

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.

SUMMARY ~~OF THE INVENTION~~

15 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.

In one aspect, 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 second population of 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-2 microglobulin
30 (*β2M*) gene to produce a third population of T cells, wherein the third population of T cells comprises T cells having the *TRAC* gene disrupted and the *β2M* 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 AAV vector comprises a nucleic acid sequence encoding a chimeric antigen receptor (CAR) and wherein the nucleic acid sequence is flanked by

homologous sequences to the *TRAC* gene locus, and wherein the fourth population of T cells comprises activated T cells expressing the CAR and having the *TRAC* gene disrupted and the *$\beta 2M$* gene disrupted; (v) expanding the fourth population of T cells thereby producing an expanded T cell population; (vi) removing TCR $\alpha\beta^+$ T cells from the expanded T cell population to produce a population of genetically engineered T cells, wherein the population of genetically engineered T cells comprises T cells expressing the CAR and having the *TRAC* gene and the *$\beta 2M$* gene disrupted; and (vii) harvesting the population of genetically engineered T cells.

Accordingly, one ~~aspect~~example 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 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 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 expressing the CAR and having the *TRAC* gene and the $\beta 2M$ gene disrupted; and (vii) harvesting the population of genetically engineered T cells.

In some [embodimentexamples](#), the first population of T cells is derived from cryopreserved T cells enriched from human blood cells. In some [embodimentexamples](#), the first population of T cells is prepared by a process comprising: (a) obtaining blood cells from a human donor; and (b) enriching CD4⁺ T cells and CD8⁺ T cells. In some [embodimentexamples](#), (b) is performed using magnetic beads conjugated with anti-CD4 and/or anti-CD8 antibodies. In some [embodimentexamples](#), 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 [embodimentexamples](#), methods further comprise (c) cryopreserving the enriched CD4⁺ T cells and CD8⁺ T cells produced in step (b).

In some [embodimentexamples](#), the T cell activating agent comprises a CD3 agonist and a CD28 agonist attached to a nanomatrix particle. In some [embodimentexamples](#), 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 [embodimentexamples](#), the ratio of the T cell activating agent to medium in the mixture is about 1:12.5 (v/v).

In some [embodimentexamples](#), 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 [embodimentexamples](#), step (iii) is performed by electroporation. In some [embodimentexamples](#), step (iii) involves one electroporation event. In some [embodimentexamples](#), the first RNP complex and the second RNP complex are introduced into the activated T cells in the one electroporation event. In some [embodimentexamples](#), 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 [embodimentexamples](#), the concentration of the first Cas9 enzyme is about 0.15 mg/mL, the concentration 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 $\beta 2M$ gene is about 0.2 mg/mL. In some [embodimentexamples](#), the cell concentration in step (iii) is about 100x10⁶ cells/mL to about 400x10⁶ cells/mL. In some [embodimentexamples](#), the cell concentration in step (iii) is about 300 x 10⁶ cells/mL. In other [embodimentexamples](#), 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 2.1×10^9 cells or about 2.7×10^9 cells), e.g., for electroporation.

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

In some [embodimentexamples](#), step (v) is performed by seeding the fourth population of T cells 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 [embodimentexamples](#), 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 [embodimentexamples](#), 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 cells/cm² to about 5×10^5 cells/cm² for about 7 days to about 9 days. In some [embodimentexamples](#), 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 [embodimentexamples](#), 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. In some [embodimentexamples](#), 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 [embodimentexamples](#), 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 [embodimentexamples](#), the first Cas9 enzyme, the second Cas9 enzyme, or both are *Streptococcus pyogenes* Cas9 nuclease (spCas9). In some [embodimentexamples](#), the first Cas9 enzyme and the second Cas9 enzyme are the same. In some [embodimentexamples](#), 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 [embodimentexamples](#), the first gRNA targeting the *TRAC* gene comprises a spacer sequence of SEQ ID NO: 4. In some [embodimentexamples](#), the first gRNA targeting the *TRAC* gene comprises the nucleotide sequence of SEQ ID NO: 2. In some [embodimentexamples](#), the second gRNA targeting the $\beta 2M$ gene comprises a spacer sequence of SEQ ID NO: 8. In some [embodimentexamples](#), the second gRNA targeting the $\beta 2M$ gene comprises the nucleotide sequence of SEQ ID NO: 6. In some [embodimentexamples](#), the first gRNA, the second gRNA, or both comprise one or more 2'-O-methyl phosphorothioate modification.

In some embodimentexamples, 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 embodimentexamples, 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 embodimentexamples, the CAR binds CD19. In some embodimentexamples, the CAR comprises the amino acid sequence of SEQ ID NO: 37. In some embodimentexamples, the CAR binds BCMA. In some embodimentexamples, the CAR comprises the amino acid sequence of SEQ ID NO: 61.

In another Aspect, of the present disclosure provides a genetically engineered T cell population, which is produced by a method described herein.

The details of one or more embodimentexamples of the inventiondisclosure are set forth in the description below. Other features or advantages of the present inventiondisclosure will be apparent from the following drawings and detailed description of several embodimentexamples, 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: