

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,
 - 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,optionally wherein the frozen input sample is a plasma apheresis sample.
2. The method of claim 1, further comprising one, two, three or all of:
 - d) depleting CD19+ cells under flow conditions;
 - e) performing density centrifugation 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 (e.g., 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 under flow conditions.
3. The method of claim 1 or 2, wherein:
 - (a) 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;
 - (b) the elutriation is performed using a flow rate of from about 30-82 mL/min or 50-80 mL/min and/or the collection volume is about 250-1250 mL or 300-1000 mL for each fraction, e.g., the elutriation is performed using a flow rate of about 30, 40, 50, 60, 70, 72, or 82 mL/min, e.g., about 70 or 72 mL/min;
the elutriation is performed using a collection volume of about 250, 400, 500, 900, or 975 mL, e.g., about 400 or 975 mL; and/or
the elutriation is performed at about 2400 rpm;
 - (c) 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;

(d) 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;

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; and/or

(e) the method has a T cell yield recovery of at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% T cells.

4. The method of any of claims 1-3, wherein:

(a) the output sample is contacted with a nucleic acid encoding a CAR, optionally 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; and/or

(b) 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.

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

a) providing an input sample comprising immune effector cells, and

b) performing density centrifugation step 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), thereby producing an output sample

comprising immune effector cells that are suitable for expression of a CAR, optionally wherein the method further comprises performing one, two, three, or all of:

- c) depleting CD19⁺ cells under flow conditions;
- d) elutriation on the input sample, wherein the input sample is optionally a thawed input sample;
- e) performing a wash step (e.g., before density centrifugation) 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

f) positive selection of CD3/CD28⁺ cells under flow conditions, optionally wherein the method does not comprise one or more of: using a solution comprising glycol, e.g., a Ficoll solution; or performing a wash step in a buffer comprising dextrose and/or sodium chloride, e.g., D5 medium, e.g., wherein the wash step is performed using a CS5 instrument; or performing a positive selection step.

6. The method of claim 5, wherein:

(a) the density centrifugation is performed using a cell separation device, e.g., a Sepax2 device;

(b) the input sample comprises:

less than 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 19%, 18%, 17%, 16%, or 15% T cells;

at least 10%, 15%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, or 70% monocytes; and/or

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

(c) the output sample comprises:

at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% T cells;

less than 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 2%, 2%, 1%, 0.5%, 0.2%, or 0.1% monocytes; and/or

less than 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 2%, 2%, 1%, 0.5%, 0.2%, 0.1%, 0.05%, or 0.01% B cells; and/or

(d) the method has a T cell yield recovery of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% T cells.

7. A method of making a population of immune effector cells (e.g., T cells) that can be engineered to express a CAR, the method comprising:
 - a) providing an input sample comprising immune effector cells, and
 - b) removing CD19+ cells from the input sample under flow conditions, e.g., using a flow-through device, thereby producing an output sample comprising immune effector cells that are suitable for expression of a CAR, optionally wherein the method further comprises performing one, two, three or all of:
 - c) elutriation on the input sample, wherein the input sample is optionally a thawed input sample;
 - d) a density centrifugation step 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),
 - e) performing a wash step (e.g., before removing CD19+ cells and/or after the input sample is thawed) with a buffer 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
 - f) positive selection of CD3/CD28+ cells under flow conditions.

8. The method of claim 7, wherein:
 - (a) the method does not comprise performing elutriation or density centrifugation, optionally wherein the CD19+ cells comprise B cells, e.g., lymphoblasts;
 - (b) the input sample comprises:
 - at least 1%, 2%, 5%, 10%, 15%, 20%, 30%, 35%, 40%, 45%, or 50% CD19+ cells;
 - at least 10%, 15%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 35%, or 40% monocytes; and/or
 - less than 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, or 20% T cells; and/or
 - (c) the output sample comprises:
 - less than 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 2%, 2%, 1%, 0.5%, 0.2%, 0.1%, 0.05%, or 0.01% CD19+ cells;
 - less than 50%, 45%, 40%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 4%, 2%, 2%, or 1% the percentage of CD19+ cells compared to the input sample;
 - less than 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 2%, 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; and/or
 - less than 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 2%, 2%, 1%, 0.5%, 0.2%, 0.1%, 0.05%, or 0.01% B cells.

