

TITLE: IN VITRO-DERIVED T CELL POPULATIONS, METHODS OF GENERATION, AND METHODS OF USE THEREOF

FIELD

5 [0001] The present invention relates generally to *in vitro* methods of generating T cell populations from stem cells and/or progenitor cells, *in vitro* generated T cell populations, and use of same.

BACKGROUND OF THE DISCLOSURE

10 [0002] Various feeder cell-based methods are available for the generation of T lineage cells from stem/progenitor cells. However, the expression of cell surface proteins by the supportive stromal cells in feeder cell-based systems is heterogenous, leading to diverse outcomes. Further, feeder cell-based systems are not easily scalable to meet the needs of clinical manufacturing.

[0003] Immobilized Notch signalling ligands, such as DL4, in combination with VCAM-1, have been shown to promote *in vitro* generation of progenitor T cells in a feeder-free and serum-free culture system (Shukla et al., 2017). Microbeads modified to present DL4 have also been shown to support *in vitro* differentiation of T-lineage cells, albeit with limited progression to mature lineages such as CD4-
15 CD8+ T cells (Trotman-Grant et al., 2021). Short-term stimulation of T cell progenitors with a CD3 antibody in the absence of Notch signalling followed by maturation without CD3 antibody has been shown to promote generation of CD8 $\alpha\beta$ + T cells (Iriguchi et al., 2021).

It is desirable to obviate or mitigate one or more of the above deficiencies.

SUMMARY OF THE DISCLOSURE

20 [0004] In a first aspect of the disclosure, a method of generating a population of CD4-CD8+ T cells is provided. The method comprises generating a population of progenitor T cells by contacting a population of hematopoietic stem/progenitor cells with an immobilized Notch signalling ligand at a first ligand concentration; generating a population of CD4+CD8+ cells by contacting the population of progenitor T cells with the immobilized Notch signalling ligand at a second ligand concentration lower
25 than the first ligand concentration; and contacting the population of CD4+CD8+ cells with a T cell activator, in the absence of the Notch signalling ligand.

[0005] In an embodiment, the immobilized Notch signalling ligand is DL4.

[0006] In an embodiment, the step of contacting the population of hematopoietic stem/progenitor cells with the immobilized Notch signalling ligand at the first ligand concentration further comprises
30 contacting the population of hematopoietic stem/progenitor cells with immobilized VCAM-1.

- [0007] In an embodiment, the first ligand concentration is 3.15×10^{11} to 1.26×10^{12} molecules/mL and the second ligand concentration is 3.94×10^{10} to 6.31×10^{11} molecules/mL.
- [0008] In an embodiment, the method further comprises contacting the population of CD4+CD8+ cells with the immobilized Notch signalling ligand at a third ligand concentration, lower than the second
5 ligand concentration, before contacting the population of CD4+CD8+ cells with a T cell activator.
- [0009] In an embodiment, the third ligand concentration is 3.94×10^{10} to 6.31×10^{11} molecules/mL.
- [0010] In an embodiment, the method further comprises enriching the CD4+CD8+ cells for CD8 α or CD8 β .
- [0011] In an embodiment, the T cell activator is a CD3 stimulator and an integrin ligand.
- 10 [0012] In an embodiment, the CD3 stimulator is an anti-CD3/anti-CD28/anti-CD2 reagent, an anti-CD3 reagent, or a peptide-major histocompatibility complex (pMHC) tetramer, and the integrin ligand is laminin, ICAM, fibronectin, a fibronectin fragment, VCAM-1, or ICOS-L.
- [0013] In an embodiment, the CD3 stimulator is an anti-CD3/anti-CD28/anti-CD2 reagent and the integrin ligand is a fibronectin fragment.
- 15 [0014] In an embodiment, the T cell activator is a chimeric antigen receptor (CAR) activator.
- [0015] In an embodiment, the CAR activator is an antigen immobilized on a substrate.
- [0016] In an embodiment, the antigen is a CD19 antigen, and the substrate is a particle.
- [0017] In an embodiment, the step of contacting the population of CD4+CD8+ cells with the T cell activator, in the absence of the Notch signalling ligand, is conducted in a cell culture medium comprising
20 IL-7, IL-15, and IL-21.
- [0018] In an embodiment, the step of contacting the population of CD4+CD8+ cells with the T cell activator, in the absence of the Notch signalling ligand, is conducted in a cell culture medium comprising one or more of IL-7, IL-15, and IL-21.
- [0019] In an embodiment, the step of contacting the population of CD4+CD8+ cells with the T cell
25 activator, in the absence of the Notch signalling ligand, is conducted in the absence of IL-2.
- [0020] In an embodiment, the method further comprises contacting the population of CD4+CD8+ T cells with a T cell activator and IL-7, IL-15, and IL-21.
- [0021] In an embodiment, the method further comprises contacting the population of CD4+CD8+ T cells with a T cell activator and one or more of IL-7, IL-15, and IL-21.
- 30 [0022] In an embodiment, the T cell activator is a CD3 stimulator.
- [0023] In an embodiment, the CD3 stimulator is an anti-CD3/anti-CD28/anti-CD2 reagent, an anti-CD3 reagent, or peptide-major histocompatibility complex (pMHC) tetramer.

- [0024] In an embodiment, the method further comprises increasing a cell density at the step of contacting the population of progenitor T cells with the immobilized Notch signalling ligand at the second ligand concentration, and/or increasing a cell density at the step of contacting the population of CD4⁺CD8⁺ cells with the T cell activator and an integrin ligand in the absence of the Notch signalling ligand.
- [0025] In an embodiment, the hematopoietic stem/progenitor cells are derived from pluripotent stem cells.
- [0026] In an embodiment, the population of CD4⁺CD8⁺ T cells comprises a nucleic acid sequence encoding a CAR or an exogenous T cell receptor (TCR).
- [0027] In an embodiment, the population of CD4⁺CD8⁺ T cells is CD3⁺.
- [0028] In an embodiment, the population of CD4⁺CD8⁺ T cells is TRAC^{-/-}.
- [0029] In an embodiment, the population of CD4⁺CD8⁺ T cells is enriched for CD8αβ⁺ cells.
- [0030] In an embodiment, the population of CD4⁺CD8⁺ T cells comprises a sub-population of TCRγδ⁺, CD49c⁺, and/or CD31⁺ cells.
- [0031] In a second aspect of the disclosure, a population of CD4⁺CD8⁺ T cells made according to the method of the first aspect is provided.
- [0032] In an embodiment, the population of CD4⁺CD8⁺ T cells comprises a nucleic acid sequence encoding a CAR or an exogenous TCR.
- [0033] In an embodiment, the population of CD4⁺CD8⁺ T cells is CD3⁺.
- [0034] In an embodiment, the population of CD4⁺CD8⁺ T cells is TRAC^{-/-}.
- [0035] In a third aspect of the disclosure, a CD4⁺CD8⁺CD3⁺ TRAC^{-/-} cell population is provided. The CD4⁺CD8⁺CD3⁺ TRAC^{-/-} cell population is derived in vitro from pluripotent stem cells.
- [0036] In an embodiment, the CD4⁺CD8⁺CD3⁺ TRAC^{-/-} cell population of the third aspect comprises a nucleic acid sequence encoding a CAR or an exogenous TCR.
- [0037] In an embodiment, the population of CD4⁺CD8⁺ T cells comprises a sub-population of TCRγδ⁺, CD49c⁺, and/or CD31⁺ cells.
- [0038] In a fourth aspect of the disclosure, a pharmaceutical composition comprising a population of CD4⁺CD8⁺ T cells and a pharmaceutically acceptable carrier is provided. The population of CD4⁺CD8⁺ T cells are CD3⁺ and TRAC^{-/-}.
- [0039] In a fifth aspect of the disclosure, a method of treating a disease or condition in a subject is provided. The method comprises generating a population of CD4⁺CD8⁺ T cells according to the method

of the first aspect; and administering an effective amount of the population of CD4-CD8+ T cells to a subject in need thereof.

[0040] In an embodiment, the population of CD4-CD8+ T cells is CD3+ and/or TRAC-/-.

5 **[0041]** In an embodiment, the disease or condition is a hematological malignancy, and the CD4-CD8+ T cells comprise a nucleic acid sequence encoding a CAR or an exogenous T cell receptor (TCR).

[0042] In a sixth aspect of the disclosure, a use of a population of CD4-CD8+ T cells in the manufacture of a medicament for the treatment of a disease or condition is provided. The population of CD4-CD8+ T cells are generated according to the method of the first aspect.

10 **[0043]** In a seventh aspect of the disclosure, a method of generating a population of CD4+CD8+ cells is provided. The method comprises generating a population of progenitor T cells by contacting a population of hematopoietic stem/progenitor cells with an immobilized Notch signalling ligand at a first ligand concentration; and contacting the population of progenitor T cells with the immobilized Notch signalling ligand at a second ligand concentration, lower than the first ligand concentration.

[0044] In an embodiment, the immobilized Notch signalling ligand is DL4.

15 **[0045]** In an embodiment, the first ligand concentration is 3.15×10^{11} to 1.26×10^{12} molecules/mL and the second ligand concentration is 3.94×10^{10} to 6.31×10^{11} molecules/mL.

[0046] In an embodiment, the population of CD4+CD8+ cells are CD1a+CD28+ and ICOS+.

[0047] In an embodiment, the population of CD4+CD8+ cells comprise a nucleic acid sequence encoding a CAR or an exogenous TCR.

20 **[0048]** In an eighth aspect of the disclosure, a population of CD4+CD8+ cells is provided. The population of CD4+CD8+ cells is made according to the method of the seventh aspect.

[0049] In a ninth aspect of the disclosure, a population of CD4+CD8+ cells derived in vitro from pluripotent stem cells is provided. The CD4+CD8+ cells are CD1a+CD28+ and ICOS+.

25 **[0050]** In a tenth aspect of the disclosure, a method of generating a population of CD4-CD8+ T cells is provided. The method comprises generating a population of progenitor T cells by contacting a population of hematopoietic stem/progenitor cells with an immobilized Notch signalling ligand; generating a population of CD4+CD8+ cells by contacting the population of progenitor T cells with a Notch signalling inhibitor and the immobilized Notch signalling ligand; and contacting the population of CD4+CD8+ cells with a T cell activator, in the absence of the Notch signalling ligand.

30 **[0051]** In an embodiment, the activity of the immobilized Notch signalling ligand is decreased in the step of contacting the population of progenitor T cells with the Notch signalling inhibitor and the immobilized Notch signalling ligand, in comparison to the activity of the immobilized Notch signalling

ligand in the step of contacting the population of hematopoietic stem/progenitor cells with the immobilized Notch signalling ligand.

[0052] In an embodiment, the Notch signalling inhibitor is a gamma-secretase inhibitor.

5 [0053] In an embodiment, the gamma-secretase inhibitor is provided at a concentration of 0.1 to 1 micromolar.

[0054] In an embodiment, the Notch signalling inhibitor is provided at a first ligand concentration of 3.15×10^{11} to 1.26×10^{12} molecules/mL in the step of contacting the population of hematopoietic stem/progenitor cells with the immobilized Notch signalling ligand; and/or the Notch signalling inhibitor is provided at a second ligand concentration of 3.15×10^{11} to 2.52×10^{12} molecules/mL in the in the step
10 of contacting the population of progenitor T cells with the Notch signalling inhibitor and the immobilized Notch signalling ligand.

[0055] In an eleventh aspect of the disclosure, a method of generating a population of CD4+CD8+ cells is provided. The method comprises: generating a population of progenitor T cells by contacting a population of hematopoietic stem/progenitor cells with an immobilized Notch signalling ligand; and
15 contacting the population of progenitor T cells with a Notch signalling inhibitor and the immobilized Notch signalling ligand.

[0056] In an embodiment, the activity of the immobilized Notch signalling ligand is decreased in the step of contacting the population of progenitor T cells with the Notch signalling inhibitor and the immobilized Notch signalling ligand, in comparison to the activity of the immobilized Notch signalling
20 ligand in the step of contacting the population of hematopoietic stem/progenitor cells with the immobilized Notch signalling ligand.

[0057] In an embodiment, the Notch signalling inhibitor is a gamma-secretase inhibitor.

[0058] In an embodiment, the gamma-secretase inhibitor is provided at a concentration of 0.1 to 1 micromolar.

25 [0059] In an embodiment, the immobilized Notch signalling ligand is provided at a first ligand concentration of 3.15×10^{11} to 1.26×10^{12} molecules/mL in the step of contacting the population of hematopoietic stem/progenitor cells with the immobilized Notch signalling ligand; and/or the immobilized Notch signalling ligand is provided at a second ligand concentration of 3.15×10^{11} to 2.52×10^{12} molecules/mL in the step of contacting the population of progenitor T cells with the Notch
30 signalling inhibitor and the immobilized Notch signalling ligand.

[0060] In a twelfth aspect of the disclosure, a method of treating a disease or condition in a subject is provided. The method comprises: generating a population of CD4-CD8+ T cells according to the method

of the eleventh aspect; and administering an effective amount of the population of CD4-CD8+ T cells to a subject in need thereof.

[0061] In an embodiment, the population of CD4-CD8+ T cells is CD3+ and/or TRAC-/-.

[0062] In a thirteenth aspect of the disclosure, a use of a population of CD4-CD8+ T cells in the
5 manufacture of a medicament for the treatment of a disease or condition is provided. The CD4-CD8+ T cells are generated according to the method of the eleventh aspect.

BRIEF DESCRIPTION OF THE DRAWINGS

[0063] In order that the subject matter may be readily understood, embodiments are illustrated by way of non-limiting examples in the accompanying drawings.

10 [0064] FIG. 1A is a graph quantifying percent expression (left axis) and median fluorescent intensity (MFI, right axis) of a chimeric antigen receptor (CAR) following lentiviral transduction or gene-editing of iPSCs.

[0065] FIG. 1B is a micrograph of G-band karyotyping of generated iPSC clones.

[0066] FIG. 2A is a graph depicting purity of CD34+ (left) and CD34+CD43+ (right) cells generated from
15 four iPSC cell lines.

[0067] FIG. 2B is a graph depicting yield of CD34+ (left) and CD34+CD43+ (right) cells generated from four iPSC cell lines.

[0068] FIG. 3A is flow cytometry plots depicting pluripotency marker (OCT4, SOX2, TRA-160, SSEA-4) and CAR expression of generated iPSC cells following thaw and expansion ("Stage 1"; culture day -10).

20 [0069] FIG. 3B is flow cytometry plots characterizing marker expression of hematopoietic stem/progenitor cells generated from iPSCs ("Stage 2", culture day 0).

[0070] FIG. 3C is flow cytometry plots depicting marker expression of progenitor T cells generated from iPSCs ("Stage 3"; culture day 10).

[0071] FIG. 3D is flow cytometry plots depicting marker expression of CD4+CD8+ double-positive (DP) T
25 lineage cells generated from iPSCs ("Stage 4"; culture day 28).

[0072] FIG. 3E is flow cytometry plots depicting marker expression of T lineage cells including DP and CD8+ single-positive (CD8 SP) cell populations generated from iPSCs ("Stage 5"; culture day 35).

[0073] FIG. 4A is a graph quantifying the percentage of CD4+CD8- immature single-positive (ISP), CD4-CD8- double-negative (DN), DP, and CD4-CD8 α + single-positive (CD8 α SP) cell populations generated
30 from iPSC-derived HSPCs with varying doses of engineered thymic niche (ETN) bead (0.1x, 0.5x, 1x bead dose) and cell densities (2×10^6 , 1×10^6 , 5×10^5 cells/mL).

- [0074] FIG. 4B is a graph showing the correlation at day 21 between ETN bead to cell ratio (bead:cell) and percentage of CD4+CD8+ cells (%DP).
- [0075] FIG. 4C is flow cytometry plots depicting representative marker expression of cells as quantified in FIG. 4A.
- 5 [0076] FIG. 5 is flow cytometry plots depicting marker expression of cells at day 15 of culture under two ETN culture conditions.
- [0077] FIG. 6A is a graph quantifying the percentage of CD56+CD7+ cells generated from iPSC-derived HSPCs at day 21 with varying doses of ETN bead (0.1x, 0.5x, 1x bead dose) and cell densities (4×10^6 , 2×10^6 , 1×10^6 cells/mL).
- 10 [0078] FIG. 6B is a graph quantifying the percentage of CD3+ cells generated from iPSC-derived HSPCs with varying doses of ETN bead (0.1x, 0.5x, 1x bead dose) and cell densities (4×10^6 , 2×10^6 , 1×10^6 cells/mL).
- [0079] FIG. 7A is a graph quantifying the percentage of CD4+ ISP, DN, DP, and CD8 α SP cell populations generated from iPSC-derived HSPCs with varying doses of ETN bead (0.1x, 0.5x, 1x bead dose) and cell
- 15 densities (4×10^6 , 2×10^6 , 1×10^6 cells/mL).
- [0080] FIG. 7B is a graph showing the correlation at day 23 between bead to cell ratio (bead:cell) and percentage of CD4+CD8+ double-positive cells (% DP).
- [0081] FIG. 7C is flow cytometry plots depicting representative marker expression of cells as quantified in FIG. 7A.
- 20 [0082] FIG. 8A is a graph quantifying the percentage of CD56+CD7+ cells at day 23, generated from iPSC-derived HSPCs with varying doses of ETN bead (0.1x, 0.5x, 1x bead dose) and cell densities (4×10^6 , 2×10^6 , 1×10^6 cells/mL).
- [0083] FIG. 8B is a graph quantifying the percentage of CD3+ cells at day 23, generated from iPSC-derived HSPCs with varying doses of ETN bead (0.1x, 0.5x, 1x bead dose) and cell densities (4×10^6 , 2
- 25 $\times 10^6$, 1×10^6 cells/mL).
- [0084] FIG. 9 is a schematic of the stages of in vitro differentiation of CD8+ cells from iPSCs, including a summary of culture conditions at each stage.
- [0085] FIG. 10 is graphs quantifying cell viability and cumulative fold expansion of cells through 28 days of differentiation from iPSC-derived HSPCs under a 2D or 3D differentiation process. Three cell lines are
- 30 shown: NTX4A1 (top), NTX6A1 (middle), and NTX4B3 (bottom).
- [0086] FIG. 11 is flow cytometry plots of marker expression of cells cultured under 2D differentiation conditions for 17 or 24 days. Two cell lines are shown: NTX4A1 (top) and NTX6A1 (middle).

- [0087] FIG. 12 is flow cytometry plots of marker expression of NTX4A1 cells cultured under 3D differentiation conditions for 17, 24, or 28 days.
- [0088] FIG. 13 is flow cytometry plots of marker expression of NTX6A1 cells cultured under 3D differentiation conditions for 17, 24, or 28 days.
- 5 [0089] FIG. 14 is flow cytometry plots of marker expression of NTX4B3 cells cultured under 2D differentiation conditions for 17 or 24 days.
- [0090] FIG. 15 is flow cytometry plots of marker expression of NTX4B3 cells cultured under 3D differentiation conditions for 17, 24, or 28 days.
- [0091] FIG. 16 is graphs quantifying cell viability (top) and cumulative fold expansion (bottom) following
10 culture of iPSC-derived DPs with either ImmunoCult™ human CD3/CD28/CD2 T Cell Activator (Immunocult) or antigen beads. Three cell lines are shown: NTX4A1, NTX6A1, and NTX4B3.
- [0092] FIG. 17 is flow cytometry plots of marker expression of NTX4A1 cells (top) and NTX6A1 cells (bottom) cultured with CD3-stimulation at day 35 or day 37.
- [0093] FIG. 18 is flow cytometry plots of marker expression of NTX4B3 cells cultured with CD3-
15 stimulation (top) or antigen beads (bottom) at day 35.
- [0094] FIG. 19 is graphs quantifying results of an *in vitro* serial restimulation assay of primary CD8+ CAR-T cells and iPSC-derived CD8+ CAR-T cells generated with antigen-coated beads (top) or CD3 stimulation (bottom). Normalized green area (vertical axis) represents the number of CD19+/+ or CD19-/- target cells for each culture condition.
- 20 [0095] FIG. 20A is a graph quantifying cumulative fold expansion of primary CD8+ CAR-T cells and iPSC-derived CD8+ CAR-T cells during an *in vitro* serial restimulation assay.
- [0096] FIG. 20B is a graph quantifying secretion of tumor necrosis factor α (TNF α) by primary CD8+ CAR-T cells and iPSC-derived CD8+ CAR-T cells following four rounds of stimulation in an *in vitro* serial restimulation assay.
- 25 [0097] FIG. 20C is a graph quantifying secretion of granzyme B by primary CD8+ CAR-T cells and iPSC-derived CD8+ CAR-T cells following four rounds of stimulation in an *in vitro* serial restimulation assay.
- [0098] FIG. 21 is a graph quantifying cumulative cytotoxicity and fold expansion of primary CD8+ CAR-T cells and iPSC-derived CD8+ CAR-T cells generated with antigen-coated beads ("3D Canonical CD19 Beads") or CD3 stimulation ("3D Canonical Immunocult") during an *in vitro* serial restimulation assay.
- 30 [0099] FIG. 22A is a graph quantifying the percentage of T cell memory cells (T stem cell memory (TSCM), CD62L+ CD45RA+ CD95+; T central memory (TCM), CD62L+ CD45RA- CD45RO+; T effector memory (TEM), CD62L- CD45RA- CD45RO+; and Terminally differentiated effector memory cells re-

expressing CD45RA (TEMRA), CD62L- CD45RA+ CD45RO+) for primary CD8+ CAR-T and iPSC-derived CD8+ CAR-T cell populations generated with antigen-coated beads ("CD19 beads") or CD3 stimulation ("Immunocult") before (left) and after (right) an *in vitro* serial restimulation assay.

