

MANUFACTURING PROCESS FOR MAKING T CELLS EXPRESSING CHIMERIC ANTIGEN RECEPTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 This application claims the benefit of priority to U.S. Provisional Patent Application No. 62/934,991, filed November 13, 2019, which is hereby incorporated by reference in its entirety.

BACKGROUND

- Chimeric antigen receptor (CAR) T-cell therapy has shown promising therapeutic effects in treating hematologic cancer. Typically, CAR-T cells are generated by genetic engineering of
10 either patient immune cells (autologous) or immune cells from unrelated human donors (allogenic). Production of high-quality, clinical grade CAR-T cells is a prerequisite for the wide application of this technology. It is therefore of great interest to develop efficient manufacturing processes for large-scale production of CAR-T cells.

15 SUMMARY OF THE INVENTION

- The present disclosure is based, at least in part, on the development of methods for manufacturing genetically engineered T cells expressing a chimeric antigen receptor (CAR) that provide several improvements over conventional manufacturing methods. Such improvements include, but are not limited to, improvements in consistency and efficiency of genetic
20 modifications described herein, which allows production of a robust supply of clinically useful CAR T-cell therapies.

- Accordingly, one aspect of the present disclosure provides a method for manufacturing genetically engineered T cells, the method comprising: (i) providing a first population of T cells; (ii) incubating the first population of T cells in the presence of a T cell activating agent in a cell
25 culture vessel to produce a second population of T cells, wherein the second population of T cells comprises activated T cells; (iii) introducing into the activated T cells a first ribonucleoprotein (RNP) complex comprising a first Cas9 enzyme and a first guide RNA (gRNA) targeting a T cell receptor alpha chain constant region (TRAC) gene, and a second RNP complex comprising a second Cas9 enzyme and a second gRNA targeting a $\beta 2M$ gene to
30 produce a third population of T cells, wherein the third population of T cells comprises T cells having the *TRAC* gene disrupted and the $\beta 2M$ gene disrupted; (iv) incubating the third population of T cells with an adeno-associated viral (AAV) vector to produce a fourth population of T cells, wherein the fourth population of T cells comprises T cells expressing a chimeric antigen receptor (CAR), wherein the AAV vector comprises a nucleic acid sequence encoding the CAR, and

wherein the CAR-encoding nucleic acid sequence is flanked by homologous sequences to the *TRAC* gene locus targeted by the first gRNA; (v) expanding the fourth population of T cells; (vi) removing TCR $\alpha\beta$ ⁺ T cells from the expanded T cells to produce a population of genetically engineered T cells, wherein the population of genetically engineered T cells comprises T cells
5 expressing the CAR and having the *TRAC* gene and the *β 2M* gene disrupted; and (vii) harvesting the population of genetically engineered T cells.

In some embodiments, the first population of T cells is derived from cryopreserved T cells enriched from human blood cells. In some embodiments, the first population of T cells is prepared by a process comprising: (a) obtaining blood cells from a human donor; and (b)
10 enriching CD4⁺ T cells and CD8⁺ T cells. In some embodiments, (b) is performed using magnetic beads conjugated with anti-CD4 and/or anti-CD8 antibodies. In some embodiments, the first population of T cells has a cell viability of at least 80% and/or a purity of at least 80% of CD4⁺ and CD8⁺ T cells. In some embodiments, methods further comprise (c) cryopreserving the enriched CD4⁺ T cells and CD8⁺ T cells produced in step (b).

15 In some embodiments, the T cell activating agent comprises a CD3 agonist and a CD28 agonist attached to a nanomatrix particle. In some embodiments, step (ii) is performed by mixing the first population of T cells with the T cell activating agent in the cell culture vessel at a cell seeding density of about 2x10⁶/cm² and a cell concentration of about 2x10⁶ cells/mL; and incubating the mixture thus formed for about 48 hours. In some embodiments, the ratio of the T
20 cell activating agent to medium in the mixture is about 1:12.5 (v/v).

In some embodiments, a method disclosed herein may further comprise diluting the T cell activating agent in the second population of T cells after step (ii) to reduce activation and to allow cells to recover before step (iii).

In some embodiments, step (iii) is performed by electroporation. In some embodiments,
25 step (iii) involves one electroporation event. In some embodiments, the first RNP complex and the second RNP complex are introduced into the activated T cells in the one electroporation event. In some embodiments, the amount of the first Cas9 enzyme in the first RNP complex is the same as the amount of the second Cas9 enzyme in the second RNA complex. In some embodiments, the concentration of the first Cas9 enzyme is about 0.15 mg/mL, the concentration
30 of the second Cas9 enzyme is about 0.15 mg/mL, the concentration of the first gRNA targeting the *TRAC* gene is about 0.08 mg/mL, and the concentration of the second gRNA targeting the *β 2M* gene is about 0.2 mg/mL. In some embodiments, the cell concentration in step (iii) is about 100x10⁶ cells/mL to about 400x10⁶ cells/mL. In some embodiments, the cell concentration in step (iii) is about 300 x 10⁶ cells/mL. In other embodiments, the total cell number in each vessel

used in step (iii) (e.g., electroporation) can be about 5×10^8 to about 1×10^9 cells, for example, about 7×10^8 cells. In some examples, multiple vessels may be used in step (iii) (e.g., electroporation), for example, about 5-10 vessels. In specific examples, as many as 7 vessels may be used in step (iii), which may contain about 1.5×10^9 to about 3×10^9 cells (e.g., about
5 2.1×10^9 cells or about 2.7×10^9 cells), e.g., for electroporation.

In some embodiments, the AAV vector has a multiplicity of infection (MOI) value of about 10,000 to about 80,000. In some embodiments, the MOI of the AAV vector is about 20,000. In some embodiments, the AAV vector is AAV serotype 6 (AAV6) vector.

In some embodiments, step (v) is performed by seeding the fourth population of T cells in
10 a cell culture vessel at a seeding density of about 2×10^5 cells/cm² to about 7×10^5 cells/cm², and culturing the cells for about 6 days to about 12 days. In some embodiments, the fourth population of T cells may be seeded in a cell culture vessel at a seeding density of about 150,000 cells/cm² to about 600,000 cells/cm². In some embodiments, step (v) is performed by culturing the fourth population of T cells in a cell culture vessel at a seeding density of about 2×10^5
15 cells/cm² to about 5×10^5 cells/cm² for about 7 days to about 9 days. In some embodiments, step (v) is performed by seeding the fourth population of T cells in a cell culture vessel at a seeding density of about 3×10^5 cells/cm² to about 5×10^5 cells/cm². In some embodiments, the cell culture vessel is a static cell culture vessel (also referred interchangeably herein as a static culture vessel) allowing for cell expansion for about 10 days to about 12 days without medium change.
20 In some embodiments, the cell culture vessel is a static cell culture vessel allowing for cell expansion for about 7 days to about 9 days without medium change

In some embodiments, step (vi) is performed by contacting the expanded cells to beads on which anti-TCR $\alpha\beta$ antibodies are immobilized, and collecting unbound cells.

In some embodiments, the first Cas9 enzyme, the second Cas9 enzyme, or both are
25 *Streptococcus pyogenes* Cas9 nuclease (spCas9). In some embodiments, the first Cas9 enzyme and the second Cas9 enzyme are the same. In some embodiments, the first Cas9 enzyme comprises the amino acid sequence of SEQ ID NO: 1, and/or wherein the second Cas9 enzyme comprises the amino acid sequence of SEQ ID NO: 1. In some embodiments, the first gRNA targeting the *TRAC* gene comprises a spacer sequence of SEQ ID NO: 4. In some embodiments,
30 the first gRNA targeting the *TRAC* gene comprises the nucleotide sequence of SEQ ID NO: 2. In some embodiments, the second gRNA targeting the *$\beta 2M$* gene comprises a spacer sequence of SEQ ID NO: 8. In some embodiments, the second gRNA targeting the *$\beta 2M$* gene comprises the nucleotide sequence of SEQ ID NO: 6. In some embodiments, the first gRNA, the second gRNA, or both comprise one or more 2'-O-methyl phosphorothioate modification.

In some embodiments, the CAR comprises an extracellular domain targeting a cancer antigen, a transmembrane domain, a co-stimulatory domain, and a CD3z cytoplasmic signaling domain. In some embodiments, the extracellular domain comprises a single-chain variable fragment (scFv), the transmembrane domain is derived from CD8a, and/or the co-stimulatory domain is derived from CD28 and/or 4-1BB. In some embodiments, the CAR binds CD19. In some embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 37. In some embodiments, the CAR binds BCMA. In some embodiments, the CAR comprises the amino acid sequence of SEQ ID NO: 61.

Aspects of the present disclosure provide a genetically engineered T cell population, which is produced by a method described herein.

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.

DETAILED DESCRIPTION OF THE DRAWINGS

FIGs. 1A-1B include diagrams showing activation and expansion of T cells under various conditions. **FIG. 1A:** a graph showing T cell activation measured as percent of cells expressing CD25 and/or CD69. **FIG. 1B:** a graph showing that the expression level of CD25 is correlated to the cell expansion rate. The expression level of CD25 was measured as the mean florescent intensity (MFI) of CD25.

FIGs. 2A-2D include diagrams showing editing efficiency and CAR expression in T cells prepared in a small scale manufacturing process in which T cells were activated in a static culture vessel using optimized conditions described herein. T cells were manufactured in parallel in a T-flask as a control. UT: untreated T cells; EP: mock electroporated T cells; Flask: T cells in T-flask; and Vessel: T cells in static culture vessel. **FIG. 2A:** a graph showing TCR $\alpha\beta$ knockout efficiency in T cells. **FIG. 2B:** a graph showing β 2M knockout efficiency in T cells. **FIG. 2C:** a graph showing double knockout (DKO) efficiency in T cells. **FIG. 2D:** a graph showing CAR percent (CAR%) expression in T cells.

FIG. 3 is a diagram showing T cell expansion post editing of T cells prepared in a small scale manufacturing process. UT: untreated T cells; EP: mock electroporated T cells; Flask: T cells in T-flask; and Vessel: T cells in static culture vessel.

FIGs. 4A-4F include diagrams showing editing efficiency and CAR expression in T cells that were electroporated at different cell concentrations. UT: untreated T cells; D3: editing efficiency on day 3; D6: editing efficiency on day 6; D9: editing efficiency on day 9; and D12:

editing efficiency on day 12. **FIG. 4A:** a graph showing β 2M knockout efficiency in T cells electroporated at cell concentrations of 100×10^6 cells/mL to 300×10^6 cells/mL. **FIG. 4B:** a graph showing TCR $\alpha\beta$ knockout efficiency in T cells electroporated at cell concentrations of 100×10^6 cells/mL to 300×10^6 cells/mL. **FIG. 4C:** a graph showing CAR percent (CAR%) expression in T cells electroporated at cell concentrations of 100×10^6 cells/mL to 300×10^6 cells/mL. **FIG. 4D:** a graph showing β 2M knockout efficiency in T cells electroporated at cell concentrations of 200×10^6 cells/mL to 400×10^6 cells/mL. **FIG. 4E:** a graph showing TCR $\alpha\beta$ knockout efficiency in T cells electroporated at cell concentrations of 200×10^6 cells/mL to 400×10^6 cells/mL. **FIG. 4F:** a graph showing CAR percent (CAR%) expression in T cells electroporated at cell concentrations of 200×10^6 cells/mL to 400×10^6 cells/mL.

FIGs. 5A-5B include diagrams showing CAR⁺ expression in T cells transduced with varying MOI. **FIG. 5A:** a graph showing CAR⁺ expression in T cells transduced with MOI ranging from 1.25K to 80K. UT: untreated T cells; D3: CAR⁺ expression 3 days after transduction; D6: CAR⁺ expression 6 days after transduction; D10: CAR⁺ expression 10 days after transduction; and D13: CAR⁺ expression 13 days after transduction. **FIG. 5B:** a graph showing CAR⁺ expression in T cells measured 11 days after transduced with MOI ranging from 0.12K to 23K. P.C.: positive control; EP: electroporation only control; and Iso Type: CAR positive isotype replaced with goat IgG.

FIGs. 6A-6C include diagrams showing effects of cell seeding density on expansion of edited T cells. **FIG. 6A:** a graph showing cell number during expansion. **FIG. 6B:** a graph showing cell density during expansion. **FIG. 6C:** a graph showing fold expansion during expansion.

FIGs. 7A-7E include diagrams showing data from manufacturing of genetically engineered T cells expressing an anti-CD19 directed chimeric T cell antigen receptor (CTX110). **FIG. 7A** includes a flow chart of an illustrative manufacturing process for making T cells expressing an anti-CD19 CAR, in accordance with some embodiments of the technology described herein. **FIGs. 7B-7C** include diagrams showing CAR⁺ expression in T cells transduced with varying MOI. **FIG. 7B:** a graph showing CAR⁺ expression in T cells transduced with rAAV-138 MOI ranging from 0K to 80K. **FIG. 7C:** a graph showing CAR⁺ expression in T cells transduced with rAAV-138 MOI ranging from 0K to 80K. Transduction with rAAV-138 MOI of 20K was used as a positive control. **FIGs. 7D-7E** include diagrams showing editing efficiency in T cells electroporated with RNP complexes formed from different concentrations of sgRNA targeting TCR (TA-1 sgRNA) or sgRNA targeting B2M (B2M-1 sgRNA). TCR $\alpha\beta$: percent of cells having TCR $\alpha\beta$ edits; β 2M: percent of cells having β 2M edits; and double

knockout (DKO): percent of cells having TCR $\alpha\beta$ edits and β 2M edits. **FIG. 7D**: a graph showing knockout efficiency in T cells electroporated with RNP complexes formed using 37.5 μ g/mL to 300 μ g/mL of TA-1. **FIG. 7E**: a graph showing knockout efficiency in T cells electroporated with RNP complexes formed using 37.5 μ g/mL to 300 μ g/mL of B2M-1.

5 **FIGs. 8A-8G** include diagrams showing data from manufacturing of genetically engineered T cells expressing an anti-BCMA directed chimeric T cell antigen receptor (CTX120). **FIG. 8A** includes a flow chart of an illustrative manufacturing process for making T cells expressing an anti-BCMA CAR, in accordance with some embodiments of the technology described herein. **FIG. 8B**: a graph showing CAR⁺ expression in T cells transduced with increasing MOI. **FIG. 8C**: a graph showing levels of exhaustion markers detected in CTX120. **FIG. 8D**: a graph showing levels of memory markers detected in CD8⁺ T cells of CTX120. **FIG. 8E**: a graph showing levels of memory markers detected in CD4⁺ T cells of CTX120. **FIG. 8F**: a graph showing production of IFN γ upon co-culture of CTX120 with BCMA⁺ tumor cells. **FIG. 8G**: a graph showing tumor killing upon co-culture of CTX120 with BCMA⁺ tumor cells.

15 **FIGs. 9A and 9B** provide graphs of cell concentration per mL as a function of days of expansion post editing.

FIGs. 10A and 10B provide graphs of calculated cell number as a function of days of expansion post editing.

FIGs. 11A and 11B provide graphs of percentage cell viability as a function of days of expansion post editing.

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FIGs. 12A-12C provide graphs of depicting editing efficiency including CAR⁺% (**FIG. 12A**), TRAC-% (**FIG. 12B**) and β 2M-% (**FIG. 12C**) assessed in the various replating and low-plating groups.

FIGs. 13A and 13B provide the ratio of CD4⁺ and CD8⁺ cells in the various replated cell populations.

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FIGs. 14A-14F provide bar graphs depicting the assessment of memory cell subtype markers in the replated populations. The cells in the replated populations were assessed as naïve T cells, central memory (CM) T cells, effector memory (EM) T cells and terminal effector (TE) T cells.

30 **FIGs. 15A-15F** provide bar graphs depicting the assessment of exhaustion markers in the replated populations of CAR⁺, CD4⁺/CAR⁺, and CD8⁺/CAR⁺ cells. The three exhaustion markers assayed were PD1, LAG3 and TIM3.

FIGs. 16A-16C provide graphs showing the ability of the CAR-T cells in replated and low-plating density groups to kill CD19 positive Raji target cells *in vitro*, which was assessed using a flow cytometry-based cytotoxicity assay.

FIGs. 17A-17D provide graphs showing the percentage of survival of tumor cells as a function of days post inoculation at three different doses of CAR cells *in vivo*.

FIGs. 18A-18D provides graphs showing the tumor mass in mice as a function of days post inoculation at three dose of CAR cells *in vivo*.

FIG. 19 shows a flow chart illustrating one embodiment of the present disclosure.

FIG. 20 shows an assay control FACS analysis by measuring CAR T-cell lysis. The CAR T-cells were CTX110 CAR T-cells. 81% of the T-cells were CAR⁺.

FIGs. 21A-21C show the results of an assay control experiment measuring cell lysis and cytokine production *in vitro*. The assay used CTX110 CAR-T cells thawed from frozen stock. The T-cells were 80% CAR⁺ day 6 post HDR.

FIGs. 22A-22C show the results of an *in vitro* efficacy analysis showing that T-cells derived from each of the three donors had varying degrees of *in vitro* efficacy among 1x, 2x and 4x culture conditions.

FIGs. 23A-23C show the results of an analysis of cell lysis at different cell concentrations, demonstrating that cells derived from donors 1 and 2 showed similar responses despite differing percentages of CAR⁺ cells.

FIGs. 24A-24B show the results of an analysis of cell lysis from the three donors when normalized for CAR⁺ cells. Donors 2 and 3 behaved similarly in the assay when CAR cells were normalized. The assay was repeated with 2x CAR-T cell number for donor 2 at the same E:T ratios.

FIGs. 25A-25C provide survival curves showing the percentage of survival of mice as a function of days post inoculation of CAR cells for all three donors and expansion conditions *in vivo*.

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure is based, at least in part, on the development of improved manufacturing processes for producing CAR-T cells, particularly allogenic CAR-T cells, including improved conditions for one or more steps of the manufacturing processes. The

improved manufacturing processes disclosed herein led to at least the following advantageous outcomes:

- (a) Improved T cell purity and improved T cell viability resulting from the improved T cell enrichment conditions provided herein.
- 5 (b) Improved consistency and improved efficiency for producing CAR-expressing T cells resulting from the improved T cell transduction conditions provided herein.
- (c) Improved consistency and improved efficiency of *TRAC* gene and *$\beta 2M$* gene disruptions in T cells resulting from the improved CRISPR-Cas9-mediated gene editing conditions provided herein.
- 10 (d) Increased supply of CAR T-cell therapy resulting from decreased production times and decreased production costs provided by the improved manufacturing processes described herein.
- (e) Reduced variability of manufactured drug product resulting from production of uniform and high quality CAR T-therapies using the improved manufacturing processes
- 15 described herein.
- (f) Simplified AAV transduction condition while maintaining high CAR expression level in T cells.

Accordingly, provided herein are methods for manufacturing genetically engineered T cells expressing a CAR construct, such as a CAR construct targeting a cancer antigen, for
20 example, CD19 or BCMA, and having *TRAC* and *$\beta 2M$* gene knocked-out. The genetically engineered T cell populations produced by methods described herein, and therapeutic uses thereof are also within the scope of the present disclosure.

I. Manufacturing Genetically Engineered T Cells

25 Aspects of the present disclosure provide methods for manufacturing genetically engineered T cells comprising a disrupted beta-2-microglobulin (*$\beta 2M$*) gene, and a disrupted T cell receptor alpha chain constant region (*TRAC*) gene, and an inserted nucleic acid encoding a chimeric antigen receptor (CAR).

Disruption of the *$\beta 2M$* gene and the *TRAC* gene renders the genetically engineered T cell
30 non-alloreactive and suitable for allogeneic transplantation. Insertion of a nucleic acid encoding a CAR enables the genetically engineered T cell to express the CAR on its surface where it targets the genetically engineered T cell to cancer cells.

Accordingly, methods for manufacturing genetically engineered T cells disclosed herein, in some embodiments, involve the use of CRISPR-Cas9 gene editing to disrupt expression of

TRAC and $\beta 2M$, and the use of adeno-associated virus (AAV) transduction to insert a nucleic acid encoding a CAR.

In general, the method for manufacturing CAR-T cells disclosed herein may comprise: (i) enriching $CD4^+/CD8^+$ T cells from a suitable human immune cell source, (ii) activating the enriched $CD4^+/CD8^+$ T cells, and (iii) genetically engineering the activated T cells to produce CAR-T cells having disrupted TRAC and B2M genes; and harvesting the genetically engineered T cells for therapeutic uses. When needed, the enriched $CD4^+/CD8^+$ T cells may be stored *via* cryopreservation for future use. Alternatively or in addition, the genetically engineered T cells may be expanded *in vitro* prior to harvesting. $TCR\alpha\beta^+$ T cells may be depleted from the CAR-T cell population thus produced.

(i) ***T Cell Enrichment***

Any of the manufacturing methods disclosed herein may use human blood cells as the starting material. For example, T cells can be obtained from a unit of blood collected from a subject using techniques known to a skilled person, such as sedimentation, *e.g.*, FICOLL™ separation. Alternatively, the T cells for use in making the genetically engineered T cells may be derived from stem cells (*e.g.*, HSCs or iPSCs) *via in vitro* differentiation. In some embodiments, blood cells can be obtained from an individual human donor. In other embodiments, blood cells can be obtained from multiple human donors (*e.g.*, 2, 3, 4, or 5 human donors).

In some examples, leukopak samples from a suitable human donor may be used. As known in the art, a leukopak sample is 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 human donor preferably is a healthy human donor. For example, a human donor candidate may be subject to screening for HBV, HCV, HIV, HTLV, WNV, trypanosoma cruzi, and/or CMV. A human subject showing negative results in the screening may be used as a donor for blood cells.

The sources of T-cells that find use in the present methods is not particularly limited. In some embodiments, T cells from a T cell bank can be used as the starting material in any of the manufacturing methods disclosed herein. A T cell bank may comprise T cells with genetic editing of certain genes (*e.g.*, genes involved in cell self renewal, apoptosis, and/or T cell exhaustion or replicative senescence) to improve T cell persistence in cell culture. A T cell bank may be produced from bonafide T cells, 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 be

produced from precursor cells such as hematopoietic stem cells (e.g., iPSCs), e.g., in vitro culture. In some examples, the T cells in the T cell bank may comprise 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, so as to disrupt or reduce expression of such genes, leading to improved persistence in culture. Examples of the edited genes in a T cell bank include, but are not limited to, Tet2, Fas, CD70, Regnase-1, or a combination thereof. Compared with the non-edited T counterpart, T cells in a T cell bank may have enhanced expansion capacity in culture, enhanced proliferation capacity, greater T cell activation, and/or reduced apoptosis levels.

Suitable T cells can be enriched from human blood cells using conventional methods or methods disclosed herein. T cells for use in making the genetically engineered T cells may express one or more of the T cell markers, including, but not limited to a CD4⁺, CD8⁺, or a combination thereof. In some embodiments, CD4⁺ T cells can be enriched from human blood cells. In other embodiments, CD8⁺ T cells can be enriched. In specific examples, both CD4⁺ and CD8⁺ T cells are purified from human blood cells.

CD4⁺ T cells and/or CD8⁺ T cells can be isolated from a suitable blood cell source, such as those described herein, using any method known in the art or those disclosed herein, for example, using antibodies capable of binding to specific cell-surface biomarkers for the target T cells, e.g., antibodies specific to CD4 and/or antibodies specific to CD8. In some embodiments, enriching CD4⁺ T cells and CD8⁺ T cells can be performed using anti-CD4 and anti-CD8 antibodies conjugated to magnetic beads. A cell population comprising CD4⁺ and CD8⁺ T cells can be incubated with such magnetic beads under suitable conditions for a suitable period allowing for binding of the target T cells to the magnetic beads via the antibodies conjugated to the beads. Non-bound cells can be washed and CD4⁺ and CD8⁺ T cells bound to the beads can be collected using routine methods.

The enriched T cells (e.g., CD4⁺ T cells and CD8⁺ T cells) may be evaluated for features such as cell viability and/or purity of the target T cells following routine practice. In some embodiments, the T cell population from the enrichment step disclosed here may have a cell viability of at least about 80% (e.g., at least about 85%, at least about 90%, at least about 95%, or above). Alternatively or in addition to, the enriched T cell population may have a purity of at least about 80% of the target T cells (e.g., CD4⁺ and/or CD8⁺ T cells), for example, at least about 85%, at least about 90%, at least about 95%, at least about 97%, about 98% or higher. Alternatively or in addition to, the enriched T cell population may have a purity of at least about 70% of the target T cells (e.g., CD4⁺ and/or CD8⁺ T cells), for

example, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, about 98% or higher.

The term “about” or “approximately” 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. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 2-fold, of a 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.

The enriched T cell population (which is also within the scope of the present disclosure) may be used immediately for further processing as disclosed herein. Alternatively, the enriched T cell population may be stored under suitable conditions for future use, for example, *via* cryopreservation. Prior to further processing, cryopreserved T cells can be thawed following routine procedures. Cell viability of the thawed cells can be assessed to determine whether the thawed cells are suitable for further processing.

(ii) **T Cell Activation**

The enriched T cells may be subject to T cell activation to allow for proliferation and expansion of the enriched CD4⁺/CD8⁺ T cells. The T cell activation step used in any of the methods disclosed herein may involve T cell activation conditions disclosed herein that provide high T cell activation efficiency. Further, the activated T cells obtained therefrom would exhibit high gene editing efficiencies and great rates of T cell expansion post editing. See Examples below.

In some embodiments, T cell activation can be achieved using a T cell activating agent or agents, for example, agents that stimulates a CD3/TCR-mediated signaling pathway and/or a co-stimulatory molecule (e.g., CD28) mediated signaling pathway. For example, a T cell activating agent may be a CD3 agonist (e.g., an agonistic anti-CD3 antibody) and activates the CD3/TCR-mediated cell signaling pathway. Alternatively or in addition, a T cell activating agent may be a CD28 agonist (e.g., an anti-CD28 antibody) and activate the co-stimulatory signaling pathway mediated by CD28. Any of the T cell activating agents for use in the method disclosed herein may be conjugated to a support member, such as a nanomatrix particle. In specific examples, the T cell activating agent for use in the method disclosed herein may comprise an anti-CD3

antibody and an anti-CD28 antibody, which may be conjugated to nanomatrix particles. In some embodiments, the T cell activating agent comprises a CD3 agonist and a CD28 agonist attached to a nanomatrix particle. In some embodiments, the CD3 agonist and a CD28 agonist are attached to the same nanomatrix particle. In some embodiments, the CD3 agonist and a CD28 agonist are attached to different nanomatrix particles.

To achieve T cell activation, the enriched T cells as disclosed herein (*e.g.*, CD4⁺/CD8⁺ T cells) may be placed in a cell culture vessel at a suitable cell seeding density and a suitable cell concentration and incubated in the presence of any of the T cell activating agents disclosed herein for a suitable period to induce T cell activation.

In some instances, ratios of the T cell activating agent to the cell culture medium in the cell culture vessel may range from about 1:10 (v/v) to about 1:15 (v/v). In some examples, the ratio of the T cell activating agent to the cell culture medium in the cell culture vessel may be about 1:10 (v/v), about 1:10.5 (v/v), about 1:11 (v/v), about 1:11.5 (v/v), about 1:12 (v/v), about 1:12.5 (v/v), about 1:13 (v/v), about 1:13.5 (v/v), about 1:14 (v/v), about 1:14.5 (v/v), or about 1:15 (v/v). In specific examples, the ratio of the T cell activating agent to the culture medium in the cell culture vessel is about 1:12.5 (v/v).

