

SUMMARY

[0005] In a first aspect, the present disclosure refers to a method of producing antigen-specific effector T cells and/or NK cells comprising (a) engineering pluripotent stem cells (PSCs) to express a chimeric antigen receptor (CAR), thereby producing CAR-PSCs; (b) differentiating the CAR-PSCs to CD34⁺ hematopoietic progenitor cells (HPCs); (c) further differentiating the CD34⁺ HPCs to T cells and/or NK cells; and (d) expanding the T cells and/or NK cells, wherein expanding comprises co-culturing with antigen-specific target cells, thereby producing antigen-specific effector T cells and/or NK cells, wherein step (b) comprises performing directed differentiation comprising (ba) generating embryoid bodies (EBs) in the presence of blebbistatin, a GSK-3 inhibitor, FGF2, and VEGF; (bb) contacting the EBs with BMP-4, VEGF, and FGF2 to induce mesoderm induction; and (bc) differentiating the EBs in the presence of Flt-3 ligand, IL3, SCF, and TPO, thereby producing HPCs. In one example, there is disclosed a method of producing antigen-specific effector T cells and/or NK cells comprising engineering pluripotent stem cells (PSCs) to express a chimeric antigen receptor (CAR), thereby producing CAR-PSCs; differentiating or forward reprogramming the CAR-PSCs to CD34⁺ hematopoietic progenitor cells (HPCs); further differentiating the CD34⁺ HPCs to T cells and/or NK cells; and expanding the T cells and/or NK cells. In some examples, expanding comprises co-culturing with antigen-specific target cells, thereby producing antigen-specific effector T cells and/or NK cells.

[0006] In some examples, the PSCs engineered to express a CAR are induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs). In another examples, the iPSCs are reprogrammed from somatic cells, such as T cells.

[0007] In some examples, the step of differentiating CAR-PSCs to CD34⁺ HPCs comprises performing directed differentiation. In certain examples, directed differentiation comprises generating embryoid bodies (EBs) in the presence of blebbistatin, a GSK-3 inhibitor, FGF2, and VEGF; contacting the EBs with BMP4, VEGF, and FGF2 to induce mesoderm induction; and differentiating the EBs in the presence of Flt-3 ligand, IL-3, SCF, and TPO, thereby producing HPCs. In some examples, the differentiating is in media essentially free of or free of BMP4. In other examples, the differentiating is in the presence of BMP4. In particular examples, the GSK-3 inhibitor is CHIR99021. In some examples, differentiating further comprises the presence of IL-11, cAMP, and/or VEGF.

[0008] In some examples, directed differentiation comprises culturing individualized PSCs on an amine-coated surface in the presence of blebbistatin, BMP4, VEGF, and bFGF; initiating differentiation by contacting the PSCs with BMP4, VEGF, and FGF2; and further differentiating the PSCs in the presence of Flt-3 ligand, IL-3, IL-6, SCF, TPO, and heparin, thereby producing HPCs, wherein the method does not comprise the formation of EBs.

[0009] In particular examples, the method comprises culturing the cells under defined, feeder-free conditions, such as for the duration of the whole method. In some examples, the PSCs are essentially transgene-free or are transgene-free. In particular examples, the PSCs are human. In certain examples, the T cells are CD4⁺ T cells, CD8⁺ T cells, cytotoxic T cells, regulatory T cells, natural killer T cells, naïve T cells, memory T cells, and/or gamma delta T cells.

[0010] In certain examples, the PSCs engineered to express a CAR are further engineered to express ERG/ETV2, GATA2, and HOXA9, such as under the control of a single inducible promoter. In some examples, the method further comprises engineering the PSCs to express HMGA2, MYCN, NR4A2, SOX17, TFEC, MEIS1, and/or HOXA4. Thus, in some examples, the step of differentiating CAR-PSCs to CD34⁺ HPCs comprises inducing expression of ERG/ETV2, GATA2, and HOXA9 for a period of time sufficient to produce HPCs and terminating the induction of expression prior to further differentiating the HPCs to T cells and/or NK cells.

[0011] In specific examples, the step of differentiating CAR-PSCs to CD34⁺ HPCs further comprises selecting for cells that express CD34 and/or CD43 prior to differentiating to antigen-specific T cells and/or NK cells. In particular examples, selecting comprises performing magnetic-activated cell sorting (MACS). In some examples, the cells that express CD34 and/or CD43 comprise at least 25, 30, 35, 40, 45, 50, 60, 65, 70, 75, 80, 85, 90, or higher percent of the total cell population. In some examples, the step of performing MACs to select for CD34 and/or CD43 positive cells is not performed.

[0012] In particular examples, at least 2, 3, 4, 5, 10, 15, 20, or higher percent of the HPCs express the CAR. In some examples, at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45 or higher percent of the HPCs express the CAR.