[0100] FIG. 22B is a graph quantifying the percentage of TIM3+LAG3+, LAG3+, TIM3+, and TIM3-LAG3- cells for primary CD8+ CAR-T and iPSC-derived CD8+ CAR-T cell populations generated with antigen-coated beads ("CD19 beads") or CD3 stimulation ("Immunocult") before (left) and after (right) an *in vitro* serial restimulation assay.

[0101] FIG. 23A is a graph quantifying the percentage and RNA expression intensity of cell marker profiles for iPSC-derived progenitor T cells (ProT), iPSC-derived CD4+CD8+ double-positive cells (DP), and iPSC-derived CD8 SP (iPSC CD8-T) cells. Cell marker profiles corresponding to each cell type were annotated according to a reference dataset (Park et al., 2020): early DN cells, DN (early); proliferating DN cells, DN (P); quiescent DN cells, DN (Q); proliferating DP cells, DP (P); quiescent DP cells, DP (Q); CD8+ T cells, CD8-T; CD8 α α + cells, CD8- α α ; and CD8+ memory T cells, CD8-Tmem.

[0102] FIG. 23B is a Uniform Manifold Approximation and Projection (UMAP) plot of iPSC-derived progenitor T cells (ProT), iPSC-derived CD4+CD8+ double-positive cells (DP), and iPSC-derived CD4-CD8+ (iPSC CD8-T) cell populations.

[0103] FIG. 23C is a graph quantifying the percentage and expression intensity of genes as noted on the vertical axis for iPSC-derived progenitor T cells (ProT), iPSC-derived CD4+CD8+ cells (DP), and iPSC-derived CD4-CD8+ T cells (iPSC CD8-T).

[0104] FIG. 24 is flow cytometry plots of marker expression for iPSC-derived cells.

[0105] FIG. 25A is a graph quantifying RNA expression of genes as noted on the vertical axis for primary T cells.

[0106] FIG. 25B is a graph quantifying the percentage of primary CD8+ T cells corresponding to DN (early), DN (P), DN (Q), DP (P), DP (Q), $\gamma\delta$ T cell (GD T), and CD8-T cell RNA expression profiles, as annotated from a reference dataset (Park et al., 2020).

[0107] FIG. 26A is a graph quantifying RNA expression of genes as noted on the vertical axis for iPSC-derived CD8+ cells from 5 cell banks.

[0108] FIG. 26B is a graph quantifying the percentage of iPSC-derived CD8+ cells corresponding to DN (early), DN (P), DN (Q), DP (Q), and CD8-T cell RNA expression profiles, as annotated from a reference dataset (Park et al., 2020).

[0109] FIG. 27A is flow cytometry plots of marker expression for TCR-engineered iPSC-derived cells (clone TCR-172) at day 25.

- [0110] FIG. 27B is a graph quantifying marker expression for TCR-engineered iPSC-derived cells (clone TCR-172) at day 25.
- [0111] FIG. 27C is flow cytometry plots of marker expression for TCR-engineered iPSC-derived cells (clone TCR-172) at day 32.
- 5 [0112] FIG. 27D is a graph quantifying marker expression for TCR-engineered iPSC-derived cells (clone TCR-172) at day 32.
- [0113] FIG. 28A is flow cytometry plots of marker expression for TCR-engineered iPSC-derived cells (clone TCR-174) at day 25.
- [0114] FIG. 28B is a graph quantifying marker expression for TCR-engineered iPSC-derived cells (clone
10 TCR-174) at day 25.
- [0115] FIG. 28C is flow cytometry plots of marker expression for TCR-engineered iPSC-derived cells (clone TCR-174) at day 32.
- [0116] FIG. 28D is a graph quantifying marker expression for TCR-engineered iPSC-derived cells (clone TCR-174) at day 32.
- 15 [0117] FIG. 29A is flow cytometry plots of marker expression for post-expansion TCR-engineered iPSC-derived cells (clone TCR-172).
- [0118] FIG. 29B is flow cytometry plots of marker expression for post-expansion TCR-engineered iPSC-derived cells (clone TCR-172) gated on CD3+ cells as shown in FIG. 29A.
- [0119] FIG. 29C is a graph quantifying marker expression for post-expansion TCR-engineered iPSC-
20 derived cells (clone TCR-172).
- [0120] FIG. 30A is flow cytometry plots of marker expression for post-expansion TCR-engineered iPSC-derived cells (clone TCR-174).
- [0121] FIG. 30B is flow cytometry plots of marker expression for post-expansion TCR-engineered iPSC-derived cells (clone TCR-1724) gated on CD3+ cells as shown in FIG. 30A.
- 25 [0122] FIG. 30C is a graph quantifying marker expression for post-expansion TCR-engineered iPSC-derived cells (clone TCR-174).
- [0123] FIG. 31 is a graph quantifying *in vitro* cytotoxicity of TCR-engineered iPSC-derived cells (TCR-172 and TCR-174) and unmodified iPSC-derived cells (NTX4A1) at varying effector:target (E:T) ratios.
- [0124] FIG. 32A is a graph quantifying the percentage of DN, DP, CD4 ISP, and CD8 SP cell populations
30 for iPSC-derived cells cultured with 3D ETN and varying concentrations of a gamma secretase inhibitor (GSI) administered once ("1 GSI dose") during the culture period.

- [0125] FIG. 32B is a graph quantifying the percentage of DN, DP, CD4 ISP, and CD8 SP cell populations for iPSC-derived cells cultured with 3D ETN and varying concentrations of a gamma secretase inhibitor (GSI) administered three times ("3 GSI Doses") during the culture period.
- [0126] FIG. 33 is graphs quantifying the percentage of CD4+CD8+ DP cells (top), cell viability (bottom left) and fold expansion (bottom right) for iPSC-derived cells cultured with 3D ETN and varying concentrations of a gamma secretase inhibitor.
- [0127] FIG. 34 is flow cytometry plots depicting marker expression for iPSC-derived cells cultured with 3D ETN and a gamma secretase inhibitor (GSI) at varying concentrations: 0.1x bead dose, no GSI (top); 2x bead dose, 1 dose of 1 μ M GSI (middle); and 2x bead dose, 3 doses of 0.3 μ M GSI (bottom).
- [0128] FIG. 35 is graphs quantifying the percentage of CD4+CD8a+ DP cells following culture with the 2D or 3D ETN and varying concentrations of GSI for two cell lines: NTX4B3 (top) and NTX4A1 (bottom).
- [0129] FIG. 36 is graphs quantifying the percentage of CD4 ISP, DN, DP, and CD8a SP cell populations following 17 days of culture with the 3D ETN or 2D ETN and varying concentrations and doses of GSI, for two cell lines, NTX4B3 ("4B3") and NTX4A1 ("4A1"), as noted.
- [0130] FIG. 37A is a graph quantifying cell viability over time for three cell lines cultured with 3D ETN.
- [0131] FIG. 37B is a graph quantifying cumulative fold expansion for three cell lines as labelled in FIG. 37A.
- [0132] FIG. 38 is flow cytometry plots of marker expression for TCR-modified cells at day 28 of culture.
- [0133] FIG. 39A is a graph quantifying cell viability for TCR-modified cells under varying conditions at 0 and 7 days post-transition to SP cells.
- [0134] FIG. 39B is a graph quantifying fold expansion for TCR-modified cells under varying conditions at 0 and 7 days post-transition to SP cells.
- [0135] FIG. 40A is flow cytometry plots of marker expression for TCR-modified cells after culture with ImmunoCult™ CD3/CD28/CD2 stimulation ("1, IC 3/28/2") or ImmunoCult™ CD3/CD28 stimulation ("3, CD3/CD28").
- [0136] FIG. 40B is flow cytometry plots of marker expression for TCR-modified cells after culture with MAGE-A4 tetramer and soluble anti-CD28 ("2, MAGE-A4 Tet. + sol. CD28"), MAGE-A4 tetramer and soluble anti-41BB ("5, MAGE-A4 Tet. + sol. 41BB"), or MAGE-A4 tetramer alone ("4, MAGE-A4 Tet.").
- [0137] FIG. 40C is flow cytometry plots of marker expression for TCR-modified cells after culture with HIV-gag tetramer and soluble anti-CD28 ("6, HIV gag Tet. + sol. CD28"), HIV-gag tetramer and soluble anti-41BB ("7, HIV gag Tet. + sol. 41BB"), or HIV-gag tetramer alone ("8, HIV gag Tet.").

[0138] FIG. 41 is a graph quantifying expression of memory markers for TCR-modified cells after culture under varying conditions (1: ImmunoCult™ activator, anti-CD3/CD28/CD2; 2: MAGE-A4 tetramer and soluble anti-CD28; 3: ImmunoCult™ activator, anti-CD3/CD28; 4: MAGE-A4 tetramer alone; 5: MAGE-A4 tetramer and soluble anti-41BB; 6: HIV gag tetramer and soluble anti-CD28; 7: HIV gag tetramer and soluble anti-41BB; 8: HIV gag tetramer alone), as assessed by flow cytometry.

[0139] FIG. 42 is a graph quantifying expression of co-stimulatory ("Co-stim") markers for TCR-modified cells after culture under varying conditions (1: ImmunoCult™ activator, anti-CD3/CD28/CD2; 2: MAGE-A4 tetramer and soluble anti-CD28; 3: ImmunoCult™ activator, anti-CD3/CD28; 4: MAGE-A4 tetramer alone; 5: MAGE-A4 tetramer and soluble anti-41BB; 6: HIV gag tetramer and soluble anti-CD28; 7: HIV gag tetramer and soluble anti-41BB; 8: HIV gag tetramer alone), as assessed by flow cytometry.

[0140] FIG. 43A is a graph quantifying cell viability for TCR-modified cells after one round of expansion under varying conditions (1: ImmunoCult™ activator, anti-CD3/CD28/CD2; 2: MAGE-A4 tetramer and soluble anti-CD28; 3: ImmunoCult™ activator, anti-CD3/CD28; 4: MAGE-A4 tetramer alone; 5: MAGE-A4 tetramer and soluble anti-41BB; 6: HIV gag tetramer and soluble anti-CD28; 7: HIV gag tetramer and soluble anti-41BB; 8: HIV gag tetramer alone).

[0141] FIG. 43B is a graph quantifying fold expansion for TCR-modified cells after one round of expansion under varying conditions (1: ImmunoCult™ activator, anti-CD3/CD28/CD2; 2: MAGE-A4 tetramer and soluble anti-CD28; 3: ImmunoCult™ activator, anti-CD3/CD28; 4: MAGE-A4 tetramer alone; 5: MAGE-A4 tetramer and soluble anti-41BB; 6: HIV gag tetramer and soluble anti-CD28; 7: HIV gag tetramer and soluble anti-41BB; 8: HIV gag tetramer alone).

[0142] FIG. 44A is flow cytometry plots of marker expression for a TCR-modified cell line ("Clone 172") after culture under with ImmunoCult™ activator, CD3/CD28/CD2 stimulation ("1, IC 3/28/2") and expansion with the anti-CD3 antibody OKT3, the fibronectin fragment RetroNectin®, and either soluble 41BB antibody (Condition B) or soluble ICOS ligand (Condition C).

[0143] FIG. 44B is flow cytometry plots of marker expression for a TCR-modified cell line ("Clone 174") after culture with ImmunoCult™ activator, CD3/CD28/CD2 stimulation ("1, IC 3/28/2") and expansion with the anti-CD3 antibody OKT3, the fibronectin fragment RetroNectin®, and soluble CD28 antibody (Condition A), soluble 41BB antibody (Condition B) or soluble ICOS ligand (Condition C), or expansion with T Cell TransAct™ CD3/CD28 stimulation (Condition D).

[0144] FIG. 44C is flow cytometry plots of marker expression for a TCR-modified cell line ("Clone 172") after culture with MAGE-A4 tetramer and soluble anti-CD28 ("2, MAGE-A4 Tet. + sol. CD28") and

expansion with the anti-CD3 antibody OKT3, the fibronectin fragment RetroNectin®, and either soluble 41BB antibody (Condition B) or soluble ICOS ligand (Condition C).

[0145] FIG. 44D is flow cytometry plots of marker expression for a TCR-modified cell line (“Clone 174”) after culture with MAGE-A4 tetramer and soluble anti-CD28 (“2, MAGE-A4 Tet. + sol. CD28”) and expansion with the anti-CD3 antibody OKT3, the fibronectin fragment RetroNectin®, and soluble CD28 antibody (Condition A), soluble 41BB antibody (Condition B) or soluble ICOS ligand (Condition C), or expansion with T Cell TransAct™ CD3/CD28 stimulation (Condition D).

[0146] FIG. 45A is a graph quantifying expression of memory markers for a TCR-modified cell line (“Clone 172”) after culture under varying conditions, as assessed by flow cytometry.

[0147] FIG. 45B is a graph quantifying expression of memory markers for a TCR-modified cell line (“Clone 174”) after culture under varying conditions, as assessed by flow cytometry.

[0148] FIG. 46A is a schematic of generation and characterization of clonal iPSC lines with a MAGE-A4 TCR integrated at the TRAC locus.

[0149] FIG. 46B is a graph quantifying TCR expression at the end of clone production for 4 selected bi-allelic iPSC clones. Vector copy number (VCN) values on top of bar chart show genetic characterization of copy number integration at the TRAC locus.

[0150] FIG. 46C is scatter plots of TCR expression for unedited (left) and selected clone (174, right).

[0151] FIG. 46D is a micrograph of G-band karyotyping for clone 174.

[0152] FIG. 46E is a table summarizing results from an iCS-digital™ assay (Stem Genomics).

[0153] FIG. 47A is a schematic of *in vitro* T cell differentiation, divided into four stages. HPC, hematopoietic progenitor cell; Pro-T cell, progenitor T cell; DP, double-positive cell; SP, single-positive cell.

[0154] FIG. 47B is flow cytometry plots depicting marker expression for TCR-modified cells at day 0 of differentiation (Stage 1 of schematic in Fig. 47A).

[0155] FIG. 47C is flow cytometry plots depicting marker expression for TCR-modified cells at day 10 of differentiation (Stage 2 of schematic in Fig. 47A).

[0156] FIG. 47D is a flow cytometry plot depicting marker expression for TCR-modified cells at day 28 of differentiation (Stage 3 of schematic in Fig. 47A).

[0157] FIG. 47E is flow cytometry plots depicting marker expression for TCR-modified cells, sub-gated on CD4+CD8α+ cells, at day 28 of differentiation (Stage 3 of schematic in Fig. 47A).

- [0158] FIG. 47F is flow cytometry plots depicting marker expression for TCR-modified cells (top) and TCR-modified cells sub-gated on CD4+CD8 α + cells (bottom) at day 35 of differentiation (Stage 4 of schematic in Fig. 47A).
- 5 [0159] FIG. 47G is flow cytometry plots depicting marker expression for TCR-modified cells, sub-gated on CD4+CD8 α + cells, at day 35 of differentiation (Stage 4 of schematic in Fig. 47A).
- [0160] FIG. 47H is a heatmap quantifying expression of co-stimulatory markers for TCR-modified cells, sub-gated on CD4+CD8 α + cells, at day 35 of differentiation (Stage 4 of schematic in Fig. 47A).
- [0161] FIG. 48 is a graph quantifying MAGE-A4 TCR expression during T cell differentiation. Pro-T, progenitor T cell; DP, double-positive cell; SP, single-positive cell.
- 10 [0162] FIG. 49A is a schematic of a biphasic iPSC-T expansion culture protocol comprised of activation and maintenance phases. OKT3, anti-CD3 antibody OKT3; pb, plate-bound.
- [0163] FIG. 49B is a graph quantifying cell viability (left axis) and fold expansion (right axis) over the 7-day expansion culture protocol.
- [0164] FIG. 49C is flow cytometry plots depicting marker expression of TCR+ iPSC-derived cells at the end of the 7-day expansion culture protocol.
- 15 [0165] FIG. 49D is flow cytometry plots depicting marker expression of TCR+ iPSC-derived cells, sub-gated on CD8A+ cells, at the end of the 7-day expansion culture protocol.
- [0166] FIG. 50A is a schematic of an in vitro serial restimulation assay.
- [0167] FIG. 50B is graphs quantifying expression of inhibitory receptors (IRs) PD-1, TIGIT, LAG3, TIM3, and CD39 on iPSC-derived TCR+ CD8+ or primary TCR-transduced CD8+ effector cells following in vitro exposure to target cells at effector to target (E:T) ratios of 2:1, 1:1 and 0.5:1 (left panel) or at baseline or following one or three rounds of antigen exposure (right panel).
- 20 [0168] FIG. 50C is a graph quantifying tumor cell cytotoxicity for TCR-transduced primary CD8+ cells and iPSC-derived TCR+ CD8+ cells at varying E:T ratios. AUC, Area under the curve; ET50, the corresponding E:T ratio to reach a relative AUC of 50%.
- 25 [0169] FIG. 50D is a graph quantifying tumor cell cytotoxicity for TCR-transduced primary CD8+ cells and iPSC-derived TCR+ CD8+ cells over four rounds of antigen exposure at a 2:1 E:T ratio.
- [0170] FIG. 50E is a graph quantifying tumor cell cytotoxicity over time for TCR-transduced primary CD8+ cells and iPSC-derived TCR+ CD8+ cells co-cultured with antigen-negative or antigen-positive tumor target cells.
- 30 [0171] FIG. 51A is a Uniform Manifold Approximation and projection (UMAP) plot based on CITEseq data (mRNA + surface protein at single cell resolution) for various cell populations as noted. PBMC-CD8T,

peripheral blood-derived CD8+ T cells; PBMC-CD4-T, peripheral blood-derived CD4+ T cells; PBMC-T-Activated, activated peripheral blood-derived T cells; PBMC-NK, peripheral blood-derived natural killer cells; iPS-TCR, iPSC-derived TCR+ CD8+ cells.

[0172] FIG. 51B is a graph quantifying the percentage of iPSC-derived TCR+ CD8+ cells (iPS-TCR) and activated primary T cells (PBMC-T-Activated) corresponding to proliferating double-negative cells (DN (P)), CD8+ T cells (CD8+ T), NK-T cells (NKT), and regulatory T cells (Treg) RNA expression profiles, as annotated from a reference dataset (Park et al., 2020).

[0173] FIG. 51C is a bubble plot quantifying RNA expression of genes as noted on the vertical axis for the cell populations in FIG. 51A.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0174] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs.

[0175] Generally, the present disclosure provides methods of generating CD4-CD8+ and CD4+CD8+ cell populations from stem/progenitor cells, CD4-CD8+ and CD4+CD8+ cell populations generated by the methods disclosed herein; a pharmaceutical composition comprising a CD4-CD8+ cell population generated by the methods disclosed herein; and a use of a CD4-CD8+ cell population in the manufacture of a medicament for the treatment of a disease or condition. Previously, there were no reports of a method of controlling *in vitro* emergence of mature T cell lineage populations via temporal control of Notch signalling and T cell activation. There were also no reports of the unique phenotype of cells that would emerge from such a process.

Definitions

[0176] As used herein, the term “stem cell” refers to a cell that can differentiate into more specialized cells and has the capacity for self-renewal. Stem cells include pluripotent stem cells (PSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), and multipotent stem cells, such as mobilized peripheral blood-derived CD34+ stem cells, umbilical cord blood stem cells, and adult stem cells, which are found in various tissues. Methods for obtaining, deriving or producing stem cells are known in the art.

[0177] As used herein, the term “progenitor cell” refers to a cell that can differentiate into one or more types of cells, but typically has a limited capacity for self-renewal. Progenitor cells are derivatives of stem cells and have more limited potency relative to their corresponding source stem cells. For example,

hematopoietic stem cells (HSCs), found in adult bone marrow, peripheral blood (in smaller numbers) and in umbilical cord blood, have the capacity to give rise to all other blood cells. Hematopoietic progenitor cells (HPCs) are multipotent or lineage-committed cells derived from HSCs that have the capacity to give rise to a more limited or specific type of blood cell lineages. Hematopoietic stem/progenitor cells (HSPCs) typically exist as a heterogeneous population *in vivo* and have use as a heterogeneous population as described herein. HPCs and HSPCs may be characterized by expression of one or more of CD34, CD43, CD31 and CD45.

