

## **CLAIMS**

1. A method of making a population of immune effector cells (e.g., T cells) that can be engineered to express a chimeric antigen receptor (CAR), the method comprising:

- a) providing a frozen input sample comprising immune effector cells, wherein the frozen input sample is from a subject having a leukemia or lymphoma,
- b) thawing the frozen input sample, to produce a thawed sample, and
- c) performing elutriation on the thawed sample and collecting immune effector cells, thereby producing an output sample comprising immune effector cells that are suitable for expression of a CAR.

2. The method of claim 1, wherein the frozen input sample is a plasma apheresis sample.

3. The method of claim 1 or 2, further comprising one, two, three or all of:

- d) depleting CD19+ cells from a washed apheresis sample under flow conditions;
- e) performing density centrifugation on the thawed sample, using a medium comprising iodixanol, e.g., 60% iodixanol in water, e.g., Optiprep medium, and/or having a density greater than Ficoll (e.g., greater than 1.077 g/ml, e.g., about 1.32 g/ml);
- f) performing a wash step on the thawed sample with a buffer comprising dextrose and/or sodium chloride, e.g., D5 medium (5% dextrose and 0.45% sodium chloride), e.g., wherein the wash step is performed using a CS5 (CellSaver5+) instrument; and
- g) performing a positive selection of CD3/CD28+ cells from the thawed sample under flow conditions.

4. The method of claim 1-3, wherein the method comprises a step of adjusting the viscosity of the thawed sample, e.g., by adding an isotonic solution, e.g., PBS, to the thawed sample.

5. The method of any of claims 1-4, wherein:

- (i) the elutriation is performed using a flow rate of 30-82 mL/min, e.g., 30, 40, 50, 60, 70, 72, or 82 mL/min;
- (ii) the elutriation is performed using a collection volume of 250-1250 mL, e.g., 250, 400, 500, 900, or 975 mL; and/or
- (iii) the elutriation is performed at 2000-2800 rpm.

6. The method of any of claims 1-5, wherein the input sample comprises:

- at least 10%, 15%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 35%, or 40% monocytes;
- less than 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, or 20% T cells; and/or

at least 1%, 2%, 5%, 10%, 15%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% B cells.

7. The method of any of claims 1-6, wherein the output sample comprises:

less than 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.2%, or 0.1% monocytes;  
at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% T cells;  
less than 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 2%, 2%, 1%, 0.5%, 0.2%, or 0.1% B cells;  
at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.7%, or 99.9% CD4+CD25+ cells; and/or  
at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.7%, or 99.9% CD8+CD25+ cells.

8. The method of any of claims 1-7, which has a T cell yield recovery of at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% T cells.

9. The method of any of claims 1-8, wherein the output sample is contacted with a nucleic acid encoding a CAR, optionally wherein, the method comprises transducing a nucleic acid encoding a CAR into one or more of the immune effector cells in the output sample, optionally wherein the CAR comprises an antigen binding domain, a transmembrane domain, and an intracellular signaling domain, e.g., comprising a primary signaling domain and/or a costimulatory signaling domain, and optionally wherein the transduction results in a transduction efficiency of at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50%.

10. The method of claim 9, wherein, after contacting the output sample with a nucleic acid encoding a CAR, the output sample comprises:

at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% CAR+CD4+ central memory cells;  
at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% CAR+ cells; and/or  
at least 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% CAR+CD8+ central memory cells.

11. The method of claim 9 or 10, wherein, after contacting the output sample with a nucleic acid encoding a CAR, the output sample:

produces less than 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, or 0.1 pg IFN-gamma per transduced cell;  
and/or

comprises a cytotoxicity level (e.g., an EC50rec) of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, or 30.

12. The method of any of the preceding claims, wherein the input sample comprises:

at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% monocytes;

at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% tumor cells, e.g., lymphoblasts; and/or

less than 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, or 20% immune effector cells, e.g., T cells.