9. The method of any of claim 7 or 8, wherein:
- (a) CD19+ cells are removed by magnetic separation, optionally wherein the magnetic separation comprises:
 - contacting the cells with a separation reagent, which separation reagent comprises a magnetic or paramagnetic member and a CD19-binding member;
 - flow cytometry; and/or
 - use of a magnetic cell separation device, e.g., CliniMACs device; or
 - (b) CD19+ cells are removed by FACS.
10. The method of any of claims 7-9, which has a T cell yield recovery of at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% T cells, optionally wherein the input sample (e.g., the input sample post-wash) comprises at least 1%, 2%, 5%, 10%, 15%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, or 80% B cells.
11. A method of making a population of immune effector cells (e.g., T cells) that can be engineered to express a CAR, the method comprising:
- a) providing an input sample comprising immune effector cells, and
 - b) positively selecting for CD3+/CD28+ cells from the input sample under flow conditions, thereby producing an output sample comprising immune effector cells that are suitable for expression of a CAR, optionally wherein the method
- further comprises performing one, two, three, or all of:
- c) depleting CD19+ cells, e.g., under flow conditions;
 - d) elutriation on the input sample, wherein the input sample is optionally a thawed input sample;
 - e) density centrifugation step 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 (e.g., before removing CD19+ cells and/or after the input sample is thawed) with a buffer 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.
12. The method of claim 11, which further comprises:
- performing elutriation, a wash step (optionally), and density centrifugation (e.g., using Ficoll or OptiPrep medium) prior to performing positive selection, and/or

performing a wash step (optionally) and density centrifugation (e.g., using Ficoll or OptiPrep medium) prior to performing positive selection.

13. The method of claim 11 or 12, wherein:

- (a) the method does not comprise performing elutriation,
- (b) the method comprises performing a wash with a buffer comprising dextrose and/or sodium chloride, e.g., D5 1/2 NS buffer, e.g., using a CS5+ instrument;
- (c) the positive selection for CD3+/CD28+ cells comprises contacting the input sample with a separation reagent, which separation reagent comprises a magnetic or paramagnetic member and a CD3 and/or CD28-binding member; and/or
- (d) the positive selection for CD3+/CD28+ cells comprises incubating the input sample with a separation reagent for about 10 to 90 minutes, about 10 to 60 minutes, about 10 to 45 minutes, about 12 to 90 minutes, about 12 to 60 minutes, about 12 to 45 minutes, about 15 to 90 minutes, about 15 to 60 minutes, about 15 to 45 minutes, e.g., about 30 minutes or about 20 minutes, optionally wherein the separation reagent comprises a bead that is coupled (e.g., covalently or non-covalently coupled) to an anti-CD3 and/or anti-CD28 antibody.

14. The method of any of claims 11-13, wherein:

- (a) the output sample comprises:
 - at least 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, or 20% T cells;
 - less than 20%, 15%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 2%, 2%, 1%, 0.5%, 0.2%, or 0.1% monocytes;
 - at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% T cells, e.g., CD3+CD45+ T cells;
- (b) the method is performed with a magnetic device, e.g., Dynamag CTS or other arrangement of magnetic elements and/or which uses an about 3:1 ratio of magnetic separation members (e.g., beads) to T cells;
- (c) the positive selection for CD3+/CD28+ cells comprises a separation or dwell time of less than about 6, 5, 6, 3, 2, or 1 minute, or less than about 50, 40, 30, 20, 10, 5, 4, 3, 2, or 1 second; and/or
- (d) the method comprises flowing a fluid that comprises the immune effector cells and magnetic separation members within an enclosed system, e.g., a chamber or a bag, where magnetic separation occurs, wherein optionally the flowing is performed at a speed such that magnetic separation of the members (optionally bound to immune effector cells) occurs.

15. The method of any of claims 11-14, which is performed using a device comprising at least one cell suspension module;

at least one flow-through magnetic separation/debeading module;
 at least one non-magnetic output module;
 at least one magnetic output module;
 optionally, at least one magnetic component, external to the magnetic separation/debeading module, that creates magnetic forces and/or gradients;
 and optionally, at least one buffer module, optionally wherein the magnetic separation/debeading module comprises:
 a chamber defined by walls and having an x-direction, a y-direction, and a z-direction;
 an inlet and an outlet arranged on opposite ends of the chamber, e.g., in the x-direction, in the y-direction, or in the z-direction;
 at least two magnets adjacent or proximate to a wall of the chamber and arranged to establish a zero gradient line within the chamber between the inlet and the outlet.

