

CLAIMS

1. A method of expanding and/or activating a population of immune cells, e.g., immune effector cells, comprising:

introducing a nucleic acid encoding a first Chimeric Antigen Receptor (CAR) molecule into the immune cell population, under conditions suitable for transient expression of the CAR molecule, thereby producing a first CAR-expressing cell population, wherein the CAR molecule comprises an antigen binding domain of an antibody molecule; and

contacting the first CAR-expressing cell population with a ligand of the CAR antigen binding domain chosen from a cognate antigen molecule (e.g., a recombinant antigen molecule), or an anti-antigen idiotype antibody molecule, under conditions such that immune cell expansion and/or activation occurs, thereby producing an expanded and/or activated immune cell population; wherein:

(i) the expansion and/or activation of the population of immune cells is carried out *in vitro* or *ex vivo*; and/or

(ii) the population of immune cells is acquired, e.g., obtained, from a blood sample from a subject (e.g., a cancer patient), e.g., by apheresis.

2. A method of expanding and/or activating a population of immune cells, e.g., immune effector cells, comprising:

providing a first CAR-expressing cell population, said first CAR-expressing cell population comprising a transiently expressed first CAR molecule, and said CAR molecule comprises an antigen binding domain of an antibody molecule; and

contacting said CAR-expressing cell population with a ligand of the CAR molecule chosen from a cognate antigen molecule (e.g., a recombinant antigen molecule), or an anti-antigen idiotype antibody molecule, under conditions such that immune cell expansion and/or activation occurs, thereby producing an expanded and/or activated immune cell population; wherein:

(i) the expansion and/or activation of the population of immune cells is carried out *in vitro* or *ex vivo*; and/or

(ii) the population of immune cells is acquired, e.g., obtained, from a blood sample from a subject (e.g., a cancer patient), e.g., by apheresis.

3. The method of any of claims 1 or 2, wherein:

(i) the population of immune cells comprises immune effector cells chosen from T cells (e.g., alpha/beta T cells and gamma/delta T cells), B cells, natural killer (NK) cells, natural killer T (NKT) cells, mast cells, myeloid-derived phagocytes, or a combination thereof;

(ii) the population of immune cells comprises primary T cells or a subset of lymphocytes chosen from anergized T cells, naïve T cells, T-regulatory cells, Th-17 cells, stem T cells, or a combination thereof;

(iii) the population of immune cells comprises peripheral blood mononucleated cells (PBMcs), cord blood cells, or a combination thereof; and/or

(iv) the population of immune cells comprises cells that express a low level of, substantially impaired, or do not have, a functional T cell receptor.

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

(i) a cognate antigen molecule;

(ii) anti-antigen idiotype antibody molecule;

(iii) immobilized or attached to a non-naturally occurring substrate, *e.g.*, a substrate surface, optionally wherein the non-naturally occurring substrate is a solid support chosen from a plate, a membrane, a matrix, a chip or a bead (*e.g.*, a magnetic bead

(iv) present on a surface of a cell, *e.g.*, a cell expressing a heterologous cognate antigen on its surface.

5. The method of any of claims 1-4, wherein the nucleic acid encoding the first CAR molecule is an RNA molecule, *e.g.*, an *in vitro* transcribed (IVT) RNA.

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

(i) the first CAR molecule is transiently expressed in the immune cell population for a finite period of time or number of cell replications, *e.g.*, less than 50 days (*e.g.*, less than 40, 30, 25, 20, 15, 10, 5 or fewer days);

(ii) the first CAR molecule is internalized post a single ligand (*e.g.*, antigen) stimulation;

(iii) the immune cell does not receive repeated ligand (*e.g.*, antigen) stimulation;

(iv) the strength of the immune cell stimulation is customized to a desired level by adjusting one or both of: the first CAR-surface density, or the affinity of the CAR antigen binding domain to the ligand;

(v) the first CAR-expressing immune cells are cultured in the presence of the ligand of the CAR molecule for about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 18, 21, 22, 23 or 24 hours, or about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 days, optionally wherein the first CAR-expressing cells are cultured for a period of 8 days or less, *e.g.*, 7, 6 or 5 days.

(vi) the CAR-expressing cells shows at least 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 or higher population doublings (e.g., a total of 8-10, or about 9 population doublings), optionally wherein the first CAR-expressing immune cell population expands to a total of 400-600, or about 500 cells, wherein the cell expansion is measured between 10 and 25 days after stimulation with the ligand; and/or

(vii) the expanded and/or activated immune cell population comprises immune effector cells having a less differentiated phenotype, e.g., a younger cell, e.g., a younger T cell, optionally wherein the younger T cell is chosen from a naïve T cell (T_N), a memory stem cell (T_{SCM}), a central memory T cell (T_{CM}), or a combination thereof.

7. The method of any of claims 1-6, further comprising contacting the expanded and/or activated immune cell population with a nucleic acid encoding a second CAR molecule, thereby producing a second CAR-expressing cell population, optionally wherein the nucleic acid encoding the second CAR molecule is selected from the group consisting of a DNA, an RNA, a plasmid, a lentivirus vector, adenoviral vector, or a retrovirus vector.

8. The method of any of claims 1-7, wherein the first and second CAR molecules are directed to the same antigen, e.g., the same cancer associated antigen, or different antigens, e.g., different cancer associated antigens, optionally wherein the cancer associated antigen is chosen from CD19, CD123, CD22, CD30, CD171, CS-1, CLL-1 (CLECL1), CD33, EGFRvIII, GD2, GD3, BCMA, Tn Ag, PSMA, ROR1, FLT3, TAG72, CD38, CD44v6, CEA, EPCAM, B7H3, KIT, IL-13Ra2, Mesothelin, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, CD24, PDGFR-beta, SSEA-4, CD20, Folate receptor alpha, ERBB2 (Her2/neu), MUC1, EGFR, NCAM, Prostase, PAP, ELF2M, Ephrin B2, FAP, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, Fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, Folate receptor beta, TEM1/CD248, TEM7R, CLDN6, TSHR, GPRC5D, CXORF61, CD97, CD179a, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-1a, MAGE-A1, MAGE A1, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, prostatein, survivin and telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, Androgen receptor, Cyclin B1, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, legumain, HPV E6, E7, intestinal carboxyl esterase, mut hsp70-2, CD79a, CD79b, CD72, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, or IGLL1.

9. The method of claim 8, wherein the cancer associated antigen is CD19.
10. The method of any of claims 1-9, wherein the first and second CAR molecules are the same or different CAR molecules, optionally wherein the first and second CAR molecules are each independently chosen from a CD19 CAR, a BCMA CAR, a CD33 CAR, a CLL-1 CAR, EGFRvIII CAR, a GFR alpha 4 CAR, an ROR1 CAR, a CD20 CAR, a CD22 CAR, a CD123 CAR, a CD10 CAR, a CD34 CAR, a FLT-3 CAR, a CD79b CAR, a CD179b CAR, a mesothelin CAR or a CD79a CAR, or any combination thereof.
11. The method of any of claims 1-10, wherein the immune cell population transiently expressing the first CAR is expanded and/or activated *in vitro* or *ex vivo*, and the immune cell population expressing the second CAR is to be administered to a subject as part of a therapeutic protocol, optionally wherein:
- (i) the immune cell population transiently expressing the first CAR is expanded and/or activated *in vitro* or *ex vivo* by contacting said immune cell population with a cancer associated antigen or an anti-antigen idiotypic antibody against the CAR binding antibody molecule (e.g., a CD19-antigen or anti-CD19 idiotypic antibody immobilized onto a non-cellular or cellular substrate as described herein); and/or
 - (ii) the method further comprising storing the expanded and/or activated immune cell population after the appropriate expansion period, e.g., cryopreserving the expanded and/or activated immune cell population.
12. Use of an expanded and/or activated immune cell population made according to one or more of claims 1-11 in the manufacture of a medicament for the treatment of a cancer, or provision of an anti-tumor immunity, in a subject, wherein the expanded and/or activated immune cell population, is/are to be administered alone or in combination with an additional therapy.
13. An expanded and/or activated immune cell population that expresses a first and/or second CAR molecule made according to one or more of claims 1-11 for use in a method of treating, or providing anti-tumor immunity to, a subject having a cancer, wherein said expanded and/or activated immune cell population is to be administered to the subject alone or in combination with an additional therapy.

