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

FIELD

[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

[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-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 CD8αβ+ T cells (Iriguchi et al., 2021).
It is desirable to obviate or mitigate one or more of the above deficiencies.

SUMMARY OF THE DISCLOSURE

[0004] In a first example 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 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. In one aspect, the disclosure provides 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; 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 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.

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

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

[0007] In an example, the first ligand concentration is 3.15 x10^{11} to 1.26 x10^{12} molecules/mL and the second ligand concentration is 3.94 x10^{10} to 6.31 x10^{11} molecules/mL.

[0008] In an example, 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 ligand concentration, before contacting the population of CD4+CD8+ cells with a T cell activator.

[0009] In an example, the third ligand concentration is 3.94 x10^{10} to 6.31 x10^{11} molecules/mL.

[0010] In an example, the method further comprises enriching the CD4+CD8+ cells for CD8α or CD8β.

[0011] In an example, the T cell activator is a CD3 stimulator and an integrin ligand.

[0012] In an example, 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 example, the CD3 stimulator is an anti-CD3/anti-CD28/anti-CD2 reagent and the integrin ligand is a fibronectin fragment.

[0014] In an example, the T cell activator is a chimeric antigen receptor (CAR) activator.

[0015] In an example, the CAR activator is an antigen immobilized on a substrate.

[0016] In an example, the antigen is a CD19 antigen, and the substrate is a particle.

[0017] In an example, 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.

[0018] In an example, 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 example, 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 the absence of IL-2.

[0020] In an example, 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 example, 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.

[0022] In an example, the T cell activator is a CD3 stimulator.

[0023] In an example, 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 example, 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 example, the hematopoietic stem/progenitor cells are derived from pluripotent stem cells.

[0026] In an example, 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 example, the population of CD4-CD8+ T cells is CD3+.

[0028] In an example, the population of CD4-CD8+ T cells is TRAC-/-.

[0029] In an example, the population of CD4-CD8+ T cells is enriched for CD8αβ+ cells.

[0030] In an example, the population of CD4-CD8+ T cells comprises a sub-population of TCRγδ+, CD49c+, and/or CD31+ cells.

[0031] In a second example of the disclosure, a population of CD4-CD8+ T cells made according to the method of the first example is provided. In one aspect, the disclosure provides a population of CD4-CD8+ T cells made according to the method as disclosed herein.

[0032] In an example, the population of CD4-CD8+ T cells comprises a nucleic acid sequence encoding a CAR or an exogenous TCR.

[0033] In an example, the population of CD4-CD8+ T cells is CD3+.

[0034] In an example, the population of CD4-CD8+ T cells is TRAC-/-.

[0035] In a third example 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. In one aspect, the disclosure provides a CD4-CD8+CD3+ TRAC-/- cell population derived in vitro from pluripotent stem cells, wherein a) the cell population optionally comprises a nucleic acid sequence encoding (i) a CAR or an exogenous TCR; and/or (ii) a CAR or an exogenous TCR; and/or b) the population of CD4-CD8+ T cells comprises a sub-population of TCRγδ+, CD49c+, and/or CD31+ cells.

[0036] In an example, the CD4-CD8+CD3+ TRAC-/- cell population of the third example comprises a nucleic acid sequence encoding a CAR or an exogenous TCR.

[0037] In an example, the population of CD4-CD8+ T cells comprises a sub-population of TCRγδ+, CD49c+, and/or CD31+ cells.

[0038] In a fourth example 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-/-. In one aspect, the disclosure provides a pharmaceutical composition comprising a population of CD4-CD8+ T cells and a pharmaceutically acceptable carrier, wherein the population of CD4-CD8+ T cells are CD3+ and TRAC-/-.

[0039] In a fifth example 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 example; and administering an effective amount of the population of CD4-CD8+ T cells to a subject in need thereof.

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

[0041] In an example, 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 example 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 example. In one aspect, the disclosure provides a 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 a method as disclosed herein.

[0043] In a seventh example 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. In one aspect, the disclosure provides 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 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.

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

[0045] In an example, the first ligand concentration is 3.15 $x10^{11}$ to 1.26 $x10^{12}$ molecules/mL and the second ligand concentration is 3.94 $x10^{10}$ to 6.31 $x10^{11}$ molecules/mL.

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

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

[0048] In an eight example 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 example.

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

[0050] In a tenth example 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. In one aspect, the disclosure provides 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, 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 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.

[0051] In an example, 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 example, the Notch signalling inhibitor is a gamma-secretase inhibitor.

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

[0054] In an example, the Notch signalling inhibitor is provided at a first ligand concentration of 3.15 $x10^{11}$ to 1.26 $x10^{12}$ 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 $x10^{11}$ to 2.52 $x10^{12}$ molecules/mL in the in the step of contacting the population of progenitor T cells with the Notch signalling inhibitor and the immobilized Notch signalling ligand.

[0055] In an eleventh example 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 contacting the population of progenitor T cells with a Notch signalling inhibitor and the immobilized Notch signalling ligand. In one aspect, the disclosure provides 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.

[0056] In an example, 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.

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

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

[0059] In an example, the immobilized Notch signalling ligand is provided at a first ligand concentration of 3.15 $x10^{11}$ to 1.26 $x10^{12}$ 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 $x10^{11}$ to 2.52 $x10^{12}$ molecules/mL in the step of contacting the population of progenitor T cells with the Notch signalling inhibitor and the immobilized Notch signalling ligand.

[0060] In a twelfth example 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 example; and administering an effective amount of the population of CD4-CD8+ T cells to a subject in need thereof.

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

[0062] In a thirteenth example 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 CD4-CD8+ T cells are generated according to the method of the eleventh example.

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.

[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 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).

[0069] FIG. 3B is flow cytometry plots characterizing marker expression of hematopoeitic 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 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-CD8α+ single-positive (CD8a SP) cell populations generated from iPSC-derived HSPCs with varying doses of engineered thymic niche (ETN) bead (0.1x, 0.5x, 1x bead dose) and cell densities (2 $x10^6$, 1 $x10^6$, 5 $x10^5$ cells/mL).