Alternatively or in addition, a suitable cell seeding density may be about 1.5×10^6 to 2.5×10^6 (*e.g.*, $2 \times 10^6/\text{cm}^2$) and a suitable cell concentration may be about 1.5×10^6 to 2.5×10^6 (*e.g.*, $2 \times 10^6/\text{ml}$). The cells may be incubated with the T cell activating agent for about 42-54 hours, for example, about 48 hours.

In some embodiments, the cell culture vessel may be a static culture vessel, which would allow for relatively large-scale production of the genetically engineered T cells as disclosed herein. Compared to conventional cell culture flasks, static cell culture vessels allow T cells to reside on a highly gas permeable membrane submerged under medium that supplies oxygen and nutrients to the T cells without mixing or shaking. Static culture vessels allow T cell manufacturing without medium change. Accordingly, in some embodiments, the T cell activation process in any of the methods disclosed herein may involve no medium change.

When needed, the activating agent may be removed from the cell culture vessel or diluted prior to the follow-on gene editing events to minimize any potential impact that the activating agent may confer during gene editing. In some embodiments, the activating agent can be removed from the cell culture vessel using routine methods, *e.g.*, centrifugation. Alternatively, the activating agent may be diluted in the cell culture vessel prior to gene editing, *e.g.*, diluted by addition of media to the cell culture vessel.

In some embodiments, the activated T cells derived from any of the T cell activation

processes disclosed herein may be cultured overnight (*e.g.*, about 16 hours) to allow T cells to recover prior to gene editing. In some instances, the activated T cell culture may still contain the T activating agent. In other instances, the activated T cells may have little or no presence of the T cell activating agent.

5 (iii) ***CRISPR-CAS9-Mediated Gene Editing of Activated T Cells***

The activated T cells prepared by any of the procedures disclosed herein may subject to gene editing to knock out host response related genes, for example, the *TRAC* gene and/or the *β2M* gene, via, for example, CRISPR-Cas9 gene editing technology.

10 The *TRAC* gene encodes a component of the TCR complex. Disruption of the *TRAC* gene 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. The *β2M* gene encodes a common (invariant) component of the major histocompatibility complex (MHC) I complexes. Disrupting the *β2M* gene can prevent host versus therapeutic allogeneic T cells responses. Knocking out both the *TRAC* gene and the *β2M* gene would result in production of
15 allogeneic T cells for use in cell therapy.

CRISPR-Cas9-Mediated 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, crRNA (crRNA) and trans-activating RNA (tracrRNA), to target the cleavage of DNA. CRISPR is an acronym for
20 Clustered Regularly Interspaced Short Palindromic Repeats, a family of DNA sequences 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
25 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 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
30 Microbiol 37:67-78).

crRNA drives sequence recognition and specificity of the CRISPR-Cas9 complex 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 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 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 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.

(i) Cas9

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 comprises a *Streptococcus pyogenes*-derived Cas9 nuclease protein that has been engineered to include C- and N-terminal SV40 large T antigen nuclear localization sequences (NLS). The resulting Cas9 nuclease (sNLS-spCas9-sNLS) is a 162 kDa protein that is produced by recombinant *E. coli* fermentation and purified by chromatography. The spCas9 amino acid sequence can be found as UniProt Accession No. Q99ZW2, which is

provided herein as SEQ ID NO: 1.

(ii) Guide RNAs (gRNAs)

CRISPR-Cas9-mediated gene editing as described herein includes the use of a guide RNA or a gRNA. As used herein, a “gRNA” refers to a genome-targeting nucleic acid that can direct the Cas9 to a specific target sequence within a *TRAC* gene or a $\beta 2M$ gene for gene editing at the specific target sequence. 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.

An exemplary gRNA targeting a *TRAC* gene is provided in SEQ ID NO: 2. See also International Application No. PCT/IB2018/001619, filed May 11, 2018, which published as WO 2019/097305A2, 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; ENSG00000277734). In some embodiments, gRNAs targeting the *TRAC* genomic region and Cas9 create breaks in the *TRAC* genomic region resulting in Indels in the *TRAC* gene disrupting expression of the mRNA or protein.

In some embodiments, gRNAs targeting the *TRAC* genomic region create Indels in the *TRAC* gene comprising at least one nucleotide sequence selected from the sequences in **Table 9**. In some embodiments, gRNA (SEQ ID NO: 2) targeting the *TRAC* genomic region create Indels in the *TRAC* gene comprising at least one nucleotide sequence selected from the sequences in **Table 9**.

An exemplary gRNA targeting a $\beta 2M$ gene is provided in SEQ ID NO: 6. See also International Application No. PCT/IB2018/001619, filed May 11, 2018, which published as WO 2019/097305A2, 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 $\beta 2M$ 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.

In some embodiments, gRNAs targeting the $\beta 2M$ genomic region create Indels in the $\beta 2M$ gene comprising at least one nucleotide sequence selected from the sequences in **Table 10**. In some embodiments, gRNA (SEQ ID NO: 6) targeting the $\beta 2M$ genomic region create Indels in the $\beta 2M$ gene comprising at least one nucleotide sequence selected from the sequences in

Table 10.

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 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 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 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 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 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.

The "target sequence" is in a target gene that is adjacent to a PAM sequence and is the sequence to be modified by 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 TRAC target sequence is 5'-AGAGCAACAGTGCTGTGGCC-3' (SEQ ID NO: 11), then the gRNA spacer sequence is 5'-AGAGCAACAGUGCUGUGGCC-3' (SEQ ID NO: 5). In another example, if the β 2M target sequence is 5'-GCTACTCTCTCTTTCTGGCC-3' (SEQ ID NO: 13), then the gRNA spacer sequence is 5'-GCUACUCUCUCUUUCUGGCC-3' (SEQ ID NO: 9). The spacer of a gRNA interacts with 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 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.

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 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'-NNNNNNNNNNNNNNNNNNNNNNNRG-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 the *S. pyogenes* PAM.

A spacer sequence in a gRNA is a sequence (*e.g.*, a 20 nucleotide sequence) that defines the target sequence (*e.g.*, a DNA target sequences, such as a genomic target sequence) of a target gene of interest. An exemplary spacer sequence of a gRNA targeting a *TRAC* gene is provided in SEQ ID NO: 4. An exemplary spacer sequence of a gRNA targeting a β 2M gene is provided in SEQ ID NO:8.

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 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 International Application No. PCT/IB2018/001619, filed May 11, 2018, which published as WO 2019/097305A2, and International Application No. PCT/IB2019/000500, filed May 10, 2019, which published as WO/2019/215500. the relevant disclosures of each of the prior applications 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 a 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 8** in Example 7.

In some embodiments, the sgRNA comprises no uracil at the 3' end of the 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.

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 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.

It should be understood that more than one suitable Cas9 and more than one suitable gRNA can be used in methods described herein, for example, those known in the art or disclosed herein. In some embodiments, methods comprise a Cas9 enzyme and/or a gRNA known in the art. Examples can be found in, *e.g.*, International Application No. PCT/IB2018/001619, filed May 11, 2018, which published as WO 2019/097305A2, and International Application No. PCT/IB2019/000500, filed May 10, 2019, which published as WO/2019/215500, the relevant disclosures of each of the prior applications are herein incorporated by reference for the purposes and subject matter referenced herein.

CRISPR-Cas9-Mediated Gene Editing of TRAC and $\beta 2M$ Genes

In some embodiments, the activated T cells as disclosed herein may subject to gene editing of both the *TRAC* gene and *$\beta 2M$* gene via CRISPR-Cas9-mediated gene editing under conditions disclosed herein, which would result in higher and more consistent gene editing efficiencies compared to those provided by conventional conditions. Further, the *TRAC*/ *$\beta 2M$* T cells obtained from the gene editing process disclosed herein showed high expression level of a chimeric antigen receptor (CAR) when a viral vector coding for the CAR construct is delivered into the *TRAC*/ *$\beta 2M$* T cells.

The Cas9 enzyme and the gRNAs targeting the *TRAC* gene and *$\beta 2M$* gene may form one or more ribonucleoprotein (RNP) complexes, which can be delivered into the activated T cells as disclosed herein. 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.

The CRISPR-Cas9-mediated gene editing process may involve two ribonucleoprotein complexes. The first RNP complex comprises a first Cas9 enzyme and a guide RNA (gRNA) targeting a *TRAC* gene. The second RNP complex comprises a second Cas9 enzyme and a
5 gRNA targeting a $\beta 2M$ gene. In some examples, the two RNP complexes may comprise different Cas9 enzymes. In other examples, the two RNP complexes comprise the same Cas9 enzyme. In specific examples, the Cas9 enzyme of SEQ ID NO:1 can be used in both the first and second RNPs.

In some embodiments, the two RNP complexes may contain the same amount of the
10 Cas9 enzyme. For example, both RNP complexes may comprise about 0.1-0.3 mg/ml (*e.g.*, about 0.1-0.2 mg/ml) of the Cas9 enzyme (*e.g.*, the Cas9 enzyme of SEQ ID NO:1). In some examples, each of the RNP complexes may comprise about 0.15 mg/ml of the Cas9 enzyme, which may be the Cas9 enzyme of SEQ ID NO:1.

In other embodiments, the two RNP complexes may contain different amounts of the
15 Cas9 enzyme. In some examples, the RNP complex targeting the *TRAC* gene may comprise a higher amount of the Cas9 enzyme relative to the RNP complex targeting the $\beta 2M$ gene. Alternatively, the RNP complex targeting the $\beta 2M$ gene may comprise a higher amount of the Cas9 enzyme relative to the RNP complex targeting the *TRAC* gene.

The two RNP complexes may comprise the same amount of the gRNAs (one targeting
20 *TRAC* and the other targeting $\beta 2M$). Alternatively, the two RNP complexes may comprise different amounts of the gRNAs. For example, the amount of the gRNA targeting the *TRAC* gene may range from about 0.035 mg/ml to about 0.8 mg/ml, for example, about 50 μ g/ml to about 80 μ g/ml. In specific examples, the amount of the gRNA targeting the *TRAC* gene is about 0.08 mg/ml. Alternatively or in addition, the amount of the gRNA targeting the $\beta 2M$ gene may
25 range from about 0.075 mg/ml to about 0.3 mg/ml, for example, about 0.1 mg/ml to about 0.3 mg/ml. In specific examples, the amount of the gRNA targeting the $\beta 2M$ gene is about 0.2 mg/ml.

In specific examples, the RNP complex targeting the *TRAC* gene may comprise about
30 0.15 mg/ml Cas9 (*e.g.*, the Cas9 of SEQ ID NO:1) and about 0.08 mg/ml of a gRNA targeting the *TRAC* gene (*e.g.*, the gRNA of TA-1). Alternatively or in addition, the RNP complex targeting the $\beta 2M$ gene may comprise about 0.15 mg/ml Cas9 (*e.g.*, the Cas9 of SEQ ID NO:1) and about 0.2 mg/ml of a gRNA targeting the $\beta 2M$ gene (*e.g.*, the gRNA of B2M-1).

In some embodiments, the two RNPs may be introduced into the activated T cell via

electroporation sequentially, *i.e.*, via two electroporation event. Alternatively, the two RNPs may be introduced into the activated T cells simultaneously, *i.e.*, via one electroporation event. In this case, the two RNPs may be combined to form a mixture prior to the electroporation event.

Any of the RNPs disclosed herein may be introduced into the activated T cells by mixing the RNP(s) with a suitable amount of the activated T cells and the mixture thus formed is subject to electroporation under suitable conditions allowing for delivery of the RNPs into the cells. In some instances, the suitable amount of the activated T cells may range from about 100×10^6 cells/mL to about 300×10^6 cells/mL. For example, suitable amount of the T cells for the electroporation step may range from about 200×10^6 cells/mL to about 300×10^6 cells/mL. In some examples, the concentration of the activated T cells may be about 100×10^6 cells/mL. In some embodiments, the concentration of activated T cells may be about 200×10^6 cells/mL. In some embodiments, the concentration of activated T cells may be about 300×10^6 cells/mL.

In some embodiments, the suitable amount of the activated T cells may range from about 1×10^8 to about 1×10^{10} cells, *e.g.*, about 5×10^8 to about 8×10^9 cells, about 1×10^9 to about 5×10^9 cells, or about 1×10^9 to about 3×10^9 cells.

The T cells for use in electroporation may be placed in multiple cell cassettes, depending upon the electroporation instrument used. Suitable electroporation instruments are known to those skilled in the art and could include static and flow electroporators, including the Lonza Nucleofector, Maxcyte GT, and MaxCyte GTx. In some instances, multiple cell cassettes may be used in an electroporation process. More details are provided in Example 10 below.

In specific examples, the two RNPs disclosed above, comprising about 0.3 mg/ml of the Cas9 enzyme in total (*e.g.*, the Cas9 enzyme of SEQ ID NO:1), about 0.08 mg/ml of the gRNA of TA-1, and about 0.2 mg/ml of the gRNA of B2M-1, may be mixed with the activated T cells in the amount of about 100×10^6 cells/mL to about 300×10^6 cells/mL (*e.g.*, about 300×10^6 cells/mL). The mixture is then subject to electroporation for delivery of the RNPs into the T cells.

After electroporation, the cells may be cultured in a fresh medium or electroporation buffer for a suitable period for recovery. Gene editing efficiency may be performed following routine practice. The genetically edited T cells thus produced may be subjected to viral vector transduction for delivery of a nucleic acid configured for CAR expression.

(iv) *T Cell Transduction*

The genetically edited T cells, having *TRAC* and $\beta 2M$ genes knocked out, may be subject to transduction with a viral vector such as an adeno-associated viral (AAV) vector that comprises a nucleic acid sequence encoding a chimeric antigen receptor (CAR) to produce a population of

5 T cells expressing the CAR.

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, disease cells such as cancer cells. A T cell that expresses a CAR polypeptide is referred to as a

10 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 advantageously do not dimerize with endogenous T-cell receptor (TCR) alpha and beta chains.

15 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 z) 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

20 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

25 recognizes a target antigen (*e.g.*, a single-chain variable 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

30 well as a signal peptide at the N-terminus for surface expression. Examples of signal peptides include MLLLVTSLLLCELPHPAFLLIP (SEQ ID NO: 44) and MALPVTALLLPLALLLHAARP (SEQ ID NO: 75). Other signal peptides may be used.

(a) 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
5 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
10 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
15 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
20 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
25 type of tumor cells.

In some examples, the CAR constructs disclosed herein comprise a scFv extracellular domain capable of binding to CD19. In some examples, the CAR constructs disclosed herein comprise a scFv extracellular domain capable of binding to BCMA. Examples of anti-CD19 CAR and anti-BCMA CAR are provided in Examples below.

30

(b) 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:

FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIW
 10 APLAGTCGVLLLSLVITLYCNHRNR (SEQ ID NO: 49); or
 IYIWAPLAGTCGVLLLSLVITLY (SEQ ID NO: 31).

Other transmembrane domains may also be used.

(c) Hinge Domain

15 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
 20 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
 25 CD8 hinge domain. Other hinge domains may be used.

(d) 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
 30 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.

It should be understood that methods described herein encompasses more than one suitable CAR that can be used to produce genetically engineered T cells expressing the CAR, for example, those known in the art or disclosed herein. Examples can be found in, *e.g.*, PCT/IB2018/001619, filed May 11, 2018, which published as WO 2019/097305A2, the relevant disclosures of which are incorporated by reference herein for the purpose and subject matter referenced herein. In another example, the CAR binds CD19 (also known as a “CD19 CAR” or an “anti-CD19 CAR”). The amino acid sequence of an exemplary CAR that binds CD19 is provided in SEQ ID NO: 37 (see Example 7 below, Table 11). In yet another example, the CAR binds BCMA (also known as a “BCMA CAR” or an “anti-BCMA CAR”). The amino acid sequence of an exemplary CAR that binds to BCMA is provided in SEQ ID NO: 61 (see Example 8 below, Tables 16 and 17).

AAV Vectors for Delivery of CAR Constructs to T Cells

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. 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 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 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.

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 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* gene and the target regions can be used for this purpose, *e.g.*, those disclosed herein.

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 examples, the gRNA target sequence, or portion thereof, is deleted (*e.g.*, SEQ ID NO: 17). In some embodiments, a disruption in the *TRAC* gene can be created with an 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 CRISPR-Cas9 gene editing technology. 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 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 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 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 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 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 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 regulatory sequences, for example, promoters, enhancers, insulators, internal ribosome entry sites, sequences encoding 2A peptides and/or polyadenylation signals.

T Cell Transduction

A suitable amount of any of the viral vectors such as an AAV vector, which encodes a

CAR construct disclosed herein (*e.g.*, an anti-CD19 CAR or an anti-BCMA CAR) may be incubated with a suitable amount of T cells, such as the genetically edited T cells disclosed herein for a suitable period to allow for entry of the viral vector into the T cells. For example, the transduction process may involve the use of a range of optimized multiplicity of infection (MOI) that increases percentages of CAR⁺ T cells. In some instances, the MOI of an AAV vector in the transduction process may range from about 1,000 to about 150,000, such as from about 10,000 to about 80,000. In some examples, the MOI of the AAV vector used in the transduction process may be about 1,000 to about 150,000, about 5,000 to about 100,000, about 10,000 to about 100,000, about 10,000 to about 90,000, about 10,000 to about 80,000, about 10,000 to about 70,000, about 10,000 to about 60,000, about 10,000 to about 50,000, about 10,000 to about 40,000, about 10,000 to about 30,000, about 10,000 to about 20,000, about 20,000 to about 80,000, about 30,000 to about 80,000, about 40,000 to about 80,000, about 50,000 to about 80,000, about 60,000 to about 80,000, or about 70,000 to about 80,000. In some examples, the MOI of the AAV vector used in the transduction process may be about 1,000, about 2,500, about 5,000, about 10,000, about 15,000, about 20,000, about 25,000, about 30,000, about 31,000, about 32,000, about 33,000, about 34,000, about 35,000, about 40,000, about 50,000, about 60,000, about 70,000, about 80,000, about 90,000, about 100,000, about 110,000, about 120,000, about 130,000, about 140,000, or about 150,000.

In some embodiments, the AAV vector encodes an anti-CD19 CAR (*e.g.*, as disclosed in Example 7 below) and the MOI of such an AAV vector for use in the transduction process is about 20,000. In other embodiments, the AAV vector encodes an anti-BCMA CAR (*e.g.*, as disclosed in Example 8 below) and the MOI of such an AAV vector for use in the transduction process is about 20,000.

After transduction, the T cells may be cultured in a suitable cell culture medium for a suitable period for recovery. The genetically engineered T cells, having TRAC and B2M genes knocked-out and expressing the CAR, may be expanded *in vitro* as disclosed below.

(v) ***T Cell Expansion***

The genetically engineered T cells as disclosed herein may be expanded *in vitro* under suitable conditions to produce a population of genetically engineered T cells to a clinically relevant scale. Cell culture conditions used in this expansion step intend to, at least in part, achieve higher final cell densities in shorter incubation periods (thereby reducing manufacturing cost) and higher potent T cells for use in cell therapy. Potency may be indicated by various T cell functions, *e.g.*, proliferation, target cell killing, cytokine production, activation, migration,

and combinations thereof.

In some embodiments, the T cell expansion step may be performed by seeding a population of T cells (*e.g.*, the genetically engineered T cells as disclosed herein) in a cell culture vessel at a seeding density of about 150,000 cells/cm² to about 600,000 cells/cm² in a cell vessel.

5 For example, the T cells may be seeded at about 300,000 cells/cm² to about 500,000 cells/cm², in a cell vessel. In some aspects, the T cell expansion is performed by seeding a population of T cells in a cell culture vessel at a seeding density of at least about 60,000 cells/cm², at least about 62,500 cells/cm², or at least about 83,000 cells/cm². In some aspects, the T cell expansion is performed by seeding a population of T cells in a cell culture vessel at a seeding density of at
10 least about 150,000 cells/cm², or at least about 250,000 cells/cm², or at least about 300,000 cells/cm², or at least about 400,000 cells/cm², or at least about 500,000 cells/cm², or at least about 600,000 cells/cm². In some aspects, the seeding density is about 250,000 cells/cm². In other aspects, the seeding density is about 500,000 cells/cm². In other aspects, the seeding density is about 600,000 cells/cm².

15 In some embodiments, the T cell expansion step may be performed by seeding a population of T cells (*e.g.*, the genetically engineered T cells as disclosed herein) in a cell culture vessel at a seeding density of about 2×10^5 cells/cm² to about 7×10^5 cells/cm², and culturing the cells for about 6 days to about 12 days. In some examples, the T cell expansion is performed by seeding a population of T cells in a cell culture vessel at a seeding density of about 2×10^5
20 cells/cm² to about 7×10^5 cells/cm², about 2×10^5 cells/cm² to about 5×10^5 cells/cm², about 2×10^5 cells/cm² to about 4×10^5 cells/cm², 2×10^5 cells/cm² to about 3×10^5 cells/cm², 3×10^5 cells/cm² to about 5×10^5 cells/cm², or 4×10^5 cells/cm² to about 5×10^5 cells/cm², and culturing the cells for about 6 days to about 12 days, about 6 days to about 11 days, about 6 days to about 10 days, about 6 days to about 9 days, about 6 days to about 8 days, about 6 days to about 7 days, about 7
25 days to about 12 days, about 7 days to about 11 days, about 7 days to about 10 days, about 7 days to about 9 days, about 7 days to about 8 days, about 8 days to about 12 days, about 8 days to about 9 days, about 9 days to about 12 days, about 10 days to about 12 days, or about 11 days to about 12 days. In some embodiments, the T cell expansion is performed by seeding a population of T cells in a cell culture vessel at a seeding density of about 3×10^5 cells/cm² to about 5×10^5
30 cells/cm² and culturing the cells for about 7 days to about 9 days.

In some embodiments, the T cell expansion step may include replating the cell culture (*i.e.*, splitting the cell culture into new culture vessels). In some embodiments, the cell culture can be replated at day 3, 4, 5, 6, or 7 post editing at a 1:4 ratio (1 vessel split into 4 new vessels) for further expansion.

T cell expansion may be performed in a static culture vessel, which allows expansion of the T cells without medium change. For example, T cells can be expanded in a static culture vessel for at about 7 days to about 12 days, or at about 7 days to about 9 days without medium change.

5 (vi) ***Depletion of TCR $\alpha\beta$ ⁺ T Cells***

In some embodiments, TCR $\alpha\beta$ ⁺ T cells may be depleted from the expanded T cell population disclosed herein to produce a population of allogenic T cells for use in cell therapy. As used herein, “TCR $\alpha\beta$ ⁺ T cell depletion” refers to depleting TCR $\alpha\beta$ ⁺ T cells from a population of cells comprising such. Following TCR $\alpha\beta$ ⁺ T cell depletion, the resultant T cell population
10 may have a substantially low level of TCR $\alpha\beta$ ⁺ T cell (*e.g.*, less than 3% in the total cell population, or less than 2%, less than 1% , or less than 0.5% in the total cell population). In some examples, the resultant T cell population may be free of TCR $\alpha\beta$ ⁺ T cell, *i.e.*, presence of TCR $\alpha\beta$ ⁺ T cell is not detectable via a conventional method (*e.g.*, in an immune assay using an antibody binding to TCR $\alpha\beta$ ⁺ or by flow cytometry).

15 TCR $\alpha\beta$ ⁺ T cell depletion may be performed using an agent that recognizes TCR $\alpha\beta$ ⁺ T cells to capture the TCR $\alpha\beta$ ⁺ T cells, thereby separating them from those lacking TCR $\alpha\beta$ ⁺, *e.g.*, by performing a magnetic cell separation. Such methods may be carried out by contacting the expanded T cells disclosed above to beads on which anti-TCR $\alpha\beta$ antibodies are immobilized, and collecting unbound cells. Unbound cells (those lacking TCR $\alpha\beta$ ⁺) thus collected may be
20 cultured to allow cell recovery prior, for example, unbound cells may be cultured overnight to allow cells to recover.

(vii) ***Harvest of Genetically Engineered T Cells***

The genetically engineered T cells produced by any of the methods disclosed herein can then be harvested for therapeutic uses using conventional methods known in the art. For
25 example, harvesting genetically engineered T cells may comprise collecting cells from which TCR $\alpha\beta$ ⁺ has been depleted. The harvested population of genetically engineered T cells may be used as the drug substance. As used herein, a “drug substance” refers to a population of genetically engineered T cells that may be administered to patients. The drug substance may be formulated for therapeutic uses, *e.g.*, formulated in storage media (*e.g.*, CryoStor CS5) and
30 cryopreserved for future use.

Drug substance may be tested for one or more contaminants, *e.g.*, mycoplasma, human viruses (*e.g.*, HIV, HBV, HCV, CMV), and bacterial endotoxins. Alternatively, or in addition to, drug substance may be tested for sterility. Contamination free drug substance may be aliquoted

into individual patient doses. Alternatively, or in addition to, contamination free drug substance may be stored for therapeutic use.

Accordingly, aspects of the present disclosure provide a population of genetically engineered T cells (drug substance). The population of genetically engineered T cells has a
5 disrupted *TRAC* gene, a disrupted $\beta 2M$ gene, and a nucleic acid encoding a CAR, *e.g.*, those described herein. In some embodiments, the CAR binds an antigen expressed on a pathological cell. In some embodiments, the CAR binds CD19. In some embodiments, the CAR binds BCMA.

In some embodiments, at least 30%, at least 40%, at least 50%, at least 60%, at least
10 70%, at least 80%, at least 90%, or at least 95% of the population of genetically engineered T cells produced by methods described herein express a CAR. In other aspects, these cells that express a CAR further do not express a detectable level of surface TCR and/or a detectable level of surface $\beta 2M$.

In other embodiments, where at least 30% of the population of genetically engineered T
15 cells produced by methods described herein express a CAR, that population of cells comprises not more than about 1.0%, not more than about 0.5%, not more than about 0.4%, or not more than about 0.15% T cells that express surface TCR (*e.g.*, TCR α/β^+ cells).

In other embodiments, where at least 30% of the population of genetically engineered T
20 cells produced by methods described herein express a CAR, that population of cells comprises not more than about 50%, not more than about 40%, or not more than about 30%, T cells that express surface $\beta 2M$.

Also within the scope of the present disclosure is a genetically engineered T cell
population produced by methods described herein comprising a Cas9 enzyme, a gRNA targeting a *TRAC* gene, a gRNA targeting a $\beta 2M$ gene, and an AAV vector comprising a nucleic acid
25 sequence encoding a CAR (*e.g.*, a CD19 CAR or a BCMA CAR).

II. Therapeutic Applications

A population of genetically engineered T cells produced by methods described herein may be administered to a subject for therapeutic purposes, for example, treatment of a cancer targeted by the CAR construct expressed by the population of genetically engineered T cells.

30 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.

Non-limiting examples of cancers that may be treated using a genetically engineered T cell population produced by methods described herein include, but are not limited to, multiple myeloma, leukemia (*e.g.*, T cell leukemia, B-cell acute lymphoblastic leukemia (B-ALL), and/or

chronic lymphocytic leukemia (C-CLL)), lymphoma (*e.g.*, B-cell non-Hodgkin's lymphoma (B-NHL), Hodgkin's lymphoma, and/or T cell lymphoma), and/or clear cell renal cell carcinoma (ccRCC), pancreatic cancer, gastric cancer, ovarian cancer, cervical cancer, breast cancer, renal cancer, thyroid cancer, nasopharyngeal cancer, non-small cell lung (NSCLC), glioblastoma, and/or melanoma.