[0178] As used herein, the terms “progenitor T cell” and “proT cell” refer to a cell that is derived from a pluripotent stem cell or a CD34+ hematopoietic stem and/or progenitor cell and expresses at least CD7+, and has the capacity to differentiate into one or more types of immature and mature T cells. Examples of progenitor T cells include, but are not limited to, CD7+ cells, CD7+CD5+ cells, CD7+CD5+CD34+ cells, CD7+CD5+CD45RA+ cells, and/or CD7+CD5+CD1a+ cells.

[0179] As used herein, an “immature T cell” or mature T cell is a T lineage cell derived from a progenitor T cell. T cell development may be characterized by the progressive expression of cell surface receptors, particularly CD4 and CD8. *In vivo*, T lineage cells progress from progenitor T cells through CD4-CD8- (double-negative, DN), CD4+CD8- (CD4 immature single-positive, CD4 ISP), early CD4+CD8+ (double-positive, DP), late DPs, and CD4-CD8+ (CD8 single-positive, CD8 SP) and CD4 single-positive (CD4 SP) stages. Late DPs are characterized by the presence of CD4+/CD8A+/CD8B+/CD3+ and TCRαβ+. The TCRγδ fate is not open at the late DP stage, the cell size decreases, and the cells are metabolically quiescent. In CD8 SPs, CD8 may be expressed as a heterodimer of CD8α and CD8β, resulting in CD8αβ+ cells, or as CD8αα homodimer, resulting in CD8αα+ cells. CD4-CD8+ T cells may also be characterized by cell-surface expression of CD3 and one of TCRγδ (γδ T cells) or TCRαβ (αβ T cells).

[0180] As used herein, “serum-free medium” refers to a cell culture medium that lacks animal serum. Serum-free medium may include specific, known serum components isolated from an animal (including human animals), such as, for example, bovine serum albumin (BSA).

[0181] As used herein, a “Notch signalling ligand” refers to any ligand capable of interacting with a Notch protein receptor for regulation of T cell lineage commitment and differentiation. Examples of Notch signalling ligand include, Delta-like 4 (DL4), Delta-like-1 (DL1), Delta-like 3 (DL3), Jagged1 and Jagged2.

[0182] As used herein, Notch signalling ligand, for example, “Delta-like-4” and “DL4” refer to a protein that in humans is encoded by the *DLL4* gene. DL4 is a member of the Notch signalling pathway and is also referred to in the art as “Delta like ligand 4” and “DLL4”. Herein, reference to DL4 is not limited to

the entire DL4 protein, but includes at least the signalling peptide portion of DL4. For example, a commercially available product (Sino Biologicals) comprising the extracellular domain (Met 1-Pro 524) of human DL4 (full-length DL4 accession number NP 061947.1; SEQ ID NO: 1) fused to the Fc region of human IgG1 at the C-terminus is a DL4 protein suitable for use in the methods provided herein.

5 **[0183]** As used herein, Notch signalling ligand also includes a variant of a known Notch signalling ligand, for example, DL4. A variant Notch signalling ligand refers to a protein molecule which differs in amino acid sequence from the wild-type amino acid sequence by one or more additions, deletions, and/or substitutions and retains the desired Notch signalling activity of the wild-type DL4. Also included within the definition are variants such as polypeptides, oligopeptides, peptides and proteins having amino acid
10 sequence identity to a given polypeptide, oligopeptide, peptide or protein. The percent identity can be, for example, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid identity to the given polypeptide, oligopeptide, peptide or protein over a specified length, for example, over the full length of the polypeptide.

[0184] As used herein, "Vascular cell adhesion molecule 1" and "VCAM-1" refer to a protein that in
15 humans is encoded by the *VCAM1* gene. VCAM-1 is a cell surface sialoglycoprotein, a type I membrane protein that is a member of the Ig superfamily. VCAM-1 is also referred to in the art as "vascular cell adhesion protein 1 and cluster of differentiation 106" (CD106). Herein, reference to VCAM-1 is not limited to the entire VCAM-1 protein, but includes at least the signalling peptide portion of VCAM-1 (QIDSPL (SEQ ID NO: 2) or TQIDSPLN (SEQ ID NO: 3)). For example, a commercially available mouse
20 VCAM-1-Fc chimeric protein (R&D) that comprises (Phe25-Glu698) region of mouse VCAM-1 (full-length murine VCAM-1 accession number CAA47989; SEQ ID NO: 4) fused with the Fc region of human IgG1 is a VCAM-1 protein suitable for use herein. Use of at least a portion of human VCAM-1 (full-length human VCAM-1 accession number P19320, NP001069, EAW72950; SEQ ID NO: 5) may also be suitable for use in the methods provided herein. Herein, reference to VCAM-1 also includes a variant, which differs in
25 amino acid sequence from the wild-type amino acid sequence of VCAM-1 by one or more additions, deletions, and/or substitutions and retains the desired activity of the wild-type VCAM-1. Also included within the definition are variants such as polypeptides, oligopeptides, peptides and proteins having amino acid sequence identity to a given polypeptide, oligopeptide, peptide or protein. The percent identity can be, for example, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%,
30 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid identity to the given polypeptide, oligopeptide, peptide or protein over a specified length, for example, over the full length of the

polypeptide. VCAM-1 has been shown to synergistically increase Notch signalling in combination with DL4 (e.g., Shukla et al., 2017).

[0185] As used herein, “integrin ligand” refers to a peptide or protein capable of binding to an integrin such as, for example, the VLA-1-VLA-5 family of integrins. Examples of integrin ligands are known in the art and include, for example, fibronectin, the fibronectin fragment RetroNectin®, VCAM, RGD peptide, laminins, including laminin-211, laminin-511, and laminin-332, intercellular adhesion molecules (ICAMs, including ICAM-1), and inducible costimulator-ligand (ICOS-L).

[0186] As used herein, “two-dimensional engineered thymic niche (2D ETN)” refers to a two-dimensional substrate immobilized with a Notch signalling ligand, for example, DL4, and optionally VCAM-1. A two-dimensional (2D) substrate can include, for example, a tissue culture plate. Methods of immobilizing Notch signalling ligands on a 2D substrate are known in the art and are described, for example in Shukla et al., 2017.

[0187] As used herein, “three-dimensional engineered thymic niche (3D ETN)” or “ETN bead” refers to a three-dimensional substrate immobilized with a Notch signalling ligand, for example, DL4 and optionally VCAM-1.

[0188] As used herein, “antigen bead”, “antigen particle”, “antigen-coated bead” or “antigen-coated particle” refers to an antigen immobilized on a substrate, for example, on a three-dimensional substrate such as a particle or bead. The antigen may be immobilized on a substrate through covalent or non-covalent interactions, affinity-based interactions, or other suitable forms of interactions. For example, the antigen may be a CD19 antigen (“CD19-coated bead”, “CD19 bead”).

[0189] A three-dimensional (3D) substrate can include, for example, micron-size particles (or beads), with or without a magnetic core, coated with one or more full proteins, protein domains (e.g., extracellular, intracellular, or other domain), peptides or protein fragments. Several approaches can be used individually or in combination to produce protein coated particles, e.g.: physisorption driven by protein affinity to the particle material, chemical conjugation by reaction with, among others, amine, carboxyl, thiol, epoxy, azide reactive groups, or by coating an appropriate ligand to capture the protein of interest by affinity. Examples of affinity tags include but are not limited to: polyhistidine (His), Fc, biotin, Halo, aldehyde, Snap, Spy-Catcher, VIPER. Particles or beads may be composed of, for example, polystyrene, iron oxide, polystyrene and magnetizable iron oxide (magnetic polystyrene), gold, or other suitable materials known in the art. ETN and antigen beads may be used for culturing cells on a tissue culture plate, flasks, or other vessels utilized for culturing cells.

[0190] As used herein, “immobilized” or “surface-bound” refers to a ligand, such as a Notch signalling ligand or integrin ligand, antigen, peptide, or protein binding to a substrate through covalent or non-covalent interactions, affinity-based interactions, or other suitable forms of interactions.

[0191] As used herein, “T cell activator” refers to a reagent that activates a T cell or T lineage cell.

5 Methods and reagents for T cell activation are known in the art and include, for example, antibody-based stimulation of CD3, CD3 and CD28, or CD3, CD28 and CD2 in a cell, or stimulation of a T cell receptor or chimeric antigen receptor (“CAR activator”) through a peptide-major Histocompatibility complex (pMHC)-tetramer, antibody, or antigen presentation.

[0192] As used herein, “CD3 stimulator” refers to a reagent that stimulates a cell through binding or
10 activation of CD3 in the cell. Methods and reagents for CD3 stimulation are known in the art and include, for example, antibody-based stimulation of CD3, CD3 and CD28, or CD3, CD28 and CD2 in a cell.

[0193] As used herein, a “Notch signalling inhibitor” refers to a reagent that inhibits Notch signalling in a cell. The Notch signalling inhibitor may be a small molecule inhibitor. One type of Notch signalling inhibitor is a gamma-secretase inhibitor (γ -secretase inhibitor, GSI), such as N-[N-(3, 5-
15 difluorophenacetyl)-l-alanyl]-s-phenylglycine t-butyl ester (DAPT). Additional reagents for inhibiting Notch signalling include an antibody directed against the Notch1 receptor and soluble (i.e., non-immobilized) DL4.

[0194] As used herein, “enriched” cell population refers to when the cell population comprising one or more cell phenotypes (for example, CD4-CD8+ (CD8SP), CD4+CD8+ (DP), CD4+CD8- (CD4 ISP), CD4-CD8-
20 (DN)) exhibits a higher absolute number or the ratio of one of the cell phenotypes (for example, CD4-CD8+ (CD8SP)) compared to other cell phenotypes, where at least 25% of the cell population is comprised of a single cell phenotype.

[0195] As used herein, the term “subject” refers to a vertebrate, preferably a mammal (e.g., a non-human mammal), more preferably a primate and still more preferably a human. Mammals include, but
25 are not limited to, primates, humans, farm animals, sport animals, and pets.

[0196] As used herein, the term “treatment”, “treat” or “treating” is an approach for obtaining beneficial or desired clinical results. For purposes of this disclosure, beneficial or desired clinical results include, but are not limited to, one or more of the following: increased immune response, increased T cell response, decreased extent of damage from a disease, condition, or disorder, decreased duration of
30 a disease, condition, or disorder, and/or reduction in the number, extent, or duration of symptoms related to a disease, condition, or disorder. The term includes the administration of the compounds, agents, drugs or pharmaceutical compositions of the present disclosure to prevent or delay the onset of

one or more symptoms, complications, or biochemical indicia of a disease or condition; lessening or improving one or more symptoms; shortening or reduction in duration of a symptom; or arresting or inhibiting further development of a disease, condition, or disorder. Treatment may be prophylactic (to prevent or delay the onset of a disease, condition, or disorder, or to prevent the manifestation of clinical or subclinical symptoms thereof) or therapeutic suppression or alleviation of symptoms after the manifestation of the disease, condition, or disorder. The beneficial or desired clinical result may be an increase or decrease (as appropriate) of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% relative to an appropriate control, for example, a subject that did not receive the therapy.

[0197] The term “administering” or “administration” as used herein refers to the placement of an agent, a drug, a compound, or a pharmaceutical composition as disclosed herein into a subject by a method or route which results in at least partial delivery of the composition to a desired site. The compounds and pharmaceutical compositions disclosed herein can be administered by any appropriate route which results in an effective treatment in the subject. Routes of administration of the compounds and pharmaceutical compositions disclosed herein include, but are not limited to, intravenous, or intraperitoneal routes of administration, or a combination thereof.

[0198] The term “effective amount” or “therapeutically effective amount”, for example an effective amount or therapeutically effective amount of a T cell lineage population as used herein is an amount sufficient to bring about any one or more beneficial or desired results. In more specific aspects, an effective amount may alleviate or ameliorate one or more symptoms of a disease; decrease the duration of time that one or more symptoms of a disease, are present in a subject; increase the survival rate of a subject having a disease. For prophylactic use, beneficial or desired results may include eliminating or reducing the risk, lessening the severity, or delaying the onset of a disease, including biochemical and/or histological symptoms of the infection, its complications and intermediate pathological phenotypes presenting during development of the disease. For therapeutic use, beneficial or desired results may include clinical results such as reducing one or more symptoms of a disease; decreasing the dose or length of administration of other medications required to treat the disease; enhancing the effect and/or reducing the toxicity of another medication; delaying the progression of the disease in a subject, decreasing the duration of time that one or more symptoms of a disease, are present in a subject, and /or increasing the overall survival rate of a subject having a disease. An effective amount can be administered in one or more than one dose, round of administration, or course of treatment.

[0199] For purposes of this disclosure, an effective dosage of a cell population or a pharmaceutical composition is an amount sufficient to accomplish prophylactic or therapeutic treatment either directly or indirectly. As is understood in the clinical context, an effective dosage of a compound, or a pharmaceutical composition may or may not be achieved in conjunction with another agent, drug, compound, or pharmaceutical composition. Thus, an “effective dosage” may be considered in the context of administering one or more therapeutic agents, and a single agent may be considered to be given in an effective amount if, in conjunction with one or more other agents, a desirable result may be or is achieved. The amount may vary from one subject to another and may depend upon one or more factors, such as, for example, subject gender, age, body weight, subject’s health history, and/or the underlying cause of the disease, condition, or disorder to be prevented, inhibited and/or treated.

[0200] The term “pharmaceutically acceptable carrier, diluent, or excipient” as used herein includes any material which, when combined with an active ingredient, allows the ingredient to retain biological activity and is non-reactive with the subject’s immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. In some embodiments, diluents for aerosol or parenteral administration are phosphate buffered saline (PBS) or normal (0.9%) saline. Compositions comprising such carriers are formulated by well-known conventional methods (see, for example, Remington’s Pharmaceutical Sciences, 18th edition, A. Gennaro, ed., Mack Publishing Co., Easton, PA, 1990; and Remington, The Science and Practice of Pharmacy 20th Ed. Mack Publishing, 2000).

[0201] As used herein, the singular forms “a,” “an,” and “the,” are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0202] The phrase “and/or” should be understood to mean “either or both” of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Thus, as a non-limiting example, a reference to “A and/or B”, when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

[0203] As used herein, the phrase “one or more,” in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This

definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “one or more” refers, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, “one or more of A and B” (or, equivalently, “one or more of A or B,” or, equivalently “one or more of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); etc.

[0204] When the term “about” is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below those numerical values. In general, the term “about” is used herein to modify a numerical value above and below the stated value by a variance of 20%, 10%, 5%, or 1%. In certain embodiments, the term “about” is used to modify a numerical value above and below the stated value by a variance of 10%. In certain embodiments, the term “about” is used to modify a numerical value above and below the stated value by a variance of 5%. In certain embodiments, the term “about” is used to modify a numerical value above and below the stated value by a variance of 1%.

[0205] When a range of values is listed herein, it is intended to encompass each value and sub-range within that range. For example, “1-5 mL” is intended to encompass 1 mL, 2 mL, 3 mL, 4 mL, 5 mL, 1-2 mL, 1-3 mL, 1-4 mL, 1-5 mL, 2-3 mL, 2-4 mL, 2-5 mL, 3-4 mL, 3-5 mL, and 4-5 mL.

[0206] It will be further understood that the terms “comprises,” “comprising,” “includes,” and/or “including,” when used herein, specify the presence of stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof.

[0207] The term “consisting of” and its derivatives, as used herein, are intended to be closed terms that specify the presence of stated features, integers, steps, operations, elements, and/or components, and exclude the presence or addition of one or more other features, integers, steps, operations, elements and/or components.

General techniques

[0208] Unless otherwise defined herein, scientific, and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Generally, nomenclatures used in connection with, and techniques of, cell and tissue culture,

molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art.

[0209] The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, 5 biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook et al., 1989) Cold Spring Harbor Press; Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J.E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R.I. Freshney, ed., 1987); Introduction to Cell and Tissue Culture (J.P. Mather and 10 P.E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-1998) J. Wiley and Sons; Methods in Enzymology (Academic Press, Inc.); Handbook of Experimental Immunology (D.M. Weir and C.C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Cales, eds., 1987); Current Protocols in Molecular Biology (F.M. Ausubel et al., eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Current 15 Protocols in Immunology (J.E. Coligan et al., eds., 1991); Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (2001); Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, NY (2002); Harlow and Lane Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1998); Coligan et al., Short Protocols in Protein Science, John Wiley & Sons, NY (2003); Short Protocols 20 in Molecular Biology (Wiley and Sons, 1999); and Immunobiology (C.A. Janeway and P. Travers, 1997).

Hematopoietic stem/progenitor cell populations

[0210] Generally, the *in vitro* methods of generating CD4+CD8+ and CD4-CD8+ cell populations provided herein involve culturing hematopoietic stem/progenitor cells in the presence of Notch signalling ligands under conditions and for a time suitable for differentiation into T cell lineage 25 populations.

[0211] Hematopoietic stem/progenitor cells (HSPCs) typically exist as a heterogeneous population *in vivo* and have use as a heterogeneous population as described herein. HPCs and HSPCs may be characterized by expression of one or more of CD34, CD43, CD31 and CD45.

[0212] In an embodiment, the HSPCs may be obtained from cord blood, peripheral blood or bone 30 marrow or they may be derived *in vitro* from pluripotent stem cells such as embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) or other intermediate stem cells. In a preferred embodiment,

the stem and/or progenitor cells are human cells. In an embodiment, the stem cells are mobilized peripheral blood-derived CD34+ cells. In a preferred embodiment, the HSPCs are derived *in vitro* from iPSCs. Methods of generating HSPCs from iPSCs are known in the art, for example, differentiation with appropriate medium conditions (e.g., Trotman-Grant et al., 2021).

- 5 [0213] Suitable techniques for analyzing cell surface markers are known to those of ordinary skill in the art, and may include, for example, flow cytometry as used herein, or immunocytochemistry. The number of cells and viability of the cells may be analyzed by techniques well known to those of ordinary skill in the art, and may include, for example use of an automated cell counter as disclosed herein.

Cell culture systems

- 10 [0214] Cells may be cultured in types of cell culture systems known in the art, for example, cell culture plates, culture dishes, and bioreactors including stirred-tank reactors (STRs), rocking bag bioreactors, and other suitable cell culture formats. Cell culture may be carried out under static conditions, dynamic or agitated conditions, or a combination of static and dynamic conditions. Bioreactors can be any type of bioreactor known in the art and can use any type of processing/culturing conditions and methods,
15 including, for example, batch processes, fed-batch processes, and perfusion culturing methods and conditions.

Notch ligand substrates

- [0215] In an embodiment, cells are cultured in a two-dimensional culture system utilizing a suitable 2D substrate, which can include, for example, a standard culture plate coated with Notch signalling ligands,
20 for example, DL4. The culture plate may also be coated with VCAM-1.

[0216] In an embodiment, cells are cultured in a three-dimensional culture system utilizing a suitable 3D substrate, which can include, for example, micron-size particles (or beads), with or without a magnetic core, coated with one or more full proteins, protein domains (e.g. extracellular, intracellular, or other domain), peptides or protein fragments to activate Notch signalling.

- 25 [0217] In one example, the Notch signalling ligand, for example, DL4, alone or in combination with VCAM-1, may be conjugated to polystyrene microbeads as described in Trotman-Grant et al., 2021, and WO2019157597.

- [0218] In another example, 3D ETN beads can be manufactured by affinity capturing DL4 and VCAM-1 carrying the appropriate affinity tag on streptavidin or protein G coated beads, where beads are diluted
30 to 0.1% solids in Dulbecco's phosphate-buffered saline (DPBS) without Ca²⁺ or Mg²⁺, supplemented with 0.05% BSA, and incubated with the protein solution (0.1x-20x protein molar excess) for 60 min at room temperature with continuous stirring. At the end of the incubation period, excess free protein is

removed by magnetic separation followed by a buffer exchange. The procedure is repeated an additional four times, after which 3D ETN beads are concentrated ten-fold for storage.

[0219] Quantification of protein immobilization may be carried out according to methods known in the art such as, for example, the colorimetric bicinchoninic acid (BCA) assay, an immunofluorescence assay, or other known detection methods.

T Cell Therapies

[0220] T cells have a broad range of therapeutic applications. T cells may be modified by, for example, conventional gene editing approaches such as nuclease editing or viral vector transduction, to express a chimeric antigen receptor (CAR), and/or an exogenous T Cell Receptor (TCR), to generate engineered T cell therapies (Weber et al., 2020). T cells derived from progenitor cells, including pluripotent stem cells, may be genetically engineered at the pluripotent or progenitor cell stage. Engineered T cell therapies have applicability in, for example, oncology and autoimmune disorders. In oncology, engineered T cell therapies have applicability in, for example, hematologic cancers, such as B cell lymphoma, B cell acute lymphoblastic leukemia and other B cell malignancies, multiple myeloma, and other hematologic cancers, as well as in solid tumors such as, for example, mesothelioma, adenocarcinomas, gliomas, and sarcomas (Weber et al., 2020). In autoimmune disorders, engineered T cell therapies have applicability in, for example, Type I diabetes, rheumatoid arthritis, multiple sclerosis, and other autoimmune disorders or conditions (Weber et al., 2020).

[0221] Engineered T cell therapies may target antigens known to be expressed on target cell types, including tumor cells or within tumor tissues. Chimeric antigen receptors (CARs) may be designed to target surface antigens or multivalent soluble antigens. The targeting ectodomain of the CAR may be a single-chain variable fragment (scFv), single-domain antibodies (single variable domain on a heavy chain, VHH), nanoantibodies, or other antigen-binding domain (Qu et al., 2022). CAR-T cell therapies may be directed towards multiple antigens using varying CAR designs or multiple CARs (Qu et al. 2022).

Exemplary oncology antigens and corresponding cancer types for CAR-T cell therapies are listed in Table 1 below (Qu et al., 2022; Guha et al., 2022; Drougkas et al., 2023; Want et al., 2023).

Table 1	
Antigen/Antigen Type	Cancer Type
5 T4 (TPBG)	ovarian cancer
AE1/2	gastric cancer
AFP	hepatocellular carcinoma (HCC)
Alpha folate receptor	serous ovarian, Fallopian tube, or primary peritoneal cancer (epithelial cancer)

ALPPL2 (PLAP-like)	ovarian cancer
ANXA2 (ANX2L4, CAL1H, LIP2, LPC2, LPC2D, P36)	ovarian cancer
ARP2/3 (p41-ARC)	gastric cancer
AXL (UFO, ARK)	breast cancer, glioma, lung cancer
B7H3 (CD276)	colorectal cancer, esophageal cancer, Ewing's sarcoma, gastric cancer, glioma, hepatocellular carcinoma (HCC), lung cancer, melanoma, pancreatic cancer, prostate cancer, osteosarcoma, ovarian cancer
B7H6	gastric cancer
BAFF-R (TNFRSF13C, CD268)	B-acute lymphoblastic leukemia (B-ALL)
BCMA (TNFRSF17, CD269)	chronic lymphocytic leukemia (CLL), multiple myeloma (MM)
BRCA	serous ovarian, Fallopian tube, or primary peritoneal cancer (epithelial cancer)
BT-001	solid tumor
CA19-9 (sialyl-Lewis)	gastric cancer
CAIX	renal cell carcinoma (RCC)
CD117 (c-kit, KIT)	acute myeloid leukemia (AML), Ewing's sarcoma, osteosarcoma
CD123 (IL3RA)	acute myeloid leukemia (AML), B-acute lymphoblastic leukemia (B-ALL)
CD126 (IL6R, gp80)	melanoma, multiple myeloma (MM), prostate cancer
CD133 (Prominin-1, PROM1, AC133)	breast cancer, colorectal cancer, Ewing's sarcoma, gastric cancer, glioma, hepatocellular carcinoma (HCC), lung cancer, osteosarcoma, ovarian cancer, pancreatic cancer
CD138 (Syndecan, SDC)	multiple myeloma (MM)
CD147 (BSG)	glioma, hepatocellular carcinoma (HCC), lung cancer
CD16 (FCG3, FCGR3, IGF3R3, IMD20)	melanoma
CD166 (ALCAM)	Ewing's sarcoma, osteosarcoma
CD171	neuroblastoma
CD177 (NB1 GP, PRV-1)	Ewing's sarcoma, osteosarcoma
CD19 (B lymphocyte surface antigen B4, T cell surface antigen LEU-12)	B-acute lymphoblastic leukemia (B-ALL), B-cell non-Hodgkin's lymphoma (B-NHL), Hodgkin's lymphoma (HL), chronic lymphocytic leukemia (CLL), multiple myeloma (MM)
CD20 (B Lymphocyte surface antigens B1, Bp35, leU-16, MS4A1)	B-acute lymphoblastic leukemia (B-ALL), B-cell non-Hodgkin's lymphoma (B-NHL), chronic lymphocytic leukemia (CLL), Mantle cell lymphoma (MCL), melanoma
CD20/CD22	Relapsed or refractory lymphoid cancers
CD22 (Siglec-2, BL-CAM, T cell surface antigen LEU-14)	B-acute lymphoblastic leukemia (B-ALL), B-cell non-Hodgkin's lymphoma (B-NHL), T-cell non-Hodgkin's lymphoma (T-NHL)
CD23 (Low affinity immunoglobulin, εFc receptor, BLAST-2, FcεRII, FCER2, CLEC4J)	chronic lymphocytic leukemia (CLL)

CD30 (TNF receptor superfamily member 8, TNFRSF8, Ki-1 antigen)	Hodgkin's lymphoma (HL), T-cell non-Hodgkin's lymphoma (T-NHL)
CD318 (CDCP 1)	pancreatic cancer
CD32b (FcγRII, FCGR2A, FCGR2B)	chronic lymphocytic leukemia (CLL)
CD33 (Siglec-3)	acute myeloid leukemia (AML), myeloid malignancies
CD38 (ADP ribocyclase 1)	acute myeloid leukemia (AML), B-acute lymphoblastic leukemia (B-ALL), multiple myeloma (MM)
CD4 (OKT4)	T-cell non-Hodgkin's lymphoma (T-NHL)
CD44 (Hermes, Pgp1, H-CAM, Hutch)	hepatocellular carcinoma (HCC), ovarian cancer
CD44v6 (Epican, HUTCH-I, LHR, ECMR-III)	acute myeloid leukemia (AML), breast cancer, gastric cancer, head and neck squamous cell carcinoma (HNSCC), lung cancer, multiple myeloma (MM)
CD47 (MER6, IAP)	lung cancer, ovarian cancer
CD5 (LEU1)	T-acute lymphoblastic leukemia (T-ALL), T-cell non-Hodgkin's lymphoma (T-NHL)
CD56 (NCAM1)	multiple myeloma (MM)
CD7 (LEU 9, GP40, TP41)	acute myeloid leukemia (AML), T-acute lymphoblastic leukemia (T-ALL), T-cell non-Hodgkin's lymphoma (T-NHL)
CD70 (CD27L)	acute myeloid leukemia (AML), breast cancer, esophageal cancer, head and neck squamous cell carcinoma (HNSCC), melanoma, ovarian cancer, pancreatic cancer, renal cell carcinoma (RCC)
CD72	B-acute lymphoblastic leukemia (B-ALL)
CDH17	colorectal cancer, gastric cancer
CDH6	ovarian cancer
CEA (CEACAM5)	breast cancer, colorectal cancer, metastatic colorectal cancer, gastric cancer, hepatocellular carcinoma (HCC), lung cancer, pancreatic cancer / pancreatic-biliary tract cancer, peritoneal cancer, prostate cancer
CLDN18.2 (CLDN18, Claudin 18, SFTA5)	esophageal cancer, gastric cancer, lung cancer, pancreatic / pancreatic-biliary tract cancer
CLDN6 (Skullin)	ovarian cancer, testicular cancer
CLEC14A (EGFR5)	lung cancer
CLL1 (CLEC12A)	acute myeloid leukemia (AML)
CLTX	glioma
c-MET (HGFR)	breast cancer, colorectal cancer, gastric cancer, glioma, hepatocellular carcinoma (HCC), lung cancer
CSPG4 (NG2, MCSP, MCSPG, MSK16, HMW-MAA, MEL-CSPG)	breast cancer, glioma, head and neck squamous cell carcinoma (HNSCC), melanoma
CXCR1	ovarian cancer
CXCR4 (CD184, Fusin, NPYR, HSY3RR, LAP3, LCR1)	lung cancer
CXCR5 (CD185)	B-cell non-Hodgkin's lymphoma (B-NHL)
DLL3 (SCDO1)	lung cancer

DR5	hepatocellular carcinoma (HCC)
DSC2	gastric cancer
EGFR and CD133	pancreatic cancer / pancreatic-biliary tract cancer
EGFR(ERBB1)	breast cancer, colorectal cancer, Ewing's sarcoma, gastric cancer, head and neck squamous cell carcinoma (HNSCC), lung cancer (malignant pleural mesothelioma), osteosarcoma, pancreatic cancer / pancreatic-biliary tract cancer
EGFR806	central nervous system tumor, pediatric glioma
EGFRIII	glioblastoma, gliosarcoma and brain tumor
EGFRVIII	glioblastoma, glioma, hepatocellular carcinoma (HCC), pancreatic cancer
EpCAM (CD326, TACSTD1)	breast cancer, colorectal cancer, gastric cancer, hepatocellular carcinoma (HCC), pancreatic cancer, prostate cancer
EphA10	breast cancer
EphA2 (ECK, CTPA, ARCC2, CTPP1, CTRCT6)	Ewing's sarcoma, glioma, lung cancer, osteosarcoma
FAP (SIMP, DPPIV, FAPA)	glioma, lung cancer (malignant pleural mesothelioma), pancreatic cancer
FcμR	chronic lymphocytic leukemia (CLL)
FLT3 (CD135, FLK2)	acute myeloid leukemia (AML)
Fn14	glioma
FR-a	ovarian cancer
FRα (FOLR1)	breast cancer, gastric cancer, lung cancer, ovarian cancer
FRβ	acute myeloid leukemia (AML), lung cancer
GD2	breast cancer, Ewing's sarcoma, glioma, lung cancer, melanoma, osteosarcoma
GD3	melanoma
GFRα4 (GDNF family receptor alpha-4)	thyroid cancer
Glypican-3	liver cancer
gp100	breast cancer, hepatocellular carcinoma (HCC), melanoma
GPC3 (DGSX, OCI5, SDYS, SGB, SGBS1, MXR7)	hepatocellular carcinoma (HCC), lung cancer, renal cell carcinoma (RCC)
GPRC5D	multiple myeloma (MM)
GUCY2C (GUC2C, STAR)	colorectal cancer, pancreatic cancer
HER2 (ERBB2)	breast cancer, colorectal cancer, esophageal cancer, Ewing's sarcoma, gastric cancer, glioblastoma, glioma / pediatric glioma, head and neck squamous cell carcinoma (HNSCC), hepatocellular carcinoma (HCC), melanoma, lung cancer, osteosarcoma, ovarian cancer, pancreatic cancer / pancreatic-biliary tract cancer
HLA-A2	ependymoma
ICAM-1 (CD54)	breast cancer, , gastric cancer, thyroid cancer
IGF1R (CD221, JTK13, MGC18216)	Ewing's sarcoma osteosarcoma
IL13Rα2 (CD213A2, IL13BP, CT19)	glioblastoma, glioma, melanoma
IM83	liver cancer

KK-LC-1	serous ovarian, Fallopian tube, or primary peritoneal cancer (epithelial cancer)
L1-CAM (CD171, HSAS, MASA)	ovarian cancer
LewisY	acute myeloid leukemia (AML), lung cancer, multiple myeloma (MM)
LILRB4 (CD85, LILRB3, ILT5, LIR3)	acute myeloid leukemia (AML)
LMP1	hematological malignancies
Mesothelin (MSLN)	acute myeloid leukemia (AML), breast cancer, cervical cancer, colorectal cancer, gastric cancer, lung cancer (malignant pleural mesothelioma), ovarian cancer, pancreatic cancer
MG7	hepatocellular carcinoma (HCC)
MPL (MPF, SMRP)	acute myeloid leukemia (AML)
MUC1 (PEM, PUM, DF3, MAM-6)	breast cancer, cervical cancer, colorectal cancer, esophageal cancer, Ewing's sarcoma, gastric cancer, glioma, head and neck squamous cell carcinoma (HNSCC), hepatocellular carcinoma (HCC), lung cancer, osteosarcoma, ovarian cancer, pancreatic cancer, renal cell carcinoma (RCC)
MUC16 (CA125)	ovarian cancer
NKG2DL	acute myeloid leukemia (AML), breast cancer, colorectal cancer, Ewing's sarcoma, gastric cancer, glioma, hepatocellular carcinoma (HCC), multiple myeloma (MM), osteosarcoma, prostate cancer, T-acute lymphoblastic leukemia (T-ALL)
NRP-1 (VEGF165R, NRP)	gastric cancer
NY-ESO-1 (CTAG1B, ESO1, LAGE2)	esophageal cancer, lung cancer, melanoma, multiple myeloma (MM)
OR2H1	lung cancer, ovarian cancer
P32	glioma
PAK4	glioma
PD-L1 (CD274, B7-H1)	breast cancer, gastric cancer, head and neck squamous cell carcinoma (HNSCC), hepatocellular carcinoma (HCC), lung cancer, melanoma
PDPN (GP36)	glioma
PODXL (PCLP, PCLP1, Gp200)	pancreatic cancer
PR1	acute myeloid leukemia (AML)
PSCA	colorectal cancer, gastric cancer, lung cancer, pancreatic cancer / pancreatic-biliary tract cancer, metastatic pancreatic cancer, prostate cancer, metastatic castration-resistant prostate cancer
PSMA, prostate-specific membrane antigen (GIG27, FOLH, NAALAD1, PSM)	prostate cancer, metastatic castration-resistant prostate cancer, salivary gland cancers
PTK7 (CCK4)	breast cancer, lung cancer, ovarian cancer
ROBO1 (DUTT1)	pancreatic cancer
ROR1 (NTRKR1)	breast cancer, chronic lymphocytic leukemia (CLL), Ewing's sarcoma, lung cancer, osteosarcoma
ROR2	gastric cancer, pancreatic cancer, renal cell carcinoma (RCC)

Siglec-6 (CD327, OB-BP1)	acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL)
SLAMF3 (CD229, Ly9)	multiple myeloma (MM)
SLAMF7 (CS1, CD319, CRACC)	multiple myeloma (MM)
TAA-T	acute myeloid leukemia (AML), Hodgkin's lymphoma (HL), myelodysplastic syndrome (MDS),
TAG 72 (CA72-4)	colorectal cancer, metastatic colorectal cancer, gastric cancer, ovarian cancer
TEM8 (ATR, GAPO)	breast cancer
TGFβ (IBDIMDE)	hepatocellular carcinoma (HCC), lung cancer
Tim3 (CD366, HAVCR2)	acute myeloid leukemia (AML)
TRBC	T-cell non-Hodgkin's lymphoma (T-NHL)
TROP2 (TACSTD2, GA733-1, M1S1)	breast cancer, gastric cancer, pancreatic cancer
TRP1/2	melanoma
TSHR (LGR3, CHNG1)	thyroid cancer
TSLPR (CRL2, CRLF2)	B-acute lymphoblastic leukemia (B-ALL)
TSPAN8	pancreatic cancer
U87	pancreatic cancer
uPAR (CD87)	gastric cancer, ovarian cancer
VEGFR-2 (FLK-1)	melanoma, renal cell carcinoma (RCC)
WT1 (WIT-1)	acute myeloid leukemia (AML)
αvβ3 (CD51, CD61, GP3A)	melanoma
κ light chain	B-cell non-Hodgkin's lymphoma (B-NHL), chronic lymphocytic leukemia (CLL), multiple myeloma (MM)
λ light chain	B-cell non-Hodgkin's lymphoma (B-NHL), chronic lymphocytic leukemia (CLL)

[0222] TCR-T cell therapies target antigens expressed as peptide-human leukocyte antigen (HLA) complexes on the surface of a target cell. These targets may include tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) (Baulu et al., 2023). Exemplary oncology antigens and corresponding cancer types for TCR-T cell therapies are listed in Table 2 below (Baulu et al., 2023; Sun et al., 2021; Want et al., 2023).

Table 2	
Antigen/Antigen Type	Cancer Type
AFP	hepatocellular carcinoma (HCC)
CEA	colorectal cancer
CTAG1A (NY-ESO-1)	bladder carcinoma, breast cancer, esophageal cancer, gastric cancer, hepatocellular carcinoma (HCC), lung cancer, myeloma, melanoma, neoplasms, serous ovarian, Fallopian tube, or primary peritoneal cancer (epithelial cancer), synovial sarcoma

EBV	acute myeloid leukemia (AML), head and neck squamous cell carcinoma (HNSCC), nasopharyngeal carcinoma, neuroblastoma
gp100	melanoma
HA-1H	juvenile myelomonocytic leukemia (JMML), recurrent acute biphenotypic leukemia, recurrent acute undifferentiated leukemia
HBV	HBV-hepatocellular carcinoma (HCC)
HERV-E	kidney cancer
HPV16-E6	human papillomavirus (HPV)-16+ cancers (cervical, vulvar, vaginal, penile, anal, and oropharyngeal cancers)
HPV16-E7	human papillomavirus (HPV)-16+ cancers (cervical, vulvar, vaginal, penile, anal, and oropharyngeal cancers), neoplasia
Kita-kyushu Lung Cancer Antigen 1/cancer testis antigen (KK-LC-1)	breast cancer, cervical cancer, gastric cancer, lung cancer
KRAS mutations e.g. KRAS G12D	gastric cancer, gastrointestinal cancer, pancreatic cancer, pancreatic ductal adenocarcinoma,
LMP2	nasopharyngeal carcinoma, recurrent and metastatic nasopharyngeal carcinoma
MAGE family	bladder cancer, breast cancer, cervical cancer, head and neck squamous cell carcinoma (HNSCC), melanoma, metastatic non-small cell lung carcinoma (NSCLC), metastatic malignant solid neoplasm, renal cell carcinoma (RCC), uveal melanoma
MAGEA10	non-small cell lung carcinoma (NSCLC)
MAGEA3	esophageal cancer, melanoma, metastatic solid tumors, myeloma, synovial sarcoma
MAGEA4	esophageal cancer, solid tumors
MAGEC2	head and neck squamous cell carcinoma (HNSCC), melanoma, uveal melanoma
MART-1 (MLANA)	melanoma
MCPyV	Merkel cell carcinoma
Mesothelin (MSLN)	Metastatic pancreatic ductal adenocarcinoma, ovarian cancer
NY-ESO-1	liposarcoma, melanoma, myeloma, malignant Peripheral Nerve Sheath Tumor (MPNST), osteosarcoma, serous ovarian, Fallopian tube, or primary peritoneal cancer (epithelial cancer), soft tissue sarcoma, synovial sarcoma
PMEL (gp100)	skin cancer, melanoma
PRAME	acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), uveal melanoma
TAC-1	HER2-positive solid tumors
TP53	breast cancer, metastatic breast cancer, kidney cancer, melanoma
TSA	malignant epithelial neoplasms

WT1	acute myeloid leukemia (AML), chronic myeloid leukemia (CML), myelodysplastic syndromes (MDS), pleural malignant, recurrent non-small cell lung carcinoma (NSCLC)
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[0223] T cell therapies, including stem cell-derived T cell therapies, may be genetically modified, for example, to eliminate endogenous TCR expression by knock-out of the T cell receptor α constant (TRAC) locus (TRAC^{-/-}).

5 **[0224]** It is contemplated that cell populations derived using the methods provided herein may be contained in pharmaceutical compositions.

[0225] It is further contemplated that the cell populations derived using the methods provided herein may be used to treat a disease or condition in a subject. By “treat” we mean administering to the subject an effective amount of cells, as provided herein, under conditions suitable for increasing the
10 number of T cells in the subject, which may result in prevention, inhibition and/or therapeutic treatment of a medical condition. By “effective amount” we mean a therapeutically effective amount such as, for example, the amount of cells that, upon administration to a subject, is sufficient to achieve the intended purpose (e.g., treatment). The amount may vary from one subject to another and may depend upon one or more factors, such as, for example, subject gender, age, body weight, subject’s health history,
15 and/or the underlying cause of the condition to be prevented, inhibited and/or treated.

[0226] For example, subjects afflicted with an oncological or autoimmune disease, condition or disorder may benefit from administration of CD4-CD8⁺ cell populations, as described herein.

[0227] The pharmaceutical composition provided herein may be administered to a subject in order to alleviate or ameliorate one or more symptoms of a disease; decrease the duration of time that one or
20 more symptoms of a disease, are present in a subject; and increase the survival rate of a subject having a disease.

[0228] The pharmaceutical composition provided herein may be administered to a subject to treat cancer or autoimmune disorders in the subject.

[0229] The pharmaceutical composition provided herein may be administered to a subject in an
25 effective amount or a therapeutically effective amount. A person of ordinary skill in the art would be able to determine such amounts based on such factors as the subject’s size (e.g., weight), age and/or sex; the severity of the subject’s symptoms; and the particular composition or route of administration selected. A person skilled the art would also know how to select the proper route of administration and to administer the compounds and compositions provided herein.

[0230] The dosage of the pharmaceutical composition of the disclosure varies depending on many factors, such as the pharmacodynamic properties of the composition, the mode of administration, the age, health and weight of the recipient, the nature and extent of the symptoms, the frequency of the treatment and the type of concurrent treatment, if any, and the clearance rate of the compound in the subject to be treated. One of skill in the art can determine the appropriate dosage based on the above factors. In some embodiments, the pharmaceutical composition is administered initially in a suitable dosage that is adjusted as required, depending on the clinical response.

