

CLAIMS:

1. An *in vitro* or *ex vivo* 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;
 - contacting said first CAR-expressing cell population with a ligand of the CAR molecule, under conditions such that immune cell expansion and/or activation occurs, thereby producing an expanded and/or activated immune cell population; and
 - 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.
2. The method of claim 1,
 - wherein providing the first CAR-expressing cell population comprises introducing a nucleic acid encoding a first Chimeric Antigen Receptor (CAR) molecule into the immune cell population, thereby producing a first CAR-expressing cell population comprising a transiently expressed first CAR molecule, wherein the CAR molecule comprises an antigen binding domain of an antibody molecule.
3. Use of a ligand of a first CAR molecule for expanding and/or activating immune cells transiently expressing the first chimeric antigen receptor (CAR) molecule, and said use being *in vitro* or *ex vivo*, wherein said use further comprises contacting the expanded and/or activated immune cell population with a nucleic acid encoding a second CAR molecule.
4. The method of claim 1 or 2 or the use of claim 3, wherein the population of immune cells:
 - (i) is acquired, e.g., obtained, from a blood sample from a subject (e.g., a cancer patient), e.g., by apheresis;

- (ii) 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;
 - (iii) 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;
 - (iv) comprises peripheral blood mononucleated cells (PBMCs), cord blood cells, or a combination thereof; and/or
 - (v) comprises cells that express a low level of, substantially impaired, or do not have, a functional T cell receptor.
5. The method of any of claims 1, 2 or 4, or the use of claim 3 or 4, wherein the ligand of the CAR molecule is:
- (i) 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); or
 - (ii) present on a surface of a cell, e.g., a cell expressing a heterologous cognate antigen on its surface.
6. The method of any of claims 1, 2, 4 or 5 or the use of any of claims 3-5, wherein:
- (i) the nucleic acid encoding the first CAR molecule is an RNA molecule, e.g., an in vitro transcribed (IVT) RNA;
 - (ii) 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);
 - (iii) the first CAR-expressing immune cells are cultured in the presence of the ligand of the CAR molecule for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 18, 21, 22, 23 or 24 hours, or 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;
 - (iv) 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 9 population doublings);

- (v) the first CAR-expressing immune cell population expands to a total of 400-600, or 500 cells, wherein the cell expansion is measured between 10 and 25 days after stimulation with the ligand; and/or
 - (vi) 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, 2 or 4-6 or the use of any of claims 3-6, wherein:
- (i) the first CAR molecule is internalized post a single ligand (e.g., antigen) stimulation;
 - (ii) the immune cell does not receive repeated ligand (e.g., antigen) stimulation; or
 - (iii) 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.
8. The method of any of claims 1, 2 or 4-7 or the use of any of claims 3-7, wherein:
- (i) 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;
 - (ii) 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, PAX3, 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, mutant hsp70-2, CD79a, CD79b, CD72, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, or IGLL1, *e.g.* the cancer associated antigen is CD19;

- (iii) the first and second CAR molecules are the same or different CAR molecules;
 - (iv) 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;
 - (v) 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 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 ligand of the CAR molecule; and/or
 - (vi) the method further comprises 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.
9. The immune cell population made according to the method of one or more of claims 1, 2 or 4-8, for use in the treatment of cancer in a subject, 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.

10. Use of an immune cell population made according to the method of one or more of claims 1, 2 or 4-8, in the manufacture of a medicament for the treatment of cancer in a subject, or for 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.
11. The use of claim 10, wherein the immune cell population 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, and wherein said immune cell population is to be administered to the subject alone or in combination with an additional therapy.
12. The use as in claim 10 or 11, wherein the *in vitro* or *ex vivo* expansion and/or activation of the immune cell population comprises contacting said immune cell population with a ligand of the CAR molecule.
13. The use as in any of claims 10-12, 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.

14. The method of any of claims 1, 2 or 4-8, the use as in any of claims 3-8, or the use as in any of claims 10-13, 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) the 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; and/or
 - (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.
15. The method of any of claims 1, 2, 4-8 or 14, the use as in any of claims 3-8 or 14, or the use as in any of claims 10-14, wherein:
- (i) 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;
 - (ii) the subject from which immune effector cells are acquired and/or the subject to be treated, is a human cancer patient;
 - (iii) 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;
 - (iv) 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);

- (v) the population of cells is expanded in the presence a cytokine, e.g., IL2 or IL-15 and IL-7;
- (vi) 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);
- (vii) 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+; and/or
- (viii) the population of the immune cells is cryopreserved after the appropriate expansion period.

16. The method of any of claims 1, 2, 4-8, 14 or 15, the use as in any of claims 3-8, 14 or 15 or the use as in any one of claims 10-15, further comprising contacting the population of immune effector cells with a nucleic acid encoding a telomerase subunit, e.g., hTERT, or wherein the immune effector cell population has been contacted with a nucleic acid encoding a telomerase subunit, e.g., hTERT.

17. An immune cell preparation or reaction mixture, that

- (i) comprises a population of immune effector cells made according to any of the methods of claims 1, 2 or 4-8; or
- (ii) 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.