Administering may include placement (*e.g.*, transplantation) of the genetically engineered T cell population into a subject by a method or route that results in at least partial localization of the genetically engineered T cell population at a desired site, such as a tumor site, such that a desired effect(s) can be produced. The genetically engineered T cell population 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 engraftment. For example, in some aspects described herein, an effective amount of the genetically engineered T cell population can be administered *via* a systemic route of administration, such as an intraperitoneal or intravenous route.

In some embodiments, the genetically engineered T cell population is administered systemically, which refers to the administration of a population of cells other than directly into a target site, tissue, 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, subcapsular, subarachnoid, intraspinal, intracerebro spinal, and intrasternal injection and infusion. In some embodiments, the route is intravenous.

An effective amount refers to the amount of a genetically engineered T cell population 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 genetically engineered T cell population 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 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.

An effective amount of a genetically engineered T cell population may comprise at least 10^2 cells, at least 5×10^2 cells, at least 10^3 cells, at least 5×10^3 cells, at least 10^4 cells, at least 5×10^4 cells, at least 10^5 cells, at least 2×10^5 cells, at least 3×10^5 cells, at least 4×10^5 cells, at least 5×10^5 cells, at least 6×10^5 cells, at least 7×10^5 cells, at least 8×10^5 cells, at least 9×10^5 cells, at least 1×10^6 cells, at least 2×10^6 cells, at least 3×10^6 cells, at least 4×10^6 cells, at least 5×10^6 cells, at least 6×10^6 cells, at least 7×10^6 cells, at least 8×10^6 cells, at least 9×10^6 cells, or multiples thereof.

The efficacy of a treatment using the genetically engineered T cell population manufactured as described herein can be determined by one of ordinary skill in the art. 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.

Genetically engineered T cell populations manufactured as described herein may also be used in combination therapies. For example, the genetically engineered T cell population manufactured as described herein may be co-used with other therapeutic agents, for treating the same indication, or for enhancing efficacy of the genetically engineered T cell population and/or reducing side effects of the genetically engineered T cell population.

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 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 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
- 5 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 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
- 10 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;
- 15 *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

20 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

In order that the invention described may be more fully understood, the following

25 examples are set forth. The examples described in this application are offered to illustrate the methods and compositions provided herein and are not to be construed in any way as limiting their scope.

EXAMPLE 1: Identification of Optimized Conditions for T Cell Enrichment.

30 This Example reports identification of optimized conditions for T cell enrichment, using an automated cell processing system to enrich CD4⁺ and CD8⁺ T cells from leukopaks.

METHODS

Leukopak and Buffer Preparation

Human leukopaks were collected from HemaCare or Stem Express and processed for T cells enrichment. PBS/EDTA Buffer (phosphate buffered saline, pH 7.2, supplemented with 1 mM EDTA) was supplemented with 0.5% Human Serum Albumin (HSA) and used for processing, priming, washing, and elution during T cell selection.

- 5 The leukopak donors were screened for the following:
- Hepatitis B Surface Antigen (HBsAg EIA)
 - Hepatitis C Virus Antibody (Anti-HCV EIA)
 - Human Immunodeficiency Virus Antibody (HIV 1/2 plus O)
 - Human T-Lymphotropic Virus Antibody (HTLV-I/II)
 - 10 • HIV-1/HCV/HBV Nucleic Acid Testing
 - WNV Nucleic Acid Testing
 - Trypanosoma Cruzi Antibody (Selective Chagas Disease Testing, a single lifetime test per donor)
 - HIV/HBV/HCV
 - 15 • CMV

Donors showing positive results of any of the above tests were excluded. Demographic information of the donors used in the Examples disclosed herein is shown in **Table 1**.

20 **Table 1.** Donor demographic and hematology parameters. All donors were male.

Batch	Supplier	Donor source ID	Age	Donor weight (lb)	BMI	Ethnicity	ABO/ Rh	Product volume (mL)	WBC (x10 ⁹)	Lymphocyte %
1	HemaCare	D327083	26	144	19.0	Hispanic/ Latino	O-POS	279	9.77	79
2	HemaCare	141402	29	160	22.9	Caucasian	A-POS	302	13.59	75.9
3	HemaCare	141121	26	154	24.8	Hispanic	O-POS	250	8.75	74.7
4	HemaCare	136723	20	130	20.9	Caucasian	A-POS	305	12.81	70.1
5	HemaCare	D64140	28	272	42.6	Hispanic/ Latino	A-POS	339	21.36	81.1
6	Stem Express	D001003 864	33	176	24.0	Caucasian	A-POS	140	8.14	70.9
7	HemaCare	141722	20	135	19.9	Hispanic	O-POS	308	13.24	78.5
8	HemaCare	D327737	36	200	26.4	African American	B-POS	310	14.57	81.3
9	HemaCare	D326737	31	225	29.7	African American	AB-POS	314	10.99	77.9

Leukopak Hematology Analysis with Sysmex

Samples from incoming leukopaks were processed for hematology analysis with Sysmex XP300 (Sysmex, Serial No: B0628) following manufacturer's instructions. White blood cell (WBC) count was used to calculate the total cell mass loaded into the automated cell processing system.

5

T cell Enrichment

Process buffer, leukopak, CD4 microbeads, and CD8 microbeads were loaded in the automated cell processing system prior to starting the run. Cells were washed and labeled in the chamber and directed to the magnet column for separation. CD4⁺ and CD8⁺ T cells were
10 captured and further eluted into the target bag in processing buffer.

Cell Count and Viability

Cell count and viability assessment were performed with COUNTESS® II (Life Technologies, Cat: AMQAX1000) using a default profile. Cells (20 µL) were mixed with
15 Trypan blue (20 µL) by pipetting up and down a few times without introducing bubbles. Cell/Trypan blue mixture (10 µL) was loaded into COUNTESS® II cell counting chamber slides.

Flow Cytometry

About 1×10^6 total nuclei cells were blocked with 5 µL of human TruStain FcX™ in 95
20 µL of staining buffer (0.5% Bovine Serum Albumin (BSA)/DPBS)) at room temperature (RT) for 10 minutes. Cells were further incubated with Pacific blue-conjugated anti-human CD45 antibody (1:50), BV510-conjugated anti-human CD3 antibody (1:50), APC-Cy7-conjugated anti-human CD4 antibody (1:50), PE-Cy7-conjugated anti-human CD8 antibody (1:50), APC-conjugated anti-human CD19 antibody (1:50), FITC-conjugated anti-human CD56 antibody
25 (1:50) and PE-conjugated anti-human CD33 antibody (1:50) at 4°C for 30 minutes. Then, 1 mL of Ammonium-Chloride-Potassium (ACK) lysis buffer containing 5 µL 7-amino-actinomycin D (7-AAD) viability staining solution was applied to each sample. After incubation with ACK lysing buffer at RT for 10 minutes, cells were acquired with NovoCyte-3000 flow cytometer.

30 RESULTS

White Blood Cells (WBCs) in Leukopak Samples

WBC in the tested leukopaks ranged from 8.14×10^9 to 21.36×10^9 cells with lymphocyte number ranging from 5.77×10^9 to 17.32×10^9 .

35 *CD4 and CD8 Enrichment – Purity, Viability, Cell recovery, and Yield*

Among the 9 batches tested, four were evaluated with program A and five were evaluated with program B. All batches yielded T cells with >90% purity and with >90% viability (**Table 2**). Cell recovery from program A was 31% whereas cell recovery from program B was 55.69%.

Table 2. CD4 and CD8 enrichment results

Batch	Program	Leukopak CD3%	Non-Target Cell CD3%	Target Cell			
				Cell Number (x10 ⁹)	CD3%	Viability (%)	Recovery (%)
1	A	73.20	50.80	1.32	96.20	96.50	29.24
2		72.30	60.40	2.76	96.30	93.50	27.00
3		64.90	46.00	2.32	96.80	95.00	39.15
4		63.50	55.00	2.59	89.70	94.00	30.77
Avg (A)		68.48	53.05	2.25	94.75	94.75	31.54
5	B	70.30	15.70	6.00	94.50	93.00	39.75
6		56.00	3.17	2.14	92.80	96.00	47.10
7		69.00	16.80	4.68	96.60	93.00	49.10
8		59.40	15.20	6.82	92.60	96.00	75.87
9		55.50	11.20	3.88	93.60	98.00	61.65
Avg (B)		62.04	12.41	4.70	94.02	95.20	54.69

5

Taken together, these results demonstrate that T cells from healthy donor (HD) leukopaks were enriched with high purity (>90%) and high viability (>90%) for CD4⁺ and CD8⁺ T cells.

EXAMPLE 2: Identification of Optimized Conditions for T Cell Activation.

10 This Example reports identification of optimized conditions for T cell activation, using a colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists.

Identification of Optimized Conditions for T Cell Activation in a Static Culture Vessel

In brief, cryopreserved T cells from healthy donor leukopaks were thawed, and activated
 15 with recombinant humanized CD3 and CD28 agonists conjugated to a polymeric nanomatrix for 48 hours in a T-flask as a control or a static culture vessel. T cell activation was evaluated by monitoring the surface expression of cell activation marker CD25 and CD69, and by monitoring cell proliferation. Various T cell activation conditions for activation in static culture vessels were tested including cell seeding density, medium volume, and recombinant humanized CD3
 20 and CD28 agonists conjugated to a polymeric nanomatrix concentration (“CD3/CD28 agonists”) (**Table 3**). T cell activation was evaluated by monitoring the surface expression of cell activation marker CD25 and CD69, and by monitoring cell proliferation. T cell activation in a T-flask was used as a positive control (PC) (**Table 3**).

Table 3. T cell activation conditions tested

Condition	Vessel	cell number per cm ²	Volume of Medium (mL)	cell density per mL	CD3/CD28 agonists (μl per 1x10 ⁶ cells)	CD3/CD28 agonists to Medium ratio
1	Static Vessel	1.00x10 ⁷	8	2.50x10 ⁶	40	1:10
2		1.00x10 ⁷	4	5.00x10 ⁶	40	1:5
3		1.00x10 ⁷	2	1.00x10 ⁷	40	1:2.5
4		1.00x10 ⁷	4	5.00x10 ⁶	8	1:25
5		1.00x10 ⁷	2	1.00x10 ⁷	4	1:25
6		2.00x10 ⁶	4	1.00x10 ⁶	40	1:25
7		2.00x10 ⁶	2	2.00x10 ⁶	40	1:12.5
8		2.00x10 ⁶	2	2.00x10 ⁶	20	1:12.5
Positive Control (PC)	T-Flask		10	1.00x10 ⁶	40	1:25

As shown in **FIG. 1A**, the percentage of cells expressing CD25 and CD69 were similar among the conditions tested. A slightly higher (~10% higher) population of CD69⁺ cells and CD25+CD69⁺ cells were observed in condition 3 (**FIG. 1A**).

The cell expansion rate, however, was not correlated with the expression level of CD69 but rather, it was correlated with the expression level of CD25, as measured by the mean florescent intensity (MFI) of CD25 (**FIG. 1B**). Among the conditions tested, condition 7 had the most similar correlation between CD25 MFI and cell expansion when compared to that of the positive control (**FIG. 1B**). CD25 and CD69 are T-cell activation markers, where early upregulation and late upregulation are correlated with activation status.

In sum, these results demonstrate that Condition 7 in **Table 3** led to superior T cell activation effect (Condition 7: 2.00x10⁶ cells/cm²; 2.00x10⁶ cells/mL; 40 μL of colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists/1x10⁶ cells; and 1:12.5 colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists to medium ratio) in a static culture vessel.

Validation of Optional T Cell Activation Conditions in a Small-Scale Manufacturing Process

Next, the identified T cell activation conditions (Condition 7: 2.00x10⁶ cells/cm²; 2.00x10⁶ cells/mL; 40 μL of colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists/1x10⁶ cells; and 1:12.5 colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists to medium ratio) were tested in a

small scale manufacturing process (in a static culture vessel) and the activated T cells were investigated for their gene editing efficiency with respect to expression of a chimeric antigen receptor (CAR), T cell receptor alpha chain constant region (*TRAC*) knock-out, and/or beta-2-microglobulin ($\beta 2M$) knock-out. Activation and editing of T cells in a T-flask (Flask) was compared to that in a static cell culture vessel (Vessel). *TRAC* and $\beta 2M$ electroporated T cells (EP) and untreated T cells (UT) were used as controls.

Small Scale Manufacturing Process

Cryovials were retrieved from liquid nitrogen storage and were thawed in a water bath until a small amount of frozen material remained. Cells were then added dropwise to a 10X volume of full growth medium (X-VIVO™ 15 (Lonza), 5% Human AB Serum, 100U/mL IL2, 100U/mL IL7), and pelleted by centrifugation at 300g for 10 minutes at room temperature. Cells were resuspended to a concentration of 1×10^6 cells/mL and subjected to colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists-mediated activation, which improved downstream modification. In brief, isolated T cells were activated with recombinant CD3 and CD28 covalently attached to a colloidal polymeric nanomatrix. The colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists was applied to cells at a 1:25 ratio or 40 μ L per 1×10^6 cells in a nontreated flask. Cells were maintained in the colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists for 2 days in an incubator at 37°C, 5% CO₂ for 48 hours. Following incubation, cells are centrifuged at 300g for 10 minutes at room temperature. Cell pellets were then resuspended in full growth media and cultured overnight at a concentration of 1×10^6 cells/mL prior to gene modification.

Following overnight culture in full media without the colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists, total cell numbers and cell viability were quantified by addition of Trypan blue and counting on the COUNTESS® cytometer. Then, cells were centrifuged at 300g for 10 minutes at room temperature. Cell pellets were washed in 10 mL of electroporation buffer and centrifuged again. While cells were being centrifuged, ribonucleoprotein (RNP) complexes were prepared. Two separate RNP complexes were formed. One RNP was formed containing B2M sgRNA and Cas9 at concentrations of 150 μ g/mL and 150 μ g/mL, respectively. The other RNP was formed containing TCR sgRNA and Cas9 at concentrations of 150 μ g/mL and 150 μ g/mL respectively. RNP complexes containing sgRNAs and Cas9 were formed by incubation at room temperature for 10 minutes. One RNP complex was formed containing Cas9 (Cas9; SEQ ID NO: 1) and a

gRNA targeting the $\beta 2M$ gene (B2M-1; SEQ ID NO: 6), and the other RNP complex was formed containing Cas9 (Cas9; SEQ ID NO: 1) and a gRNA targeting the *TCR* gene (TA-1; SEQ ID NO: 2). Following centrifugation, cell pellets were resuspended in electroporation buffer to a concentration of 400×10^6 cells/mL. Using the resulting cell suspension, further dilutions were generated bearing final cell concentrations of 300×10^6 cells/mL, 200×10^6 cells/mL, 150×10^6 cells/mL, and 100×10^6 cells/mL. Separate RNP complexes were combined and pipetted into electroporation cuvettes. Cells at the varying concentrations were added to the RNP complexes and pipetted up and down 5 times.

Cells were electroporated using a transfection system based on flow electroporation. Once each individual cuvette was electroporated, the cell and RNP solution was aliquoted into a non-treated 12-well plate, with each well containing 500 μ L of X-VIVO™ 15 media (without Human AB serum, IL2 and IL7). Cells were allowed to rest for 20 minutes in the incubator. Total cell numbers and cell viability were quantified by addition of Trypan blue and counting on the COUNTESS® cytometer or NC-200.

Based on total cell numbers after resting, cells may need to be further diluted with X-VIVO™ 15 (without Human AB serum, IL2 or IL7) to reach the desired concentration. Total cell numbers are needed to calculate the volume of AAV needed to perform the transduction.

$$\mu\text{L of AAV needed} = \frac{\text{(Total cell numbers)} \times \text{(desired MOI (i.e., 20,000))}}{\text{(virus vgc/mL (i.e., } 1.5 \times 10^{13} \text{))}}$$

AAV and cell suspension was mixed and allowed to incubate in a non-treated flask at 37°C and 5% CO₂ for 1 hour. The entire volume, including AAV, was added to a static culture vessel containing 100 mL of full media. The static culture vessel was incubated for 3 days to allow cell expansion.

After electroporation, each well of a static culture vessel was filled with 100 mL of full growth media. Gene modified cells were seeded at a concentration of 5×10^5 cells/mL to 1×10^6 cells/mL in full growth media. IL2 or IL7 were replenished every three to four days to a final working concentration of 100U/mL. Total cell numbers were quantified every three to four days by addition of Trypan blue and counting on the COUNTESS® cytometer. Cells were maintained in culture for nine to twelve days after electroporation to achieve maximal total cell numbers based on a saturation concentration of 30×10^6 cells/mL. Once cells reached this threshold, depletion of any remaining unedited cells that expressed TCR alpha or beta was performed to remove these cellular impurities.

During the expansion phase in a static culture vessel, cells may reach a plateau phase, thereby attaining a maximal number of cells in the static culture vessel. At this stage, the total cell population comprised 6% or less of TCR alpha and beta positive expressing cells. TCR alpha and beta positive cells may be depleted from the population because they may contribute to graft versus host response. Volume reduction was performed on the static culture vessel to remove 90% of the volume, with the remaining 10% of the volume containing cells. Cells were loaded into a transfer bag which was sterile welded to the tubing set used to perform the depletion. TCR alpha and beta positive cells were removed from the main population using a TCR alpha beta depletion kit comprising biotin anti-TCR alpha beta, which may be captured by anti-biotin beads. Cells depleted of TCR alpha beta positive were eluted into the target bag and are transferred back into a static culture vessel and cultured for an additional day. Cells were then cryopreserved in CS5 and stored at -145°C.

Cells fresh from culture or thawed from cryovials were washed in staining buffer and centrifuged at 1500 rpm for 5 minutes. As a negative control, 1×10^6 cells were incubated with Fab-Biotin or IgG-Biotin antibodies. Cells were washed with staining buffer and incubated with mouse anti-IgG to capture excess primary antibodies. Cells were washed again and incubated with the full panel of secondary antibodies (CD45, CD5, CD4, CD8, B2M, TCR, Streptavidin-APC) and viability dye. Cells were washed a final time with staining buffer and run on the flow cytometer to capture various stained populations.

Flow cytometry was used to quantify the diverse populations present in in-process samples as well as cryopreserved product. The gating strategy described herein was used to differentiate subpopulations. In brief, the strategy used is based on initially gating the lymphocyte population, selecting singlet cell populations, and gating CD45⁺ or CD5⁺ populations. Editing efficiency was determined by visualizing B2M⁻ and TRAC⁻ stained cells as a proportion of the parental CD45⁺ or CD5⁺ population. Similarly, ratios of CD4 and CD8 subpopulations were plotted as a proportion of the CD45⁺ or CD5⁺ population. Isotype controls were used to set the gate for CAR⁺ expression in the APC channel.

Results

In the small-scale manufacturing process disclosed herein, T cells were activated in a static culture vessel and in a T-flask under the same activation conditions (Condition 7) and the resultant activated T cells were then electroporated in the presence of two ribonucleoprotein (RNP) complexes using a transfection system based on flow electroporation. After electroporation, the cells were transduced with a rAAV vector for expressing an anti-CD19 CAR

(anti-CD19 CAR; SEQ ID NO: 53) at multiplicity of infection (MOI) of 20,000 and expanded. Knockout efficiency of TCR $\alpha\beta$ and β 2M, anti-CD19 CAR expression, and cell expansion were assessed during cell expansion. TCR $\alpha\beta$ depletion was performed using the automated cell processing system. Process buffer, cell product, and a TCR $\alpha\beta$ kit that includes anti-TCR $\alpha\beta$ monoclonal antibodies conjugated to biotin were loaded in the automated cell processing system prior to the run. Cells were washed and labeled in the chamber and directed to the magnet column for separation. Unbound cells (TCR $\alpha\beta$ ⁻) were collected into the target bag in processing buffer.

The cells thus obtained were analyzed by flow cytometry to examine T cell activation efficiency (as represented by CD25⁺%, CD69⁺% and fluorescence intensity or MFI), gene editing efficiency ($\alpha\beta$ % and β 2M%), TCR $\alpha\beta$ depletion efficiency, and CAR expression efficiency. See **Table 4** below.

Table 4. Flow panels for flow cytometry.

Panel	Purpose	Unconjugated Antibody	Unconjugated Antibody
T cell activation panel	T cell activation status: CD25%, CD25 mean fluorescence intensity (MFI), CD69%		CD45-Pacific Blue; CD5-FITC; CD4-APC-Cy7; CD3-BV510; CD4-APC-Cy7; CD8-Percp5.5; CD25-PE; CD69-APC; 7-AAD
CAR full panel (In-process)	Editing outcome and TCR $\alpha\beta$ depletion efficiency: $\alpha\beta$ ⁻ %, β 2M ⁻ % and CAR ⁺ %	Biotin-Anti-Mouse Fab'	CD45-Pacific Blue; CD5-FITC; CD4-APC-Cy7; CD8-Percp5.5; TCRab-PE; B2M-PE-Cy7; Live-Dead-HV500
CAR reduced panel (Post Thaw)	Editing outcome: CAR ⁺ %	Biotin-Anti-Mouse Fab'	CD45-Pacific Blue; Streptavidin-APC; Live-Dead-HV500
TCR panel (Post thaw)	Editing outcome: TCR $\alpha\beta$ ⁻ %, β 2M ⁻ %		CD45-Pacific Blue; CD5-FITC; CD4-APC-Cy7; CD8-Percp5.5; TCRab-PE; B2M-PE-Cy7; Live-Dead-HV500

Briefly, a total 0.5x10⁶ to 1x10⁶ cells were incubated in primary un-conjugated antibody for CAR full panel and CAR reduced panel at 4°C for 20 min. Unbound antibody was removed by washing with 1 mL of staining buffer (DPBS/0.5% BSA), and then cells were incubated with 1 μ g control mouse IgG in 100 μ L of staining buffer at room temperature (RT) for 10 min. Then, cells were stained with conjugated antibodies (all panels) including LIVE/DEAD™ Fixable Dead Cell Stain (Thermo Fisher) (except for T cell activation panel) at 4°C for 30 min protected from light. After incubation, cells were washed with staining buffer and resuspended in staining buffer except for T cell activation panel, which was resuspended in staining buffer

containing 7-AAD.

T cells activated in the static culture vessel showed comparable or higher TCR $\alpha\beta$ and β 2M knockout efficiency, and CAR% expression as compared to T cells activated in the T-flask (FIGs. 2A-2D). Editing remained persistent over a 12-day time period in which editing efficiency was monitored (FIGs. 2A-2D). An elevated level of CAR% expression in untreated T cells (UT) on day 9 resulted from a technical issue during flow cytometry and was inconsistent with the CAR% expression measured on days 3, 6, and 12 (FIG. 2D).

T cells activated in the static culture vessel showed significantly higher fold expansion post editing compared to T cells activated in T-flasks (98.66 fold compared to 58.46 fold; FIG. 3). Fold expansion post editing of untreated T cells (84.61 fold) and mock electroporated T cells (71.77 fold) was higher than that observed for T cells activated in the T-flask (58.46 fold), but lower than that observed for T cells activated in the static culture vessel (98.66 fold) (FIG. 3).

Taken together, these results demonstrate that the identified optimal conditions for T cell activation showed similar high T cell activation efficiency in a small scale manufacturing process (represented by a static culture vessel) as compared with a control T-flask. Further, the resultant activated T cells produced in the static culture vessel showed comparable or higher editing efficiency, CAR expression efficiency, and greater cell expansion post editing compared to activated T cells manufactured in T-flasks.

20 **EXAMPLE 3: Identification of Optimized Conditions for T Cell Electroporation.**

This Example reports identification of optimized conditions for gene editing of T cells via electroporation, including the range of T cell concentrations for optimal CRISPR-Cas9-dependent gene editing at the TRAC and the β 2M loci. In this Example, fixed concentrations of gsRNA and Cas9 were introduced into increasing T cell concentrations by electroporation, and editing efficiency was determined by flow cytometry.

Cell Concentrations of 100×10^6 cells/mL to 300×10^6 cells/mL Permits Efficient Editing

Using a fixed concentration of B2M sgRNA (B2M-1; SEQ ID NO: 6), TRAC sgRNA (TA-1, SEQ ID NO: 2) and CAS9 (SEQ ID NO: 1) at 150 μ g/mL, 150 μ g/mL and 300 μ g/mL respectively, electroporation was performed with an increasing cell concentration (100×10^6 cells/mL to 400×10^6 cells/mL). Editing efficiency was monitored every three days after gene editing using flow cytometry. Concentrations in each sample are summarized in Table 5.

Table 5. Cell concentrations for electroporation.

Cell Concentration (10 ⁶ /mL)	B2M-1 256117 (μg/mL)	TA-1 256116 (μg/mL)	CAS9 E0417 (μg/mL)	Cassette	Volume (μL)
100	150	150	150	100 μL volume	100
150	150	150	150	100 μL volume	100
200	150	150	150	100 μL volume	100
300	150	150	150	100 μL volume	100
200	150	150	150	100 μL volume	50
300	150	150	150	100 μL volume	50
400	150	150	150	100 μL volume	50

At cell concentrations ranging from 100x10⁶ cells/mL to 300x10⁶ cells/mL, B2M(-) and TCR(-) subpopulations in edited cells were >80% and >98%, respectively (**FIGs. 4A-4B**).

5 CAR⁺ expression was >40% when cells were electroporated at concentrations ranging from 100x10⁶ cells/mL to 300x10⁶ cells/mL (**FIG. 4C**). For a cell concentration of 400x10⁶ cells/mL, B2M(-) and TCR(-) subpopulations were <80% and <87%, respectively (**FIGs. 4D-4E**). CAR⁺ expression was also slightly reduced in cells electroporated at a density of 400x10⁶ cells/mL (**FIG. 4F**).

10 In sum, these results demonstrate that a range of cell concentration between 100x10⁶ cells/mL to 300x10⁶ cells/mL allows efficient editing at the endogenous β2M and TCR loci.

EXAMPLE 4: Identification of Optimized Conditions for T Cell Transduction.

This Example reports identification of the range of MOI for optimal T cell transduction
15 of an rAAV vector coding for a chimeric antigen receptor, leading to CAR⁺ expression in T cells. In this Example, T cells were transduced by the rAAV vector with increasing MOI, and CAR⁺ expression was quantified by flow cytometry.

In brief, cryopreserved T cells from healthy donor leukopak were thawed and activated for 48 hours. Cells were electroporated in bulk at a cell concentration of 1x10⁶ in the presence of
20 RNP complexes comprising Cas9 and sgRNA targeting TCR (TA-1; SEQ ID NO: 2/Cas9; SEQ ID NO: 1), and Cas9 and sgRNA targeting β2M (B2M-1; SEQ ID NO: 6/Cas9; SEQ ID NO: 1), with 150 μg/mL of sgRNA and 150 μg/mL in each complex (**Table 6**). See also **Examples 1-3** above.

Following electroporation, cells were resuspended and allowed to rest in the incubator for
25 20 minutes. Electroporated cells were then separated into various aliquots and transduced with

increasing MOI of rAAV for 1 hour at 37°C (**Table 6**). CAR⁺ expression was determined by flow cytometry after electroporation and transduction on days 3, 6, 10, and 13.

Table 6. T cell transduction conditions tested.