Kits

[0231] The invention also provides kits comprising the pharmaceutical composition described herein.

10 Kits of the invention include one or more containers comprising the pharmaceutical composition described herein and instructions for use in accordance with any of the methods of the invention described herein. Generally, these instructions comprise a description of administration of the pharmaceutical composition for the above-described therapeutic treatments. In some embodiments, kits are provided for producing a single-dose administration unit.

15 [0232] The instructions relating to the use of the pharmaceutical composition generally include information as to dosage, dosing schedule, and route of administration for the intended treatment. The containers may be unit doses, bulk packages (e.g., multi-dose packages) or sub-unit doses. Instructions supplied in the kits of the invention are typically written instructions on a label or package insert (e.g., a paper sheet included in the kit), but machine-readable instructions (e.g., instructions carried on a magnetic or optical storage disk) are also acceptable.

20 [0233] The disclosure is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the disclosure should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

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EXAMPLE 1: Materials and Methods

DL4 and VCAM-1 Production

[0234] Recombinant DL4-Fc fusion protein was purchased from Sino Biological or manufactured in-house using HEK-293T cells and purified with HiTrap™ Protein G affinity columns (GE Healthcare) as previously described (e.g., Trotman-Grant et al., 2017). Recombinant VCAM-1-FC fusion protein was

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purchased from R&D Systems. DL4 and VCAM-1 that are suitable for preparation of 2D ETN and 3D ETN as further described below, are shown in Table 3.

Table 3		
Protein	Species	Sequence
DL4	Human	MAAASRSASGWALLLLVALWQQRAAGSGVFQLQLQEFINERGVLASGRPCEPGCRTFFRV CLKHFQAVVSPGPCTFGTVSTPVLGTNSFAVRDDSSGGGRNPLQLPFNFTWPGTFSLIIEA WHAPGDDLPEALPPDALISKIAIQGSLAVGQNWLLDEQTSTLRLRYSYRVICSDNYYGDN CSRLCKKRNDHFGHYVCQPDGNLSCLPGWTGEYCQQPICLSGCHEQNGYCSKPAECLCRP GWQGRCLCNECIPHNGCRHGTCSTPWQCTCDEGWGGLFCDQDLNYCTHHSPCKNGATCS NSGQRSYTCTCRPGYTGVDCLELSECDSNPCRNGGSKDQEDGYHCLCPPGYGLHCEH STLSCADSPCFNGGSCRERNQGANACECPNFTGSNCEKKVDRCTSNPCANGGQCLNR GPSRMCRCRPGFTGTCELHVSDCARNPCAHHGTCHDLENGLMCTCPAGFSGRRCVET SIDACASSPCFNATCYTDLSTDTFVCNCPYGFVGSRCFVGLPSPFWVAVSLVGLAVL LVLLGMVAVAVRQLRLRRPDDGSTREAMNNLSDFQKDNLPAAQLKNTNQKKELEVDCGL DKSNCGKQQNHTLDYNLAPGLGRGTMPGKFPKSDKSLGEKAPRLRHSEKPECRISAICSP RDSMYQSVCLISEERNECVIATEV (SEQ ID NO: 1)
VCAM-1	Human	QIDSPL (SEQ ID NO: 2)
VCAM-1	Human	TQIDSPLN (SEQ ID NO: 3)
VCAM-1	Mouse	MPVKMVAVLGASTVLWILFAVSQAFKIEISPEYKTIAQIGDSMALTCTTGCESPFWSWRTQI DSPLNAKVRTEGSKSVLTMEPVSFENEHSYLCTATCGSGKLESIHVDIYSFPKDPEIQFSGPL EVGKPVTVKCLAPDIYPVYRLEIDLFGDQLMNRQEFSSSEMTKSLETKSLEVTFTPVIEDIGK ALVCRAKLHIDQIDSTLKERETVKELQVYISPRNTTISVHPSTRLQEGGAVTMTCSSEGLPAP EIFWGRKLDNEVLQLLSGNATLTLIAMRMEDSGVYVCEGVNLIGRDKAEVELVVEKPFIV DISPGSQVAAQVGDVSVLTCAAGCDSPSFSWRTQTDSPNLNGVVRNEGAkstLVSSVGF DEHSYLCAVTCLQRTLEKRTQVEVYSFPEDPVIKMSGPLVHGRPVTVNCTVPNVYPFDHLEI ELLKGETTLMKKYFLEEMGKISLETKILETTFTIPTIEDTGKSLVCLARLHSGEMESEPKQRQSV QPLYVNVAPKETTIWVSPSILEEGSPVNLTCSSDGIPAPKILWSRQLNNGELQPLSENTTLT FMSTKRDDSGIYVCEGINEAGISRSKVELIIQVSPKDIQLTVFPSKSVKEGDTVIISCTCGNVPE TWIILKKAKTGDMVLKSDGSYTIRQAQLQDAGIYECESKTEVGSQRLSLTLDVKGEHNK DYFSPPELLALYCASSLIPAIGMIVYFARKANMKGYSYSLVEAQKSKV (SEQ ID NO: 4)
VCAM-1	Human	MPGKMVVILGASNILWIMFAASQAFKIETTPESRYLAQIGDSVSLTCTTGCESPFWSWRTQ IDSPLNGKVTNEGTTSTLTMPVSVFGNEHSYLCTATCESRKLEKGIQVEIYSFPKDPEIHLSP LEAGKPITVKCSVADVYPFDRLEIDLKGDHLMKSQEFLEDADRKSLETKSLEVTFTPVIEDIG KVLVCRAKLHIDEMDSVPTVRQAVKELQVYISPKNTVISVNPSTKLQEGGSVTMTCSSEGLP APEIFWSKKLDNGLQHLSGNATLTLIAMRMEDSGIYVCEGVNLIKNRKEVELIVQEKPF VEISPGPRIAAQIGDSVMLTCSVMGCESPSFSWRTQIDSPLSGKVRSEGTNSTLTLSPVSFEN EHSYLCTVTCGHKKLEKGIQVELYSFPRDPEIEMSGGLVNGSSVTVSCKVPVYPLDRLEIELL KGETILENIEFLEDTMKSLENKSLEMTFTIPTIEDTGKALVCQAKLHIDDMEFEPKQRQSTQT LYVNVAPRDTTLVSPSSILEEGSSVNMTCLSQGFPAPKILWSRQLPNEGELQPLSENATLTLI STKMEDSGVYLCEGINQAGRSRKEVELIIQVTPKDIKLTAFPSVKEGDTVIISCTCGNVPE WIIILKKAEtGDTVLKSIDGAYTIRKAQLKDAGVYECESKNKVGSLRSLTLDVQGRENNKD YFSPPELLVLYFASSLIIPAIGMIYFARKANMKGYSYSLVEAQKSKV (SEQ ID NO: 5)

Preparation of 2D ETN

[0235] Tissue culture plates with 6-well, 12-well, 24-well, 48-well or 96-well were coated with the Notch signalling ligand, DL4, and VCAM-1 overnight at 4 °C or for 3 hours at 37 °C. Tissue culture plates may be stored at 4 °C for up to 2 weeks after coating. To coat, a solution of 20 µg/mL DL4 and 10µg/mL VCAM-1 was prepared in Dulbecco’s phosphate-buffered saline (DPBS) (-/-). The appropriate coating volume per well of DL4 and VCAM-1 diluted in DPBS were added to the tissue culture plates as shown in Table 4.

Table 4		
Plate format	Surface area (cm2/well)	Coating volume (µL/well)
96-well	0.32	50
48-well	0.95	150
24-well	1.9	300
12-well	4	650
6-well	9.5	1500

[0236] The tissue culture plate(s) were tapped gently to ensure that the coating solution comprising the Notch signalling ligand, DL4, and VCAM-1 is evenly spread out throughout the well surface. The tissue culture plates were sealed with Parafilm® prior to being stored at 4 °C overnight or at 37 °C for 3 hours.

10 Tissue culture plates coated with Notch signalling ligand DL4, and VCAM-1 overnight at 4 °C were placed in a 37 °C incubator for equilibration for three hours before plating cells. Following equilibration, or coating the tissue culture plates with the Notch signalling ligand DL4, and VCAM-1 for 3 hours at 37 °C, the coating solution was aspirated from the wells. The wells were rinsed with DPBS (-/-) using the volumes shown in Table 5, immediately followed by addition of the cell suspension into the tissue

15 culture plates.

Table 5	
Plate format	DPBS (-/-) wash volume (µL/well)
96-well	200
48-well	800
24-well	1600
12-well	3200
6-well	7500

Preparation of 3D ETN

[0237] The dose of 3D ETN may be calculated to scale with the bead diameter, and may be expressed as: a dose proportional to the surface area of the culturing surface of the culture plate or flask (e.g., as in Table 6), the number of beads per unit volume of culture, or the bead surface area per unit volume of

20

culture. As calculated on a per unit volume basis, the bead per mL concentration does not change for different vessels. A “1x” bead dose denotes complete coverage of the plate surface by one layer of beads; as the beads are approximately spherical, the total surface area of the beads is 4 times the surface area of the surface of the plate or culture vessel. Table 6 provides a range of bead concentrations for a 3.05 μm diameter polystyrene bead.

Table 6			
Bead dose	Bead concentration (beads/mL)	Bead surface area per unit volume (cm^2/mL)	Ratio of bead surface area to culture vessel area
0.02	1.00E+06	0.29210	0.08:1
0.05	2.70E+06	0.78867	0.2:1
0.1	5.40E+06	1.57733	0.4:1
0.25	1.35E+07	3.94333	1:1
0.5	2.70E+07	7.88666	2:1
1	5.40E+07	15.77332	4:1

[0238] The Notch signalling ligand density (such as, for example, the density of surface-bound DL4) on the bead may vary from, for example, 100 molecules per square micrometer ($100 \text{ molecules}/\mu\text{m}^2$) to 3000 molecules per square micrometer ($3000 \text{ molecules}/\mu\text{m}^2$). Table 7 provides a calculation of the Notch signalling ligand concentration for a range of bead doses and Notch signalling ligand densities for 3.05 μm polystyrene beads.

Table 7					
Bead dose	Bead concentration (beads/mL)	Bead surface area per unit volume (cm^2/mL)	Notch ligand concentration (molecules/mL media) for bead with surface density of 100 molecules/ μm^2	Notch ligand concentration (molecules/mL media) for bead with surface density of 800 molecules/ μm^2	Notch ligand concentration (molecules/mL media) for bead with surface density of 3000 molecules/ μm^2
0.05	2.70E+06	0.78867	7.89E9	6.31E10	2.37E11
0.1	5.40E+06	1.57733	1.58E10	1.26E11	4.73E11
0.25	1.35E+07	3.94333	3.94E10	3.15E11	1.18E12
0.5	2.70E+07	7.88666	7.89E10	6.31E11	2.37E12
1	5.40E+07	15.77332	1.58E11	1.26E12	4.73E12

[0239] Table 8 provides the Notch signalling ligand (such as, for example, DL4) surface area per unit volume for a range of bead doses and Notch signalling ligand densities for both 3.05 μm and 3.29 μm diameter polystyrene beads.

Table 8						
Bead dose	Bead concentration (beads/mL)	Bead surface area per unit volume (cm ² /mL)	Notch ligand concentration (molecules/mL) for beads with surface density of 100 molecules /μm ²	Notch ligand concentration (molecules/mL) for beads with surface density of 800 molecules /μm ²	Notch ligand concentration (molecules/mL) for beads with surface density of 3000 molecules /μm ²	Bead diameter (μm)
0.5	2.70E+07	7.88666	7.89E10	6.31E11	2.37E12	3.05
2	1.08E+08	31.54664	3.15E11	2.52E12	9.46E12	
0.5	2.25E+07	7.64723	7.65E10	6.12E11	2.29E12	3.29
2	9.00E+07	30.58891	3.06E11	2.45E12	9.18E12	

[0240] The 3D ETN may also comprise surface-bound VCAM-1. VCAM-1 may be immobilized to 3D ETN at an input molar ratio ranging from 1:6 to 10:1 DL4:VCAM-1. In an embodiment, the 3D ETN is prepared with an input molar ratio of 2.5:1 DL4:VCAM-1. In an embodiment, the final density of the surface-bound VCAM-1 on the bead is equivalent to the density of the Notch ligand. For example, the VCAM-1 surface area per unit volume may be equivalent to the Notch ligand surface area per unit volume shown in Table 8 above.

[0241] Cells may be cultured at densities appropriate for culture scale and format. In microplate culture cells may be cultured at, for example, $2.5 \times 10^5 - 2 \times 10^6$ cells/mL. In STR culture cells may be cultured at, for example $5 \times 10^4 - 6 \times 10^6$ cells/mL.

Preparation of Antigen Beads

[0242] The CD19 wild-type antigen (AcroBiosystems) or an engineered variant, CD19.1 (Klesmith et al., 2019) was functionalized with a polyhistidine (-His) or human IgG1a Fc (-Fc) tag. The CD19 antigen was then immobilized onto carboxyl group functionalized, magnetic polystyrene beads (4.5 μm) using carbodiimide crosslinker conjugation with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide HCl (NHC) and N-hydroxysuccinimide (NHS). Based on the total moles of carboxyl groups available for protein conjugation on the beads, 0.5 X moles, 1.0X moles, 2.0X moles, or 5.0X moles of CD19.1 protein were added, respectively, into the reaction mixture. The total conjugation reaction volume was 200 μL. A maximum CD19.1 protein loading on beads of 69.4% of theoretical maximum coverage was observed when the protein concentration was the highest in the reaction mixture i.e., 5.0X moles. Sequences of wtCD19 and CD19.1 are shown in Table 9 below.

Table 9		
Protein	Species	Sequence
etCD19 (wtCD19)	Human	PEEPLVVKVEEGDNAVLQCLKGTSDGPTQQLTWSRESPLKPFLKLSLGLPGLGIHMRPLAI WLFIFNVSQQMGGFYLCQPGPPSEKAWQPGWTVNVEGSGELFRWNVSDLGGLGCGLK NRSSEGPSSPSGKLMSPKLYVWAKDRPEIWEGEPPCLPPRDSLNLQSLSDLTMAPGSTLW LSCGVPPDSVSRGPLSWTHVHPKGPKSLLSELEKDDRPARDMWVWVETGGLLPRATAQDA GKYYCHRGNTMSFHLEITARPVLWHWLLRTGGWK (SEQ ID NO: 6)
CD19.1	Human	PEEPLVVKVEEGDEAWLPCLKGTSDGPTQQLTWSRESPLKPFLKVSFGVPGLVHVRPNA VSLVISNVSQQMGGFYLCQPGPPSEKAWQPGWTVNVEGSGELFRWNVSDLGGLGCGLK NRSSEGPSSPSGKLMSPKLYVWAKDRPEIWEGEPPCLPPRDSLNLQSLSDMTVAPGSTLW LSCGVPPDSVSRGPLSWTHVHPKGPKSLLSELEKDDRPARDMWVWVETGTRFLPRATAQDAG KYYCHRGNTMSFHLEVKARPVSAHTKLRTGGWK (SEQ ID NO: 7)
CD19.1- Fc	Human	MPPPRLLFFLLFTPMFVRPEEPLVVKVEEGDEAWLPCLKGTSDGPTQQLTWSRESPLKPFL KVSFGVPGLVHVRPNAVSLVISNVSQQMGGFYLCQPGPPSEKAWQPGWTVNVEGSGE LFRWNVSDLGGLGCGLKLRNRSSEGPSSPSGKLMSPKLYVWAKDRPEIWEGEPPCLPPRDSL NQSLSRDMTVAPGSTLWVSCGVPPDSVSRGPLSWTHVHPKGPKSLLSELEKDDRPARDM WVTGTRFLPRATAQDAGKYYCHRGNTMSFHLEVKARPVSAHTKLRTGGWKGGGSGG GSPKSSDKTHTCPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFN WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPV LDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 8)

Generation and characterization of a clonal iPSC line with CAR integrated at the TRAC locus.

[0243] An induced pluripotent stem cell line was genetically modified to integrate a chimeric antigen receptor (CAR) at the T cell receptor α constant (TRAC) locus. CAR expression was evaluated at the end of clone production from 1 parental iPSC line (Fig. 1A, depicting the percentage of CAR positive cells (bar chart) and median fluorescent intensity (MFI, dots)). The parent line (unedited) and a clonal iPSC line expressing CD19-CAR by random LVV integration (LVV CAR) were used as controls. Genetic characterization of copy number integration at TRAC locus was assessed by digital droplet polymerase chain reaction (ddPCR) copy number variation (CNV) Assay (Biorad) by amplifying genomic TRAC locus - CAR transgene junction. Genomic stability analysis of selected clone was evaluated by G-band karyotyping conducted by WiCell, demonstrating a normal karyotype (Fig. 1B). An iCS-digital assay also showed an expected copy number in 24 genomic regions of recurrent iPSC abnormalities (data not shown).

[0244] Next, multipotent lymphoid-competent CD34+ hematopoietic progenitor cells were generated from engineered iPSCs. High purity and yield of CD34+ cells and CD34+CD43+ HPCs was obtained from iPSC lines derived from 2 independent donors as well as gene-edited clonal iPSC lines prior to enrichment (Fig. 2A, B).

[0245] Prior to differentiation, engineered iPSCs were positive for undifferentiated cell makers OCT4, SOX2, SSEA4 and TRA-160. 100% CAR expression was detected in iPSCs upon thaw and expansion of a seed bank of an iPSC clone with CAR integrated into TRAC locus, as described above (Fig. 3A, "Stage 1"). iPSCs were differentiated to CD34⁺ cells in scalable agitated suspension cultures and enriched HPCs were also positive for CD43 and CD45 (Fig. 3B, "Stage 2"). Expression of erythroid progenitor marker CD235a was negative, indicating lack of early lineage commitment (Fig. 3B). CAR expression was reduced upon CD34 differentiation (Fig. 3B). CD34⁺ HPCs were then cultured with ETN beads presenting DLL4 and VCAM-1 with conditions identified to provide high level of Notch signalling and ProT phenotype was assessed by measuring CD5 and CD7. T-cell markers (CD4 and CD8) were low at this stage, typical for ProT cells (Fig. 3C, "Stage 3"). A gradual reduction in Notch signalling after ProT stage resulted in the gain of T lineage fate during week 4 and 5, and primarily CD4⁺CD8⁺ DP cells were produced (Fig. 3D, "Stage 4"). CAR expression was gradually increased as cells were committed to T-cell lineage fate (Fig. 3D). CAR engagement, as described further in Example 3, facilitated maturation of DP cells to CD8 SP T-cells. iPSC-derived CD8 SP cells were a mix of CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ T-cells (Fig. 3E). CD8SPs had high levels of expression of stem cell memory markers CD45RA, CD62L and CD95 (data not shown). CAR-stimulated cells expressed the T-cell activation/NK-cell marker CD56 but lacked canonical NK markers such as NKP44 and NKP46. CAR expression was 100% after SP transition.

EXAMPLE 2: Generation of CD4⁺CD8⁺ cells through control of Notch signalling

[0246] In this Example, iPSC-derived CD34⁺ cells were seeded at a density of 5×10^4 cells/mL in LEM, and after 24 hours ETN beads were added at a relative bead dose of 0.5x (2.7×10^7 beads/mL). Cells were harvested and reseeded at day 10 at a density of 5×10^5 cells/mL in LEM, and after 4 hours of resting, a 0.5x ETN bead dose (2.7×10^7 beads/mL) was added. Cells were again harvested at day 15, and reseeded at densities of 1×10^6 , 2×10^6 and 4×10^6 cells/mL, in a matrix with bead doses of 0.1x (5.4×10^6 beads/mL), 0.5x (2.7×10^7 beads/mL), and 1x (5.4×10^7 beads/mL). After 21 days, cell phenotype was assessed by flow cytometry. The proportion of double negative (DN), CD4 immature single positive (CD4 ISP), double positive (DP) and CD8 single positive (CD8 SP) cells varied as a function of both cell density and bead dose, with lower bead dose (0.1x) and higher cell densities (4×10^6 cells/mL) resulting in a larger fraction of CD4 ISP and DP, whereas higher bead dose (1x) and lower cell density (1×10^6 cells/mL) generated more CD8 SP cells (Fig. 4A, 4C). The percentage of DP cells was inversely proportional to bead to cell ratio (Fig. 4B). Modulation of Notch signalling via ETN bead dose and cell density was shown to control cell fate trajectory.

[0247] In a further experiment, cells were differentiated to day 15 as described above. On day 15, cells were reseeded at a cell density of 2×10^6 cells/mL and ETN bead dose of 0.5x (2.7×10^7 beads/mL) with either standard ETN bead ($800 \text{ molecules}/\mu\text{m}^2$, Fig. 5, top row), or beads with lower protein density (0.1x density or $100 \text{ molecules}/\mu\text{m}^2$, Fig. 5, bottom row). Flow cytometric analysis revealed that beads with lower protein density produced a higher proportion of CD4 ISP and DP cells, and fewer DN and CD8 SP cells than standard ETN beads (Fig. 5). Total CD8 β expression was similar between the two beads, though a lower percentage of CD8 $\alpha\alpha^+$ cells were observed with the lower density beads.