14. Use of an immune cell population that expresses a first CAR molecule and a second CAR molecule, simultaneously or sequentially, wherein the first CAR molecule is transiently expressed, and the second CAR molecule is stably expressed in the manufacture of a medicament for use in the treatment, or provision of anti-tumor immunity to, a subject having a cancer.

15. Use of an immune cell population expressing a second CAR molecule, wherein the immune cell population was previously obtained by expanding and/or activating *in vitro* or *ex vivo* an immune cell population transiently expressing a first CAR molecule, said first CAR molecule comprising an antigen binding domain of an antibody molecule, e.g., an antigen binding domain as described herein in the manufacture of a medicament for use in the treatment, or provision of an anti-tumor immunity to, a subject having a cancer.

16. The use of claim 15, wherein the *in vitro* or *ex vivo* expansion and/or activation of the immune cell population comprises contacting said immune cell population with a cancer associated antigen recognized by the first CAR molecule, or an anti-antigen idiotype antibody against the first CAR binding domain, optionally wherein the cancer associated antigen or the anti-antigen idiotype antibody against the binding domain of the CAR molecule is immobilized onto a non-cellular, e.g., a bead (e.g., a magnetic bead).

17. The use of any of claims 12-16, wherein:

(i) the second CAR-expressing cell population comprises a nucleic acid encoding the second CAR molecule selected from the group consisting of a DNA, a RNA, a plasmid, a lentivirus vector, adenoviral vector, or a retrovirus vector;

(ii) the first and second CAR molecules are directed to the same or different cancer associated antigen, optionally wherein the cancer associated antigen is chosen from CD19, CD123, CD22, CD30, CD171, CS-1, CLL-1 (CLECL1), CD33, EGFRvIII, GD2, GD3, BCMA, Tn Ag, PSMA, ROR1, FLT3, TAG72, CD38, CD44v6, CEA, EPCAM, B7H3, KIT, IL-13Ra2, Mesothelin, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, CD24, PDGFR-beta, SSEA-4, CD20, Folate receptor alpha, ERBB2 (Her2/neu), MUC1, EGFR, NCAM, Prostase, PAP, ELF2M, Ephrin B2, FAP, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, Fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, Folate receptor beta, TEM1/CD248, TEM7R, CLDN6, TSHR, GPRC5D, CXORF61, CD97, CD179a, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-1a, MAGE-A1, MAGE A1, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53,

p53 mutant, prostatein, survivin and telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, Androgen receptor, Cyclin B1, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, legumain, HPV E6, E7, intestinal carboxyl esterase, mut hsp70-2, CD79a, CD79b, CD72, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, or IGLL1; and/or

(iii) the first and second CAR molecules are the same CAR molecule, or different CAR molecules, optionally wherein:

(a) the first and/or second CAR molecules are each independently chosen from a CD19 CAR, a BCMA CAR, a CD33 CAR, a CLL-1 CAR, EGFRvIII CAR, a GFR alpha 4 CAR, an ROR1 CAR, a CD20 CAR, a CD22 CAR, a CD123 CAR, a CD10 CAR, a CD34 CAR, a FLT-3 CAR, a CD79b CAR, a CD179b CAR, a mesothelin CAR or a CD79a CAR, or any combination thereof; or

(b) the first and second CAR molecules are mesothelin CAR and CD19 CAR molecules, respectively.

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

(i) the first and/or second CAR-expressing immune effector cell comprises a CD19 CAR, a BCMA CAR, a CD33 CAR, a CLL-1 CAR, EGFRvIII CAR, a GFR alpha 4 CAR, an ROR1 CAR, a CD19 CAR, a CD20 CAR, a CD22 CAR, a CD123 CAR, a CD10 CAR, a CD34 CAR, a FLT-3 CAR, a CD79b CAR, a CD179b CAR, a mesothelin CAR or a CD79a CAR;

(ii) first and/or second CAR-expressing immune effector cell comprises a CD19 CAR;

(iii) the CD19 CAR comprises a sequence according to Table 1 or Table 4;

(iv) the CD19 CAR comprises the amino acid sequence of the antigen binding domain of CTL019, e.g., SEQ ID NO: 51, or an amino acid sequence substantially identical thereto;

(v) the CD19 CAR comprises the amino acid sequence of CTL019, e.g., SEQ ID NO: 89 (including the signal sequence) or without the signal sequence, or an amino acid sequence substantially identical thereto;

(vi) at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96 %, 97%, 98%, 99% or 100% of the immune effector cells in the immune effector cell population express the first and/or the second CAR molecule on their cell surface;

(vii) the subject from which immune effector cells are acquired and/or the subject to be treated, is a human cancer patient.