	B2M-1 256162 (μg/mL)	TA-1 256161 (μg/mL)	CAS9 E0417 (μg/mL)	Cuvette	Volume	MOI (vg/cell)
1	150	150	150	400 μL volume	400	80K
2	150	150	150	400 μL volume	400	40K
3	150	150	150	400 μL volume	400	20K
4	150	150	150	400 μL volume	400	10K
5	150	150	150	400 μL volume	400	5K
6	150	150	150	400 μL volume	400	1.25K
7	Untreated	Untreated	Untreated	NA	NA	NA

5 As shown in **FIG. 5A**, a MOI of 20K was sufficient to achieve CAR⁺ expression of at least 50% over the time period tested. CAR⁺ expression was saturated at MOIs of 10K, 20K, 40K, and 80K (**FIG. 5A**). Varying MOI had no effect on cell viability and cell expansion (data not shown). Differences in CAR⁺ expression were not due to inefficiencies in gene editing, as bulk electroporation was performed, and B2M and TRAC knockdown was consistent across all
10 samples, with the exception of untreated cells (data not shown). MOIs between 1.25K and 10K appeared to correlate linearly with decreased CAR⁺ expression (**FIG. 5A**). Additional experiments in which T cells were incrementally transduced at MOI between 0K to 23K showed a linear correlation between CAR⁺ expression and MOIs in the range of 0.12K to 4.7K (**FIG. 5B**).

15 Taken together, these results demonstrate that CAR⁺ expression was saturated in T cells transduced at an MOI between 10K and 80K, and that CAR⁺ expression was linearly correlated to MOI in T cells transduced at an MOI between 0.12K and 4.7K.

EXAMPLE 5: Identification of Optimized Conditions for T Cell Expansion.

20 This Example reports identification of optional cell seeding densities for superior T cell expansion. In this Example, T cells were seeded at increasing densities and cell expansion was monitored over time.

In brief, cryopreserved T cells from healthy donor leukopak were thawed and activated for 48 hours. Cells were then electroporated in the presence of RNP complexes comprising Cas9
25 and sgRNA targeting TCR (TA-1; SEQ ID NO: 2/Cas9; SEQ ID NO: 1) and Cas9 and sgRNA

targeting $\beta 2M$ (B2M-1; SEQ ID NO: 6/Cas9; SEQ ID NO: 1), with 150 $\mu\text{g/mL}$ of sgRNA and 150 $\mu\text{g/mL}$ in each complex. After electroporation, cells were transduced with the rAAV at MOI of 20,000, and then expanded in a static culture vessel. See **Examples 1-4** above for details.

After editing, cells were seeded in a static culture vessel at 5×10^4 cells/cm² (50,000),
5 1×10^5 cells/cm² (100,000), 2×10^5 cells/cm² (200,000), 3×10^5 cells/cm² (300,000), and 5×10^5 cells/cm² (500,000) for expansion of up to 12 days. Cell count and viability were assessed every 3 days. Fold expansion was calculated as the ratio of ending cell number and starting cell number.

As shown in **FIGs. 6A-6B**, cells seeded at a density of 5×10^5 cells/cm² reached a growth
10 plateau in 9 days. By day 12, cells seeded at a density of 3×10^5 cells/cm² reached cell numbers comparable to those reached by cells seeded at 5×10^5 cells/cm² on day 9 (**FIGs. 6A-6B**). Cells seeded at densities of 2×10^5 cells/cm² and 1×10^5 cells/cm² showed modest expansion without reaching a growth plateau by day 12 (**FIGs. 6A-6B**). Among the tested seeding densities, the lowest levels of proliferation were observed for cells seeded at a density of 5×10^4 cells/cm² cells
15 (**FIGs. 6A-6B**). Cells seeded at a density of 1×10^5 cells/cm² showed more robust fold expansion rate (223.4 fold) compared to cells seeded at densities of 2×10^5 cells/cm² (185.5 fold) and 3×10^5 cells/cm² (164.6 fold) (**FIG. 6C**), although they resulted in smaller total cells. Fold expansion rate for cells seeded at either 5×10^5 cells/cm² and 5×10^4 cells/cm² were around 100-fold (**FIG. 6C**).

20 In sum, these results demonstrate that a range of cell seeding densities between 3×10^5 cells/cm² to 5×10^5 cells/cm² provided efficient T cell expansion post editing.

EXAMPLE 6: Identification of Optimized Conditions for TCR $\alpha\beta$ Depletion.

This Example reports identification of conditions for optimal depletion of TCR $\alpha\beta$ ⁺ cells
25 that remain after editing. CRISPR-Cas9-mediated gene-editing typically leads to an ablation of TCR $\alpha\beta$ expression in >90% of T cells. To minimize the potential of graft versus host disease (GvHD), the remaining TCR $\alpha\beta$ ⁺ T cells may be further reduced through a TCR $\alpha\beta$ ⁺ depletion process.

In brief, cells were incubated with biotin conjugated-TCR $\alpha\beta$ antibody and anti-biotin
30 microbeads. After removal of excess unbound antibody and microbeads, cells were passed through a magnet column, and labeled TCR $\alpha\beta$ ⁺ cells were captured on the column. Unbound TCR $\alpha\beta$ ⁻ cells are eluted into a target bag with 0.5% HSA in PBS/EDTA buffer. Eluted cells were cultured overnight to allow cell recovery, and then harvested for drug product formulation.

Four batches of CAR-expressing T cell product were processed for TCR $\alpha\beta$ depletion. Three batches were generated from a full scale process, and one batch (CTX110-18-01) was generated from a medium size process. Input cell number varied from 7.4×10^9 cells to 32.0×10^9 cells due to donor variation and expansion scale (**Table 7**). Post depletion cell number recovery ranged from 75% to 113.33% (**Table 7**). Cell number recovery of 100% or 113% may have been caused by an under estimation of input cell number (**Table 7**). Viability of input and output cells were above 90%, except input cells from the CTX110-18-01 batch (84.5%) (**Table 7**). Average percent of TCR $\alpha\beta$ ⁺ in input cells and output cells was 2.06% and 0%, respectively.

Table 7. TCR $\alpha\beta$ Depletion of Four Batches of Drug Product.

Input Cells					Output Cells				
Batch	Cell number ($\times 10^9$)	Viability (%)	TCR $\alpha\beta$ ⁺ %	TCR $\alpha\beta$ ⁺ cell number ($\times 10^9$)	Cell number ($\times 10^9$)	Viability (%)	Cell Recovery (%)	TCR $\alpha\beta$ ⁺ %	TCR $\alpha\beta$ ⁺ cell number ($\times 10^9$)
CTX110-18-01	7.40	84.50	1.98	0.15	6.32	93.00	85.41	0.000	0.00
CTX110-18-02	32.00	93.50	0.71	0.23	24.00	96.00	75.00	0.000	0.00
CTX110-18-03	8.48	93.50	2.76	0.23	9.61	97.00	113.33	0.000	0.00
CTX110-18-04	14.30	92.00	2.78	0.40	14.40	96.00	100.70	0.003	0.00
Average	15.55	90.88	2.06	0.25	13.58	95.50	93.61	0.00	0.00

In sum, these results demonstrate efficient depletion of TCR $\alpha\beta$ from CAR-expressing T cells in which the *TRAC* gene and the *$\beta 2M$* gene has been genetically disrupted.

15 **EXAMPLE 7: Manufacturing Process Development for Making Genetically Engineered T Cells Expressing an Anti-CD19 CAR and Having Genetically Disrupted *TRAC* and *$\beta 2M$* Genes (CTX110).**

Overview

20 CTX110 is a CD19-directed T cell immunotherapy comprised of allogeneic T cells that are genetically modified *ex vivo* using CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9) gene editing components (sgRNA and Cas9 nuclease).

25 The modifications include targeted disruption of the *TRAC* and *$\beta 2M$* genes. The disruption of the *TRAC* locus results in loss of expression of the T cell receptor (TCR) and is intended to reduce the probability of Graft versus Host Disease (GvHD), while the disruption of the *$\beta 2M$* locus results in lack of expression of the major histocompatibility complex type I (MHC

I) proteins and is intended to improve persistence by reducing the probability of host rejection. The addition of the anti-CD19 CAR directs the modified T cells towards CD19-expressing tumor cells.

The CAR is composed of an anti-CD19 scFv, the CD8 transmembrane domain, a CD28 co-stimulatory domain, and a CD3 ζ signaling domain. Expression of the CTX110 CAR is driven by the EF-1 α promoter.

An exemplary manufacturing process for CTX110 is depicted in **FIG. 7A**.

Evolution of Manufacturing Process

The CTX110 manufacturing process was performed at three production scales including research scale, development scale, and clinical scale. The Research Scale Process was performed at small scale, and the Research Scale Process was scaled up and transferred for Development Scale Process and Clinical Scale Process. Initial development campaigns (4 lots) were conducted using laboratory-grade starting materials for the drug substance for feasibility and adjustment of the operating parameters. Subsequently, use of GMP-sourced starting materials (sgRNAs, Cas9 and rAAV-138) and quantitative acceptance criteria were implemented for the Clinical Scale Process, which is operationally identical to the Development Scale Process.

Selection of the Starting Materials

The starting materials for production of CTX110 include:

- leukopaks collected from healthy donors,
- bacterially-derived Cas9 nuclease,
- two single guide RNAs (sgRNA), TA-1 which targets the TRAC locus and β 2M-1 which targets the β 2M locus, and
- the recombinant AAV-6 vector (rAAV-138), which encodes the anti-CD19 CAR gene.

Structure information for the components used in making the genetic modifications of CTX110, as well as edited *TRAC* and *β 2M* gene loci, is provided below:

Amino acid sequence of Cas9 nuclease (SEQ ID NO:1):

MDKKYSIGLDIGTNSVGWAVITDEYKVP SKKFKVLGNTDRHS IKKNLIGALLFDSGETAEATRL
KRTARRRYTRRKNRICYLQEIFS NEMAKVDDSFHRL EESFLVEEDKKHERHPIFGNIVDEVAY
HEKYPTIYHLRKKLVDSTDKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVKLF IQLVQTY
NQLFEENPINASGVDAKAILSARLSKSRRLLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNF
DLAEDAKLQLSKD TYDDDLNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSAS

MIKRYDEHHQDLTLLKALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMD
 GTEELLVKLNRDILLRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPPFLKDNREKIEKILTFRI
 PYYVGPLARGNSRFAWMTRKSEETITPWNFEVVDKGASAQSFIERMTNFDKNLPNEKVLPHKS
 LLYEYFTVYNELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFD
 5 SVEISGVEDRFNASLGTYHDLKIIKDKDFLDNEENEDILEDIVLTTLTFEDREMIEERLKTIA
 HLFDDKVMKQLKRRRYTGWGRLSRKLINGIRDQSGKTIIDFLKSDGFANRNFQLIHDDSLTF
 KEDIQKAQVSGQDLSLHEHIANLAGSPAIIKKGILQTVKVDELVKVMGRHKPENIVIEARENQ
 TTQKGQKNSRERMKRIIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINR
 LSDYDQVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKMKKNYWRQLLNAKLITQRK
 10 FDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKVITLKS
 KLVSDFRKDFQFYKREINNYHHAHDAYLNAVVGTAIIKKYPKLESEFVYGDYKVDVVRKMIK
 SEQEI GKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDFATVRKVL
 MPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAYSVLVVAKEKG
 KSKKLKSVKELLGITIMERSSEFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLEFELNGRKRMLAS
 15 AGELQKGNELALPSKYVNFYLLASHYEKLKGSPEDEQKQLFVEQHKHYLDEIIEQISEFSKRV
 ILADANLDKVL SAYNKHDKPIREQAENIIHLFTLTNLGAPAAFKYFDTTIDRKRYTSTKEVLD
 ATLIHQSI TGLYETRIDLSQLGGD

Table 8. sgRNA Sequences and Target Gene Sequences.

sgRNA Sequences			SEQ ID NO:
<i>TRAC</i> sgRNA (TA-1)	Modified	A*G*A*GCAACAGUGCUGUGGCCguuuuagagcua gaaauagcaaguuaaaaaaaggcuaguccguuau aacuugaaaaaguggcaccgagucggugcU*U*U*U	2
	Unmodified	AGAGCAACAGUGCUGUGGCCguuuuagagcua gaaauagcaaguuaaaaaaaggcuaguccguuau aacuugaaaaaguggcaccgagucggugcUUUU	3
<i>TRAC</i> sgRNA spacer	Modified	A*G*A*GCAACAGUGCUGUGGCC	4
	Unmodified	AGAGCAACAGUGCUGUGGCC	5
<i>β2M</i> sgRNA (B2M-1)	Modified	G*C*U*ACUCUCUCUUUCUGGCCguuuuagagcua gaaauagcaaguuaaaaaaaggcuaguccguuau aacuugaaaaaguggcaccgagucggugcU*U*U*U	6
	Unmodified	GCUACUCUCUCUUUCUGGCCguuuuagagcua gaaauagcaaguuaaaaaaaggcuaguccguuau aacuugaaaaaguggcaccgagucggugcUUUU	7
<i>β2M</i> sgRNA spacer	Modified	G*C*U*ACUCUCUCUUUCUGGCC	8
	Unmodified	GCUACUCUCUCUUUCUGGCC	9
Target Sequences (PAM)			
<i>TRAC</i> sgRNA	AGAGCAACAGTGCTGTGGCC (TGG)		10
<i>TRAC</i> sgRNA	AGAGCAACAGTGCTGTGGCC		11

β 2M sgRNA	GCTACTCTCTCTTTCTGGCC (TGG)	12
β 2M sgRNA	GCTACTCTCTCTTTCTGGCC	13
Exemplary sgRNA Formulas		
sgRNA sequence	nnnnnnnnnnnnnnnnnnnnnguuuagagcuagaaa <u>uagcaaguua</u> aaauaaggcuaguccguuaucacuugaaaaaguggcaccgagucg gugcuuuu	14
sgRNA sequence	nnnnnnnnnnnnnnnnnnnnnguuuagagcuagaaa <u>uagcaaguua</u> aaauaaggcuaguccguuaucacuugaaaaaguggcaccgagucg gugc	15
sgRNA sequence	n ₍₁₇₋₃₀₎ guuuuagagcuagaaa <u>uagcaaguua</u> aaaaa <u>aaggcuaguc</u> cguaucacuugaaaaaguggcaccgagucgqqugc ₍₁₋₈₎	16

* indicates a nucleotide with a 2'-O-methyl phosphorothioate modification

“n” refers to the spacer sequence at the 5’ end

Table 9. Edited *TRAC* Gene Sequence

Description	Sequence (Deletions indicated by dashes (-); insertions indicated by bold)	SEQ ID NO:
<i>TRAC</i> gene edit	AA-----GAGCAACAAATCTGACT	17
<i>TRAC</i> gene edit	AAGAGCAACAGTGCTGT-GCCTGGAGCAACAAATCTGACT	18
<i>TRAC</i> gene edit	AAGAGCAACAGTG-----CTGGAGCAACAAATCTGACT	19
<i>TRAC</i> gene edit	AAGAGCAACAGT-----GCCTGGAGCAACAAATCTGACT	20
<i>TRAC</i> gene edit	AAGAGCAACAGTG-----CTGACT	21
<i>TRAC</i> gene edit	AAGAGCAACAGTGCTGT G GGCCTGGAGCAACAAATCTGACT	22
<i>TRAC</i> gene edit	AAGAGCAACAGTGC--TGGCCTGGAGCAACAAATCTGACT	23
<i>TRAC</i> gene edit	AAGAGCAACAGTGCTGT G GCCTGGAGCAACAAATCTGACT	24

Table 10. Edited $\beta 2M$ Gene Sequence.

Description	Sequence (Deletions indicated by dashes (-); insertions indicated by bold)	SEQ ID NO:
$\beta 2M$ gene-edit	CGTGGCCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCT-GCCTGGAGGCTATCCAGCGTGAGTCTCTCCTACCCCTCCCGCT	25
$\beta 2M$ gene-edit	CGTGGCCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCT--GCCTGGAGGCTATCCAGCGTGAGTCTCTCCTACCCCTCCCGCT	26
$\beta 2M$ gene-edit	CGTGGCCCTTAGCTGTGCTCGCGCTACTCTCTCTTT-----CTGGAGGCTATCCAGCGTGAGTCTCTCCTACCCCTCCCGCT	27
$\beta 2M$ gene-edit	CGTGGCCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTG GATAG CCTGGAGGCTATCCAGCGTGAGTCTCTCCTACCCCTCCCGCT	28
$\beta 2M$ gene-edit	CGTGGCCCTTAGCTGTGCTCGC----- ---GCTATCCAGCGTGAGTCTCTCCTACCCCTCCCGCT	29

<i>β2M</i> gene-edit	CGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGTGGCC TGGAGGCTATCCAGCGTGAGTCTCTCCTACCTCCCGCT	30
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Table 11. Sequences of Anti-CD19 CAR Construct Components

Name Description	Sequence	SEQ ID NO:
CD8a transmembrane domain	IYIWAPLAGTCGVLLLSLVITLY	31
CD28 nucleotide sequence	TCAAAGCGGAGTAGGTTGTTGCATTCCGATTACATGAATATGACT CCTCGCCGGCCTGGGCGGACAAGAAAACATTACCAACCTATGCC CCCCACGAGACTTCGCTGCGTACAGGTCC	32
CD28 amino acid sequence	SKRSRLHSDYMNTPRRPGPTRKHYPYAPPRDFAAYRS	33
CD3-zeta nucleotide sequence	CGAGTGAAGTTTTCCCGAAGCGCAGACGCTCCGGCATATCAGCAA GGACAGAATCAGCTGTATAACGAACTGAATTTGGGACGCCGCGAG GAGTATGACGTGCTTGATAAACGCCGGGGGAGAGACCGGAAATG GGGGGTAAACCCCGAAGAAAGAATCCCCAAGAAGGACTCTACAAT GAACTCCAGAAGGATAAGATGGCGGAGGCCTACTCAGAAATAGGT ATGAAGGGCGAACGACGACGGGAAAAGGTCACGATGGCCTCTAC CAAGGGTTGAGTACGGCAACCAAGATACGTACGATGCACTGCAT ATGCAGGCCCTGCCTCCAGA	34
CD3-zeta amino acid sequence	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDRRRGRDPEM GGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLY QGLSTATKDTYDALHMQALPPR	35
Anti-CD19 CAR FMC63-28Z (FMC63- CD8[tm]-CD28[co- stimulatory domain]- CD3z) Nucleic Acid	ATGCTTCTTTTGGTTACGTCTCTGTTGCTTTGCGAAGTTCTCAT CCAGCGTTCTTGCTGATCCCCGATATTAGATGACTCAGACCACC AGTAGCTTGTCTGCCTCACTGGGAGACCGAGTAACAATCTCCTGC AGGGCAAGTCAAGACATTAGCAAATACCTCAATTGGTACCAGCAG AAGCCCGACGGAACGGTAAACTCCTCATCTATCATACTCAAGG TTGCATTCCGGAGTACCGTCACGATTTTCAGGTTCTGGGAGCGGA ACTGACTATTCTTGACTATTTCAAACCTCGAGCAGGAGGACATT GCGACATATTTTTGTCAACAAGGTAATACCTCCCTTACACTTTC GGAGGAGGAACCAACTCGAAATTACCGGGTCCACAGTGGCTCT GGGAAGCCTGGCAGTGGAGAAGGTTCCACTAAAGGCGAGGTGAAG CTCCAGGAGAGCGGCCCCGGTCTCGTTGCCCCCAGTCAAAGCCTC TCTGTAACGTGCACAGTGAGTGGTGATCATTGCCTGATTATGGC GTCTCCTGGATAAGGCAGCCCCGCGAAAGGGTCTTGAATGGCTT GGGGTAATATGGGGCTCAGAGACAACGTATTATAACTCCGCTCTC AAAAGTCGTTGACGATAATAAAAGATAACTCCAAGAGTCAAGTT TTCCTTAAAATGAACAGTTTGCAGACTGACGATACCGCTATATAT TATTGTGCTAAACATTATTACTACGGCGGTAGTTACGCGATGGAT TATTGGGGGCAGGGGACTTCTGTACAGTCAGTAGTGCTGCTGCC TTTGTCCCGGTATTTCTCCAGCCAAACCGACACGACTCCCGCC CCGCGCCCTCCGACACCCGCTCCCACCATCGCCTCTCAACCTCTT AGTCTTCGCCCCGAGGCATGCCGACCCGCCCGGGGTGCTGTT CATACGAGGGGCTTGGACTTCGCTTGTGATATTTACATTTGGGCT CCGTTGGCGGGTACGTGCGGCGTCTTTTGTGTGCTCACTCGTTATT ACTTTGTATTGTAATCACAGGAATCGCTCAAAGCGGAGTAGGTTG	36

Name Description	Sequence	SEQ ID NO:
	TTGCATTCCGATTACATGAATATGACTCCTCGCCGGCCTGGGCGG ACAAGAAAACATTACCAACCCTATGCCCCCCCACGAGACTTCGCT GCGTACAGGTCCCGAGTGAAGTTTTCCCGAAGCGCAGACGCTCCG GCATATCAGCAAGGACAGAATCAGCTGTATAACGAACTGAATTTG GGACGCCGCGAGGAGTATGACGTGCTTGATAAACGCCGGGGGAGA GACCCGGAATGGGGGGTAAACCCCGAAGAAAGAATCCCCAAGAA GGACTCTACAATGAACTCCAGAAGGATAAGATGGCGGAGGCCTAC TCAGAAATAGGTATGAAGGGCGAACGACGACGGGGAAAAGGTCAC GATGGCCTCTACCAAGGGTTGAGTACGGCAACCAAGATACGTAC GATGCACTGCATATGCAGGCCCTGCCTCCCAGA	
Anti-CD19 CAR FMC63-28Z (FMC63- CD8[tm]-CD28[co- stimulatory domain]- CD3z) Amino Acid	MLLLVTSLLLCELPHPAFLIPDIQMTQTSSLSASLGDRVTISC RASQDISKYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSG TDYSLTISNLEQEDIATYFCQQGNTLPYTFGGGKLEITGSTSGS GKPGSGEGSTKGEVKLQESGPGLVAPSQLSVTCTVSGVSLPDYG VSWIRQPPRKLEWLGVWGETTYNSALKSRLTIKDNSKSQV FLKMNSLQTDDBTAIYYCAKHYYYGGSYAMDYWGQTSVTVSSAAA FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAV HTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRSKRSRL LHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSRVKFSRSADAP AYQQGQNQLYNELNLGRREEYDVLDRKRRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGGHGLYQGLSTATKDTY DALHMQALPPR	37
Left ITR (5' ITR) (alternate)	CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGG CGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCG CGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTTCT	38
Right ITR (3' ITR) (alternate)	AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTC GCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCCGACGCCCGG GCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCTGCC TGCAGG	39
TRAC-LHA (800bp)	GAGATGTAAGGAGCTGCTGTGACTTGCTCAAGGCCTTATATCGAG TAAACGGTAGTGCTGGGGCTTAGACGCAGGTGTTCTGATTTATAG TTCAAAACCTCTATCAATGAGAGAGCAATCTCCTGGTAATGTGAT AGATTTCCCAACTTAATGCCAACATACCATAAACCTCCCATTCTG CTAATGCCAGCCTAAGTTGGGGAGACCACTCCAGATTCCAAGAT GTACAGTTTGCTTTGCTGGGCCTTTTCCCATGCCTGCCTTTACT CTGCCAGAGTTATATTGCTGGGGTTTTGAAGAAGATCCTATTAAA TAAAAGAATAAGCAGTATTATTAAAGTAGCCCTGCATTTTCAGGTTT CCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATC ATGGCCTCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTC CCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTATTTCCCGTA TAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCT TGTCCATCACTGGCATCTGGACTCCAGCCTGGGTTGGGGCAAAGA GGGAAATGAGATCATGTCCTAACCCCTGATCCTCTTGTCCACAGA TATCCAGAACCCTGACCTGCCGTGTACCAGCTGAGAGACTCTAA ATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCA AACAAATGTGTACAAAAGTAAGGATTCTGATGTGTATATCACAGA CAAACTGTGCTAGACATGAGGTCTATGGACTTCA	40
TRAC-RHA (800bp)	TGGAGCAACAAATCTGACTTGCATGTGCAACGCCTTCAACAAC AGCATTATTCCAGAAGACACCTTCTCCCCAGCCCAGGTAAGGGC	41

Name Description	Sequence	SEQ ID NO:
	AGCTTTGGTGCCCTTCGCAGGCTGTTTCCTTGCTTCAGGAATGGCC AGGTTCTGCCCAGAGCTCTGGTCAATGATGTCTAAAACTCCTCTG ATTGGTGGTCTCGGCCTTATCCATTGCCACAAAACCTCTTTTT ACTAAGAAACAGTGAGCCTTGTCTGGCAGTCCAGAGAATGACAC GGGAAAAAAGCAGATGAAGAGAAGGTGGCAGGAGAGGGGCACGTGG CCCAGCCTCAGTCTCTCCAAGTTCCTGCCTGCCTGCCTTTG CTCAGACTGTTTGCCCTTACTGCTCTTCTAGGCCTCATTCTAAG CCCCTTCTCCAAGTTGCCTCTCCTTATTTCTCCCTGTCTGCCAAA AAATCTTTCCCAGCTCACTAAGTCAGTCTCACGCAGTCACTCATT AACCACCAATCACTGATTGTGCCGGCACATGAATGCACCAAGTG TTGAAGTGGAGGAATTAAGAGTCAGATGAGGGGTGTGCCAGAG GAAGCACCATTCTAGTTGGGGGAGCCCATCTGTGAGCTGGGAAAA GTCCAAATAACTTCAGATTGGAATGTGTTTTAACTCAGGGTTGAG AAAACAGCTACCTTCAGGACAAAAGTCAGGGAAGGGCTCTCTGAA GAAATGCTACTTGAAGATACCAGCCCTACCAAGGGCAGGGAGAGG ACCCTATAGAGGCCTGGGACAGGAGCTCAATGAGAAAAGG	
EF1 α	GGCTCCGGTGCCCGTCACTGGGCGAGCGCACATCGCCACAGTC CCCGAGAAGTTGGGGGGAGGGGTGGGCAATTGAACCGGTGCCTAG AGAAGGTGGCGCGGGGTAACTGGGAAAAGTGATGTGCTGTACTGG CTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGCA GTAGTCGCCGTGAACGTTCTTTTTTCGCAACGGGTTTGCCGCCAGA ACACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTT ACGGGTTATGGCCCTTGCGTGCCTTGAATTACTTCCACTGGCTGC AGTACGTGATTCTTGATCCCGAGCTTCGGGTTGGAAGTGGGTGGG AGAGTTCGAGGCCTTGCGCTTAAGGAGCCCCTTCGCCTCGTGCTT GAGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCGCTGCGAATCT GGTGGCACCTTCGCGCTGTCTCGCTGCTTTTCGATAAGTCTCTAG CCATTTAAAATTTTTGATGACCTGCTGCGACGCTTTTTTTCTGGC AAGATAGTCTTGTAATGCGGGCCAAGATCTGCACACTGGTATTT CGGTTTTTGGGGCCGCGGGCGGCGACGGGGCCCGTGCCTCCAGC GCACATGTTCCGCGAGGCGGGGCTGCGAGCGCGGCCACCGAGAA TCGGACGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTG GCCTCGCGCCGCGGTGTATCGCCCCGCCCTGGGCGGCAAGGCTGG CCCGGTTCGGCACCAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCG GCCCTGCTGCAGGGAGCTCAAAATGGAGGACGCGCGCTCGGGAG AGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGGGCCTTTCCGT CCTCAGCCGTGCTTCATGTGACTCCACGGAGTACCGGGCGCCGT CCAGGCACCTCGATTAGTTCTCGAGCTTTTGGAGTACGTGCTCTT TAGGTTGGGGGGAGGGGTTTTATGCGATGGAGTTTCCCACACTG AGTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGTAAT TCTCCTTGGAATTTGCCCTTTTGGAGTTGGATCTTGGTTCAATC TCAAGCCTCAGACAGTGGTTCAAAGTTTTTTTCTTCCATTTACAG TGTCGTGA	42
GM-CSF signal peptide	ATGCTTCTTTTGGTTACGTCTCTGTTGCTTTGCGAACTTCCTCAT CCAGCGTTCTTGCTGATCCCC	43
GM-CSF signal peptide	MLLLVTSLLLCELPHPAFLIP	44
Anti-CD19 scFv	GATATTCAAGATGACTCAGACCACCAGTAGCTTGTCTGCCTCACTG GGAGACCGAGTAACAATCTCCTGCAGGGCAAGTCAAGACATTAGC	45