[0248] Cells were differentiated to day 21, as described above. Expression of CD56 varied with bead to cell ratio, as high bead dose (1x, 5.4×10^7 beads/mL) and low cell density (1×10^6 cells/mL) produced the highest %CD56+ cells (Fig. 6A). Lower bead doses (0.5x and 0.1x) and higher cell densities (2×10^6 and 4×10^6 cells/mL) resulted in fewer CD56+ cells (Fig. 6A). On day 21, CD3 displayed the inverse trend as CD56, with low bead dose (0.1x) and high cell density (4×10^6) resulting in the highest CD3 expression; however, CD3 expression was quite low in all conditions ($< 3\%$) (Fig. 6B).

[0249] Cell differentiation was further extended past day 21 to day 23. Similar to day 21, the phenotype on day 23 was a function of both cell density and bead dose, with low bead doses and high cell density producing the most double positive cells, and CD8 SP cells most abundant with high bead dose (1x, 5.4×10^7 beads/mL) and low cell density (1×10^6 cells/mL) (Fig. 7A). Notably, more DPs and fewer DN and CD4 ISP were present in the low bead:cell ratio conditions on day 23 compared to day 21 (Figs. 7A, 7C). The percentage of DPs was inversely correlated with bead:cell ratio, with a slightly steeper slope compared to day 21 (Fig. 7B).

[0250] Cells were differentiated to day 23, as described in Figure 7. Expression of CD56 varied with bead to cell ratio, as high bead dose (1x, 5.4×10^7) and low cell density (1×10^6 cells/mL) produced the highest %CD56+ cells (Fig. 8A). Lower bead doses (0.5x and 0.1x) and higher cell densities (2×10^6 and 4×10^6) resulted in fewer CD56+ cells (Fig. 8A). On day 23, CD3 displayed the inverse trend as CD56, with low bead dose (0.1x, 5.4×10^6 beads/mL) and high cell density (4×10^6) resulting in the highest CD3 expression (Fig. 8B). CD3 expression on day 23 was higher than that observed on day 21, though still less than 6% in all conditions (Fig. 8B).

[0251] The differentiation conditions and resulting cell phenotypes for days 21 and 23 are summarized in Table 10 below.

Table 10

Bead Dose	Bead concentration (beads/mL)		DN (%)	ISP (%)	DP (%)	CD8a SP (%)	Cell density (cells/ml)	Bead: Cell Ratio
0.1X	5.4 x10 ⁶	Day 21	8.14	41.91	45.15	3.80	4 x10 ⁶	1.35:1
			9.54	39.67	44.59	6.20	2 x10 ⁶	2.7:1
			15.59	37.68	36.41	10.33	1 x10 ⁶	5.4:1
0.5x	2.7 x10 ⁷	Day 21	11.93	40.53	37.36	10.18	4 x10 ⁶	6.75:1
			20.47	31.47	29.01	19.05	2 x10 ⁶	13.5:1
			30.08	18.83	19.55	31.54	1 x10 ⁶	27:1
1X	5.4 x10 ⁷	Day 21	19.92	28.03	29.80	22.24	4 x10 ⁶	13.5:1
			26.18	19.00	20.85	33.96	2 x10 ⁶	27:1
			30.83	9.33	13.17	46.67	1 x10 ⁶	54:1
0.1X	5.4 x10 ⁶	Day 23	7.52	34.43	54.83	3.21	4 x10 ⁶	1.35:1
			7.30	32.35	53.44	6.91	2 x10 ⁶	2.7:1
			10.79	27.95	47.49	13.78	1 x10 ⁶	5.4:1
0.5x	2.7 x10 ⁷	Day 23	8.78	36.03	46.01	9.18	4 x10 ⁶	6.75:1
			18.09	25.66	34.31	21.93	2 x10 ⁶	13.5:1
			24.26	18.69	26.31	30.74	1 x10 ⁶	27:1
1X	5.4 x10 ⁷	Day 23	17.58	22.2	32.80	27.42	4 x10 ⁶	13.5:1
			21.03	18.76	26.38	33.83	2 x10 ⁶	27:1
			26.37	9.23	17.14	47.26	1 x10 ⁶	54:1

EXAMPLE 3: Generation of CD8+ cells through temporal control of Notch signalling

[0252] iPSC-derived CD34⁺ HPCs were differentiated into CD8SP T cells using a 5-stage protocol (protocol schematic, Fig. 9). During Stage # 1, CD34 HPCs were seeded onto DL4 and VCAM-1 coated culture vessels ("2D") or mixed with 3D ETN beads at 0.5x bead dose (2.7×10^7 beads/mL, "3D") in progenitor expansion medium (SFEM II + LEM) and cultured for 10 days to generate CD34-CD7⁺CD5⁺ ProT cells.

[0253] At this point, cells moved onto Stage # 2 where ProT cells were differentiated into early DPs (CD4⁺CD8A⁺) for 7 days by two methods. In one method, cells (unmodified or TCR-modified cell lines) were re-seeded at an intermediate seeding density of 2×10^6 cells/mL and mixed with 3D ETN beads at 0.25x dose in progenitor maturation medium (SFEM II + LMM). In an alternate method, cells (CAR-modified) were re-seeded at an intermediate seeding density of 2×10^6 cells/mL and mixed with 3D ETN at 0.1x protein density and 0.5X dose in progenitor maturation medium (SFEM II + LMM). At the end of stage # 2, early DPs require further maturation as they do not have sufficient surface CD3/TCR expression for stimulation.

[0254] During Stage # 3, in one method early DPs were allowed to mature into late DPs (CD4⁺CD8A⁺CD8B⁺CD3⁺TCR $\alpha\beta$ ⁺/TCR $\gamma\delta$ ⁺/-) by re-seeding them at a higher seeding density of 6×10^6 cells/mL, mixed with 3D ETN beads at 0.25x bead dose (TCR-modified cell lines). In an alternate method, early DPs were re-seeded with 3D ETN beads at 0.1x protein density and 0.25x bead dose (unmodified or CAR-modified cell lines) in SFEM II + LMM and cultured for an additional 8-13 days (timing may be cell line dependent).

[0255] Stage # 4 was started with the enrichment of Late DPs, e.g. via a CD8 Positive Selection kit (STEMCELL Technologies), followed by re-seeding in DP-SP medium containing IL-21, adding an appropriate T cell activator, and culturing for 7 days to allow for the DP to CD8SP conversion to occur. In one method, the T cell activator is a CD3 stimulator such as anti-CD3/CD28/CD2 or anti-CD3/CD28, for example Immunocult™, in combination with an integrin ligand such as the fibronectin fragment RetroNectin®, fibronectin, laminin, or ICOS-L. It is also contemplated that the CD3 stimulator is ICAM-1. In an alternate method, e.g., for CAR-modified cell lines, the T cell activator is antigen-coated beads, such as, for example, CD19-coated beads.

[0256] Lastly, newly transitioned CD8SPs were moved onto Stage # 5, where they were activated and expanded (for unmodified or TCR-modified cell lines). CD8SPs were seeded at 5×10^5 cells/mL in activation medium with soluble anti-CD28 antibody on plate-bound CD3 antibody (OKT3) and fibronectin fragment (RetroNectin®)-coated non TC-treated vessels for 3 days (activation step), reseeded onto non TC-treated vessels in expansion medium at 1×10^5 cells/mL for 2 days and reseeded once more

onto non TC-treated vessels in expansion medium at 3×10^5 cells/mL for an additional 2 days (expansion step). It is also contemplated that the CD8SPs may be activated/expanded by contacting with ICAM-1.

[0257] Viability and cumulative-fold expansion kinetics were evaluated during Stages 1-3 of the 3D differentiation protocol. During the first 10 days of the process, the emerging proT cells remained highly viable and cumulative fold-expansion was at its highest (between 200-600 fold) (Fig. 10). Once proT cells entered stage # 2 (Day 10-17), a substantial decrease in viability was observed with minimal increase in fold-expansion (Fig. 10). During Stage # 3 (Day 17 onwards), while viability continued to decrease slightly due to improvement in DP maturity and phenotype, cells were able to sustain viabilities >10% for approximately 1 week with minimal decrease in fold-expansion, which was an improvement from previous protocols.

[0258] In one method, day 10 ProT cells (unmodified lines) generated in Stage # 1 were seeded in progenitor maturation medium (SFEM II + LMM) at 1×10^6 cells/mL in non-tissue culture (TC)-treated plates previously coated with 1.37 $\mu\text{g}/\text{mL}$ human DL4 and 1 $\mu\text{g}/\text{mL}$ human VCAM-1 (intermediate 2D ETN). At day 17, sacrificial wells were harvested, and flow cytometry analysis was performed. On day 22, cultures were harvested and replated in SFEM II + LMM at 3×10^6 cells/mL in non TC-treated plates previously coated with 0.68 $\mu\text{g}/\text{mL}$ human DL4 and 0.5 $\mu\text{g}/\text{mL}$ human VCAM-1 (lower 2D ETN). At day 24, sacrificial wells were harvested, and flow cytometry analysis was performed. Lowering the 2D ETN coating and increasing seeding densities from Day 10 to Day 24 resulted in successful generation of CD4+CD8 α + Early DPs by Day 17, and effective maturation of this population as evidenced by >70% CD4+CD8 α / β +, 20-30% surface CD3 and 4-8% TCR α / β + expression at day 24 (Fig. 11). Cells were 23.37% and 15.32% CD1a+ at day 24 (NTX4A1, NTX6A1 lines, data not shown).

[0259] In an alternative method, day 10 ProT cells (unmodified cell lines) generated in Stage # 1 were seeded in SFEM II + LMM at 2×10^6 cells/mL, rested for 2-4hrs at 37°C, mixed with 0.25x dose of 3D ETN beads, and cultured for 7 days. At day 17, cells were harvested, flow cytometry analysis performed and the remaining cells were reseeded in SFEM II + LMM at 5×10^6 cells/mL, rested for 2-4 hours at 37°C, mixed with 0.25x dose of 0.1x protein density 3D ETN beads (Example 2), and cultured for an additional 11-13 days. At day 24, sacrificial wells were harvested and flow cytometry analysis was performed. In contrast to the 2D process, the 3D protocol improves CD4+ ISP to CD4+CD8A+ DP conversion, resulting in an earlier emergence of CD4+CD8 α / β + Late DPs by Day 17 for both unmodified cell lines, NTX4A1 (Fig. 12) and NTX6A1 (Fig. 13). While the cultures were predominantly CD4+CD8 α / β + DPs at Day 24, these Late DPs were more immature than the ones generated through the 2D protocol due to lower endogenous TCR α / β surface expression at this timepoint (Fig. 12, Fig. 13). Extending the cultures for an

additional 4-6 days resulted in improved surface CD3 and TCR α/β for both cell lines (Fig. 12, Fig. 13). CD1a expression also increased from 4.04% to 12.17% from day 24 to day 28 for the NTX4A1 cell line, and from 4.73% to 15.36% for the NTX6A1 cell line.

5 [0260] For 2D culture, day 10 ProT cells generated in Stage # 1 from CAR-modified HPCs were seeded in progenitor maturation medium (SFEM II + LMM) at 1×10^6 cells/mL in non-TC-treated plates previously coated with 1.37 $\mu\text{g}/\text{mL}$ human DL4 and 1 $\mu\text{g}/\text{mL}$ human VCAM-1 (intermediate 2D ETN). At day 17, sacrificial wells were harvested, and flow cytometry analysis was performed. On day 22, cultures were harvested and replated in SFEM II + LMM at 3×10^6 cells/mL in non-TC-treated plates previously coated with 0.68 $\mu\text{g}/\text{mL}$ human DL4 and 0.5 $\mu\text{g}/\text{mL}$ human VCAM-1 (lower 2D ETN). At day 24, sacrificial wells
10 were harvested, and flow cytometry analysis was performed. Lowering the 2D ETN coating and increasing seeding densities from Day 10 to Day 24 resulted in successful generation of CD4+CD8 α + Early DPs by Day 17, and effective maturation of this population as evidenced by >70% CD4+CD8 α/β +, >20% surface CD3 and >70% CAR+ expression at Day 24 (Fig. 14). CD1a expression at Day 24 was 23.25%.

15 [0261] For 3D culture, day 10 ProT cells generated in Stage # 1 from CAR-modified HPCs were seeded in SFEM II + LMM at 2×10^6 cells/mL, rested for 2-4hrs at 37°C, mixed with 0.5X dose of 0.1x protein density 3D ETN beads, and cultured for 7 days. At day 17, cells were harvested, flow cytometry analysis performed, and the remaining cells were reseeded in SFEM II + LMM at 5×10^5 cells/mL, rested for 2-4 hours at 37°C, mixed with 0.25X dose of 0.1x protein density 3D ETN beads, and cultured for an
20 additional 11 days. At day 24, sacrificial wells were harvested, and flow cytometry analysis was performed. In contrast to the 2D protocol, the 3D protocol improved CD4+ ISP to CD4+CD8A+ DP conversion, resulting in an earlier emergence of CD4+CD8 α/β + Late DPs by Day 17 (Fig. 15, top row). While the cultures were predominantly CD4+CD8 α/β + DPs at Day 24 (Fig. 15, middle row), these Late DPs were more immature than the ones generated through the 2D protocol due to lower CD1a+ expression
25 at this timepoint (2.61%). Extending the cultures for an additional 4 days resulted in improved surface CD3 and CAR expression (Fig. 15, bottom row) and increased CD1a expression (8.97%).

[0262] Next, Late DPs were generated with either 2D ETN or 3D ETN beads. Enriched Late DPs from two unmodified lines, NTX4A1 and NTX6A1, were seeded onto Stage # 4 conditions at 1×10^6 cells/mL in medium containing IL-21 and 1.25% ImmunoCult™ activator anti-CD3/CD28/CD2 for 7 days. Enriched
30 Late DPs from CAR-modified lines were seeded onto Stage # 4 conditions at 1×10^6 cells/mL and stimulated for 7 days with either CD3 stimulation by 1.25% ImmunoCult™ activator (anti-CD3/CD28/CD2 beads) or antigen stimulation with a 1:1 bead:cell ratio of CD19 antigen-coated beads (Example 1). The

enriched DPs generated using the 3D protocol had a higher viability than those generated using the 2D protocol. When the anti-CD3/CD28/CD2 transition protocol was used (Fig. 16A, B, left), a decline in cell viability (Fig. 16A, left) and cell numbers (Fig. 16B, left) was observed during the 7-day DP-SP transition regardless of the cell line or protocol used to generate the input DPs. In contrast, when the CD19 bead transition protocol was used (Fig. 16A, B, right) for the CAR-modified cell line, an increase in both cell number and viability was observed.

[0263] Next, enriched Late DPs from two unmodified lines, NTX4A1 and NTX6A1, were seeded onto Stage # 4 conditions at 1×10^6 cells/mL in DP-SP medium containing IL-21 and 1.25% ImmunoCult™ activator (anti-CD3/CD28/CD2) for 7 days. At the end of this stage (Day 35 for NTX4A1 – Fig. 17, top panel, and Day 37 for NTX6A1 – Fig. 17, bottom panel), cultures were harvested and stained for selected markers of T cell maturation, activation, and TCR expression. For both cell lines, a partial DP-SP transition was observed, evidenced by the presence of residual DPs in the cultures (Fig. 17, left panel, top right quadrant). Taking a closer look at the CD8SP populations (Fig. 17, gray overlay), these cells were highly positive for CD8 α/β (82-85%) and expressed surface CD3 and TCR α/β (30-58%), as well as key integrins such as LFA-1 and CD49c (VLA-3) and activation marker CD25.

[0264] Enriched Late DPs from CAR-modified lines were seeded onto Stage # 4 conditions at 1×10^6 cells/mL and stimulated for 7 days with either CD3 stimulation by 1.25% ImmunoCult™ activator (anti-CD3/CD28/CD2) (Fig. 18, top panel) or antigen stimulation with a 1:1 bead:cell ratio of CD19 antigen beads (Fig. 18, bottom panel). At the end of this stage (Day 35), cultures were harvested and stained for selected markers of T cell maturation, activation, and surface CD3 expression. Both methods of conversion resulted in the effective generation of high MFI CAR+ CD8SPs (60-63%). Anti-CD3/CD28/CD2 transitioned cells had more residual DPs post-transition than the CD19 bead condition (Fig. 18A, B). Taking a closer look at the CD8SP populations (gray overlay), these cells had intermediate levels of CD8 α/β (43%), moderate to high surface CD3 expression (86% for anti-CD3/CD28/CD2, 56% for CD19 beads), as well as key integrins such as LFA-1 and CD49c (VLA-3). The activation marker CD25 was highly expressed in anti-CD3/CD28/CD2-transitioned CD8SPs (78.57%, Fig. 18, top panel) and lower in CD19 bead conditions (56%, Fig. 18, bottom panel). Both CD3 activation via anti-CD3/CD28/CD2 and CAR activation via CD19 beads were effective methods of conversion that successfully transitioned CAR+ DPs into CD8SPs. Differentiation of unmodified cell lines yielded CD4-CD8 α +CD8 β +CD25+CD69+LFA-1+/CD49c+ CD3+ TCR $\alpha\beta$ + TCR $\gamma\delta$ + CD56lo cells. Differentiation of CAR-modified, TRAC-/- cell lines yielded CD4-CD8 α +CD8 β +CD25+CD69+LFA-1+/CD49c+ CD3+ CAR+ TCR $\gamma\delta$ + CD56lo cells.

[0265] A summary of the differentiation conditions and resultant cell phenotypes are provided in Tables 11-14 below for the unmodified cell lines, NTX4A1 and NTX6A1 (Table 11, Table 12), the CAR-modified cell line, NTX4B3 (Table 13) and the TCR-modified cell line, TCR-174 (Table 14). Cell seeding density is reported prior to bead (ETN or CD19 antigen-coated bead) addition.

Table 11								
3D ETN	Seeding density (cells/mL)	Bead: Cell Ratio	Day	Pro T CD5+CD7+	DN	ISP CD4+CD8-	DP CD4+CD8+	SP CD8+
Standard, 0.25x bead dose	2x10 ⁶ /mL	6.18	Day 10 [start of Stage 2]	13.78	54.01	32.22	4.93	8.85
0.1x density, 0.25x bead dose	5x10 ⁶ /mL	2.48	Day 17 [start of Stage 3]	39.79	5.46	36.17	53.09	5.29
			Day 24	22.71	1.09	14.56	83.06	1.28
N/A	1x10 ⁶ /mL	N/A	Day 28 [start of Stage 4, pre-enrichment]	29.31	2.25	8.29	83.89	5.57
		N/A	Day 35 [end of Stage 4]	N/A	0.33	0.04	69.86	29.77

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Table 12								
3D ETN	Seeding density (cells/mL)	Bead: Cell	Day	Pro T CD5+CD7+	DN	ISP CD4+CD8-	DP CD4+CD8+	SP CD8+
Standard, 0.25x bead dose	2x10 ⁶ /mL	6.18	Day 10	13.57	75.53	15.06	1.81	7.60
0.1x density, 0.25x bead dose	5x10 ⁶ /mL	2.48	Day 17	69.23	17.97	38.36	35.97	7.69
			Day 24	38.47	6.25	12.92	79.90	0.92
N/A	1x10 ⁶ /mL	N/A	Day 30	36.01	24.68	1.46	69.37	4.49
		N/A	Day 37	N/A	0.99	0.19	65.24	33.58

Table 13										
3D ETN	CD3 stimulator	Seeding density (cells/mL)	Bead: Cell	Day	Pro T CD5+CD7+	DN	ISP CD4+CD8-	DP CD4+CD8+	SP CD8+	CAR

0.1x density, 0.5x bead dose	N/A	2x10 ⁶ /mL	12.38	Day 10	13.96	61.08	26.52	3.61	8.80	32.42
0.1x density, 0.25x bead dose	N/A	5x10 ⁶ /mL	2.48	Day 17	51.96	4.18	47.41	45.83	2.58	47.00
	N/A			Day 24	24.89	0.92	13.97	83.92	1.19	68.02
N/A	N/A	1x10 ⁶ /mL	N/A	Day 28	31.33	2.42	2.78	90.60	4.21	79.31
	anti-CD3/CD28/CD2		N/A	Day 35	N/A	1.93	0.21	37.26	60.61	99.76
	Antigen bead		N/A	Day 35	N/A	3.06	0.52	33.03	63.39	99.91

Table 14									
3D ETN	Seeding density (cells/mL)	Bead: Cell	Day	Pro T CD5+CD7+	DN	ISP CD4+CD8-	DP CD4+CD8+	SP CD8+	MAGE-A4 Tetramer+
Standard, 0.25x bead dose	2x10 ⁶ /mL	6.18	Day 10	21.19	88.28	8.56	0.38	2.78	51.48
Standard, 0.25x bead dose	6x10 ⁶ /mL	2.06	Day 17	88.16	26.36	34.49	28.76	10.40	53.10
			Day 24	92.78	8.12	17.95	69.77	4.15	N/A
N/A	1x10 ⁶ /mL	N/A	Day 28	75.29	15.24	5.24	78.51	1.01	67.98
		N/A	Day 35	N/A	1.73	2.03	22.25	73.99	87.34

EXAMPLE 4: In Vitro Function of CAR+ CD8+ SP Cells

[0266] At the end of the CD8SP stage (Day 35 from HPC), iPSC-derived cell cultures transitioned to CD8SPs with ImmunoCult™ activator anti-CD3/CD28/CD2 or antigen-coated beads (Example 3) were harvested and cultured with target cells with and without CD19 expression. A serial restimulation assay was performed to measure cytotoxic activity using an Incucyte®-based assay (Sartorius), with GFP-expressing CD19+ cells as target cells (n= 3 technical replicates). T cells were co-cultured at a 2:1 E:T every 5 days with target cells and exogenous cytokine support. Target clearance was measured by GFP surface area reduction. CD8SP cells generated with both methods had some degree of non-specific activity during the first stimulation (Fig. 19A, B, dark gray inverted triangles) potentially due to being

activated during the DP-SP stage of differentiation. iPSC-derived CD8SPs were able to serially engage target cells over 4 rounds of stimulation (Fig. 19A, B).