19. The method or use of any one of the preceding claims, wherein the cancer is a hematological cancer chosen from one or more of: a B-cell acute lymphocytic leukemia (B-ALL), T-cell acute lymphocytic leukemia (T-ALL), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), B cell promyelocytic 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 (MCL), marginal zone lymphoma, multiple myeloma, myelodysplasia and myelodysplastic syndrome, non-Hodgkin's lymphoma (NHL), Hodgkin's lymphoma (HL), plasmablastic lymphoma, plasmacytoid dendritic cell neoplasm, and Waldenstrom macroglobulinemia.
20. The method or use of any one of the preceding claims, wherein the population of immune effector cells are from a subject having a haematological cancer chosen from a leukemia, e.g., chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), or a lymphoma, e.g., mantle cell lymphoma (MCL).
21. The method or use of any of the preceding claims, wherein the population of cells is expanded in the presence a cytokine, e.g., IL2 or IL-15 and IL-7.
22. The method or use of any one of the preceding claims, further comprising removing T regulatory cells, e.g., CD25+ T cells, from the immune cell population, to thereby provide a population of T regulatory-depleted cells, e.g., CD25+ depleted cells, optionally wherein the population of T regulatory-depleted cells contains less than 30%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, 1% of CD25+ cells.
23. The method or use of any one of the preceding claims, wherein the acquired immune effector cell population are cells of a subject having cancer, e.g., a subject having a CD25 expressing cancer such as, e.g., chronic lymphocytic leukemia (CLL), wherein the population of T regulatory-depleted cells contains less than 30%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, 1% of the leukemia cells, e.g., CLL cells, ALL cells, or lymphoma cells, e.g., MCL cells, HL cells.
24. The method or use of any of claims 1-23, further comprising:
- (i) removing cells from the immune effector cell population which express a tumor antigen, e.g., a tumor antigen that does not comprise CD25, e.g., CD19, CD30, CD38, CD123, CD20, CD14 or

CD11b, to thereby provide a population of T regulatory-depleted, e.g., CD25+ depleted, and tumor antigen depleted cells that are suitable for expression of a CAR, e.g., a CAR described herein; and/or

(ii) removing cells from the acquired immune effector cell population which express a check point inhibitor, e.g., a check point inhibitor described herein, e.g., one or more of PD1+ cells, LAG3+ cells, and TIM3+ cells, to thereby provide a population of T regulatory-depleted, e.g., CD25+ depleted cells, and check point inhibitor depleted cells, e.g., PD1+, LAG3+ and/or TIM3+ depleted cells.

25. The method or use of any one of claims 1-24, wherein the acquired immune effector cell population has been selected based upon the expression of one or more markers, e.g., CD3, CD28, CD4, CD8, CD45RA, and CD45RO, e.g., the provided population of immune effector cells (e.g., T cells) are CD3+ and/or CD28+.

26. The method or use of any one of the preceding claims, further comprising:

(i) activating the population of T regulatory-depleted cells, e.g., CD25+ depleted cells, e.g., by a method described herein;

(ii) transducing a cell from the population of T regulatory-depleted cells, e.g., the population of CD25+ depleted cells, with a vector comprising a nucleic acid encoding a CAR, e.g., a CAR described herein, e.g., a CD19 CAR described herein; and/or

(iii) expanding the population of T regulatory-depleted cells, e.g., engineered to express a CAR, a CAR described herein, e.g., a CD19 CAR described herein, e.g., by a method described herein, optionally wherein the population of cells is expanded for a period of 8 days or less, e.g., 7, 6 or 5 days, and/or wherein the population of the immune cells is cryopreserved after the appropriate expansion period;

(iv) contacting the population of immune effector cells with a nucleic acid encoding a telomerase subunit, e.g., hTERT.

27. An immune cell preparation or reaction mixture, comprising a population of immune effector cells made according to any of the methods described herein.

28. An immune effector cell preparation or reaction mixture that expresses a first and a second CAR molecule, simultaneously or sequentially, wherein the first CAR molecule is transiently expressed, and the second CAR molecule is stably expressed.