Name Description	Sequence	SEQ ID NO:
	AAATACCTCAATTGGTACCAGCAGAAGCCCCGACGGAACGGTAAAA CTCCTCATCTATCATACGTCAAGGTTGCATTCCGGAGTACCGTCA CGATTTTCAGGTTCTGGGAGCGGAAGTACTATTCCTTGACTATT TCAAACCTCGAGCAGGAGGACATTGCGACATATTTTGTCAACAA GGTAATACCCTCCCTTACACTTTCGGAGGAGGAACCAAACCTCGAA ATTACCGGGTCCACCAGTGGCTCTGGGAAGCCTGGCAGTGGAGAA GGTTCCACTAAAGGCGAGGTGAAGCTCCAGGAGAGCGGCCCGGT CTCGTTGCCCCCAGTCAAAGCCTCTCTGTAACGTGCACAGTGAGT GGTGTATCATTCGCTGATTATGGCGTCTCCTGGATAAGGCAGCCC CCGCGAAAGGGTCTTGAATGGCTTGGGGTAATATGGGGCTCAGAG ACAACGTATTATAACTCCGCTCTCAAAAGTCGCTTGACGATAATA AAAGATAACTCCAAGAGTCAAGTTTTCTTAAAAATGAACAGTTTG CAGACTGACGATACCGCTATATATTATTGTGCTAAACATTATTAC TACGGCGGTAGTTACGCGATGGATTATTGGGGGCAGGGGACTTCT GTCACAGTCAGTAGT	
CD19 scFv amino acid sequence Linker underlined	DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVK LLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQ GNTLPYTFGGGKLEITGSTSGSKPGSGEGSTKGEVKLQESGPG LVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVWGSSE TTYNSALKSRLTIIDKNSKSQVFLKMNSLQTDITAIYYCAKHYY YGGSYAMDYWGQTSVTVSS	46
CD8a extracellular + CD8a transmembrane + 5' Linker (underlined)	GCTGCTGCCTTTGTCCCGGTATTTCTCCAGCCAAACCGACCACG ACTCCCGCCCCGCGCCCTCCGACACCCGCTCCACCATCGCCTCT CAACCTCTTAGTCTTCGCCCCGAGGCATGCCGACCCGCCGCCGGG GGTGCTGTTTCATACGAGGGGCTTGGACTTCGCTTGTGATATTTAC ATTTGGGCTCCGTTGGCGGGTACGTGCGGCGTCCTTTTGTGTGCA CTCGTTATTACTTTGTATTGTAATCACAGGAATCGC	47
CD8a extracellular + CD8a transmembrane (without linker)	TTTGTCCCGGTATTTCTCCAGCCAAACCGACCACGACTCCCGCC CCGCGCCCTCCGACACCCGCTCCACCATCGCCTCTCAACCTCTT AGTCTTCGCCCCGAGGCATGCCGACCCGCCGCCGGGGGTGCTGTT CATACGAGGGGCTTGGACTTCGCTTGTGATATTTACATTTGGGCT CCGTTGGCGGGTACGTGCGGCGTCCTTTTGTGTGCTCACTCGTTATT ACTTTGTATTGTAATCACAGGAATCGC	48
CD8a extracellular + CD8a transmembrane	FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAV HTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNR	49
CD19 VH	EVKLQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGL EWLGVIWGSETTYNSALKSRLTIIDKNSKSQVFLKMNSLQTDIT AIYYCAKHYYYGGSYAMDYWGQTSVTVSS	50
CD19 VL	DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNWYQQKPDGTVK LLIYHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQ GNTLPYTFGGGKLEIT	51
CD19 linker	GSTSGSKPGSGEGSTKG	52
rAAV	CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGG CGTCGGGCGACCTTTGGTCGCCCCGGCCTCAGTGAGCGAGCGAGCG CGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTTCTGCGGC CGCACGCGTGAGATGTAAGGAGCTGCTGTGACTTGCTCAAGGCCT TATATCGAGTAAACGGTAGTGCTGGGGCTTAGACGAGGTGTTCT GATTTATAGTTCAAAACCTCTATCAATGAGAGAGCAATCTCCTGG TAATGTGATAGATTTCCCAACTTAATGCCAACATACCATAAACCT	53

Name Description	Sequence	SEQ ID NO:
	CCCATTCTGCTAATGCCAGCCTAAGTTGGGGAGACCACTCCAGA TTCCAAGATGTACAGTTTGTCTTGCTGGGCCTTTTTCCCATGCCT GCCTTTACTCTGCCAGAGTTATATTGCTGGGGTTTTGAAGAAGAT CCTATTAAATAAAAAGAATAAGCAGTATTATTAAGTAGCCCTGCAT TTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTT ACTGAAATCATGGCCTCTTGCCCAAGATTGATAGCTTGTGCCTGT CCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTTCTAAGATGCTA TTTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAG CCCC GCCCTTGTCATCACTGGCATCTGGACTCCAGCCTGGGTTG GGGCAAAGAGGGAAATGAGATCATGTCCTAACCCCTGATCCTCTTG TCCCACAGATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAG AGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTACCGATT TGATTCTCAAACAAATGTGTCAAAAAGTAAGGATTCTGATGTGTA TATCACAGACAAAACGTGCTAGACATGAGGTCTATGGACTTCAG GCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCACAGTCC CCGAGAAGTTGGGGGGAGGGGTCCGCAATTGAACCGGTGCCTAGA GAAGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGC TCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGCAG TAGTCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAA CACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTA CGGGTTATGGCCCTTGCGTGCCCTGAATTACTTCCACTGGCTGCA GTACGTGATTCTTGATCCCGAGCTTCGGGTTGGAAGTGGGTGGGA GAGTTCGAGGCCTTGCGCTTAAGGAGCCCCCTTCGCCTCGTGCTTG AGTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTG GTGGCACCTTCGCGCCTGTCTCGCTGCTTTCGATAAGTCTCTAGC CATTTAAATTTTTGATGACCTGCTGCGACGCTTTTTTTCTGGCA AGATAGTCTTGTAATGCGGGCCAAGATCTGCACACTGGTATTTT GGTTTTTGGGGCCGCGGGCGGCGACGGGGCCCGTGCGTCCCAGCG CACATGTTCCGGCAGGCGGGGCCTGCGAGCGCGGCCACCGAGAAT CGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGG CCTCGCGCCGCCGTGTATCGCCCCGCCCTGGGCGGCAAGGCTGGC CCGGTCCGCACCAAGTTGCGTGAGCGGAAAGATGGCCGCTTCCCGG CCCTGCTGCAGGGAGCTCAAAATGGAGGACGCGGCGCTCGGGAGA GCGGGCGGGTGAGTACCCACACAAAGGAAAAGGGCCTTTCCGTC CTCAGCCGTGCTTTCATGTGACTCCACGGAGTACCGGGCGCCGTC CAGGCACCTCGATTAGTTCTCGAGCTTTTGAGTACGTGCTCTTT AGGTTGGGGGGAGGGTTTTATGCGATGGAGTTTCCCCACACTGA GTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGTAATT CTCCTTGGAATTTGCCCTTTTGAGTTTGATCTTGTTTCAATTCT CAAGCCTCAGACAGTGGTTCAAAGTTTTTTCTTCCATTTACAGGT GTCGTGACCACCATGCTTCTTTGGTTACGTCTCTGTTGCTTTGC GAACTTCCTCATCCAGCGTTCTTGCTGATCCCCGATATTACAGATG ACTCAGACCACCAAGTAGCTTGTCTGCCTCACTGGGAGACCCAGTA ACAATCTCCTGCAGGGCAAGTCAAGACATTAGCAAATACCTCAAT TGGTACCAGCAGAAGCCCGACGGAACGGTAAAACTCCTCATCTAT CATACGTCAAGGTTGCATTCCGGAGTACCGTCACGATTTTACAGGT TCTGGGAGCGGAAGTACTATTCTTGACTATTTCAAACCTCGAG CAGGAGGACATTGCGACATATTTTGTCAACAAGGTAATACCCCTC CCTTACACTTTCGGAGGAGGAACCAACTCGAAATTACCGGGTCC	

Name Description	Sequence	SEQ ID NO:
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Name Description	Sequence	SEQ ID NO:
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Name Description	Sequence	SEQ ID NO:
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(i) Cell editing performance across healthy donors

T cells from healthy donors (male, n = 10) were isolated from leukopaks and frozen in cryotubes. Editing efficiency was evaluated on thawed cells of each donor using the following concentrations of the gene-editing components: Cas9 (300 µg/mL), TA-1 (75 µg/mL), and B2M-1 (150 µg/mL and 200 µg/mL).

Greater than 40% of the edited cells from all donors expressed CAR. β2M and TRAC knockout rates were greater than 80% and 95% of the total cell population, respectively (**Table 12**). All of the T cell isolations across donors were deemed acceptable for CTX110 manufacturing, indicating a robust production process.

Table 12. Editing Outcomes across 10 Male Donors.

Donor	Blood Type	Age	BMI	%TCRαβ ⁺	% B2M ⁺	% CAR ⁺	Fold Expansion
TA-1 = 75 µg/mL, B2M-1 = 150 µg/mL, Cas9 = 300 µg/mL							
1	A+	49	40.1	99.00	89.00	46.30	13.41
2	A+	28	42.6	99.99	95.00	68.20	62.64
3	A+	36	29	99.00	85.00	66.30	63.00
4	A+	33	24	99.96	91.00	53.10	42.16
TA-1 = 75 µg/mL, B2M-1 = 200 µg/mL, Cas9 = 300 µg/mL							
40	A-	19	25.2	95.00	82.33	49.00	53.57
41	A+	31	24	98.00	86.33	59.67	76.07
43	O+	29	25.7	96.00	81.67	59.00	69.83
A	O+	23	24.4	98.00	86.67	63.00	63.53
44	O+	30	26.5	98.00	83.00	60.00	76.37
45	A+	35	27.2	97.00	84.33	62.00	59.40
Average				98.00	86.43	58.66	58.00
SD				1.54	3.98	6.78	17.72
% CV				1.57	4.61	11.55	30.56

Abbreviations. B2M = β2 microglobulin, BMI = Body mass index, CAR = Chimeric antigen receptor, CV = Coefficient of variation, SD = Standard deviation, TCRαβ = T cell receptor alpha chain + T cell receptor beta chain.

(ii) Cas9 nuclease

The Cas9 nuclease of SEQ ID NO:1 was used in this Example. The results, summarized in **Table 14** below, indicate that similar levels of TCRαβ⁺ and B2M⁺ cells were present, as well as double-negative cells.

(iii) rAAV-138 vector

rAAV-138 vector as disclosed above was used for evaluation of the impact of MOI to achieve desired CAR⁺ expression. Cells were transduced with increasing MOI and CAR⁺

expression was quantified. See **Example 4** above. The data, presented in **FIG. 7B**, support the selection of a MOI of 20,000.

For scale-up development, a study was conducted to verify the suitability of the selected MOI with the starting materials noted above. Cells were transduced at MOIs ranging from 0 to 23,000. After electroporation and viral infection followed by 11 days of expansion, CAR⁺ expression was quantified by flow cytometry. The results are presented in **FIG. 7C**. CAR⁺ expression, which is AAV dose-dependent, ranged from 2.1% CAR⁺ at MOI of 0 to 56.2% CAR⁺ at MOI of 23,000. CAR⁺ expression saturated at MOI of 4,700.

CAR expression for cells infected with the GMP rAAV-138 (MOI of 23,000) was comparable to that obtained with the non-GMP vector (MOI of 20,000), 56.2% and 55.4%, respectively. The MOI of 20,000 was selected for scale-up manufacture.

(iv) In situ formation of the ribonucleoprotein complex (RNP)

Using a fixed concentration of cells, electroporation was performed with increasing concentrations of RNP complexes formed by incubation of Cas9 (SEQ ID NO: 1) with a sgRNA targeting TCR (TA-1, SEQ ID NO: 2) and a sgRNA targeting β 2M (B2M-1; SEQ ID NO 6). *In situ* formation of the TA-1/Cas9 and β 2M/Cas9 complexes was evaluated using a final combined concentration of 300 μ g/mL Cas9 nuclease (equivalent to a final concentration of 150 μ g/mL Cas9 nuclease combined with each guide). Final concentrations of TA-1 and B2M-1 varied from 37.5 μ g/mL to 300 μ g/mL.

As shown in **FIG. 7D**, TA-1 sgRNA concentrations from 37.5 μ g/mL to 75 μ g/mL resulted in higher editing of TCR $\alpha\beta$. TA-1 sgRNA concentrations from 150 μ g/mL or 300 μ g/mL did not provide additional editing of TCR $\alpha\beta$, and decreased editing of B2M. Among the concentrations tested, 75 μ g/mL of TA-1 sgRNA provided the highest efficiency for editing of TCR $\alpha\beta$, β 2M, and TCR $\alpha\beta$ and β 2M double knockout (DKO). As shown in **FIG. 7E**, B2M-1 sgRNA concentrations from 75 μ g/mL to 150 μ g/mL resulted in higher editing of β 2M suggesting that efficient editing may be achieved using a concentration of B2M-1 sgRNA that is higher than the concentration of TA-1 sgRNA.

In sum, these results demonstrate efficient editing of T cells using final concentrations of Cas9, TA-1 sgRNA, and B2M-1 sgRNA of 0.3 mg/mL, 0.08 mg/mL, and 0.2 mg/mL, respectively. To achieve these concentrations, TA-1 sgRNA/Cas9 and B2M-1 sgRNA/Cas9 mixtures were prepared at molar ratios of 2.7:1 and 6.7:1, respectively.

To determine the percent of free Cas9 detected in RNP complexes, TA-1 sgRNA (TA-1; SEQ ID NO: 2) and Cas9 (Cas9; SEQ ID NO: 1), and B2M-1 sgRNA (B2M-1; SEQ ID NO: 6)

and Cas9 (Cas9; SEQ ID NO: 1) mixtures were prepared at molar ratios of 2.7:1 and 6.7:1, respectively. Mixtures were incubated for 10 minutes and then analyzed by CEX HPLC to quantify the amount of free Cas9. As shown in **Table 13**, the low percent of free Cas9 detected in the mixtures suggests that RNP complexes were efficiently formed.

5 **Table 13.** Percent of Free Cas9 in RNP Complexes.

	Free Cas9 (%)
B2M-1	16 ± 2 (n = 9)
TA-1	3 ± 2 (n = 9)

These results demonstrate that incubation of Cas9 and sgRNA results in the majority of Cas9 comprised within the RNP complex.

10 *Development of Manufacturing Process*

(i) Research Process

A total of 22 research lots were produced by Research Scale Processes using T cells from 17 healthy volunteers. Conditions identified for the Research Scale Process were verified and adjusted for scale-up to perform the Development Scale Process. Finally, GMP-sourced critical starting materials were evaluated for the preparation of clinical materials in the Clinical Scale Process. Effectively, the Development Scale Process and Clinical Scale Process are operationally identical.

The research scale process followed the same steps as the process illustrated in **FIG. 7A**. Briefly, T cells from either frozen vials of PBMC (Lots 1-14, 16, 21, 22) or frozen T cells enriched from leukapheresis products (Lots 12-15, 17-20) were activated with colloidal nanomatrix particles conjugated to CD3/CD28 agonists for 2-3 days in “T-cell media” consisting of X-VIVO™ 15 without gentamicin or phenol red, 5% human AB serum, rhIL-2 and rhIL-7. On the 2nd or 3rd day, the colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists was either diluted with fresh media or removed by washing the cells and centrifugation. On the next day, T cells were electroporated with Cas9 and sgRNAs using electroporation-based transfection systems, including a transfection system based on flow electroporation.

Approximately 20-60 minutes after electroporation, cells were left untreated or infected with AAV6 rAAV-138 at a MOI of either 20,000 or 50,000 genocopies per cell. After approximately 1 hour of infection, cells were washed and plated in T-cell media.

Approximately 1 week after genome editing, cells were assessed for TCR $\alpha\beta$ /B2M knockout and CAR expression by flow cytometry. The percentage of cells that were TCR $\alpha\beta$ ⁺, B2M⁺, CD4⁺, CD8⁺ and CAR⁺ was subsequently calculated.

The percentage of cells that lost surface expression of the TCR $\alpha\beta$ and B2M after gene editing, and that expressed cell-surface detectable CAR was evaluated by flow cytometry for each process (**Table 14**). Across the research lots (n = 22), 43 $\pm$ 16% (18-72%) of the cells achieved the desired surface expression of the anti-CD19 CAR, while also exhibiting surface loss of TCR $\alpha\beta$ (98 $\pm$ 0.66%, 97-99%) and B2M (79 $\pm$ 9.6%, 54-86%) (**Table 14**). Similar results were obtained for the Development Scale Process and Clinical Scale Process (**Table 14**). On average the percentage of cells that were fully edited (TCR $\alpha\beta$ ⁺B2M⁺CAR⁺) in the research lots was 31 $\pm$ 13% (15-59%).

CD3 ζ surface expression is dependent on the formation of a complex with the TCR. As such, it serves as a functional biochemical marker for loss of the TCR in addition to that of TCR $\alpha\beta$. Loss of CD3 ζ surface expression averaged 96 $\pm$ 3.5% (85-99%) in the research lots.

CD4/CD8 frequencies for subpopulations were compared to control T cells processed by electroporation with no gene-editing components. For research lots, edited cells (TCR $\alpha\beta$ ⁺B2M⁺CAR⁺, Lots 1-11, 16-22) contained, on average, 50 $\pm$ 12% CD4 cells and 45 $\pm$ 14% CD8 cells. Electroporated and control T cells (Lots 1-11, 16-21) contained 57 $\pm$ 12% CD4 cells and 40 $\pm$ 12% CD8 cells. No statistically-significant differences were observed between CD4 or CD8 frequencies when comparing edited to control T cells (unpaired 2-tailed Students t-test).

(ii) Development and Clinical Processes

The Research Process was transferred to a GMP facility, for scale-up and manufacture of the clinical material. Conditions identified for the Research Process were verified and adjusted for scale-up (Development Process). Finally, GMP-sourced critical starting materials were evaluated (Clinical Process) for the preparation of clinical materials. Effectively, the Development and Clinical Processes are operationally identical. Results are presented in **Table 14** below.

(iii) Comparability of Gene Editing Across the Manufacturing Processes

The comparison of results from the clinical lots to the research process and initial scale-up and non-clinical lots is presented in **Table 14**.

Table 14. Results Across Manufacturing Processes.

Parameter	Research Scale Process	Development Scale Process	Clinical Scale Process
Cell Viability (%)	N.D.	86.4 ± 7.1	92.5 (average, n = 2)
%CAR ⁺ T Cells	43.0 ± 16.0	49.0 ± 12.7	58.0 ± 12.0
CD3 ζ	96 ± 3.5	N.D.	N.D.
%TCR $\alpha\beta$ ⁺	98.0 ± 0.7	99.8 ± 0.2	99.8 ± 0.1
%B2M ⁺	79.0 ± 9.6	84.8 ± 1.6	83.9 ± 1.1

Evaluation of the Process Parameters

The manufacturing operating parameters were evaluated in a series of small-scale and full-scale experiments, which are summarized in **Table 15**.

Table 15. Process Development Study Results

Parameter	Results and Conclusions
T Cell Enrichment	
CD4_CD8 T cell Isolation with the automated cell processing system	T cells isolated using a CD4_CD8 enrichment program were of high purity and viability
T Cell Activation	
T cell activation in a gas permeable rapid expansion system: Cell density, cell concentration, colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists dose and dilution	Conditions below were identified to achieve desirable editing and cell expansion for CTX110 manufacture: 1) cell seeding density: $2 \times 10^6/\text{cm}^2$ 2) cell seeding concentration: $2 \times 10^6/\text{mL}$ 3) colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists: $40\mu\text{L}/1 \times 10^6$ cells 4) 10X colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists dilution 48 hours post-activation
Electroporation and Transduction	
Concentration of sgRNAs and Cas9	TA-1 sgRNA concentrations from 37.5 to 150 $\mu\text{g}/\text{mL}$, B2M-1 sgRNA concentrations from 75 to 150 $\mu\text{g}/\text{mL}$ and Cas9 concentration of 300 $\mu\text{g}/\text{mL}$, achieves comparable TCR $\alpha\beta$ and B2M editing efficiency. The following conditions were selected for the final manufacturing process: 0.08 mg/ml TA-1, 0.2 mg/ml B2M-1 and 0.3 mg/ml Cas9

Cell concentration	Cell concentrations ranging from $100\text{-}400 \times 10^6$ cells/mL were incubated with fixed concentrations of RNP (B2M-1, TA-1 gRNAs and Cas9 at 150 $\mu\text{g/mL}$, 150 $\mu\text{g/mL}$ and 300 $\mu\text{g/mL}$, respectively). At cell concentrations of $100\text{-}300 \times 10^6$ cells/mL, B2M ⁺ and TCR $\alpha\beta$ ⁺ subpopulations were > 80% and > 98% respectively. At a cell concentration of 400×10^6 cells/mL, the B2M ⁺ and TCR $\alpha\beta$ ⁺ subpopulations were < 80% and < 87%, demonstrating lower efficiency.
Impact of EP medium during electroporation	Comparable TCR $\alpha\beta$ and B2M editing efficiency and CAR expression were achieved with up to 10% medium during electroporation, indicating no negative impact of residual medium on CTX110 editing.
rAAV-138 transduction	The clinical AAV transduction process includes: 1) removal of wash step before and after AAV transduction 2) Cell density of AAV Transduction: 10×10^6 /mL 3) AAV MOI: 20,000 (determined on a lot by lot basis)
Cell Expansion	
Seeding density for the gas permeable rapid expansion system culture vessel	Seeding densities between 3×10^5 cells/cm ² to 5×10^5 cells/cm ² after electroporation were examined. Cells seeded between $3\text{-}5 \times 10^5$ cells/cm ² achieved a final cell density about 30×10^6 cells/cm ² and up to 50×10^6 cells/cm ² after culture for 7-9 days.
Cell Expansion in different sized gas permeable rapid expansion system culture vessels	The scalability of cell expansion with the gas permeable rapid expansion system was assessed by comparing cell expansion when seeded in 60 cm ² , 100 cm ² , and 500 cm ² gas permeable membrane surface gas permeable cell culture devices. Results indicate that modified T cells can achieve comparable fold-expansion in different vessel sizes with the same cell density at harvest, thus supporting the procedure of using satellite plating in 60 cm ² gas permeable membrane surface gas permeable cell culture device to monitor cell expansion in the 500 cm ² gas permeable membrane surface gas permeable cell culture device for the CTX110 manufacturing process.
TCR$\alpha\beta$⁺ Cell Depletion	
Evaluation of depletion performance	A customized program for TCR $\alpha\beta$ depletion was developed using an automated cell processing system. The performance of the depletion process with this program was evaluated over 4 batches, demonstrating high depletion efficiency (input 2% → output <LOQ TCR $\alpha\beta$ ⁺ cells) with an average of 70% cell recovery and > 90% viability.

EXAMPLE 8: Methods for Manufacturing Genetically Engineered T Cells Expressing an Anti-BCMA CAR and Having Genetically Disrupted *TRAC* and *$\beta 2M$* Genes (CTX120).

5 CTX120 is a BCMA-directed T cell immunotherapy comprised of human allogeneic T cells that are genetically modified *ex vivo* using CRISPR/Cas9 (Clustered Regularly Interspaced

Short Palindromic Repeats/CRISPR associated protein 9) gene editing components (sgRNA (single guide RNA) and Cas9 nuclease).

- The modifications include targeted disruption of the TRAC and B2M loci, and the insertion of an anti-BCMA chimeric antigen receptor (CAR) transgene into the TRAC locus
- 5 using a recombinant adeno-associated virus vector (rAAV166, a serotype 6 rAAV encoding anti-BCMA directed chimeric T cell antigen receptor).

- The CAR is composed of a humanized single-chain variable fragment (scFv) specific for BCMA, followed by a CD8 hinge and transmembrane region that is fused to the intracellular signaling domains for CD137 (4-1BB) and CD3 ζ . Expression of the CTX120 CAR is driven by
- 10 the elongation factor 1 alpha (EF-1 α) promoter.

- The manufacturing process of CTX120 is illustrated in **FIG. 8A**. Structural information of the starting materials, including bacterially-derived Cas9 nuclease; two single guide RNAs (sgRNA), TA-1 which targets the TRAC locus and B2M-1 which targets the β 2M locus, is provided in **Example 7** above. Amino acid sequences and nucleotide sequences of the anti-
- 15 BCMA CAR in a rAAV vector are provided below (Tables 16 and 17):

Table 16. Nucleotide Sequences of Anti-BCMA CAR Construct Components.

