

CLAIMS

What Is Claimed Is:

1. 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 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 (*$\beta 2M$*) gene to produce a third population of T cells, wherein the third population of T cells comprises ~~activated~~ 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 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 ~~activated~~ 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.
2. The method of claim 1, wherein the first population of T cells is derived from cryopreserved T cells enriched from human blood cells.

3. The method of claim 1 or claim 2, wherein 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/or CD8⁺ T cells from the blood cells.
4. The method of claim 3, wherein step (b) is performed using magnetic beads conjugated with anti-CD4 and/or anti-CD8 antibodies.
5. The method of any one of claims 1-4, wherein the first population of T cells has a cell viability of at least about 80% and/or a purity of at least about 80% of CD4⁺ and CD8⁺ T cells.
6. The method of any one of claims 3-5, further comprising (c) cryopreserving the enriched CD4⁺ T cells and CD8⁺ T cells produced in step (b).
7. The method of any one of claims 1-6, wherein the T cell activating agent comprises a CD3 agonist and a CD28 agonist, and wherein the CD3 agonist and CD28 agonist are attached to a nanomatrix particle.
8. The method of any one of claims 1-7, wherein step (ii) is performed by incubating the first population of T cells with the T cell activating agent in the cell culture vessel at a cell seeding density of about $2 \times 10^6/\text{cm}^2$ and a cell concentration of about 2×10^6 cells/mL for about 48 hours.
9. The method of any one of claims 1-8, wherein the ratio of the T cell activating agent to medium in the mixture is about 1:12.5 (v/v).
10. The method of any one of claims 1-9, further comprising 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).
11. The method of any one of claims 1-10, wherein step (iii) is performed by electroporation.
12. The method of claim 11, wherein step (iii) involves one electroporation event.

13. The method of claim 1, wherein the first RNP complex and the second RNP complex are introduced into the activated T cells in the one electroporation event.
14. The method of any one of claims 11-13, wherein 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.
15. The method of any one of claims 11-14, wherein 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.
16. The method of any one of claims 11-15, wherein the cell concentration in step (iii) is about 100×10^6 cells/mL to about 400×10^6 cells/mL, optionally about 300×10^6 cells/mL.
17. The method of any one of claims 11-16, wherein the cell number in each vessel ~~concentration~~ in step (iii) is about 3×10^8 cells.
18. The method of any one of claims 1-15, wherein the AAV vector has a multiplicity of infection (MOI) value of about 10,000 to about 80,000.
19. The method of claim 16, wherein the MOI of the AAV vector is about 20,000.
20. The method of claim 16 or claim 17, wherein the AAV vector is AAV serotype 6 (AAV6) vector.
21. The method of any one of claims 1-20, wherein 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 7×10^5 cells/cm² for about 6 days to about 12 days.
22. The method of any one of claims 1-20, wherein 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.

23. The method of claim 22, wherein the fourth population of T cells is cultured at a seeding density of about 3×10^5 cells/cm² to about 5×10^5 cells/cm².
24. The method of claim 22 or claim 23, wherein the cell culture vessel is a static cell culture vessel allowing for cell expansion for about 10 days to about 12 days without medium change.
25. The method of any one of claims 21-23, wherein 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.
26. The method of any one of claims 1-25, wherein step (vi) is performed by contacting the expanded cells to beads on which anti-TCR $\alpha\beta$ antibodies are immobilized, and collecting unbound cells.
27. The method of any one of claims 1-25, wherein the expanding step comprises seeding the T cells at a density between about 150,000 cells/cm² and about 500,000 cells/cm², optionally between about 300,000 cells/cm² and about 500,000 cells/cm², in a cell vessel.
28. The method of any one of claims 1-26, wherein the first Cas9 enzyme, the second Cas9 enzyme, or both are *Streptococcus pyogenes* Cas9 nuclease (spCas9).
29. The method of any one of claims 1-27, wherein the first Cas9 enzyme and the second Cas9 enzyme are the same.
30. The method of any one of claims 1-26, wherein 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.
31. The method of any one of claims 1-29, wherein the first gRNA targeting the *TRAC* gene comprises a spacer sequence of SEQ ID NO: 4.

32. The method of claim 32, wherein the first gRNA targeting the *TRAC* gene comprises the nucleotide sequence of SEQ ID NO: 2.
33. The method of any one of claims 1-33, wherein the second gRNA targeting the *β2M* gene comprises a spacer sequence of SEQ ID NO: 8.
34. The method of claim 34, wherein the second gRNA targeting the *β2M* gene comprises the nucleotide sequence of SEQ ID NO: 6.
35. The method of any one of claims 32-35, wherein the first gRNA, the second gRNA, or both comprise one or more 2'-O-methyl phosphorothioate modification.
36. The method of any one of claims 1-36, wherein the CAR comprises an extracellular domain targeting a cancer antigen, a transmembrane domain, a co-stimulatory domain, and a CD3z cytoplasmic signaling domain.
37. The method of any one of claims 1-37, wherein the CAR binds CD19.
38. The method of claim 38, wherein 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.
39. The method of claim 39, wherein the CAR comprises the amino acid sequence of SEQ ID NO: 37.
40. The method of any one of claims 1-37, wherein the CAR binds BCMA.
41. The method of claim 41, wherein 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 4-1BB.
42. The method of claim 42 wherein the CAR comprises the amino acid sequence of SEQ ID NO: 61.

43. A genetically engineered T cell population, which is produced by a method of any one of claims 1-43.