOs: 272, 300, 308, and 314.~~

~~49. The method of any one of claims 40-48, wherein the gRNA targeting the *Reg1* gene and the gRNA targeting the *TGFBR2* gene further comprises a scaffold sequence.~~

~~5022. The method of claim 4921, wherein the gRNA targeting the *Reg1* gene comprises a the nucleotide sequence of SEQ ID NO: 50, 14, 18, 22, 26, 30, 34, 38, 42, or 46, optionally SEQ ID NO: 50, 22, 30, or 34; and/or wherein the gRNA targeting the *TGFBR2* gene comprises a the nucleotide sequence of SEQ ID NO: 312, 264, 270, 276, 282, 288, 294, 300, or 306, optionally SEQ ID NO: 312, 270, 300, or 306; SEQ ID NOs: 270, 300, 306, or 312.~~

~~5123. The method of any one of claims 4019-50 or 20, wherein the plurality of T cells in step (a) comprises one or more of the following genetic modifications:~~

- ~~(i) engineered to express a chimeric antigen receptor (CAR);~~
- ~~(ii) has a disrupted T cell receptor alpha chain constant region (*TRAC*) gene;~~
- ~~(iii) has a disrupted $\beta 2M$ gene; and~~
- ~~(iv) has a disrupted *CD70* gene.~~

~~5224.~~ The method of ~~any one of~~ claims ~~40-51~~19 or claim 20, wherein the method further comprises:

- (i) delivering to the T cells a nucleic acid encoding a chimeric antigen receptor (CAR), which optionally is in an AAV vector;
- (ii) genetically editing a T cell receptor alpha chain constant region (TRAC) gene to ~~knockout~~disrupt its expression;
- (iii) genetically editing a beta-2-microglobulin ($\beta 2M$) gene to ~~knockout~~disrupt its expression;
- (iv) genetically editing a *CD70* gene to ~~knockout~~disrupt its expression; or
- (v) a combination thereof.

25. The method of claim 24, wherein the CAR binds CD19, which optionally is set forth in claim 10 or claim 11, or where the CAR binds CD70, which optionally is set forth in claim 12 or claim 13.

~~5326.~~ The method of claim ~~5224~~ or claim 25, wherein one or more of (i)-(iv) are performed by one or more CRISPR/Cas-mediated gene editing system comprising one or more RNA-guided nucleases and one or more gRNAs targeting the *TRAC* gene, the $\beta 2M$ gene, and/or the *CD70* gene; optionally wherein:-

~~54.—The method of claim 53, wherein~~ the gRNA targeting the *TRAC* gene comprises the nucleotide sequence of SEQ ID NO: 59;;

~~55.—The method of claim 53 or claim 54, wherein~~ the gRNA targeting the $\beta 2M$ gene comprises the nucleotide sequence of SEQ ID NO: 63; and/or:-

~~56.—The method of any one of claims 53-55, wherein~~ the gRNA targeting the *CD70* gene comprises the nucleotide sequence of SEQ ID NO: 55.

27. A guide RNA (gRNA) targeting a Reg1 gene or a TGFBR2 gene, which is set forth in claim 21 or claim 22.

28. The gRNA of claim 27, wherein the gRNA comprises one or more 2'-O-

methyl phosphorothioate residues at the 5' and/or 3' terminus of the gRNA; optionally wherein the gRNA that targets Reg-1 is set forth as SEQ ID NO: 51, 15, 19, 23, 27, 31, 35, 39, 43, or 47, optionally SEQ ID NO: 51, 23, 31, or 35; and the gRNA that targets TGFBR2 is set forth as SEQ ID NO: 313, 265, 271, 277, 283, 289, 295, 301, or 307; optionally SEQ ID NO: 313, 271, 301, or 307.

~~57.—The method of any one of claims 50-56, wherein the method comprises delivering to the T cells one or more ribonucleoprotein particles (RNP), comprising the RNA-guided nuclease, one or more of the gRNAs, and the nucleic acid encoding the CAR.~~

~~58.—The method of any one of claims 50-57, wherein the RNA-guided nuclease is a Cas9 nuclease, which optionally is a *S. pyogenes* Cas9 nuclease.~~

~~59.—The method of any one of claims 50-58, wherein the nucleic acid encoding the CAR is in an AAV vector.~~

~~60.—The method of any one of claims 50-59, wherein the nucleic acid encoding the CAR comprises a left homology arm and a right homology arm flanking the nucleotide sequence encoding the CAR; and wherein the left homology arm and the right homology arm are homologous to a genomic locus in the T cells, allowing for insertion of the nucleic acid into the genomic locus.~~

~~61.—The method of claim 60, wherein the genomic locus is in the *Reg1* gene, the *TGFBR2* gene, the *TRAC* gene, the $\beta 2M$ gene, or the *CD70* gene; optionally wherein the genomic locus is the *TRAC* gene.~~

~~62.—The method of any one of claims 50-61, wherein the method comprising disrupting the *TRAC* gene by a CRISPR/Cas-mediated gene editing system comprising a gRNA comprising nucleotide sequence of SEQ ID NO: 47 and the nucleic acid encoding the CAR is inserted at the site targeted by the gRNA.~~

~~63.—The method of any one of claims 40-62, wherein the method comprising delivering to the T cells a nucleic acid encoding a CAR, which is specific to CD70, and~~

genetically editing the *CD70* gene to disrupt its expression.

64. — The method of any one of claims 40–63, wherein the T cells of step (a) are derived from primary T cells of one or more human donors.

65. — A population of genetically engineered T cells, which is prepared by a method of any one of claims 40–64.

66. — A method for eliminating undesired cells in a subject, the method comprising administering to a subject in need thereof a population of genetically engineered T cells set forth in any one of claims 4–39 and 65.

67. — The method of claim 66, wherein the undesired cells are cancer cells, which optionally are hematopoietic cancer cells or solid tumor cells.

68. — The method of claim 66 or claim 67, wherein the undesired cells are $CD19^{+}$, $BCMA^{+}$, $CD70^{+}$, $CD33^{+}$, or $PTK7^{+}$.

69. — A guide RNA (gRNA) targeting a *Reg1* gene, comprising a nucleotide sequence specific to a fragment in exon 1, exon 2, exon 3, or exon 4, optionally in exon 2 or exon 4 of the *Reg1* gene.

70. — The gRNA of claim 69, wherein the gRNA comprises a spacer listed in Table 22, which optionally is selected from the group consisting of SEQ ID NO: 24, 32, 36, and 52.

71. — The gRNA of claim 69 or claim 70, wherein the gRNA further comprises a scaffold sequence.

72. — The gRNA of any one of claims 69–71, wherein the gRNA comprises one or more modified nucleotides.

73. — The gRNA of claim 72, wherein the gRNA comprises one or more 2'-O-methyl phosphorothioate residues at the 5' and/or 3' terminus of the gRNA.

~~74. — The gRNA of claim 73, which comprises the nucleotide sequence listed in Table 22, which optionally is any of SEQ ID NOs: 22, 23, 30, 31, 34, 35, 50, or 51.~~

~~75. — A guide RNA (gRNA) targeting a *TGFBR1* gene, comprising a nucleotide sequence specific to a fragment in exon 1, exon 2, exon 3, exon 4, or exon 5 of the *TGFBR1* gene, preferably wherein the gRNA comprises a nucleotide sequence specific to exon 4 or exon 5 of the *TGFBR1* gene.~~

~~76. — The gRNA of claim 75, wherein the gRNA comprises a spacer listed in Table 32, which optionally has the nucleotide sequence selected from the group consisting of SEQ ID NOs: 272, 302, 308, and 314.~~

~~77. — The gRNA of claim 76, wherein the gRNA further comprises a scaffold sequence.~~

~~78. — The gRNA of any one of claims 75-77, wherein the gRNA comprises one or more modified nucleotides.~~

~~79. — The gRNA of claim 78, wherein the gRNA comprises one or more 2'-O-methyl phosphorothioate residues at the 5' and/or 3' terminus of the gRNA.~~

~~80. — The gRNA of claim 79, which comprises the nucleotide sequence listed in Table 39, optionally wherein the gRNA comprises the nucleotide sequence of any one of SEQ ID NO: SEQ ID NOs: 270, 271, 300, 301, 306, 307, 312, and 313.~~