Name Description	Nucleotide Sequence	SEQ ID NO:
CTX-166b rAAV	CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCCGGGCG TCGGGCGACCTTTGGTCGCCCCGGCCTCAGTGAGCGAGCGAGCGCGCA GAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCTGCGGCCGCACG CGTGAGATGTAAGGAGCTGCTGTGACTTGCTCAAGGCCTTATATCGA GTAAACGGTAGTGCTGGGGCTTAGACGCAGGTGTTCTGATTTATAGT TCAAAACCTCTATCAATGAGAGAGCAATCTCTGGTAATGTGATAGA TTTCCCAACTTAATGCCAACATACCATAAACCTCCCATTCTGCTAAT GCCCAGCCTAAGTTGGGGAGACCACTCCAGATTCCAAGATGTACAGT TTGCTTTGCTGGGCCTTTTTCCCATGCCTGCCTTTACTCTGCCAGAG TTATATTGCTGGGGTTTTGAAGAAGATCCTATTAAATAAAAGAATAA GCAGTATTATTAAGTAGCCCTGCATTTCAAGTTTCCTTGAGTGGCAG GCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCCTCTTGGCCA AGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCA GCTGGTTTCTAAGATGCTATTTCCCGTATAAAGCATGAGACCGTGAC TTGCCAGCCCCACAGAGCCCCGCCCTTGTCATCACTGGCATCTGGA CTCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAAC CCTGATCCTCTTGTCACAGATATCCAGAACCCTGACCCTGCCGTG TACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATT CACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTG ATGTGTATATCACAGACAAAACCTGTGCTAGACATGAGGTCTATGGAC TTCAGGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCACAG TCCCCGAGAAGTTGGGGGGAGGGGTGCGCAATTGAACCGGTGCCTAG	55

Name Description	Nucleotide Sequence	SEQ ID NO:
	AGAAGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCT CCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGCAGTAG TCGCCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAACACAG GTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTACGGGTTA TGGCCCTTGCGTGCCTTGAATTACTTCCACTGGCTGCAGTACGTGAT TCTTGATCCCGAGCTTCGGGTGGAAGTGGGTGGGAGAGTTCGAGGC CTTGCGCTTAAGGAGCCCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGG CCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGCGC CTGTCTCGCTGCTTTCGATAAGTCTCTAGCCATTTAAATTTTTGAT GACCTGCTGCGACGCTTTTTTCTGGCAAGATAGTCTTGTAATGCG GGCCAAGATCTGCACACTGGTATTTTCGGTTTTTGGGGCCGCGGGCGG CGACGGGGCCCGTGCCTCCAGCGCACATGTTCCGCGAGGCGGGGCC TCGGAGCGCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGC CGGCCTGCTCTGGTGCCTGGCCTCGCGCCGCCGTGTATCGCCCCGCC CTGGGCGGCAAGGCTGGCCCGGTCCGGCACCAGTTGCGTGAGCGGAAA GATGGCCGCTTCCCGGCCCTGCTGCAGGGAGCTCAAAATGGAGGACG CGGCGCTCGGGAGAGCGGGCGGGTGAGTACCCACACAAAGGAAAAG GGCCTTTCGTCCTCAGCCGTGCTTCAATGTGACTCCACGGAGTACC GGGCGCCGTCCAGGCACCTCGATTAGTTCTCGAGCTTTTGGAGTACG TCGTCTTTAGGTTGGGGGGAGGGGTTTTATGCGATGGAGTTTCCCA CACTGAGTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGT AATTCTCCTTGGAATTTGCCCTTTTTGAGTTTGATCTTGTTTCATT CTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTCTTCCATTTCCAGGT GTCGTGACCACCATGGCGCTTCCGGTGACAGCACTGCTCCTCCCCTT GCGCTGTTGCTCCACGCAGCAAGGCCGAGGTGCAGCTGGTGCAGA GCGGAGCCGAGCTCAAGAAGCCCGGAGCCTCCGTGAAGGTGAGCTGC AAGGCCAGCGGCAACACCCTGACCAACTACGTGATCCACTGGGTGAG ACAAGCCCCCGGCCAAAGGCTGGAGTGGATGGGCTACATCCTGCCCT ACAACGACCTGACCAAGTACAGCCAGAAGTCCAGGGCAGGGTGACC ATCACCAGGGATAAGAGCGCCTCCACCGCTATATGGAGCTGAGCAG CCTGAGGAGCGAGGACACCGCTGTGTACTACTGTACAAGGTGGGACT GGGACGGCTTCTTTGACCCCTGGGGCCAGGGCACAACAGTGACCGTC AGCAGCGGCGGCGGAGGCAGCGGCGGCGGCGGAGCGGCGGAGGCGG AAGCGAAATCGTGATGACCCAGAGCCCCGCCACACTGAGCGTGAGCC CTGGCGAGAGGGCCAGCATCTCCTGCAGGGCTAGCCAAAGCCTGGTG CACAGCAACGGCAACACCCACCTGCACTGGTACCAGCAGAGACCCGG ACAGGCTCCCAGGCTGCTGATCTACAGCGTGAGCAACAGGTTCTCCG AGGTGCCTGCCAGGTTTAGCGGCAGCGGAAGCGGCACCGACTTTACC CTGACCATCAGCAGCGTGGAGTCCGAGGACTTCGCCGTGTATTACTG CAGCCAGACCAGCCACATCCCTTACACCTTCGGCGGCGGCACCAAGC TGGAGATCAAAAGTGCTGCTGCCTTTGTCCCGGTATTTCTCCCAGCC AAACCGACCACGACTCCCGCCCCGCGCCTCCGACACCCGCTCCCAC CATCGCCTCTCAACCTCTTAGTCTTCGCCCCGAGGCATGCCGACCCG CCGCCGGGGTGCTGTTTATACGAGGGGCTTGGACTTCGCTTGTGAT ATTTACATTTGGGCTCCGTTGGCGGGTACGTGCGGCGTCCCTTTGTT GTCACCTCGTTATTACTTTGTATTGTAATCACAGGAATCGCAAACGGG GCAGAAAGAACTCCTGTATATATTCAAACAACCATTTATGAGACCA GTACAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGA	

Name Description	Nucleotide Sequence	SEQ ID NO:
	AGAAGAAGAAGGAGGATGTGAACTGCCGAGTGAAGTTTTCCCGAAGCG CAGACGCTCCGGCATATCAGCAAGGACAGAATCAGCTGTATAACGAA CTGAATTTGGGACGCCGCGAGGAGTATGACGTGCTTGATAAACGCCG GGGGAGAGACCCGAAATGGGGGTAAACCCCGAAGAAAGAATCCCC AAGAAGGACTCTACAATGAACTCCAGAAGGATAAGATGGCGGAGGCC TACTCAGAAATAGGTATGAAGGGCGAACGACGACGGGGAAAAGGTCA CGATGGCCTCTACCAAGGGTTGAGTACGGCAACCAAAGATACGTACG ATGCACTGCATATGCAGGCCCTGCCTCCCAGATAATAATAAAATCGC TATCCATCGAAGATGGATGTGTGTTGGTTTTTGTGTGTGGAGCAAC AAATCTGACTTTGCATGTGCAAACGCCCTCAACAACAGCATTATTCC AGAAGACACCTTCTTCCCCAGCCCAGGTAAGGGCAGCTTTGGTGCCT TCGCAGGCTGTTTTCTTGCTTCAGGAATGGCCAGGTTCTGCCAGAG CTCTGGTCAATGATGTCTAAAACTCCTCTGATTGGTGGTCTCGGCCT TATCCATTGCCACCAAAACCCCTCTTTTTACTAAGAAAACAGTGAGCCT TGTTCTGGCAGTCCAGAGAATGACACGGGAAAAAAGCAGATGAAGAG AAGGTGGCAGGAGAGGGCACGTGGCCAGCCTCAGTCTCTCCAACTG AGTTCTGCCTGCCTGCCTTTTGCTCAGACTGTTGCCCCCTTACTGCT CTTCTAGGCCTCATTCTAAGCCCCTTCTCCAAGTTGCCTCTCCTTAT TTCTCCCTGTCTGCCAAAAAATCTTTCCCAGCTCACTAAGTCAGTCT CACGCAGTCACTCATTAAACCCACCAATCACTGATTGTGCCGGCACAT GAATGCACCAGGTGTTGAAGTGGAGGAATTAAGAGTCAGATGAGGG GTGTGCCAGAGGAAGCACCATTCTAGTTGGGGGAGCCCATCTGTCA GCTGGGAAAAGTCCAATAACTTCAGATTGGAATGTGTTTTAACTCA GGGTTGAGAAAACAGCTACCTTCAGGACAAAAGTCAGGGAAGGGCTC TCTGAAGAAATGCTACTTGAAGATACCAGCCCTACCAAGGGCAGGGA GAGGACCTATAGAGGCCGTTGGGACAGGAGCTCAATGAGAAAGGTAAC CACGTGCGGACCGAGGCTGCAGCGTCGTCTCCTAGGAACCCCTAG TGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAG GCCGGGCGACCAAAGGTGCCCCGACGCCCGGGCTTTGCCCGGGCGGC CTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAGG	
5' ITR	CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCCGGGCG TCGGGCGACCTTTGGTTCGCCCCGGCCTCAGTGAGCGAGCGAGCGCGCA GAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCTT	38
3' ITR	AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGC TCGCTCACTGAGGCCGGGCGACCAAAGTTCGCCCCGACGCCCGGGCTT TGCCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAGG	39
LHA to RHA	GAGATGTAAGGAGCTGCTGTGACTTGCTCAAGGCCTTATATCGAGTA AACGGTAGTGCTGGGGCTTAGACGCAGGTGTTCTGATTTATAGTTCA AAACCTCTATCAATGAGAGAGCAATCTCCTGGTAATGTGATAGATTT CCAACTTAATGCCAACATAACCATAAACCTCCCATTCTGCTAATGCC CAGCCTAAGTTGGGGAGACCACTCCAGATTCCAAGATGTACAGTTTG CTTTGCTGGGCCTTTTTCCCATGCCTGCCTTTACTCTGCCAGAGTTA TATTGCTGGGGTTTTGAAGAAGATCCTATTAATAAAAGAATAAGCA GTATTATTAAGTAGCCCTGCATTTTCAGGTTTCCTTGAGTGGCAGGCC AGGCCTGGCCGTGAACGTTCACTGAAATCATGGCCTCTTGCCCAAGA TTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCAGGACGAGCT GGTTCCTAAGATGCTATTTCCCGTATAAAGCATGAGACCGTGACTTG CCAGCCCCACAGAGCCCCGCCCTTGTCATCACTGGCATCTGGACTC	76

Name Description	Nucleotide Sequence	SEQ ID NO:
	CAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCTT GATCCTCTTGTCCACAGATATCCAGAACCCTGACCCTGCCGTGTAC CAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTAC CGATTTTGATTCTCAAACAAATGTGTACAAAAGTAAGGATTCTGATG TGTATATCACAGACAAAACCTGTGCTAGACATGAGGTCTATGGACTTC AGGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCACAGTCC CCGAGAAGTTGGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGA AGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCCG CCTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGTGACAGTAGTCG CCGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAACACAGGTA AGTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTACGGGTATATGG CCCTTGCCTGCCCTTGAATTACTTCCACTGGCTGCAGTACGTACTTCT TGATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTTCGAGGCCTT GCGCTTAAGGAGCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCT GGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGCGCCTG TCTCGCTGCTTTCGATAAGTCTCTAGCCATTTAAAATTTTGTATGAC CTGCTGCGACGCTTTTTTCTGGCAAGATAGTCTTGTAATGCGGGC CAAGATCTGCACACTGGTATTTTCGGTTTTTGGGGCCGCGGGCGGCGA CGGGGCCCGTGCGTCCAGCGCACATGTTCCGGCGAGGCGGGGCTGC GAGCGCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGCCGG CCTGCTCTGGTGCCTGGCCTCGCGCCCGCGTGTATCGCCCCGCCCTG GCGGCAAGGCTGGCCCGGTCCGCACCAAGTTGCGTGAGCGGAAAGAT GGCCGCTTCCCGCCCTGCTGCGAGGAGCTCAAAATGGAGGACGCGG CGCTCGGGAGAGCGGGCGGGTGAGTACCCACACAAAGGAAAAGGGC CTTTCCGTCCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGG CGCCGTCACGGCACCTCGATTAGTTCTCGAGCTTTTGAGTACGTCG TCTTTAGGTTGGGGGGAGGGGTTTTATGCGATGGAGTTTCCCCACAC TGAGTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGTAAT TCTCCTTGGAATTTGCCCTTTTTGAGTTGGATCTTGGTTTCACTCTC AAGCCTCAGACAGTGGTTCAAAGTTTTTTCTTCCATTTTCAGGTGTC GTGACCACCATGGCGCTTCCGGTGACAGCACTGCTCCTCCCCTTGGC GCTGTTGCTCCACGCAGCAAGGCCGAGGTGCAGCTGGTGCAGAGCG GAGCCGAGCTCAAGAAGCCCGGAGCCTCCGTGAAGGTGAGCTGCAAG GCCAGCGGCAACACCCTGACCAACTACGTGATCCACTGGGTGAGACA AGCCCCCGGCCAAAGGCTGGAGTGGATGGGCTACATCCTGCCCTACA ACGACCTGACCAAGTACAGCCAGAAGTTCCAGGGCAGGGTGACCATC ACCAGGGATAAGAGCGCTCCACCGCCTATATGGAGCTGAGCAGCCT GAGGAGCGAGGACACCGCTGTGTACTACTGTACAAGGTGGGACTGGG ACGGCTTCTTTGACCCCTGGGGCCAGGGCACAACAGTGACCGTCAGC AGCGGCGGCGGAGGAGCGGCGGCGGCGGCGGCGGCGGAGGCGGAAG CGAAATCGTGATGACCCAGAGCCCCGCCACACTGAGCGTGAGCCCTG GCGAGAGGGCCAGCATCTCCTGCAGGGCTAGCCAAAGCCTGGTGCAC AGCAACGGCAACACCCACCTGCACTGGTACCAGCAGAGACCCGGACA GGCTCCAGGCTGCTGATCTACAGCGTGAGCAACAGGTTCTCCGAGG TGCTGCCAGGTTTAGCGGCAGCGGAAGCGGCACCGACTTTACCCCTG ACCATCAGCAGCGTGGAGTCCGAGGACTTCGCCGTGTATTACTGCAG CCAGACCAGCCACATCCCTTACACCTTCGGCGGCGGCACCAAGCTGG AGATCAAAAGTGCTGCTGCCTTTGTCCCGGTATTTCTCCAGCCAAA	

Name Description	Nucleotide Sequence	SEQ ID NO:
	CCGACCACGACTCCCGCCCCGCGCCCTCCGACACCCGCTCCCACCAT CGCCTCTCAACCTCTTAGTCTTCGCCCCGAGGCATGCCGACCCGCCG CCGGGGGTGCTGTTTCATACGAGGGGCTTGGACTTCGCTTGTGATATT TACATTGGGCTCCGTTGGCGGGTACGTGCGGCGTCTTTTTGTTGTC ACTCGTTATTACTTTGTATTGTAATCACAGGAATCGCAAACGGGGCA GAAAGAACTCCTGTATATATTCAAACAACCATTTATGAGACCAGTA CAAATACTCAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGA AGAAGAAGGAGGATGTGAACTGCGAGTGAAAGTTTTCCCGAAGCGCAG ACGCTCCGGCATATCAGCAAGGACAGAATCAGCTGTATAACGAACTG AATTTGGGACGCCGAGGAGTATGACGTGCTTGATAAACGCCGGGG GAGAGACCCGGAATGGGGGGTAAACCCCGAAGAAAGAATCCCCAAG AAGGACTCTACAATGAACTCCAGAAGGATAAGATGGCGGAGGCCTAC TCAGAAATAGGTATGAAGGGCGAACGACGACGGGGAAAAGGTCACGA TGGCCTCTACCAAGGGTTGAGTACGGCAACCAAAGATACGTACGATG CACTGCATATGCAGGCCCTGCCTCCCAGATAATAATAAAATCGCTAT CCATCGAAGATGGATGTGTGTTGGTTTTTTGTGTGTGGAGCAACAAA TCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGA AGACACCTTCTTCCCCAGCCCAGGTAAGGGCAGCTTTGGTGCCCTTCG CAGGCTGTTTCTTTCCTTCCGAAATGGCCAGGTTCTGCCAGAGCTC TGGTCAATGATGTCTAAAACTCCTCTGATTGGTGGTCTCGGCCTTAT CCATTGCCACCAAAAACCTCTTTTTTACTAAGAAACAGTGAGCCTTGT TCTGGCAGTCCAGAGAATGACACGGGAAAAAAGCAGATGAAGAGAAG GTGGCAGGAGAGGGCACGTGGCCCAGCCTCAGTCTCTCCAAGTGTG TCCTGCCCTGCTGCCTTTGCTCAGACTGTTTGGCCCTTACTGCTCTT CTAGGCCTCATTCTAAGCCCCCTTCTCCAAGTTGCCTCTCCTTATTTT TCCCTGTCTGCCAAAAAATCTTTCCAGCTCACTAAGTCAGTCTCAC GCAGTCACTCATTAACCCACCAATCACTGATTGTGCCGGCACATGAA TGCACCAGGTGTTGAAGTGGAGGAATTA AAAAGTCAGATGAGGGGTG TGCCCAAGGAAGCACCATTCTAGTTGGGGGAGCCCATCTGTCACTG GGGAAAAGTCCAAATAACTTCAGATTGGAATGTGTTTTAACTCAGGG TTGAGAAAAACAGCTACCTTCAGGACAAAAGTCAGGGAAGGGCTCTCT GAAGAAATGCTACTTGAAGATAACAGCCCTACCAAGGGCAGGGAGAG GACCCTATAGAGGCCTGGGACAGGAGCTCAATGAGAAAGG	
TRAC- LHA (800bp)	GAGATGTAAGGAGCTGCTGTGACTTGCTCAAGGCCTTATATCGAGTA AACGGTAGTGCTGGGGCTTAGACGCAGGTGTTCTGATTTATAGTTCA AAACCTCTATCAATGAGAGAGCAATCTCCTGGTAATGTGATAGATTT CCCAACTTAATGCCAACATACCATAAACCTCCCATTCTGCTAATGCC CAGCCTAAGTTGGGAGACCACTCCAGATTTCAAAGATGTACAGTTTG CTTTGCTGGGCTTTTTTCCCATGCCTGCCTTTACTCTGCCAGAGTTA TATTGCTGGGGTTTTTGAAGAAGATCCTATTAATAAAAAGAATAAGCA GTATTATTAAGTAGCCCTGCATTTTCAGGTTTCCTTGAGTGGCAGGCC AGGCCTGGCCGTGAACGTTCACTGAAATCATGGCCTCTTGGCCAAGA TTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCT GGTTTCTAAGATGCTATTTCCCGTATAAAGCATGAGACCGTGACTTG CCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGACTC CAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCTTAACCTT GATCCTCTTGTCCCACAGATATCCAGAACCCTGACCTGCGCGTGATC CAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCTCTATTTCAC	40

Name Description	Nucleotide Sequence	SEQ ID NO:
	CGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATG TGTATATCACAGACAAAACCTGTGCTAGACATGAGGTCTATGGACTTC A	
<i>TRAC-</i> <i>RHA</i> (800bp)	TGGAGCAACAAATCTGACTTTGTCATGTGCAAACGCCTTCAACAACAG CATTATTCCAGAAGACACCTTCTTCCCCAGCCCAGGTAAGGGCAGCT TTGGTGCCCTTCGCAGGCTGTTTCCTTGCTTCAGGAATGGCCAGGTTC TGCCAGAGCTCTGGTCAATGATGTCTAAACTCCTCTGATTGGTGG TCTCGGCCTTATCCATTGCCACCAAAACCCTCTTTTTACTAAGAAAC AGTGAGCCTTGTTCTGGCAGTCCAGAGAATGACACGGGAAAAAAGCA GATGAAGAGAAGGTGGCAGGAGAGGGCACGTGGCCCAGCCTCAGTCT CTCCAACCTGAGTTCCTGCCTGCCTGCCTTTGCTCAGACTGTTTGCCC CTTACTGCTCTTCTAGGCCTCATTCTAAGCCCCTTCTCCAAGTTGCC TCTCCTTATTTCTCCCTGTCTGCCAAAAAATCTTTCCCAGCTCACTA AGTCAGTCTCACGCAGTCACTCATTAACCCACCAATCACTGATTGTG CCGGCACATGAATGCACCAGGTGTTGAAGTGGAGGAATTA AAAAGTC AGATGAGGGGTGTGCCAGAGGAAGCACCATTCTAGTTGGGGGAGCC CATCTGTCAGCTGGGAAAAGTCCAAATAACTTCAGATTGGAATGTGT TTTAACTCAGGGTTGAGAAAACAGCTACCTTCAGGACAAAAGTCAGG GAAGGGCTCTCTGAAGAAATGCTACTTGAAGATACCAGCCCTACCAA GGGCAGGGAGAGGACCCTATAGAGGCCTGGGACAGGAGCTCAATGAG AAAGG	41

Name Description	Nucleotide Sequence	SEQ ID NO:
Anti- BCMA CAR (CTX- 166b)	ATGGCGCTTCCGGTGACAGCACTGCTCCTCCCCTTGGCGCTGTTGCT CCACGCAGCAAGGCCGAGGTGCAGCTGGTGCAGAGCGGAGCCGAGC TCAAGAAGCCCGGAGCCTCCGTGAAGGTGAGCTGCAAGGCCAGCGGC AACACCCTGACCAACTACGTGATCCACTGGGTGAGACAAGCCCCCGG CCAAAGGCTGGAGTGGATGGGCTACATCCTGCCCTACAACGACCTGA CCAAGTACAGCCAGAAGTTCCAGGGCAGGGTGACCATCACCAGGGAT AAGAGCGCCTCCACCGCTATATGGAGCTGAGCAGCCTGAGGAGCGA GGACACCGCTGTGTACTACTGTACAAGGTGGGACTGGGACGGCTTCT TTGACCCCTGGGGCCAGGGCACAACAGTGACCGTCAGCAGCGGCGGC GGAGGCAGCGGCGGCGGCGGCGAGCGGCGAGGCGGAAGCGAAATCGT GATGACCCAGAGCCCCGCCACACTGAGCGTGAGCCCTGGCGAGAGGG CCAGCATCTCCTGCAGGGCTAGCCAAAGCCTGGTGCACAGCAACGGC AACACCCACCTGCACTGGTACCAGCAGAGACCCGGACAGGCTCCAG GCTGCTGATCTACAGCGTGAGCAACAGGTTCTCCGAGGTGCCTGCCA GGTTTAGCGGCAGCGGAAGCGGCACCGACTTTACCCTGACCATCAGC AGCGTGGAGTCCGAGGACTTCGCCGTGTATTACTGCAGCCAGACCAG CCACATCCCTTACACCTTCGGCGGCGGCACCAAGCTGGAGATCAAAA GTGCTGCTGCCTTTGTCCCGGTATTTCTCCAGCCAAACCGACCACG ACTCCCGCCCCGCGCCCTCCGACACCCGCTCCCACCATCGCCTCTCA ACCTCTTAGTCTTCGCCCCGAGGCATGCCGACCCGCGCGGGGGTG CTGTTTCATACGAGGGGCTTGGACTTCGCTTGTGATATTTACATTTGG GCTCCGTTGGCGGGTACGTGCGGCGTCCTTTTGTGTCACTCGTTAT TACTTTGTATTGTAATCACAGGAATCGCAAACGGGGCAGAAAGAAC TCCTGTATATATTCAAACAACCATTTATGAGACCAGTACAACTACT CAAGAGGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAAGAAGG AGGATGTGAAGTGCAGTGAAGTTTTCCCGAAGCGCAGACGCTCCGG CATATCAGCAAGGACAGAATCAGCTGTATAACGAAGTGAATTTGGGA CGCCGCGAGGAGTATGACGTGCTTGATAAACCGCGGGGGAGAGACCC GGAAATGGGGGGTAAACCCCGAAGAAAGAAATCCCCAAGAAGGACTCT ACAATGAAGTCCAGAAGGATAAGATGGCGGAGGCCTACTCAGAAATA GGTATGAAGGGCGAAGCAGCAGCGGGGAAAAGGTACGATGGCCTCTA CCAAGGGTTGAGTACGGCAACCAAAGATACGTACGATGCACTGCATA TGCAGGCCCTGCCTCCAGA	56
Anti- BCMA scFv (CTX-166 & CTX- 166b)	CAGGTGCAGCTGGTGCAGAGCGGAGCCGAGCTCAAGAAGCCCGGAGC CTCCGTGAAGGTGAGCTGCAAGGCCAGCGGCAACACCCTGACCAACT ACGTGATCCACTGGGTGAGACAAGCCCCCGGCCAAAGGCTGGAGTGG ATGGGCTACATCCTGCCCTACAACGACCTGACCAAGTACAGCCAGAA GTTCCAGGGCAGGGTGACCATCACCAGGGATAAGAGCGCCTCCACCG CCTATATGGAGCTGAGCAGCCTGAGGAGCGAGGACACCGCTGTGTAC TACTGTACAAGGTGGGACTGGGACGGCTTCTTTGACCCCTGGGGCCA GGGCACAACAGTGACCGTCAGCAGCGGCGGCGGAGGCAGCGGCGGC GCGGCAGCGGCGGAGGCGGAAGCGAAATCGTGATGACCCAGAGCCCC GCCACACTGAGCGTGAGCCCTGGCGAGAGGGCCAGCATCTCCTGCAG GGCTAGCCAAAGCCTGGTGCACAGCAACGGCAACACCCACCTGCACT GGTACCAGCAGAGACCCGGACAGGCTCCCAGGCTGCTGATCTACAGC GTGAGCAACAGGTTCTCCGAGGTGCCTGCCAGGTTTAGCGGCAGCGG AAGCGGCACCGACTTTACCCTGACCATCAGCAGCGTGAGTCCGAGG	57

Name Description	Nucleotide Sequence	SEQ ID NO:
	ACTTCGCCGTGTATTACTGCAGCCAGACCAGCCACATCCCTTACACC TTCGGCGGCGGCACCAAGCTGGAGATCAAA	
4-1BB nucleotide sequence	AAACGGGGCAGAAAGAAACTCCTGTATATATTCAAACAACCATTTAT GAGACCAGTACAACTACTCAAGAGGAAGATGGCTGTAGCTGCCGAT TTCCAGAAGAAGAAGAAGGAGGATGTGAACTG	58
4-1BB amino acid sequence	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	59
CD3-zeta	CGAGTGAAGTTTTCCCGAAGCGCAGACGCTCCGGCATATCAGCAAGG ACAGAATCAGCTGTATAACGAAGTGAATTTGGGACGCCGCGAGGAGT ATGACGTGCTTGATAAACGCCGGGGGAGAGACCCGGAAATGGGGGGT AAACCCCGAAGAAAGAATCCCAAGAAGGACTCTACAATGAATCCA GAAGGATAAGATGGCGGAGGCCTACTCAGAAATAGGTATGAAGGGCG AACGACGACGGGGAAAAGGTCACGATGGCCTCTACCAAGGGTTGAGT ACGGCAACCAAAGATACGTACGATGCACTGCATATGCAGGCCCTGCC TCCCAGA	31
EF-1 α promoter	GGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCACAGTCCC CGAGAAGTTGGGGGGAGGGGTTCGGCAATTGAACCGGTGCCTAGAGAA GGTGGCGCGGGGTAACTGGGAAAGTGATGTCGTGTACTGGCTCCGC CTTTTTCCCGAGGGTGGGGGAGAACCCTATATAAGTGCAGTAGTCGC CGTGAACGTTCTTTTTCGCAACGGGTTTGCCGCCAGAACACAGGTAA GTGCCGTGTGTGGTTCCCGCGGGCCTGGCCTCTTTACGGGTTATGGC CCTTGCGTGCCTTGAATTACTTCCACTGGCTGCAGTACGTGATTCTT GATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGAGGCCCTTG CGCTTAAGGAGCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCTG GGCGCTGGGGCCGCGCGTGCGAATCTGGTGGCACCTTCGCGCCTGT CTCGCTGCTTTGATAAGTCTCTAGCCATTTAAAAATTTTGATGACC TGCTGCGACGCTTTTTTTCTGGCAAGATAGTCTTGTAATGCGGGCC AAGATCTGCACACTGGTATTTTCGGTTTTTGGGGCCGCGGGCGGCGAC GGGGCCCGTGCCTCCAGCGCACATGTTTCGGCGAGGCGGGGCCTGCG AGCGCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGCCGGC CTGCTCTGGTGCCTGGCCTCGCGCCCGCGTATCGCCCCGCCCTGG GCGGCAAGGCTGGCCCGGTCCGCACCAAGTTGCGTGAGCGGAAAGATG GCCGCTTCCCGGCCCTGCTGCAGGGAGCTCAAAATGGAGGACGCGGC GCTCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGGCC TTTCCGTCCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGGC GCCGTCCAGGCACCTCGATTAGTTCTCGAGCTTTTGGAGTACGTGCT CTTTAGGTTGGGGGGAGGGTTTTATGCGATGGAGTTTCCCCACACT GAGTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGTAATT CTCCTTGAATTTGCCCTTTTTGAGTTTGGATCTTGGTTCATTCTCA	42

Name Description	Nucleotide Sequence	SEQ ID NO:
	AGCCTCAGACAGTGGTTCAAAGTTTTTTTCTTCCATTTTCAGGTGTCG TGA	
3' poly A	AATAAAATCGCTATCCATCGAAGATGGATGTGTGTTGGTTTTTTGTG TG	60

Table 17. Amino Acid Sequences of Anti-BCMA CAR Construct Components.