[0267] Fold-expansion of cells was calculated by performing a count at the end of each round of target exposure. iPSC-derived CAR-T cells proliferated 28,000-fold over 4 rounds of antigen exposure (Fig. 20A).

5 Comparable effector cytokine production 24 hours after stimulation 1 was measured by MSD in iPSC and primary CD8⁺ CAR-Ts co-cultured with CD19^{+/+} and CD19^{-/-} target cells (Fig. 20B, C).

[0268] Cumulative cytotoxicity and fold expansion was evaluated for primary and iPSC-derived cells over four rounds of target exposure (supplemented with IL-2, IL-7 and IL-21, or IL-15, IL-7, and IL-21, Figure 21). Fold expansion and cytotoxicity was calculated after each stimulation. Cumulative fold

10 expansion was calculated by taking the product of the cumulative fold expansion from the previous stimulation and the fold expansion for the current stimulation. Cytotoxicity was calculated via the relative reduction in area under the curve (AUC) from the target-only control: cytotoxicity = $1 - \text{AUC} / \text{AUC}_{\text{control}}$ (cytotoxicity of 0 corresponds to no tumor control while cytotoxicity of 1 corresponds to instant, total tumor control). Cumulative cytotoxicity was calculated by taking the sum of the cumulative

15 cytotoxicity from the previous stimulation and the cytotoxicity of the current stimulation. Comparable or higher cytotoxicity was observed for iPSC-derived cells compared to primary CAR-T cells, with primary cells exhibiting greater cumulative fold expansion (Figure 21, horizontal dashed lines indicate the maximum cumulative cytotoxicity attainable over each stimulation).

[0269] The expression of T cell memory markers was assessed at baseline and end of the 4th round of stimulation using flow cytometry. Cells were assigned into T cell memory subsets based on the following expression profile: T stem cell memory (TSCM), CD62L⁺ CD45RA⁺ CD95⁺; T central memory (TCM), CD62L⁺ CD45RA⁻ CD45RO⁺; T effector memory (TEM), CD62L⁻ CD45RA⁻ CD45RO⁺; Terminally differentiated effector memory cells re-expressing CD45RA (TEMRA), CD62L⁻ CD45RA⁺ CD45RO⁺. Anti-CD3/CD28/CD2-transitioned and CD19 bead-transitioned CD8SPs had increased memory phenotypes at

25 baseline and post-stimulation 4, when compared to primary T cells (Figure 22A). The expression of various exhaustion markers was also assessed at baseline and end of stimulation 4 using flow cytometry. Anti-CD3/CD28/CD2-transitioned and CD19 bead-transitioned CD8SPs had reduced expression of exhaustion markers at baseline and post-stimulation 4, when compared to primary T cells (Figure 22B).

30 **EXAMPLE 5: Characterization of CD8⁺ SP Cells**

[0270] Deep characterization of iPSC-derived cells at the ProT stage (Day 10), DP stage (Day 22) and iPSC CD8-T (end-of-process cells; Figure 23, y-axis labels, Example 3) was performed based on CITEseq data (single cell mRNA + protein) using published datasets on thymic cells as a reference (Park et al., 2020). Gene sets corresponding to DN (early), DN(P), DN(Q), DP(P), DP(Q), CD8-T, CD8 $\alpha\alpha$ and CD8-Tmem (23A, x-axis labels) were drawn from the reference dataset (Park et al., 2020). The gene signature enrichment was calculated with single-sample gene set enrichment analysis (ssGSEA) at a single-cell level. Overall enrichment patterns were summarized as a bubble plot, with the area of each circle representing the percentage of cells expressing the signatures and the shading representing the average expression level (Figure 23A). From this analysis, it was observed that the iPSC-derived ProT (D10) cells were equivalent to DN(early) cells in human thymus (Figure 23A). Similarly, iPSC-derived day 22 cells are equivalent to an *in vivo* DP cell stage, and end-of-process cells are equivalent to *in vivo* T cells. Further analysis was performed by generating a uniform manifold approximation plot (UMAP) based on single cell mRNA and protein expression with iPSC-derived cells from D10 (ProT), D22(DP), end-of-process (iPSC CD8-T) and *in vivo* primary CD8 T cells (Figure 23B). The overlap of cells shaded light blue (end-of-process) and purple (primary T) demonstrate that iPSC-derived end-of-process cells were equivalent to in-vivo primary T cells based on single cell mRNA and protein expression. Single cell protein expression profiles were generated of different T cell markers for iPSC-derived ProT (D10), DP(D22), CD8-T (end-of-process) and primary CD8-T cells (Figure 23C).

[0271] Next, iPSC-derived end-of-process cells were characterized by flow cytometry (Figure 23) and gene expression profiles were compared to primary T cells. Gene expression profiles of cell markers were filtered with a data-driven analysis strategy using Seurat (Stuart et al., 2019) (heatmap shown in Figure 25A, columns represent cells and rows represent the filtered markers). Cell populations, as referenced to the thymic atlas dataset (Park et al., 2020) were annotated with SingleR (Aran et al., 2019) (Figure 25B). A majority of the primary T cells (above 90%) were assigned as CD8 T cells (Figure 25B).

[0272] The above approach was also applied for iPSC-derived end-of-process cells (Figure 26A, B). A majority (above 80%) of iPSC-derived end-of-process cells were assigned as DPs, and 20% of cells were assigned as CD8 T cells (Figure 26B).

EXAMPLE 6: Generation of CD8+ SP Cells with Exogenous TCR

[0273] Successful generation of Late DPs and CD8SPs in TCR-modified cell lines was carried out using the 3D protocol (n=2 clones). TCR-modified CD34+ banks were differentiated into Late DPs (Stage # 3)

via the 3D protocol as previously described (Example 3). On day 25, cultures were harvested, enriched for CD8 α using EasySep™ CD8 Positive Selection kit, and subsequently stained with CD4, CD8A, CD8B, CD3, TCRV β 1 antibodies and APC-conjugated MAGE-A4 tetramer to assess DP % and surface TCR expression (Fig. 27A, Fig. 28A). Enriched Late DPs were seeded onto Stage # 4 conditions (Example 3) at 1 x10⁶ cells/mL in medium containing IL-21 and 1.25% ImmunoCult™ activator anti-CD3/CD28/CD2 for 7 days. At the end of this stage (Day 32), flow cytometric immunophenotyping of the cultures was performed to assess expression of selected markers of T cell maturation, activation, and TCR expression. Late DPs derived from TCR-modified lines successfully transitioned into CD8 α / β + SP T cells (89% for clone 172, Fig. 27B and 78% for clone 174, Fig. 28B), with higher CD3 and TCR surface expression compared to the DP stage. Surface TCR detection was most accurate when using MAGE-A4 tetramer staining across timepoints.

[0274] TCR-modified CD8SPs (n=2 clones) generated in Stage # 4 were activated and expanded twice for a total of 14 days using the anti-CD3 antibody OKT3 and the fibronectin fragment RetroNectin® (both coated onto culture plates), and soluble CD28 co-stimulation. At the end of this stage, immunophenotyping of the expanded CD8SPs was performed through flow cytometry for selected markers of T cell memory and exhaustion. Expanded TCR-modified CD8SPs had a low exhaustion profile (14-24% LAG3+), and retained CD8 α (74-84%), surface CD3 (85-90%) and MAGE-A4 TCR expression (84-90% CD3+TCRV β 1+, >96% Tetramer+) over two rounds of expansion (Figure 29, 30). Out of the CD3+ cells, 81-87% of the population was T_{SCM}-like (CD95+CD62L+CD45RA+e) and 10-14% was T_{EFF}-like (CD95+CD62L-CD45RA+) (Figure 29, 30). Cells had greater than 80% viability, with 26X and 126X fold expansion after 2 rounds (data not shown).

[0275] At the end of this stage, cells were assessed for *in vitro* function using a flow cytometry-based cytotoxicity assay and compared to unmodified iPSC-derived CD8SPs and primary CD8SPs engineered to express the MAGE-A4 TCR. A375 wild-type (WT) cells expressing GFP were used as the target line to evaluate target-specific cytotoxicity and A375 β -2-microglobulin (B2M) KO cells expressing GFP were used to evaluate non-specific killing. A375 B2M KO cells were labelled with CellTrace™ Blue reagent and mixed at a 1:1 ratio with the A375 WT target cells. Target cells were plated, and effector cells were added at 6 different E:T ratios between 0.03125:1 and 1:1 based on the A375 WT target cell numbers. Control wells contained no effector cells. Cells were cultured for 24 hours before harvesting and were stained with a viability dye and analysed by flow cytometry. The number of viable A375 WT cells (GFP+CellTrace-) and the number of A375 B2M KO cells (GFP+CellTrace+) was quantified using counting beads for normalization. The proportion surviving of A375 WT and A375 B2M KO was calculated

separately in each well by normalizing to the control wells without effector cells (WT or B2M KO cells / average WT or B2M KO cells with 0 E:T). The percent Normalized Specific Killing was calculated by $[1 - (\text{proportion surviving WT} / \text{proportion surviving B2M KO})] \times 100$. The iPSC-derived TCR SSI CD8SPs had similar or better Normalized Specific Killing compared to Primary TCR-T cells (Figure 31), but also a higher level of background non-specific killing (not shown). iPSC-derived CD8SPs lacking the MAGE-A4 TCR did not demonstrate target-specific killing.

EXAMPLE 7: Combined Notch Inhibition and Notch Stimulation for Control of Cell Phenotype

[0276] Modulation of Notch signalling using Gamma-Secretase inhibition (GSI) was found to alter cell differentiation trajectory. iPSC-derived CD34 cells (TRAC-/- cell line, "NTX4B1") were differentiated for 10 days in LEM using an ETN bead dose of $0.5 \times (2.7 \times 10^7 \text{ beads/mL})$. On day 10, cells were harvested and re-seeded at a cell density of $1 \times 10^6 \text{ cells/mL}$ with an ETN dose of $2 \times (1.08 \times 10^8 \text{ beads/mL})$. On day 11, the gamma-secretase inhibitor DAPT was added at doses of 0.1, 0.3, 0.5, and 1 μM . DAPT was added either at day 11 only (Figure 32 panel A; 1 GSI Dose) or on day 11 as well as during media half-exchanges on day 13 and day 15 (Figure 32 panel B; 3 GSI Doses). In the absence of GSI, cell were primarily CD8 SP (~40%) or DN (~50%). The addition of a single GSI dose on day 11 resulted in a dose-dependent decrease in CD8 SP as well as increase in both CD4 ISP and DP populations (Figure 32A). With 3 GSI doses (Figure 32B), a sharper decrease in CD8 SP percentage was observed at the 0.1 μM dose. The DP percentage increased with GSI dose, while CD4 ISP peaked at 0.3-0.5 μM dose, above which the percentage declined.

[0277] Cell viability, expansion, and DP differentiation were responsive to GSI dose. Cells were differentiated in the presence of varying doses of GSI from day 11 to day 17, as described in Figure 32. The percentage of DP cells generated was dependent on both GSI dose and the number of treatments, as higher concentration and more frequent treatment resulted in more DP cells (Figure 33A). Cell viability was also responsive to GSI dose, with lower viabilities observed at doses above 0.3 μM for single GSI treatment or above 0.1 μM for 3-time GSI addition (Figure 33B). Cell expansion (relative to day 10 input) was reduced at dose above 0 or 0.1 μM for 3-time or single treatment, respectively (Figure 33C).

[0278] Between day 10 and day 17, cells were differentiated using 0.1x ETN bead dose (Figure 34, top row), 2x bead dose + 1 μM GSI (single addition; Figure 34, middle row), or 2x bead dose + 0.3 μM GSI (3 time addition; Figure 34, bottom row). All three conditions generated similar percentages of CD5+/CD7+, CD4 ISP, DP, and CD8ab+ cells, suggesting that different, orthogonal methods of controlling

Notch signalling levels – ETN bead dose, GSI concentration, and frequency of GSI addition – can produce equivalent cell populations.

[0279] A GSI dose-response was performed as described in Figure 32. As a control, cells seeded on 2D ETN were also treated with GSI (single GSI addition only). For both CAR and WT cells, 2D ETN had more DP cells at 0 GSI dose than 3D ETN (Figure 35A, B). Cells differentiated on 2D ETN had peak DP levels at 0.3 μ M GSI dose, while DPs were most abundant at 1.0 μ M and 0.75 μ M for one-time (3D-1T) and three-time (3D-3T) GSI addition with 3D ETN, respectively. The differences in dose-response curves for 2D and 3D ETN are likely related to higher level of Notch signalling imparted by the 3D ETN. Both CAR and WT cells differentiated with 2D and 3D ETN demonstrated a GSI dose-dependent increase in DP cell population.

[0280] Cells were differentiated as described in Figure 35. The percentage of CD8 α SP, DP, DN, and CD4 ISP as a function of GSI dose is shown for CAR (Figure 36, left side) and unmodified (Figure 36, right side) cells. The distribution of cells in each quadrant is shown for 3D ETN with 3 GSI dose (Figure 36, top row), 3D ETN with single GSI dose (Figure 36, middle row), and 2D ETN with single GSI dose (Figure 36, bottom row). CAR and WT cells differentiated with 2D and 3D ETN demonstrated a GSI dose-dependent increase in DP population.

EXAMPLE 8: TCR Activation

[0281] Viability and cumulative-fold expansion kinetics for iPSC-derived TCR-modified cells were assessed during Stages 1-3 of the 3D differentiation protocol (Example 3). During the first 10 days of the process, the emerging TCR-modified proT cells remained highly viable and expansion was at its highest (between 200-340 fold; Figure 37A, B). Once proT cells entered stage # 2 (Day 10-17), a substantial decrease in viability was observed with minimal increase in fold-expansion (Figure 37A, B). During Stage # 3 (Day 17 onwards), viability decreased gradually due to DP commitment and maturation (Figure 37A, B). Despite constitutive TCR surface expression, TCR-modified lines had similar viability and expansion kinetics as the NTX6A1 parental unmodified line.

[0282] TCR-modified iPSC-derived CD34+ banks were differentiated into Late DPs (Stage # 3) via the 3D protocol as previously described (Example 3). On day 28, cultures were harvested, stained for selected markers of maturation (Figure 38, top panel) and with the remaining cells a CD8 α enrichment step was performed using EasySep™ CD8 Positive Selection kit. Flow cytometry analysis for CD4/CD8A/CD8B was performed to confirm purity of enrichment (Figure 38, bottom panel). At this timepoint, TCR-modified CD4+CD8 α /β+ DPs expressed high levels of key maturation markers like CD28 and CD2, suggesting that

the cells were at an optimal time to move onto Stage # 4 (DP-SP transition). In addition, CD137 (41BB) expression was not detected at this stage. The CD8 α enrichment resulted in the effective depletion of CD4-CD8 α / β - DN from the culture, increasing the purity of the late DP population (n=2 clones, only one is shown).

- 5 **[0283]** Enriched Late DPs were seeded onto Stage # 4 conditions at 1×10^6 cells/mL in DP-SP medium containing IL-21 for 7 days. Multiple activation conditions were tested to determine the optimal method of transition for TCR-modified lines. Two main methods were tested: two kinds of ImmunoCult™ activator (i.e. CD3-based activation) and pHC-tetramer activation (MAGE-A4 and unrelated HIV gag tetramer as control) with different co-stimulatory reagents (i.e. antigen-based activation). At the end of
- 10 Stage # 4, ImmunoCult™ activator CD3/CD28 was the best performing method of conversion as viabilities were similar to input and fold-expansions were above 0.7-fold (Figure 39, n=2 clones). In addition to the improvement in viability and fold-expansion, ImmunoCult™ activator CD3/CD28 resulted in very efficient DP-SP conversion as virtually all the Late DPs were transitioned to >90% CD8 α / β + SPs (Figure 40, conditions 1 & 3). These CD8SPs also expressed key markers of activation like CD25 and
- 15 CD69, expected to be expressed post-conversion. However, MAGE-A4 tetramer-based transition was less effective at converting DPs into CD8SP, evidenced by the high proportion of residual CD4+ CD8 α / β + DPs in culture and low expression of CD25 and CD69 (Figure 40, conditions 2, 5 and 4). Note that while the CD8 α / β + populations remained high in these conditions (>90%), it is likely because both DPs and CD8SPs expressed these markers. As expected, unrelated tetramer HIV-gag was ineffective at converting
- 20 DPs into SPs as virtually all cells were still DP cells (Figure 40, conditions 6-8).
- [0284]** Enriched Late DPs were seeded onto Stage # 4 conditions as previously described (Figures 39, 40). At the end of this stage, cell phenotype was assessed through flow cytometry for selected memory and co-stimulatory markers of interest (Figures 41, 42). CD8SPs generated using ImmunoCult™ activator (conditions 1 & 3) highly expressed key maturation markers such as CD95 (>98%), CD45RA (>80%),
- 25 CD62L (>66%), and CD27 (>92%) (Figure 41). CD2 was also expressed and was higher in condition 3 (ImmunoCult™ activator without CD2) (Figure 42). CD45RO expression was lower compared to tetramer-transitioned conditions. Other co-stimulatory molecules such as CD137, ICOS and CD28 were expressed at low levels (Figure 42). MAGE-A4 Tetramer transitioned samples (Conditions 2, 5 & 4) expressed more CD45RO (>87%) and less CD45RA (15-27%) and CD95 (69-79%) compared to the
- 30 ImmunoCult™ activator conditions (Figure 41), suggesting that this mixed population was different and phenotypically more immature than ImmunoCult™-transitioned conditions. CD2 was expressed (>67%), as well as CD27 (>80%) and in some conditions CD28 (>57%). The latter could be attributed to the

presence of DPs that also expressed this marker. Similarly, to the ImmunoCult™ activator conditions, CD137 and ICOS were lowly expressed (Figure 42). Finally, the addition of different co-stimulatory reagents during Stage # 4 did not substantially phenotype or viability and expansion kinetics post-transition (data not shown).

5 **[0285]** TCR-modified CD8SPs generated in Stage # 4 using ImmunoCult™ activator anti-CD3/CD28/CD2 (Condition # 1) or MAGE-A4 Tetramer and soluble CD28 (Condition # 2) were activated and expanded using 4 different methods: OKT3 and RetroNectin® coating and soluble anti-CD28 co-stimulation ("A"); OKT3 and RetroNectin® coating and soluble 41BB co-stimulation ("B"), OKT3 and RetroNectin® coating and soluble ICOS-L Fc co-stimulation ("C"); and T Cell TransAct™ CD3/CD28 activator ("D"). Cell counts
10 were performed at day 3 and 7 to assess viability and fold-expansion during this stage. The addition of different co-stimulatory reagents did not make a difference in viability and expansion kinetics (Figure 43). T Cell TransAct™ CD3/CD28 stimulation was the least successful condition tested. Cells generated in Condition # 1 showed a more consistent improvement in viability and fold-expansion overtime, demonstrated by the good performance observed in both TCR-modified cell lines used. In contrast, cells
15 generated in Condition # 2 had a clone-dependent response to the activation conditions tested. TCR-modified Clone 172 showed poor overall performance compared to Clone 174.