13. The method of any of the preceding claims, wherein the output sample comprises:

less than 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 2%, 2%, 1%, 0.5%, 0.2%, or 0.1% monocytes;

less than 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 2%, 2%, 1%, 0.5%, 0.2%, or 0.1% tumor cells; at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, 99.8%, or 99.9% immune effector cells, e.g., T cells;

less than 50%, 45%, 40%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 4%, 2%, 2%, or 1% the percentage of monocytes compared to the input sample;

less than 50%, 45%, 40%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 4%, 2%, 2%, or 1% the percentage of tumor cells compared to the input sample; and/or

at least 50%, 45%, 40%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 4%, 2%, 2%, or 1% the percentage of immune effector cells, e.g., T cells, compared to the input sample.

14. The method of claim 9-13, which further comprises a step of assaying the transduction efficiency and/or performing a wash step on the input sample with a buffer comprising dextrose and/or sodium chloride, e.g., D5 medium, e.g., using a CS5+ instrument.

15. The method of any of the preceding claims, wherein the immune effector cells are human immune effector cells.

16. The method of any of the preceding claims, wherein the output sample comprises CD8+ T cells and/or CD4+ T cells.

17. The method of any of the preceding claims, wherein the input sample is from a patient that has a cancer selected from the group consisting of one or more acute leukemias including B-cell

acute lymphoblastic leukemia (BALL), T-cell acute lymphoblastic leukemia (TALL), acute lymphoblastic leukemia (ALL); one or more chronic leukemias including chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL); additional hematologic cancers or hematologic conditions including B cell prolymphocytic leukemia, blastic plasmacytoid dendritic cell neoplasm, Burkitt's lymphoma, diffuse large B cell lymphoma, follicular lymphoma, hairy cell leukemia, small cell- or a large cell-follicular lymphoma, malignant lymphoproliferative conditions, MALT lymphoma, mantle cell lymphoma, Marginal zone lymphoma, multiple myeloma, myelodysplasia and myelodysplastic syndrome, non- Hodgkin lymphoma, Hodgkin lymphoma, plasmablastic lymphoma, plasmacytoid dendritic cell neoplasm, Waldenstrom macroglobulinemia, preleukemia, atypical and/or non-classical cancers, malignancies, precancerous conditions or proliferative diseases, and any combination thereof.

18. The method of any of the preceding claims, which further comprises:

a step of assaying one or more cell surface markers on cells in the output sample, e.g., CD45, CD19, CD3, CD28, CD25, or CD14; and/or

a step of stimulating the immune effector cells, e.g., by contacting the cells with a reagent that binds CD3 and/or CD28, e.g., a substrate (such as a bead) coupled to CD3 and/or CD28 antibodies, e.g., prior to transduction.

19. The method of any one of claims 1-17, further comprising collecting five fractions from the elutriation (e.g., a first fraction, a second fraction, a third fraction, a fourth fraction, and a fifth fraction), optionally wherein:

(i) the first fraction is collected using a flow rate of from about 30-50 mL/min (e.g., about 30 mL/min), the second fraction is collected using a flow rate of from about 0-50 mL/min (e.g., about 30 mL/min), the third fraction is collected using a flow rate of from about 50-80 mL/min (e.g., about 70 mL/min), the fourth fraction is collected using a flow rate of from about 50-80 mL/min (e.g., about 72 mL/min), and/or

the fifth fraction is collected using a flow rate of from about 80-150 mL/min (e.g., about 82 mL/min);

(ii) the first, third, or fourth fraction is collected using centrifugation at about 1800-2400 rpm (e.g., about 2400 rpm), and/or the second fraction is collected using centrifugation at about 0-2400 rpm (e.g., about 2400 rpm); and/or

(iii) the first fraction is collected using a collection volume of about 900 mL, the second fraction is collected using a collection volume of about 500 mL, the third fraction is collected using a collection volume of about 975 mL, the fourth fraction is collected using a collection volume of about 400 mL, and/or the fifth fraction is collected using a collection volume of about 250 mL.

20. A reaction mixture obtained using the method of any of claims 1-19, wherein the

reaction mixture comprises:

(a) at least 80%, 85%, 90%, or 95% T cells and less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% monocytes, wherein the total number of cells in the reaction mixture adds up to 100%, wherein one or more of the T cells expresses a CAR; and

(b) a nucleic acid encoding a CAR, e.g., wherein the nucleic acid is present inside a T cell or outside a T cell.