Name Description	Amino Acid Sequence	SEQ ID NO:
CAR (CTX-166b)	MALPVTALLLPLALLLHAARPQVQLVQSGAELKKPGASVKVSC KASGNTLTNYVIHWVRQAPGQRLEWMGYILPYNDLTKYSQKFQ GRVTITRDKSASTAYMELSSLRSEDVAVYYCTRWDWDGFFDPW GQGTTVTVSSGGGGSGGGGGSGGGGSEIVMTQSPATLSVSPGER ASISCRASQSLVHSNGNTHLHWYQQRPGQAPRLLIYSVSNRFS EVPARFSGSGSGTDFTLTISSVESEDFAVYYCSQTSHIPYTFG GGTKLEIKSAAAFVPVFLPAKPTTTPAPRPTTPAPTIASQPLS LRPEACRPAAGGAVHTRGLDFACDIYWAPLAGTCGVLLLSLV ITLYCNHRNRKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFP EEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDV LDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMK GERRRGKGGHDGLYQGLSTATKDTYDALHMQALPPR	61
scFv (CTX-166 (BCMA-11, & CTX-166b)	QVQLVQSGAELKKPGASVKVSCKASGNTLTNYVIHWVRQAPGQ RLEWMGYILPYNDLTKYSQKFQGRVTITRDKSASTAYMELSSL RSEDVAVYYCTRWDWDGFFDPWGQGTTVTVSSGGGGSGGGGGSG GGGSEIVMTQSPATLSVSPGERASISCRASQSLVHSNGNTHLH WYQQRPGQAPRLLIYSVSNRFSEVPARFSGSGSGTDFTLTISS VESEDFAVYYCSQTSHIPYTFGGGTKLEIK	62
V _H (CTX-166)	QVQLVQSGAELKKPGASVKVSCKASGNTLTNYVIHWVRQAPGQ RLEWMGYILPYNDLTKYSQKFQGRVTITRDKSASTAYMELSSL RSEDVAVYYCTRWDWDGFFDPWGQGTTVTVSS	63
V _L (CTX-166)	EIVMTQSPATLSVSPGERASISCRASQSLVHSNGNTHLHWYQQR RPGQAPRLLIYSVSNRFSEVPARFSGSGSGTDFTLTISSVESE DFAVYYCSQTSHIPYTFGGGTKLEIK	64
V _L CDR1	(Kabat or Chothia) RASQSLVHSNGNTHLH	65

Name Description		Amino Acid Sequence	SEQ ID NO:
V _L CDR2		SVSNR	66
V _L CDR3		SQTSHIPYT	67
V _H CDR1		NYVIH	68
V _H CDR2	(Kabat)	YILPYNDLTKYSQKFQG	69
V _H CDR3		WDWDGFFDP	70
V _H CDR1		GNTLTNY	71
V _H CDR2	(Chothia)	LPYNDL	72
V _H CDR3		WDWDGFFDP	73
linker		GGGGSGGGSGGGGS	74
CD8 signal peptide		MALPVTALLLPLALLLHAARP	75
CD8a transmembrane domain		IYIWAPLAGTCGVLLLSLVITLY	34
4-1BB		KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFEEEEGGCEL	59
CD3-zeta		RVKFSSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDP EMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRRGKGH DGLYQGLSTATKDTYDALHMQALPPR	35

- Manufacture of the CTX120 Drug Substance involved thawing enriched T cells followed by activation and electroporation/transduction, at which point cells were expanded. After expansion, TCR $\alpha\beta$ ⁺ cells were depleted. Cells were cultured overnight, harvested and sampled for Drug Substance testing. See **FIG. 8A**. Reprocessing was not performed in any step of CTX120 manufacturing.

T Cell Enrichment

- T cells were enriched from the leukapheresis materials (Leukopaks) *via* magnetic separation using a mixture of anti-CD8 and anti-CD4 antibody-coated magnetic beads using the automated cell processing system. Prior to enrichment, leukopaks were sampled for cell count and viability ($\geq 80\%$). Enriched cells were isolated in PBS/EDTA Buffer with HSA, and then sampled for cell count, viability ($\geq 80\%$), T cell purity ($\geq 70\%$ CD3), and sterility. The cells were then centrifuged at $4 \pm 1^\circ\text{C}$ and resuspended in CryoStor CS5 at a target concentration of 50×10^6 viable cells/mL.

T Cell Cryopreservation

Cells were sampled for cell count, viability ($\geq 80\%$) and then aliquoted into ethyl vinyl acetate cryobags at the target cell number of $2,500 \times 10^6$ cells/bag (30-70 mL of cell suspension). One Leukopak was sufficient to produce 1-2 bags of T cells. Each bag was heat-sealed, labeled, stored at $2-8^\circ\text{C}$ until transferred to a controlled-rate freezer and subsequently transferred to vapor phase liquid nitrogen for storage.

T Cell Thawing and Activation

One frozen bag of enriched T cells was thawed, transferred into a 3L bag and diluted into Supplemented X-VIVO™ 15 media (X-VIVO™ 15, 5% Human Serum, 100 IU/mL rhIL2, 100 IU/mL rhIL7). Cells were sampled for cell count and viability ($\geq 70\%$). Cells were centrifuged at 540 g at $20 \pm 1^\circ\text{C}$ for 15 minutes. Cells were then resuspended in the Supplemented X-VIVO™ 15 media and sampled for cell count and viability ($\geq 70\%$). Soluble colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists solution was added at the ratio of 1:12.5 (v/v) to activate the cells.

Cells were seeded to a target density 2×10^6 viable cells/mL into two static cell culture vessels, each at a total volume of approximately 500 mL of Supplemented X-VIVO™ 15 media/colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists. Static culture vessels were incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO_2 for 48 ± 4 hours. Throughout the process, whenever the static culture vessels were handled, they were inspected for tears and leaks, and the presence of clear, yellow medium.

Dilution

Two (2) days later, supplemented X-VIVO™ 15 media was added to each static culture vessel to 5 L. Cells were further incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO_2 overnight.

Electroporation and Transduction

The volume of Supplemented X-VIVO™ 15 media was reduced to a final volume of approximately 500 mL using a pump connected to dip-tube in the static culture vessel, which was gently swirled to allow resuspension of cells to in the media. Cells were sampled for cell count, and viability ($\geq 70\%$). Cells were transferred to 500 mL centrifuge tubes and centrifuged at 540 g, at $20 \pm 1^\circ\text{C}$ for 15 minutes. Cell pellets were resuspended in Electroporation Buffer and centrifuged again under the same conditions. Cells were resuspended in Electroporation Buffer a second time to a target concentration of 300×10^6 cells/mL.

Cas9 nuclease was mixed with TA-1 sgRNA (targeting TCR) and with B2M-1 sgRNA (targeting β 2M) in separate microcentrifuge tubes. Each solution was incubated for no less than 10 minutes at room temperature to form each ribonucleoprotein complex. The two Cas9/gRNA mixtures were combined, and mixed with the cells, bringing Cas9, TA-1 and B2M-1 to a final concentration of 0.3 mg/mL, 0.08 mg/mL and 0.2 mg/mL, respectively. The mixture was aliquoted and loaded into an electroporation cassette by pipetting. Cassettes were capped and sequentially electroporated using the transfection system based on flow electroporation. After electroporation, cells were pooled from each cassette in a 125 mL Erlenmeyer flask and incubated at 37°C for no less than 20 minutes. Cells were sampled for viability ($\geq 70\%$) and count. Cells were diluted to 10^7 cells/mL with X-VIVO™ 15 media and freshly thawed rAAV-166b was added at a MOI of 20,000 vg/cell. Cells were incubated at 37°C 5% CO₂ for no less than 60 minutes.

The impact of MOI to achieve desired CAR⁺ expression, was assessed using a development lot of vector (rAAV-166b). Cells were transduced with increasing MOI and %CAR⁺ was quantified. As shown in **FIG. 8B**, AAV dose-dependent CAR expression was observed. Expression of CAR was saturated around MOI of 10,000, supporting the selection of a MOI of 20,000.

Formation of the two ribonucleoprotein complexes (RNP) *in situ* was performed following the descriptions in **Example 7** above. See also results provided in **Table 13** above.

Homology directed-repair (HDR) is a high-fidelity cell repair mechanism for DNA double strand breaks. HDR is used to introduce a CAR gene from the AAV template into the desired TRAC locus by using a homologous sequence on each end of the CAR gene.

To assess the anti-BCMA CAR at the TRAC locus, a ddPCR assay was developed. A TRACsite specific PCR primer set was designed to amplify the integrated anti-BCMA CAR sequence and determine the percent of cells with the CAR gene insertion. Three lots of CTX120 were evaluated by ddPCR and the %HDR is shown in **Table 18**. These results confirm insertion of the anti-BCMA CAR at the TRAC locus.

Table 18. Percent HDR in Development Lots of CTX120

Lot Number	% HDR
CTX120-L-3	46.2%
CTX120-L-4	43.2%
P22T090	34.6%

Cell Expansion

Cells were diluted with Supplemented X-VIVO™ 15 media, sampled for cell viability ($\geq 70\%$) and count, and seeded to a density between $0.2\text{--}0.5 \times 10^6$ viable cells/cm² into two static culture vessels, and one additional static culture vessel (satellite culture for cell monitoring). The static culture vessels were incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO₂. The cell cultures were incubated for up to 9 days. During this time, the cultures were supplemented every 3 to 4 days with 100 IU of rhIL2 and rhIL7 per mL of culture volume. The satellite static cell culture vessel was tested for cell count, viability, and T cell purity throughout expansion. When the cell density in the satellite culture vessel reached approximately 30×10^6 /cm², the TCR $\alpha\beta$ depletion was performed. If cell density in the satellite culture vessel did not reach 30×10^6 /cm², TCR $\alpha\beta$ depletion on the main cultures was performed on Day 9.

TCR $\alpha\beta$ Depletion

The medium of each static cell culture vessel was reduced to a final volume of approximately 500 mL using a pump connected to the dip-tube in the static culture vessel. After the bulk of the media was removed, the static culture vessels were gently swirled to resuspend the cells in the media.

The cells were transferred to 500 mL centrifuge tubes fitted with dip-tubes that connect to the static culture vessels. Cells were sampled for viability ($\geq 70\%$), count, and %CAR⁺ cells. Cells were then centrifuged at $540 \times g$ at $20 \pm 1^\circ\text{C}$ for 15 minutes. Cell pellets were resuspended and pooled in less than 650 mL PBS/EDTA containing 0.5% HSA. Cell suspensions were transferred to a sterile bag which is connected to the automated cell processing system. The automated cell processing system incubated cells with a biotin-conjugated anti-TCR $\alpha\beta$ antibody. Cells were washed and incubated with anti-biotin magnetic beads to allow for depletion of the TCR $\alpha\beta$ ⁺ cells using the automated cell processing system. Cells were tested for cell count, viability ($\geq 70\%$), and %CAR⁺ cells.

Cell Recovery

The depleted cells were resuspended in Supplemented X-VIVO™ 15 media and transferred into 3L bag(s), seeded into static cell culture vessel(s) and incubated overnight at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO₂.

Cell Harvest (Drug Substance)

To harvest cells, static culture vessels were removed from the incubator and allowed to rest for sedimentation of cells. Growth medium was removed from each static culture vessel to a

final volume of approximately 500 mL using a pump. Removed media was sampled for sterility. Static culture vessels were gently swirled to allow the cells to resuspend in the media. The contents of each static culture vessel were transferred in a 3L transfer bag using a pump and was filtered through a 40 µm blood transfusion filter by gravity into a separate sterile 3L bag. Cells were sampled for concentration and viability.

Cell Phenotypes of CTX120 Produced by the Manufacturing Process Disclosed Herein

CTX120 Drug Product development lots were analyzed for T cell populations. Flow panels are shown in Table 19.

Table 19. Flow Panels for Characterization of T Cell Populations

Exhaustion	Subset
CD4	CD4
CD8	CD8
CD95	CD45RO
CAR	CD45RA
CD57	CD62L
Lag3	CD27
PD1	CCR7

Exhaustion Markers

CD57, Lag3 and PD1 are markers associated with T cell exhaustion. The exhaustion status of the CTX120 Drug Product was assessed using the markers defined in Table 19. As shown in FIG. 8C and Table 20, low levels of exhaustion markers were found in CAR+ CTX120 Drug Product cells.

Table 20. Percent CAR+ Cells with Various Exhaustion Markers

Marker	CTX120-L-3	CTX120-L-4	P22T090
%Lag3+	8.19	5.99	2.46
%CD57+	4.86	5.31	0.62
%PD1+	2.83	4.36	2.66
%Tim3+	5.15	4.03	7.01

The CTX120 Drug product was assessed for memory cell markers. In the CD45RO gate, CD62L, CCR7, and CD27 were markers associated with central memory. In the CD45RA gate CD62L was a marker for stem cell memory. Cells that were CD62L and CCR7+ were markers for central memory and stem cell memory. The results for CD8+ T cells are shown in FIG. 8D and Table 21. The results for CD4+ T cells are shown in FIG. 8E and Table 22.

Table 21. Central Memory and Stem Cell Memory Markers in CD8⁺ T Cells

Marker	T Cell Population	CTX120-L-3	CTX120-L-4	P22T090
CD45RA/CD62L	Stem Cell Memory	36.97	37.63	66.13
CD45RO/CD27	Central Memory	48.93	50.77	59.90
CD45RO/CD62L	Central Memory	28.63	44.63	83.03
CD45RO/CCR7	Central Memory	9.28	15.20	41.47
CD62L/CCR7	Stem Cell Memory/Central Memory	36.40	39.93	57.03

Table 22. Central Memory and Stem Cell Memory Markers in CD4⁺ T Cells

Marker	T Cell Population	CTX120-L-3	CTX120-L-4	P22T090
CD45RA/CD62L	Stem Cell Memory	50.13	41.00	54.10
CD45RO/CD27	Central Memory	24.30	26.13	45.33
CD45RO/CD62L	Central Memory	34.63	55.03	71.87
CD45RO/CCR7	Central Memory	15.03	17.90	52.53
CD62L/CCR7	Stem Cell Memory/Central Memory	51.17	39.60	58.73

5

Biological Activities of CTX120

Two assays which measure the biological activity of the CAR T cell Drug Product upon stimulation with BCMA antigen were developed. First, IFN γ secretion upon T cell activation was determined. In brief, CAR T cells were incubated with recombinant human BCMA. Upon CAR T cell activation, the level of secreted IFN γ was measured by Meso-Scale Delivery (MSD). Results are shown in **FIG. 8F**.

Next, the ability of CTX120 CAR-T cells to kill BCMA positive MM.1S target cells was assessed using a flow cytometry-based cytotoxicity assay. In brief, target cells were labeled with eFluor670 and incubated with CTX120 cells at varying ratios. CTX120 cytotoxicity was analyzed at 4 hours by assessing labeled cells in the live gate compared to control sample. The results are shown in **FIG. 8G**. All 3 development lots of CTX120 showed dose dependent target cell cytotoxicity.

Taken together, these results demonstrated production of anti- BCMA CAR expressing T cells. The anti-BCMA CAR expressing T cells manufactured, as described herein, displayed low expression levels of TCR and β 2M, thereby reducing the probability of host rejection. Further, the anti-BCMA CAR expressing T cells displayed targeted cell killing of BCMA positive cells upon T cell activation.

20

EXAMPLE 9: Identification of Optimized Conditions for T Cell Expansion for Scale up.

This Example reports identification of optimal plating or replating conditions for superior T cell expansion and increasing yields. In this Example, T cells were either plated at lower density than 500K/cm² on the same day post editing or seeded with 500K/cm² densities and replated at different days post editing. Cell expansion was monitored over time.

In brief, cryopreserved T cells from healthy donor leukopak were thawed and activated for 48 hours. Cells were then electroporated in the presence of RNP complexes comprising Cas9 (150 µg/mL) and sgRNA targeting TCR (TA-1; SEQ ID NO: 2/Cas9; SEQ ID NO: 1) (80 µg/mL) and Cas9 (150 µg/mL) and sgRNA targeting β2M (B2M-1; SEQ ID NO: 6/Cas9; SEQ ID NO: 1) (200 µg/mL). After electroporation, cells were transduced with the rAAV at MOI of 20,000, and then expanded in a static culture vessel. See **Examples 1-4** above for details.

After editing, cells were seeded at 166K/cm², 125K/cm², or 83K/cm² in a static culture vessel for expansion. Another set of cells were seeded at 500K/cm² post editing and replated at day 3, 4, 5, 6, or 7 post editing at a 1:4 ratio (1 vessel split into 4 new vessels) for further expansion. Cells that replated at 500K/cm² without replating were used as CTX110 reference group. All groups expanded until the cell density reached 3-4x10⁶/mL at which point the cells were harvested (**Table 23**). Cell count and viability were assessed every 1-3 days.

Table 23

Groups		Harvest point	Expected Yield	Harvest date
1	CTX110 500K reference	3x10 ⁶ /mL	1x	day 7
		4x10 ⁶ /mL		
2	CTX110 166K	4x10 ⁶ /mL	3X	day 10 (+3 days)
3	CTX110 125K	4x10 ⁶ /mL	4X	day 11 (+3 days)
4	CTX110 83K	4x10 ⁶ /mL	6X	day 14 (+7 days)
5	CTX110 D3 replate 1:4 split	4x10 ⁶ /mL	4X	day 10 (+3 days)
6	CTX110 D4 replate 1:4 split	4x10 ⁶ /mL		day 10 (+3 days)
7	CTX110 D5 replate 1:4 split	3x10 ⁶ /mL		day 10 (+3 days)
		4x10 ⁶ /mL		
8	CTX110 D6 replate 1:4 split	3x10 ⁶ /mL		day 18 (+11 days)
		4x10 ⁶ /mL		day 14 (+7 days)
9	CTX110 D7 replate 1:4 split	3x10 ⁶ /mL		day 17(+10 days)
		4x10 ⁶ /mL		day 18 (+11 days)

The control CTX110 reference (which was not replated) reached a concentration of 3-4x10⁶/mL in 7 days (**FIGs. 9A-9B**). Cells replated at Day 3, 4 and 5 ("D3, D4, D5") reached ~3-4x10⁶/mL cell concentration in 10 days (**FIGs. 9A-9B**). Cells replated at Day 6 ("D6") and

Day 7 (“D7”) reached $3\text{--}4 \times 10^6/\text{mL}$ cell concentration at about 14 to 18 days (**FIGs. 9A-9B**). The D3, D4, and D5 reached the target of $3.0\text{--}4.0 \times 10^6/\text{mL}$ cell concentration about 4 to 8 days earlier than the D6 and D7. Cells plated at $166\text{K}/\text{cm}^2$, $125\text{K}/\text{cm}^2$, or $83\text{K}/\text{cm}^2$ took 10, 11 and 14 days to reach harvest point, which were 3, 4, and 7 days longer than the reference group.

5 **FIGs. 10A and 10B** show that a total of $4\text{--}4.5 \times 10^8$ cells were harvested from the CTX110 reference at day 7 while the total cell number harvested from day 3, 4, 5, 6, and 7 replated groups were between 1.3×10^9 to 2×10^9 , which were 3-5 fold more cells than CTX110 control reference.

10 Total 1.2×10^9 , 1.64×10^9 and 2.32×10^9 cells were harvested from $166\text{K}/\text{cm}^2$, $125\text{K}/\text{cm}^2$, or $83\text{K}/\text{cm}^2$ plated groups, which were 3-6 fold more cells compared with CTX110 control reference.

Cell viability from all the replated groups and low-density plating groups were similar to the CTX110 reference. (**FIGs. 11A and 11B**)

It was determined that the D3, D4, and D5 replating, $166\text{K}/\text{cm}^2$ plating and $125\text{K}/\text{cm}^2$ plating, provided the expected number of cells in the lowest number of days.

15 Editing efficiency including CAR⁺%, TRAC⁻% and B2M⁻% were assessed from all the replating and low-plating groups. (**FIGs. 12A-12C**) CAR⁺% in CTX110 reference was 55.9%. D3, D4, and D5 replated groups maintained CAR⁺% at 57.9%, 56% and 52.62% while D6 and D7 resulted in the decreased CAR⁺% at 38.65% and 35.45%. CAR⁺% from $166\text{K}/\text{cm}^2$, $125\text{K}/\text{cm}^2$, or $83\text{K}/\text{cm}^2$ were 59.3%, 54.9% and 52.9% without significant changes from CTX110 reference. TRAC⁻% in CTX110 reference group was 94.24%. D3, D4, and D5 replated groups and $166\text{K}/\text{cm}^2$ plating group maintained comparable TRAC⁻% as 93.5%, 93.6%, 93.15% and 93.9%. Slight decreases in TRAC⁻% were observed in $125\text{K}/\text{cm}^2$ and $83\text{K}/\text{cm}^2$ at 91.5% and 91.2%. Greater decreases in TRAC⁻% were seen in D6 and D7 replating groups as 88% and 87.2%. The similar trend was demonstrated in B2M⁻% as well. B2M⁻% of CTX110 reference group was 77.93%. D3, D4, and D5 replated groups and $166\text{K}/\text{cm}^2$ plating group maintained comparable TRAC⁻% as 75.51%, 75.39%, 76.77% and 76.31%. Slight decreases in B2M⁻% were observed in $125\text{K}/\text{cm}^2$ and $83\text{K}/\text{cm}^2$ at 71.74% and 69.19%. Greater decreases in B2M⁻% were seen in D6 and D7 replating groups as 60.37% and 58.29%.

25 It was determined that the D3, D4, and D5 replating, $166\text{K}/\text{cm}^2$ plating and $125\text{K}/\text{cm}^2$ plating provided the most comparable editing efficiency as CTX110 reference.

30 The cellular phenotypes of the replated populations were determined using the flow panels, as described in Example 8 and **Table 19** excluding CD95, CD45RO, CD57, CD27 and CCR7, and including Tim3. **FIGs. 13A and 13B** show the ratio of CD4⁺ and CD8⁺ cells in the replated populations as well as CTX110 reference. Ratio of CD4 and CD8 was well maintained

in D3, D4, and D5 replating groups and 166k and 125k low density plating groups. Increased CD8⁺ cells were seen in D6 and D7 replating groups as well as 83K low density plating group.

The replated populations were assessed for memory cell markers. Within CAR⁺, CD4⁺CAR⁺, and CD8⁺CAR⁺ population, CD45RA⁺CD62L⁺ cells, CD45RA⁺CD62L⁺ cells, CD45RA⁺CD62L⁻ cells, and CD45RA⁺CD62L⁻ cells were defined as Naïve T cells, central memory (CM) T cells, effector memory (EM) T cells, and terminal effector (TE) T cells, respectively. These populations within the CTX110 product were defined as subsets. **FIGs. 14A-14F** show the subset composition found in the replated populations and low density plating groups. Within CAR⁺, CD4⁺CAR⁺ and CD8⁺CAR⁺ populations, most of replated and low density plating groups demonstrated reduced naïve T cells. Decreased central memory T cells was detected in D6 and D7 replated groups but not significant in D3, D4, and D5 replated groups. Most groups showed increased effector memory T cells. Increased terminal differentiated cells were seen in CAR⁺ and CD8⁺CAR⁺ cells but not in most of CD4⁺CAR⁺ cells.

As shown in **FIGs. 15A-15F**, low levels of exhaustion markers were found in the CAR⁺, CD4⁺/CAR⁺, and CD8⁺/CAR⁺ replated populations. Compared with CTX110 reference, there were increased LAG3 expression on D6 and D7 replated cells (**FIGs. 15A** and **15C**) in one of the experiments. Overall, there was not increased expression of all three exhaustion markers (PD1, LAG3 and TIM3). There was no or very low PD1 expression in all groups.

In vitro Cell Kill Assay

Next, the ability of the CAR-T cells in replated and low-plating density groups to kill CD19 positive Raji target cells was assessed using a flow cytometry-based cytotoxicity assay. In brief, target cells were labeled with eFluor670 and incubated with CAR-T cells at varying ratios. CTX110 cytotoxicity was analyzed at 24 hours by assessing labeled cells in the live gate compared to control sample. The results are shown in **FIGs. 16A-16C**. All replated and low-density plating groups of CTX110 showed dose dependent target cell cytotoxicity at comparable level as CTX110 reference.

In sum, these *in vitro* results demonstrate that D3, D4, and D5 replating and seeding densities of 166K/cm² and 125K/cm² provided sufficient expansion, editing efficiency and cytotoxicity as CTX110 reference.

In vivo Study

Next, the ability of the CAR-T cells in the replated and low-density plating groups to kill tumors in mice was studied *in vivo* in two independent studies (**Tables 24** and **25**). Nalm6-Fluc-

- GFP tumor cells were inoculated into CIEA NOG mice 4 days prior to CAR-T administration. Weekly Bioluminescence (BLI, photons/s) assessment allows to assess tumor burden in mice. In *in vivo* study #1, D5, D6 and D7 replated, as well as CTX110 reference, were administrated at dose of 2e6, 4e6, and 10e6 CAR⁺ cells per mouse. Six mice were included per group and per dose. Untreated mice were used as negative control. In *in vivo* study #2, low-density plating group (166K/cm², 125K/cm², and 83K/cm²) and replating groups (D3, D4, and D6 replating) were administrated at dose of 4e6 CAR⁺ cells per mouse. 4 recipients were included per group.

Table 24: In vivo study 1

	Groups	Dose	No. of recipient
1	Untreated	0	5
2	CTX110 Reference	2x10 ⁶ CAR ⁺	6
3		4x10 ⁶ CAR ⁺	6
4		10x10 ⁶ CAR ⁺	6
5	day 5 replating 1:4 split	2x10 ⁶ CAR ⁺	6
6		4x10 ⁶ CAR ⁺	6
7		10x10 ⁶ CAR ⁺	6
8	day 6 replating 1:4 split	2x10 ⁶ CAR ⁺	6
9		4x10 ⁶ CAR ⁺	6
10		10x10 ⁶ CAR ⁺	6
11	day 7 replating 1:4 split	2x10 ⁶ CAR ⁺	6
12		4x10 ⁶ CAR ⁺	6
13		10x10 ⁶ CAR ⁺	6

10

Table 25: In vivo study 2

	Groups	Dose	No. of recipient
1	Untreated		3
2	110 Reference	4x10 ⁶ CAR ⁺	4
3	166K/cm ² (3x)		4
4	125K/cm ² (4x)		4
5	83K/cm ² (6x)		4
6	day 3 replating 1:4 split		4
7	day 4 replating 1:4 split		4
8	day 5 replating 1:4 split		4

- In vivo study#1 indicated comparable survival between D5 replating group and CTX110 reference at all three doses. (**FIGs. 17A-17C**) The 83K/cm² and 166K/cm² plating group and D4 replating group had compromised survival compared with other testing groups as well as CTX110 reference. (**FIG. 17D**) Medium survival is listed in **Tables 26** and **27**.