[0286] TCR-modified CD8SPs generated in Stage # 4 using ImmunoCult™ activator CD3/CD28/CD2 (Condition # 1) or MAGE-A4 Tetramer and soluble anti-CD28 (Condition # 2) were activated and expanded using the 4 different methods described previously (see Fig. 43). At the end of this stage, cell
20 phenotype was assessed through flow cytometry for selected markers of interest. The addition of different co-stimulatory reagents did not make a difference in CD8SP phenotype (Figure 44A, B, C, D). CD8α/β+ surface expression was retained in all conditions tested, however a steeper decline in this population was observed in cells derived from Condition # 2 (Fig. 44C, D). Anti-CD3/CD28/CD2-transitioned CD8SPs had less CD25 and CD69 surface expression compared to CD8SPs derived from
25 Condition # 2, suggesting that these cells were in a less activated state at the end of 1 round of expansion. Interestingly, the T Cell TransAct™ CD3/CD28 activation condition ("D") generated cells with a more activated profile compared to conditions A, B & C that used plate-coated OKT3 and RetroNectin®. In terms of memory markers, anti-CD3/CD28/CD2-transitioned CD8SPs in activation conditions A, B and C expressed a higher percentage of CD45RA (>95%) and CD62L (>88%) and
30 lower percentage of CD45RO (47-70%) compared to CD8SPs derived from Condition # 2 (Figure 45A, B). T Cell TransAct™ (Condition D) had higher % CD45RO (87.57%) compared to OKT3 and RetroNectin®-based conditions. Virtually all cells were CD95+. Anti-CD3/CD28/CD2-transitioned CD8SPs showed less

activated phenotypes, higher expression of CD45RA and CD62L, and lower expression of CD45RO after 1 round of expansion compared to MAGE-A4 tetramer transitioned cells (Figure 45A, B).

Example 9: Generation, Characterization, and Function of iPSC-derived TCR-T cells

- 5 **[0287]** Clonal iPSC lines were generated with a MAGEA4 T cell receptor (TCR) integrated at the TRAC locus (schematic, Figure 46A). Clones were generated with a Namocell™ single cell deposition system and screened for targeted bi-allelic insertion at TRAC locus using digital droplet polymerase chain reaction (ddPCR). TCR expression was characterized at the end of clone production for 4 selected bi-allelic iPSC clones (Figure 46B). Vector copy number (VCN) values were determined for genetic
- 10 characterization of copy number integration at the TRAC locus using a ddPCR copy number variation (CNV) assay (Biorad) by amplifying the genomic TRAC locus - TCR transgene junction (Figure 46B). A representative scatter plot of selected clone (174) and unedited control is shown in Figure 46C. Genomic stability analysis was performed on selected clone 174 (Figure 46D, E). G-band karyotyping conducted by WiCell showed normal karyotype (Figure 46D) and a summary of iCS-digital™ assay (Stem Genomics)
- 15 showed expected copy number in 24 genomic regions of recurrent iPSC abnormalities (Figure 46E).
- [0288]** iPSC-derived MAGE-A4 TCR-expressing CD34⁺ hematopoietic progenitor cells (HPCs) were differentiated into CD8SP T cells using a 4-stage protocol (schematic, Figure 47A). During Stage 1, CD34 HPCs were seeded onto 2D ETN coated vessels (Example 1) in progenitor expansion medium (SFEM II + LEM) and cultured for 10 days to generate CD34-CD7⁺CD5⁺ ProT cells (Stage 2). At this point, cells were
- 20 moved onto Stage 3, where ProT cells were cultured with DL4 and VCAM-1 paramagnetic beads at various doses for 18 days (Example 3) to allow for the emerge of mature Late DPs (CD4⁺CD8A⁺CD8B⁺CD3⁺TCRαβ⁺). Lastly, Stage 4 started with the enrichment of Late DPs via a CD8 Positive Selection kit (STEMCELL Technologies), and activation using ImmunoCult™ activator anti-CD3/CD28 for 7 days to allow for DP to SP conversion to occur.
- 25 **[0289]** Stage 1 CD34⁺ HPCs expressed high levels of CD43 and CD45 hematopoietic markers and intermediate expression of TCR was detected using a TCRVβ1 antibody (Figure 47B). Stage 2 ProT cells were predominantly CD7⁺ (93.41%), co-expressing CD34 (13.88%), CD5 (21.19%) and CD56 (24.78%) (Figure 47C). Stage 3 Late DPs were primarily CD4⁺CD8A⁺CD8B⁺ (approximately 86%) (Figure 47D,E), with high levels of CD5⁺CD7⁺ and CD28⁺CD2⁺ co-stimulatory molecules (Figure 47E). Stage 4 CD8SPs
- 30 were 73.14% CD4⁺CD8A⁺, co-expressing high levels of CD8B (Figure 47F). The resulting CD8SPs also expressed key activation markers (CD25 and CD69) (Figure 47F), a low exhaustion profile (12.47%

LAG3+) (Figure 47F), a mix of T_{SCM}, T_{CM} and T_{EM} memory phenotypes (Figure 47G) and were predominantly CD2+ and CD27+ (Figure 47H).

[0290] MAGE-A4 TCR expression kinetics were analyzed during T cell differentiation. Cells were stained with APC-conjugated MAGE-A4 Tetramer reagent on day 10 (ProT), day 28 (Late DP) and day 35 (CD8SP) to detect surface TCR expression levels during T cell differentiation. While MAGE-A4 TCR expression was lower at the CD34 HPC stage (26.07%), as cells became more T-lineage committed, TCR surface expression increased as a result (Figure 48).

[0291] TCR+ CD8 SP iPSC-T cells were amenable for downstream *in vitro* expansion while maintaining key bona fide T cell phenotypic markers. A bi-phasic iPSC-T expansion culture protocol was developed, comprised of activation and maintenance phases (Figure 49A). Viability and fold expansion (relative to day 0) was analyzed over a 7 day culture period (Figure 49B). As assessed by flow cytometry, key bona fide T cell phenotypic markers were maintained by end of the expansion protocol (Figure 49C). CD8SP cells still retained predominantly stem cell memory phenotype while largely lacking common exhaustion markers (Figure 49D).

[0292] Next, the cytotoxicity and specificity of TCR+ CD8 SP iPSC-T cells was assessed in an *in vitro* serial restimulation assay (schematic, Figure 50A). Effector cells, either TCR+ CD8 SP iPSC-T cells or primary CD8+ cells transduced with an exogenous TCR using adeno-associated virus (AAV), were seeded with Nuclight™ green-labeled tumor targets at multiple effector to target (E:T) ratios and co-cultured for 5 days in an Incucyte® to monitor cytotoxicity (Stim 1). Following harvest at day 5, effector cells from the 2:1 E:T ratio were counted and reseeded at a 2:1 E:T for a subsequent stimulation with new tumor targets. This process was repeated for a total of 4 rounds of activity (19 days total).

[0293] T cell phenotype was monitored by flow cytometry throughout the restimulation assay. E:T ratio and assay duration (rounds of activation) influenced co-expression of inhibitory receptors PD-1, TIGIT, LAG3, TIM3 and CD39 on effector cells, as a measure of CD8 T cell exhaustion (Figure 50B).

[0294] Using an Incucyte® to monitor target cell killing, cells were challenged for a single round of activation at progressively lower E:T ratios. The difference in cytotoxicity between iPSC-derived CD8+ TCR+ and primary CD8+ TCR+ cells was most pronounced at lower E:T ratios, showing a 3-fold difference in activity (Figure 50C). Using an optimal E:T ratio for iPSC-derived CD8+ TCR+ cells (2:1), iPSC-derived CD8+ TCR+ cells demonstrated repeated killing of tumor targets comparable to Primary CD8+ TCR+ cells (Figure 50D).

[0295] To assess specificity in an Incucyte® cytotoxicity assay, iPSC-derived CD8+ TCR+ or Primary CD8+ TCR+ cells were seeded with tumor target cells with (antigen positive) or without (antigen negative)

β 2M expression. iPSC-derived CD8⁺ TCR⁺ and Primary CD8 TCR cells both demonstrated similar specificity for antigen positive cells (Figure 50E).

[0296] CITESeq was used to perform a deep characterization of iPSC-derived and primary cell populations. A Uniform Manifold Approximation and projection (UMAP) plot was generated based on
5 CITEseq data (mRNA and surface protein expression at single cell resolution) for peripheral blood-derived CD8⁺ T cells (PBMC-CD8T); peripheral blood-derived CD4⁺ T cells (PBMC-CD4-T); peripheral blood-derived T cells activated with anti-CD3/anti-CD28 beads (PBMC-T-Activated); peripheral blood-derived natural killer cells (PBMC-NK); and iPSC-derived TCR⁺ CD8⁺ cells (iPS-TCR) (Figure 51A). Further, the cell populations “iPS-TCR” and “PBMC-T-Activated” were annotated with SingleR (Aran et al., 2019)
10 in reference to the thymic atlas dataset (Park et al., 2020) (Figure 51B). Cell markers were screened based on a statistical threshold of expression and presented as a bubble plot with Seurat (Stuart et al., 2019) (Figure 51C).

[0297] Although the disclosure has been described with reference to certain specific embodiments,
15 various modifications thereof will be apparent to those skilled in the art. Any examples provided herein are included solely for the purpose of illustrating the disclosure and are not intended to limit the disclosure in any way. Any drawings provided herein are solely for the purpose of illustrating various aspects of the disclosure and are not intended to be drawn to scale or to limit the disclosure in any way. The scope of the claims appended hereto should not be limited by the preferred embodiments set forth
20 in the above description, but should be given the broadest interpretation consistent with the present specification as a whole. The disclosures of all art recited herein are incorporated herein by reference in their entirety.

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5 **CLAIMS**

We claim:

1. A method of generating a population of CD4-CD8+ T cells, comprising:
 - contacting a population of hematopoietic stem/progenitor cells with an immobilized Notch signalling ligand at a first ligand concentration, thereby generating a population of progenitor T cells;
 - 10 - contacting the population of progenitor T cells with the immobilized Notch signalling ligand at a second ligand concentration, thereby generating a population of CD4+CD8+ cells, wherein the second ligand concentration is less than the first ligand concentration; and
 - 15 - contacting the population of CD4+CD8+ cells with a T cell activator, in the absence of the Notch signalling ligand, thereby generating a population of CD4-CD8+ T cells.
2. The method of claim 1, wherein the immobilized Notch signalling ligand is DL4.
3. The method of claim 2, wherein the step of contacting the population of hematopoietic stem/progenitor cells with the immobilized Notch signalling ligand at the first ligand concentration
20 further comprises contacting the population of hematopoietic stem/progenitor cells with immobilized VCAM-1.
4. The method of one of claims 1 to 3, wherein the first ligand concentration is 3.15×10^{11} to 1.26×10^{12} molecules/mL and the second ligand concentration is 3.94×10^{10} to 6.31×10^{11} molecules/mL.
5. The method of any one of claims 1 to 4, further comprising:
 - 25 - contacting the population of CD4+CD8+ cells with the immobilized Notch signalling ligand at a third ligand concentration before contacting the population of CD4+CD8+ cells with a T cell activator, wherein the third ligand concentration is less than the second ligand concentration.
6. The method of claim 5, wherein the third ligand concentration is 3.94×10^{10} to 6.31×10^{11}
30 molecules/mL.
7. The method of any one of claims 1 to 6, further comprising enriching the CD4+CD8+ cells for CD8 α or CD8 β .

8. The method of any one of claims 1 to 7, wherein the T cell activator is a CD3 stimulator and an
35 integrin ligand.
9. The method of claim 8, wherein the CD3 stimulator is an anti-CD3/anti-CD28/anti-CD2 reagent, an anti-CD3 reagent, or a peptide-major histocompatibility complex (pMHC) tetramer, and the integrin ligand is laminin, ICAM, fibronectin, a fibronectin fragment, VCAM-1, or ICOS-L.
10. The method of claim 9, wherein the CD3 stimulator is an anti-CD3/anti-CD28/anti-CD2 reagent
40 and the integrin ligand is a fibronectin fragment.
11. The method of any one of claims 1 to 7, wherein the T cell activator is a chimeric antigen receptor (CAR) activator.
12. The method of claim 11, wherein the CAR activator is an antigen immobilized on a substrate.
13. The method of claim 12, wherein the antigen is a CD19 antigen, and wherein the substrate is a
45 particle.
14. The method of any one of claims 1 to 13, wherein the step of contacting the population of CD4+CD8+ cells with the T cell activator, in the absence of the Notch signalling ligand, is conducted in a cell culture medium comprising IL-7, IL-15, and IL-21.
15. The method of any one of claims 1 to 14, wherein the step of contacting the population of
50 CD4+CD8+ cells with the T cell activator, in the absence of the Notch signalling ligand, is conducted in the absence of IL-2.
16. The method of any one of claims 1 to 15, further comprising:
- contacting the population of CD4-CD8+ T cells with a T cell activator and IL-7, IL-15, and IL-21.
- 55 17. The method of claim 16, wherein the T cell activator is a CD3 stimulator.
18. The method of claim 17, wherein the CD3 stimulator is an anti-CD3/anti-CD28/anti-CD2 reagent, an anti-CD3 reagent, or a peptide-major histocompatibility complex (pMHC) tetramer.
19. The method of any one of claims 1 to 18, further comprising increasing a cell density at the step of contacting the population of progenitor T cells with the immobilized Notch signalling ligand at the
60 second ligand concentration, and/or increasing a cell density at the step of contacting the population of

CD4+CD8+ cells with the T cell activator and an integrin ligand in the absence of the Notch signalling ligand.

20. The method of any one of claims 1 to 19 wherein the hematopoietic stem/progenitor cells are derived from pluripotent stem cells.
- 65 21. The method of any one of claims 1 to 20, wherein the population of CD4-CD8+ T cells comprises a nucleic acid sequence encoding a CAR or an exogenous T cell receptor (TCR).
22. The method of any one of claims 1 to 21, wherein the population of CD4-CD8+ T cells is CD3+.
23. The method of any one of claims 1 to 21, wherein the population of CD4-CD8+ T cells is TRAC-/-.
24. The method of any one of claims 1 to 22, wherein the population of CD4-CD8+ T cells is enriched
70 for CD8αβ+ cells.
25. The method of any one of claims 1 to 22, wherein the population of CD4-CD8+ T cells comprises a sub-population of TCRγδ+, CD49c+, and/or CD31+ cells.
26. A population of CD4-CD8+ T cells made according to the method of any one of claims 1 to 25.
27. The population of claim 26, wherein the population of CD4-CD8+ T cells comprises a nucleic acid
75 sequence encoding a CAR or an exogenous TCR.
28. The population of claim 26 or claim 27, wherein the population of CD4-CD8+ T cells is CD3+.
29. The population of any one of claims 26 to 28, wherein the population of CD4-CD8+ T cells is TRAC-/-.
30. A CD4-CD8+CD3+ TRAC-/- cell population derived in vitro from pluripotent stem cells.
- 80 31. The CD4-CD8+CD3+ TRAC-/- cell population of claim 30, comprising a nucleic acid sequence encoding a CAR or an exogenous TCR.
32. The CD4-CD8+CD3+ TRAC-/- cell population of claim 30 or claim 31, wherein the population of CD4-CD8+ T cells comprises a sub-population of TCRγδ+, CD49c+, and/or CD31+ cells.
33. A pharmaceutical composition comprising a population of CD4-CD8+ T cells and a
85 pharmaceutically acceptable carrier, wherein the population of CD4-CD8+ T cells are CD3+ and TRAC-/-.
34. A method of treating a disease or condition in a subject comprising:
 - a) generating a population of CD4-CD8+ T cells according to any one of claims 1 to 25; and

- b) administering an effective amount of the population of CD4-CD8+ T cells to a subject in need thereof.

90 35. The method of claim 34, wherein the population of CD4-CD8+ T cells is CD3+ and/or TRAC-/-.

36. The method of claim 34 or claim 35, wherein the disease or condition is a hematological malignancy, and wherein the CD4-CD8+ T cells comprise a nucleic acid sequence encoding a CAR or an exogenous T cell receptor (TCR).

37. A use of a population of CD4-CD8+ T cells in the manufacture of a medicament for the treatment
95 of a disease or condition, wherein the population of CD4-CD8+ T cells are generated by a method according to any one of claims 1 to 25.

38. A method of generating a population of CD4+CD8+ cells, comprising:

- contacting a population of hematopoietic stem/progenitor cells with an immobilized Notch signalling ligand at a first ligand concentration, thereby generating a population
100 of progenitor T cells; and
- contacting the population of progenitor T cells with the immobilized Notch signalling ligand at a second ligand concentration, thereby generating a population of CD4+CD8+ cells, wherein the second ligand concentration is less than the first ligand concentration.

39. The method of claim 38, wherein the immobilized Notch signalling ligand is DL4.

105 40. The method of claim 38 or claim 39, wherein the first ligand concentration is 3.15×10^{11} to 1.26×10^{12} molecules/mL and the second ligand concentration is 3.94×10^{10} to 6.31×10^{11} molecules/mL.

41. The method of any one of claims 38 to 40, wherein the population of CD4+CD8+ cells are CD1a+CD28+ and ICOS+.

42. The method of any one of claims 38 to 41, wherein the population of CD4+CD8+ cells comprise a
110 nucleic acid sequence encoding a CAR or an exogenous TCR.

43. A population of CD4+CD8+ cells made according to the method of any one of claims 38 to 42.

44. A population of CD4+CD8+ cells derived in vitro from pluripotent stem cells, wherein the CD4+CD8+ cells are CD1a+CD28+ and ICOS+.

45. A method of generating a population of CD4-CD8+ T cells, comprising:

- 115 - contacting a population of hematopoietic stem/progenitor cells with an immobilized Notch signalling ligand, thereby generating a population of progenitor T cells;
- contacting the population of progenitor T cells with a Notch signalling inhibitor and the immobilized Notch signalling ligand, thereby generating a population of CD4+CD8+ cells; and
- 120 - contacting the population of CD4+CD8+ cells with a T cell activator, in the absence of the Notch signalling ligand, thereby generating a population of CD4-CD8+ T cells.

46. The method of claim 45, wherein the activity of the immobilized Notch signalling ligand is decreased in the step of contacting the population of progenitor T cells with the Notch signalling inhibitor and the immobilized Notch signalling ligand, in comparison to the activity of the immobilized

125 Notch signalling ligand in the step of contacting the population of hematopoietic stem/progenitor cells with the immobilized Notch signalling ligand.

47. The method of claim 45 or 46, wherein the Notch signalling inhibitor is a gamma-secretase inhibitor.

48. The method of claim 47, wherein the gamma-secretase inhibitor is provided at a concentration

130 of 0.1 to 1 micromolar.

49. The method of any one of claims 45 to 48, wherein:

- the immobilized Notch signalling ligand is provided at a first ligand concentration of 3.15×10^{11} to 1.26×10^{12} molecules/mL in the step of contacting the population of hematopoietic stem/progenitor cells with the immobilized Notch signalling ligand;
- 135 and/or
- the immobilized Notch signalling ligand is provided at a second ligand concentration of 3.15×10^{11} to 2.52×10^{12} molecules/mL in the step of contacting the population of progenitor T cells with the Notch signalling inhibitor and the immobilized Notch signalling ligand.

140 50. A method of generating a population of CD4+CD8+ cells, comprising:

- contacting a population of hematopoietic stem/progenitor cells with an immobilized Notch signalling ligand, thereby generating a population of progenitor T cells; and

- contacting the population of progenitor T cells with a Notch signalling inhibitor and the immobilized Notch signalling ligand, thereby generating a population of CD4⁺CD8⁺ cells.

- 145 51. The method of claim 50, wherein the activity of the immobilized Notch signalling ligand is decreased in the step of contacting the population of progenitor T cells with the Notch signalling inhibitor and the immobilized Notch signalling ligand, in comparison to the activity of the immobilized Notch signalling ligand in the step of contacting the population of hematopoietic stem/progenitor cells with the immobilized Notch signalling ligand.
- 150 52. The method of claim 50 or claim 51, wherein the Notch signalling inhibitor is a gamma-secretase inhibitor.
53. The method of claim 52, wherein the gamma-secretase inhibitor is provided at a concentration of 0.1 to 1 micromolar.
54. The method of any one of claims 50 to 53, wherein:
- 155 - the immobilized Notch signalling ligand is provided at a first ligand concentration of 3.15×10^{11} to 1.26×10^{12} molecules/mL in the step of contacting the population of hematopoietic stem/progenitor cells with the immobilized Notch signalling ligand; and/or
- 160 - the immobilized Notch signalling ligand is provided at a second ligand concentration of 3.15×10^{11} to 2.52×10^{12} molecules/mL in the step of contacting the population of progenitor T cells with the Notch signalling inhibitor and the immobilized Notch signalling ligand.
55. A method of treating a disease or condition in a subject comprising:
- a) generating a population of CD4-CD8⁺ T cells according to any one of claims 45 to 49; and
- 165 b) administering an effective amount of the population of CD4-CD8⁺ T cells to a subject in need thereof.
56. The method of claim 55, wherein the population of CD4-CD8⁺ T cells is CD3⁺ and/or TRAC^{-/-}.
57. Use of a population of CD4-CD8⁺ T cells in the manufacture of a medicament for the treatment of a disease or condition, wherein the population of CD4-CD8⁺ T cells are generated by the method
- 170 according to any one of claims 45 to 49.