16. The method of any of claims 11-15, wherein:

(a) the input sample comprises:

about 1×10^7 cells/ml;

at least about 5%, 10%, 15%, 18%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% B cells, e.g., CD45+CD19+ B cells; and/or

at least about 5%, 10%, 15%, 18%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% B cells, e.g., CD45-CD19+ B cells; and/or

(b) the output sample comprises:

less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% B cells, e.g., CD45+CD19+ B cells;

less than about 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% B cells, e.g., CD45-CD19+ B cells.

17. The method of any of the preceding claims, wherein:

(a) 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; and/or

(b) 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; and/or

(c) the method further comprises introducing, e.g., by transduction, a nucleic acid encoding a CAR into one or more of the immune effector cells in the output sample, wherein optionally 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 wherein optionally the transduction results in a transduction efficiency of at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50%, optionally wherein

the method 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.

18. The method of any of the preceding claims, wherein:

(a) the immune effector cells are human immune effector cells;

(b) the output sample comprises CD8⁺ T cells and/or CD4⁺ T cells;

(c) the input sample is from a patient that has a cancer selected from the group consisting of one or more acute leukemias including but not limited to B-cell acute lymphoid leukemia (BALL), T-cell acute lymphoid leukemia (TALL), acute lymphoid leukemia (ALL); one or more chronic leukemias including but not limited to chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL); additional hematologic cancers or hematologic conditions including, but not limited to 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; and/or

(d) wherein the method 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. 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:

- i) providing an input sample, e.g., a frozen input sample or a fresh input sample comprising immune effector cells;
- ii) optionally, wherein the input sample is a frozen input sample, thawing the frozen input sample, to produce a thawed sample;
- iii) performing an enrichment step, wherein the enrichment step comprises:
 1. performing elutriation on the input sample, wherein the input sample is optionally a thawed input sample; or
 2. performing density centrifugation step 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); and
- iv) performing a selection step, wherein the selection is a positive selection, e.g., for CD3/CD28⁺ cells, or a negative selection, e.g., for CD19⁺, CD25⁺, or CD14⁺ cells; thereby producing an output sample comprising immune effector cells that are suitable for expression of a CAR.

20. 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 an input sample, e.g., a frozen input sample or a fresh input sample, comprising immune effector cells;
- b) performing an enrichment step, wherein the enrichment step comprises:

performing elutriation or density centrifugation (e.g., using Ficoll or a Optiprep medium);
- c) performing a selection step under flow conditions, e.g., by using a flow-through device, wherein the selection is a positive selection, e.g., for CD3/CD28⁺ cells, or a negative selection, e.g., for CD19⁺, CD25⁺, or CD14⁺ cells;

thereby producing an output sample comprising immune effector cells that are suitable for expression of a CAR.

21. The method of claim 19 or 20, further comprising:

performing a wash step with a buffer comprising dextrose and/or sodium chloride, e.g., D5 medium (5% dextrose and 0.45% sodium chloride), e.g., wherein the washing step is performed using a CS5 (CellSaver5+) instrument;

stimulating the output sample with an agent that stimulates proliferation of the immune effector cells, e.g., stimulates a CD3/TCR complex associated signal and/or a ligand that stimulates a costimulatory molecule on the surface of the T cells, e.g., an anti-CD3 antibody and an anti-CD28 antibody; and/or

introducing a nucleic acid encoding a CAR, e.g., by transduction, transfection, or electroporation.

22. A reaction mixture obtained using a method according to any of claims 1-21.

23. A reaction mixture comprising 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 optionally one or more of the T cells expresses a CAR, optionally wherein the reaction mixture comprises:

(a) at least 1×10^6 , 2×10^6 , 5×10^6 , 1×10^7 , 2×10^7 , 5×10^7 , 1×10^8 , 2×10^8 , or 5×10^8 cells total;

less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% B cells; and/or

less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% cancer cells, e.g. lymphoblasts;

and/or

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