Table 26. In vivo study 1

	Groups	Dose	Medium Survival
1	Untreated	0	26
2	CTX110 Reference	2x10 ⁶ CAR ⁺	34
3		4x10 ⁶ CAR ⁺	40.5
4		10x10 ⁶ CAR ⁺	66.5
5	day 5 replating 1:4 split	2x10 ⁶ CAR ⁺	33.5
6		4x10 ⁶ CAR ⁺	40.5
7		10x10 ⁶ CAR ⁺	79
8	day 6 replating 1:4 split	2x10 ⁶ CAR ⁺	28
9		4x10 ⁶ CAR ⁺	32
10		10x10 ⁶ CAR ⁺	34
11	day 7 replating 1:4 split	2x10 ⁶ CAR ⁺	30
12		4x10 ⁶ CAR ⁺	34
13		10x10 ⁶ CAR ⁺	43.5

5

Table 27. In vivo study 2

	Groups	Dose	Medium Survival
1	Untreated	4x10 ⁶ CAR ⁺	25
2	110 Reference		70.5
3	166K/cm ² (3x)		68.5
4	125K/cm ² (4x)		Undefined
5	83K/cm ² (6x)		51.5
6	day 3 replating 1:4 split		68.5
7	day 4 replating 1: 4 split		61.5
8	day 5 replating 1:4 split		Undefined

BLI from untreated mice in both studies reached peri-morbidity condition indicating high tumor burden on day 25 and day 18. In study #1, D6 and D7 replated groups demonstrated earlier increase in BLI compared with D5 and CTX110 reference at all 3 doses (2e6, 4e6 and 10e6 CAR⁺ cells per mouse; **FIGs. 18A-18C**, respectively). D5 and CTX110 reference demonstrated the similar tumor growth kinetics. In study #2, 83K/cm² plating group showed quicker tumor growth than CTX110 reference. All other testing groups demonstrated similar or even delayed tumor growth compared with CTX110 reference (**FIG. 18D**).

According to medium survival and BLI, D5 replating group but not D6 and D7 replating groups maintained the in vivo efficacy as CTX110 reference.

Expansion duration, yield, editing, exhaustion/subset markers, in vitro and in vivo potency were used to determine optimal seeding densities and/or replating conditions. A summary of the analysis is shown in **Table 28**. Replating at 1:4 ratio at Day 5 provided beneficial expansion and editing efficiency.

5

Table 28

Group	Yield	Expansion period	Editing	Exhaustion (CAR ⁺)	Subsets (CAR ⁺)	In vitro efficacy	In vivo efficacy & persistency
CTX110 166K	3x	+3 days	Maintained			Maintained	Maintained
CTX110 125K	4x	+3 days	Decreased but within spec				Maintained
CTX110 83K	6x	+7days	Decreased, slightly below spec				Compromised
CTX110 D3 replate 1:4 split	4x	+3 days	Maintained				Maintained
CTX110 D4 replate 1:4 split		+3 days	Maintained				Pending Compromised
CTX110 D5 replate 1:4 split		+3 days	Maintained	Decrease TIM3; increase TAG3, Increase PD1	Comparable	Maintained	Maintained
CTX110 D6 replate 1:4 split		+7 or 11 days	Decreased	Decrease Tim3, Increase LAG3	Increase TE	Maintained	Compromised
CTX110 D7 replate 1:4 split		+10 or 11 days	Decreased	Decrease Tim3	Decrease Naive	Maintained	Compromised

EXAMPLE 10: Improved Cell Expansion*(A) Optimized Electroporation for Increased CTX110 & CTX 120 Cell Expansion Output*

10 The methods as described in the present disclosure utilize electroporation to deliver various nucleic acids and polypeptides to recipient T-cells, including, for example, various ribonucleoprotein (RNP) complexes comprising Cas9 and guide RNA complexes. The instrumentation used in the electroporation process is not particularly limited, as any suitable electroporation instrument from various manufacturers can find use in the methods described

15 herein. The cell seeding density used in the electroporation is not particularly limited.

The present example uses an electroporation instrument capable of electroporating increased numbers of cells in cassettes capable of retaining larger volumes while maintaining efficient editing. The larger electroporation capacity increases, for example as much as doubling, the output of any given engineered T-cell, for example the CTX110 or CTX120 engineered T-cell product, by providing a greater number of edited cells for transduction and expansion. This is a benefit in manufacturing, as this increased capacity comes without the need to extend the process duration and or cell doublings.

For example, additional cells are available to seed additional T-cell culture vessels (500 cm² gas permeable membrane surface area with 5000 mL media capacity), such as 2 or more additional culture vessels. For example, with the increase number of cells, up to 4x culture vessels can be seeded, where $300 \times 10^6 \leq x \leq 600 \times 10^6$ cells can be seeded in 2x culture vessels, $600 \times 10^6 \leq x \leq 800 \times 10^6$ cells can be seeded in 3x culture vessels, or $\leq 800 \times 10^6$ cells can be seeded in 4x culture vessels.

In some aspects, between about 400,000 cells/cm² and 500,000 cells/cm² are seeded per culture vessel. Alternatively, between about 250,000 cells/cm² and 500,000 cells/cm² are seeded per culture vessel, or between about 300,000 cells/cm² and 500,000 cells/cm² are seeded per culture vessel, or between about 150,000 cells/cm² and 250,000 cells/cm² are seeded per culture vessel, or between about 150,000 cells/cm² and 500,000 cells/cm² are seeded per culture vessel, or between about 150,000 cells/cm² and 600,000 cells/cm² are seeded per culture vessel.

In some aspects, a target seeding density is at least about 150,000 cells/cm², or at least about 250,000 cells/cm², or at least about 300,000 cells/cm², or at least about 400,000 cells/cm², or at least about 500,000 cells/cm².

In some aspects, a target seeding density is about 250,000 cells/cm². In other aspects, a target seeding density is about 500,000 cells/cm².

Electroporation cassettes capable of retaining volumes of up to 1 mL can be used. Using this system, 2.7×10^9 cells can be electroporated in up to seven G1000 cassettes. Retrieval of the cells from cassettes with a single-use blunt tip needles attached to a 3 mL syringe will also eliminate the risk of micropipette tip ejection into the Erlenmeyer.

Use of a system with larger capacity also facilitates the cell transduction step. Doubling the current maximum of 7×10^8 cells for transduction to 1.4×10^9 cells produces sufficient material to seed up to four cell culture vessels for expansion. Therefore, a fixed day 9 depletion can be maintained, effectively up to doubling the output per run in the same amount of processing time.

Other steps in the example were unchanged from the above.

(B) *Method Optimization and Comparison Using Three T-Cell Donor Lots to Increase Drug Product Yield*

This section describes the generation of CAR T cells at 1x, 2x, 4x, and 4x with day 4 split to demonstrate robustness of increased expansion methods and generate material for comparability analysis between current drug product (DP) like material and selected cell culture conditions.

Starting material for this batch was CD4/CD8 T cell selection from three healthy donor apheresis lots. Selected expansion conditions of CTX110 CAR T cells were seeded in G-Rex 500M-CS chambers.

Previous CTX110 CAR T expansion seeding and harvest cell density in the G-Rex500M-CS was 500,000/cm² and 30 x 10⁶/cm², respectively, producing $\leq 30 \times 10^9$ CAR T cells. To increase the DP yield of CAR T cells in the G-Rex500M-CS culture vessel, the present example was developed. Three select conditions, described in **Table 29** below, were selected to evaluate CTX110 DP-like comparability with three different donors, and to generate enough sample cells for multiple downstream analytical assays.

Table 29. selected cell culture conditions to increase CAR T yield during expansion unit operation

Sample ID	Condition	Seeding density (total cells to seed)	Split (Days of Expansion)	Passage 1 (volume seeded)	Harvest for TCRab Depletion (days of expansion)
S1	1x manufacturing process	250.0x10 ⁶	-	-	7
S2	2x	125.0x10 ⁶	-	-	8
S3	4x	62.5x10 ⁶	-	-	9
S4	4x with day 4 split	250.0x10 ⁶	4	¼ harvested volume	9

The detailed protocol is provided below.

- a. Prepared all culture medium, GMP IL-2 and GMP IL-7 for the entire CTX110 DS process, where:
 1. T cells were activated according to the Full Large Scale (3x G-Rex500M-CS)
 2. CAR T cells were seeded for expansion in 1x G-Rex500M-CS and Small Scale (1x Well GRex6M) with 1x well at the selected seeding density.
 3. CAR T cells were seeded post depletion according to one-half large scale, 1x G-Rex500M-CS per condition.

- b. Thawed the appropriate number of T cells to perform Full Large Scale (3x G-Rex500M-CS).
- c. Activated the appropriate number of T cells to perform Full Large Scale (3x G-Rex500M-CS).
- 5 d. Diluted the activating agent.
- e. Harvested cells for electroporation (EP) at Full Large Scale (3x G-Rex500M-CS).
- f. Electroporation of $2,040 \times 10^6$ to $2,160 \times 10^6$ of T cells (17 – 18 OC400 cassettes).
- g. Transferred cells equally between 2 x wells of a 6 well Falcon plate and incubated for 20 minutes.
- 10 h. Diluted EP T cells and seeded 5.0×10^6 total cells in Small Scale (1x well G-Rex6M) for *in vitro* efficacy +EP -AAV control.
- i. Transduced $1,000 \times 10^6$ T cells.
- j. Seeded the appropriate number of T cells in the appropriate culture vessel for expansion:
 1. S1: 250×10^6 total cells seeded into G-Rex500M-CS, 5.0×10^6 total cells in Small Scale (1x well G-Rex6M).
 - 15 2. S2: 125×10^6 total cells seeded into G-Rex500M-CS, 2.5×10^6 total cells in Small Scale (1x well G-Rex6M).
 3. S3: 62.5×10^6 total cells seeded into G-Rex500M-CS, 1.25×10^6 total cells in Small Scale (1x well G-Rex6M).
 - 20 4. S4: 250×10^6 total cells seeded into G-Rex500M-CS, 5.0×10^6 total cells in Small Scale (1x well G-Rex6M)
- k. Performed CAR T expansion according to each condition specifications:
 1. S1: supplement 100IU/mL of IL-2 and 100IU/mL of IL-7 to the G-Rex500M-CS and G-Rex6M well once every three days. Pulled a sample from the G-Rex6M for TCRab Flow panel and proceeded to TCRab depletion on day 7 of expansion.
 - 25 2. S2: supplement 100IU/mL of IL-2 and 100IU/mL of IL-7 to the G-Rex500M-CS and G-Rex6M well once every three days. Pulled a sample from the G-Rex6M for TCRab flow panel and proceeded to TCRab depletion on day 8 of expansion.
 3. S3: supplement 100IU/mL of IL-2 and 100IU/mL of IL-7 to the G-Rex500M-CS and G-Rex6M well once every three days. Pulled a sample from the G-Rex6M for TCRab flow panel and proceed to TCRab depletion on day 9 of expansion.
 - 30 4. S4:
 - a. Day 4 of expansion:

- a. Removed supernatant and harvested cells from G-Rex500M-CS using a GathRex pump, recorded volume of cells.
- b. By gravity, filled a new G-Rex500M-CS with 5000mL of culture medium and seeded one quarter of harvested cell volume into the filled culture vessel. Returned culture vessel to incubator.
- c. With a serological pipet, filled a new single well of a G-Rex6M with 75mL of culture medium. With a serological pipet, homogenized cells in G-Rex6M well and transferred 25mL of cells to filled culture vessel. Returned culture vessel to incubator.
- b. Supplemented 100IU/mL of IL-2 and 100IU/mL of IL-7 to the G-Rex500M-CS and G-Rex6M well once every three days. Pulled a sample from the G-Rex6M for TCRab flow panel and proceeded to TCRab depletion on day 9 of expansion post-transduction.
- l. Performed TCRab depletion at one-half large scale (1x G-Rex500M-CS). Obtained a pre-depletion sample for the appropriate flow analysis.
- m. Obtained a post-depletion sample for the appropriate flow analysis and seeded post-depletion target T cells to perform one-half Large Scale (1x G-Rex500M-CS).
- n. Performed harvest at one-half large scale (1x G-Rex500M-CS). Based on harvest cell counts and post-depletion obtained %CAR⁺:
 1. Calculated DP formulation viable cell concentration:

$$\frac{\text{Pre - Spin harvest total viable cell number}}{1} * \frac{\%CAR +}{25 \times 10^6 CAR + \text{cells/mL}} = \frac{\text{DP formulation concentration}}{[\text{total viable cells}]}$$
 2. Divided the harvest total viable cell number by DP formulation concentration to calculated the volume needed to reach target cell concentration:

$$\frac{\text{Pre - Spin harvest total viable cell number}}{1} * \frac{\text{mL}}{\text{DP formulation total viable cells}} = \text{target volume CS5}$$
 3. Resuspended cells to 0.5x target volume.
 4. Performed second cell count on resuspended cell pellet.
 5. Calculated remaining volume to resuspend cells to reach target viable cell concentration.
 6. Diluted down to target cell concentration based on harvest cell count calculation.
- o. Cryopreserved the appropriate number of cells for additional flow characterization, and comparability analysis.

(C) *In Vitro Efficacy of Cell Expansion Optimization Assessed by a Cell Toxicity Assay*

This example describes an *in vitro* efficacy cell toxicity assay of the cells prepared in the example (B) above. The assay measured the absolute amount of viable cells in a co-culture assay.

- 5 Raji target cancer cells (CD19⁺) were labeled with eFluor 670 (APC channel) proliferation dye and plated @ 50K cells per well. Various ratios of unlabeled effector CAR T cells were added for each condition tested. Cell killing of target cells by effector CAR T cells was measured following 24 hours of culture by DAPI live/dead staining (Pacific Blue channel). Counting beads were added during flow analysis to normalize between samples. The number of
- 10 viable cells (DAPI negative) in the test samples were enumerated and normalized to the number of viable cell in wells containing target cells alone to calculate percentage of cell lysis. Cytokine release into the culture media by CAR-T was analyzed in a multiplex ELISA assay (Luminex).

- These experiments evaluated 1x versus 2x and 4x CTX110 manufacturing conditions. T-cells from three different donors were analyzed in parallel. *In vitro* efficacy was ascertained by
- 15 two metrics, which were the 24 hour cell toxicity assay and by cytokine production.

Table 30

Sample ID	Sample Description	Seeding Density (k cells/cm ²)	Day Split (if applicable)	% CAR ⁺ (Fresh)	% CAR ⁺ (post thaw)
DP20-07-S1	Donor #1 – Std. (1x)	500	N/A	64.16	66.6
DP20-07-S2	Donor #1 – 2x	250	N/A	63.13	58.5
DP20-07-S3	Donor #1 – 4x	125	N/A	54.28	67.6
DP20-07-S4	Donor #1 – HY D4 split	500	Day 4	62.70	
DP20-08-S1	Donor #2 – Std. (1x)	500	N/A	34.65	36.6
DP20-08-S2	Donor #2 – 2x	250	N/A	34.07	35.8
DP20-08-S3	Donor #2 – 4x	125	N/A	33.99	34.0
DP20-09-S1	Donor #3 – Std. (1x)	500	N/A	68.66	64.7
DP20-09-S2	Donor #3 – 2x	250	N/A	66.70	68.9
DP20-09-S3	Donor #3 – 4x	125	N/A	65.34	63.9

FIG. 20 shows an assay control FACS analysis by measuring CAR T-cell lysis. The CAR T-cells were CTX110 CAR T-cells. 81% of the T-cells were CAR⁺.

- 20 **FIGs. 21A-21C** show the results of an assay control experiment measuring cell lysis and cytokine production *in vitro*. The assay used CTX110 CAR-T cells thawed from frozen stock. The T-cells were 80% CAR⁺ day 6 post HDR.

FIGs. 22A-22C show the results of an *in vitro* efficacy analysis showing that T-cells derived from each of the three donors had varying degrees of *in vitro* efficacy among 1x, 2x and 4x culture conditions.

FIGs. 23A-23C show the results of an analysis of cell lysis at different cell concentrations, demonstrating that cells derived from donors 1 and 2 showed similar responses despite differing percentages of CAR⁺ cells.

FIGs. 24A-24B show the results of an analysis of cell lysis from the three donors when normalized for CAR⁺ cells. Donors 2 and 3 behaved similarly in the assay when CAR cells are normalized. The assay was repeated with 2x CAR-T cell number for donor 2 at the same E:T ratios.

IFN γ production was also measure in the supernatant by ELISA. The IFN γ cytokine analysis mirrored the cell killing results in terms of dose response related to E:T ratios and there was some variability between donor responses. IL2 measurement was more variable among the donors. Significantly less IL2 production was observed in the media for donor 2 cells.

In summary, for each donor assessed, both 2x and 4x culture conditions show similar *in vitro* efficacy to the 1x manufacturing protocol.

(D) In Vivo Efficacy of Cell Expansion Optimization (In Vivo Survival Analysis)

CTX110 cells prepared according to the Example (C) above and were administered at a dose of 4e6 CAR⁺ T cells to mice in a Nalm6 xenograft tumor model, as shown in **Table 31**, below. Nalm6-Fluc-GFP tumor cells were inoculated into CIEA NOG mice 4 days prior to CAR-T administration. Weekly Bioluminescence (BLI, photons/s) assessment allows to assess tumor burden in mice.

Table 31

group	Group description	Tumor cells	Cells to be dosed	Recipient per group
1	No treatment	5.0e5	4.0e6 CAR-T	10
2	DP20-07-S1	5.0e5	4.0e6 CAR-T	10
3	DP20-07-S2	5.0e5	4.0e6 CAR-T	10
4	DP20-07-S3	5.0e5	4.0e6 CAR-T	10
5	DP20-07-S4	5.0e5	4.0e6 CAR-T	10
6	DP20-08-S1	5.0e5	4.0e6 CAR-T	10
7	DP20-08-S2	5.0e5	4.0e6 CAR-T	10
8	DP20-08-S3	5.0e5	4.0e6 CAR-T	10
9	DP20-09-S1	5.0e5	4.0e6 CAR-T	10
10	DP20-09-S2	5.0e5	4.0e6 CAR-T	10
11	DP20-09-S3	5.0e5	4.0e6 CAR-T	10
Total				110

For all three donors and expansion conditions, the animals dosed with CAR⁺ cells continue to survive at day 38, unlike animals dosed with untreated control cells, which did not

Table 32: Median Survival

1. Untreated	2. Donor #1, 1x	3. Donor #1, 2x	4. Donor #1, 4x	5. Donor #1, HY 4x, D4	6. Donor #2, 1x	7. Donor #2, 2x	8. Donor #2, 4x	9. Donor #3, 1x	10. Donor #3, 2x	11. Donor #3, 4x
23	Undefined	Undefined	38.5	39	Undefined	Undefined	Undefined	Undefined	Undefined	Undefined

5

EXAMPLE 11: Methods for Manufacturing Genetically Engineered T Cells Expressing a Chimeric Antigen Receptor and Having Genetically Disrupted *TRAC* and $\beta 2M$ Genes.

The following describes an exemplary process for the manufacture of a T cell immunotherapy comprised of human allogeneic T cells that are genetically modified *ex vivo* using CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9) gene editing components (sgRNA (single guide RNA) and Cas9 nuclease).

The modifications included targeted disruption of the *TRAC* and $\beta 2M$ loci, and the insertion of an chimeric antigen receptor (CAR) transgene into the *TRAC* locus using a recombinant adeno-associated virus vector (*e.g.*: a serotype 6 rAAV encoding an antigen directed chimeric T cell antigen receptor).

The manufacturing process is illustrated in **FIG. 19**. Structural information of the starting materials, including bacterially-derived Cas9 nuclease; two single guide RNAs (sgRNA), one sgRNA which targets the *TRAC* locus (*e.g.*: TA-1) and a second sgRNA which targets the $\beta 2M$ locus (*e.g.*: B2M-1), is provided herein. Exemplary amino acid sequences and nucleotide sequences of CARs in a rAAV vector are also provided.

T Cell Enrichment

T cells were enriched from the leukapheresis materials (Leukopaks) *via* magnetic separation using a mixture of anti-CD8 and anti-CD4 antibody-coated magnetic beads using an automated cell processing system. Prior to enrichment, leukopaks were sampled for cell count and viability ($\geq 80\%$). Enriched cells were isolated in PBS/EDTA buffer with HSA, and then sampled for cell count, viability ($\geq 80\%$), T cell purity ($\geq 70\%$ CD3), and sterility.

T Cell Cryopreservation

The cells were then centrifuged at $4 \pm 1^\circ\text{C}$ and resuspended in CryoStor CS5 at a target concentration of 50×10^6 viable cells/mL. Cells were sampled for cell count, viability ($\geq 80\%$) and then aliquoted into ethyl vinyl acetate cryobags at the target cell number of $2,500 \times 10^6$

cells/bag (30-70 mL of cell suspension). One Leukopak was sufficient to produce 1-2 bags of T cells. Each bag was heat-sealed, labeled, stored at 2-8°C until transferred to a controlled-rate freezer and subsequently transferred to vapor phase liquid nitrogen for storage.

5 *T Cell Thawing and Activation*

One frozen bag of enriched T cells was thawed, transferred into a 3L bag and diluted into Supplemented X-VIVO™ 15 media (X-VIVO™ 15, 5% Human Serum, 100 IU/mL rhIL2, 100 IU/mL rhIL7). Cells were sampled for cell count and viability ($\geq 70\%$). Cells were centrifuged at 540 g at $20 \pm 1^\circ\text{C}$ for 15 minutes. Cells were then resuspended in the Supplemented X-VIVO™ 15 media and sampled for cell count and viability ($\geq 70\%$). Soluble colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists solution was added at the ratio of 1:12.5 (v/v) to activate the cells.

Cells were seeded to a target density 2×10^6 viable cells/mL into two static cell culture vessels, each at a total volume of approximately 500 mL of Supplemented X-VIVO™ 15 media/colloidal polymeric nanomatrix conjugated to recombinant humanized CD3 and CD28 agonists. Static culture vessels were incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO₂ for 48 ± 4 hours. Throughout the process, whenever the static culture vessels were handled, they were inspected for tears and leaks, and the presence of clear, yellow medium.

Dilution

Two (2) days later, supplemented X-VIVO™ 15 media was added to each static culture vessel to 5 L. Cells were further incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO₂ overnight.

Electroporation and Transduction

In preparation for electroporation, the volume of Supplemented X-VIVO™ 15 media was reduced to a final volume of approximately 500 mL using a pump connected to dip-tube in the static culture vessel, which was gently swirled to allow resuspension of cells into the media. Cells were sampled for cell count, and viability ($\geq 70\%$). Cells were transferred to 500 mL centrifuge tubes and centrifuged at 540 g, at $20 \pm 1^\circ\text{C}$ for 15 minutes. Cell pellets were resuspended in Electroporation Buffer and centrifuged again under the same conditions. Cells were resuspended in Electroporation Buffer a second time to a target concentration of 300×10^6 cells/mL.

Cas9 nuclease was mixed with an sgRNA targeting TRAC or Cas9 nuclease was mixed with an sgRNA targeting $\beta 2\text{M}$ in separate microcentrifuge tubes. Each solution was incubated for no less than 10 minutes at room temperature to form each ribonucleoprotein complex

(RNPs). The two Cas9/gRNA mixtures were combined, and mixed with the cells, bringing Cas9, TRAC sgRNA and B2M sgRNA to a final concentration of 0.3 mg/mL, 0.08 mg/mL and 0.2 mg/mL, respectively. The mixture was aliquoted and loaded into an electroporation cassette by pipetting. Cassettes were capped and sequentially electroporated by static electroporation using a transfection system. After electroporation, cells were pooled from each cassette in a 125 mL Erlenmeyer flask and incubated at 37°C for no less than 20 minutes. Cells were sampled for viability ($\geq 70\%$) and count.

Transduction was carried out as follows. Cells were diluted to 10^7 cells/mL with X-VIVO™ 15 media and freshly thawed rAAV was added at a MOI of 20,000 vg/cell. Cells were incubated at 37°C 5% CO₂ for no less than 60 minutes.

Homology directed-repair (HDR) is a high-fidelity cell repair mechanism for DNA double strand breaks. HDR is used to introduce a CAR gene from the AAV template into the desired TRAC locus by using a homologous sequence on each end of the CAR gene.

15 *Cell Expansion*

Cells were diluted with Supplemented X-VIVO™ 15 media, sampled for cell viability ($\geq 70\%$) and count, and seeded to a density between $0.2-0.5 \times 10^6$ viable cells/cm² into two static culture vessels, and one additional static culture vessel (satellite culture for cell monitoring). The static culture vessels were incubated at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO₂. The cell cultures were incubated for up to 9 days. During this time, the cultures were supplemented every 3 to 4 days with 100 IU of rhIL2 and rhIL7 per mL of culture volume. The satellite static cell culture vessel was tested for cell count, viability, and T cell purity throughout expansion. When the cell density in the satellite culture vessel reached approximately 30×10^6 /cm², the TCR $\alpha\beta$ depletion was performed. If cell density in the satellite culture vessel did not reach 30×10^6 /cm², TCR $\alpha\beta$ depletion on the main cultures was performed on Day 9.

TCR $\alpha\beta$ Depletion

The medium of each static cell culture vessel was reduced to a final volume of approximately 500 mL using a pump connected to the dip-tube in the static culture vessel. After the bulk of the media was removed, the static culture vessels were gently swirled to resuspend the cells in the media.

The cells were transferred to 500 mL centrifuge tubes fitted with dip-tubes that connect to the static culture vessels. Cells were sampled for viability ($\geq 70\%$), count, and %CAR⁺ cells. Cells were then centrifuged at $540 \times g$ at $20 \pm 1^\circ\text{C}$ for 15 minutes. Cell pellets were resuspended

and pooled in less than 650 mL PBS/EDTA containing 0.5% HSA. Cell suspensions were transferred to a sterile bag which is connected to the automated cell processing system. The automated cell processing system incubated cells with a biotin-conjugated anti-TCR $\alpha\beta$ antibody. Cells were washed and incubated with anti-biotin magnetic beads to allow for depletion of the

5 TCR $\alpha\beta$ ⁺ cells using the automated cell processing system. Cells were tested for cell count, viability ($\geq 70\%$), and %CAR⁺ cells ($\geq 30\text{-}40\%$).

Cell Recovery

The depleted cells were resuspended in Supplemented X-VIVO™ 15 media and

10 transferred into 3L bag(s), seeded into static cell culture vessel(s) and incubated overnight at $37 \pm 1^\circ\text{C}$ and $5 \pm 1\%$ CO₂.

Cell Harvest (Drug Substance)

To harvest cells, static culture vessels were removed from the incubator and allowed to

15 rest for sedimentation of cells. Growth medium was removed from each static culture vessel to a final volume of approximately 500 mL using a pump. Removed media was sampled for sterility. Static culture vessels were gently swirled to allow the cells to resuspend in the media. The contents of each static culture vessel were transferred in a 3L transfer bag using a pump and was filtered through a 40 μm blood transfusion filter by gravity into a separate sterile 3L bag. Cells

20 were sampled for concentration and viability.

EQUIVALENTS

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

25 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

30 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, 5 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.

10 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.”

15 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 20 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 25 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, additional unlisted 30 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 “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
5 selected from any one or more of the elements in the list of elements, but not necessarily 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
10 elements specifically identified. Thus, as a non-limiting example, “at least 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);
15 in yet another embodiment, to at least one, 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 are recited.