[0013] In some examples, differentiating of HPCs to T cells and/or NK cells comprises culturing the HPCs on a retronectin and Notch DLL-4 coated surface in the

presence of ascorbic acid and nicotinamide under hypoxic conditions. In some examples, the culture further comprises SCF, FLT-3 ligand, TPO, and IL-7, and optionally comprises a GSK inhibitor (*e.g.*, CHIR99021), IL-2, and/or IL-12. In certain examples, the expanding step further comprises culturing the antigen-specific T cells in the presence of anti-CD3 antibody and IL-2. In further examples, the expanding step further comprises culturing the antigen-specific T cells in the presence of anti-CD3 antibody, IL-2, IL-15, and IL-21. In some examples, the expanding step comprises culturing the antigen-specific T cells in the presence of anti-CD3 antibody, FLT3-ligand, IL-7, IL-2, and/or IL-15, and optionally further comprises SCF, TPO, and/or IL-21. In particular examples, at least 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50 or higher percent of the differentiated CD34⁺ HPCs are CD3⁺CAR⁺ T cells. In some examples, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 50, or higher percent of the differentiated CD34⁺ HPCs are CD3⁺CAR⁺ NK cells. In some examples, at least 2 percent of the expanded CD34⁺ HPCs are CD3⁺CAR⁺ T cells. In particular examples, at least 10 percent of the expanded CD34⁺ HPCs are CD3⁺CAR⁺ NK cells.

[0014] In certain examples, the CAR and the antigen-specific target cells are directed to the same antigen. In one specific examples, the antigen is CD19. In particular examples, the antigen-specific target cells are tumor cells. In some examples, the antigen-specific target cells are human. In particular examples, the antigen-specific target cells are HLA class I negative. In some examples, at least 5, such as 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 50, or higher, percent of the antigen-specific effector T cells display cytotoxic activity against target cells. In certain examples, at least 10, such as 15, 20, 25, 30, 35, 40, 45, 50, or higher, percent of the antigen-specific effector NK cells display cytotoxic activity against target cells.

[0015] In some examples, the CAR is encoded by DNA integrated into the genome of the PSCs. In certain examples, the CAR comprises an intracellular signaling domain, a transmembrane domain, and an extracellular domain comprising an antigen binding region. In specific examples, the intracellular signaling domains comprise CD3 ζ and CD28. In some examples, the antigen binding region is a F(ab')₂, Fab', Fab, Fv, or scFv.

[0016] In certain examples, the PSCs are HLA homozygous. In some examples, the HLA homozygous PSCs are homozygous for one or more of the loci alleles HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DP or HLA-DQ. In certain examples, the HLA homozygous PSCs are homozygous for two of the loci alleles HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DP or

HLA-DQ. In particular examples, the HLA homozygous PSCs are homozygous for HLA-A and HLA-B. In certain examples, the HLA homozygous PSCs are homozygous for HLA-A, HLA-B, and HLA-C.

5 [0017] Another example describes a method of producing antigen-specific effector T cells and/or NK cells comprising engineering PSCs to express a CAR, thereby producing CAR-PSCs; culturing the CAR-PSCs in the presence of blebbistatin, a GSK-3 inhibitor, FGF2, and VEGF, thereby generating EBs; contacting the EBs with BMP4, VEGF, and FGF2 to induce mesoderm induction; differentiating the EBs in the presence of Flt-3 ligand, BMP4, IL-3, SCF, and TPO, thereby producing CD34⁺ HPCs; further differentiating the
10 CD34⁺ HPCs to T cells and/or NK cells; and expanding the T cells and/or NK cells, wherein expanding comprises co-culturing with antigen-specific target cells, thereby producing antigen-specific effector T cells and/or NK cells. In particular examples, the GSK-3 inhibitor is CHIR99021.

15 [0018] In yet another example, there is described a method of producing antigen-specific effector T cells and/or NK cells comprising engineering PSCs to express a CAR, thereby producing CAR-PSCs; culturing individualized CAR-PSCs on an amine-coated surface in the presence of blebbistatin, BMP4, VEGF, and initiating differentiation by contacting the CAR-PSCs with BMP4, VEGF, and FGF2; further differentiating the CAR-PSCs in the presence of Flt-3 ligand, IL-3, IL-6, SCF, TPO, and heparin, thereby producing
20 CD34⁺ HPCs; differentiating the CD34⁺ HPCs to T cells and/or NK cells; and expanding the T cells and/or NK cells, wherein expanding comprises co-culturing with antigen-specific target cells, thereby producing antigen-specific effector T cells and/or NK cells, wherein the method does not comprise the formation of EBs.

[0019] A further example describes a method of producing antigen-specific effector T cells and/or NK cells comprising engineering PSCs to express a CAR, thereby producing CAR-PSCs; differentiating the CAR-PSCs to CD34⁺ HPCs; selecting for CD34⁺CD43⁺ HPCs; further differentiating the CD34⁺CD43⁺ HPCs to T cells and/or NK cells; and
5 expanding the T cells and/or NK cells, wherein expanding comprises co-culturing with antigen-specific target cells, thereby producing antigen-specific effector T cells and/or NK cells.

[0020] In another examples, there is described a population of antigen-specific effector T cells and/or NK cells produced according to the examples described above. Also
10 provided herein is a pharmaceutical composition comprising the antigen-specific effector T cells and/or NK cells produced according to the examples described herein. Further provided herein is a composition comprising the antigen-specific effector T cells and/or NK cells produced according to the examples for the treatment of cancer in a subject. Also provided herein is the use of the antigen-specific effector T cells and/or NK cells produced according
15 to the examples for the treatment of cancer.

[0021] In yet another example, there is disclosed a method of treating cancer in a subject comprising administering an effective amount of the antigen-specific effector T cells and/or NK cells produced according to the examples described herein to the subject. In some examples, the cancer expresses a tumor antigen and the antigen-specific effector T cells
20 and/or NK cells are directed to said tumor antigen. In particular examples, the subject is human.

[0022] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the

CLAIMS

1. A method of producing antigen-specific effector T cells and/or NK cells comprising:
 - (a) engineering pluripotent stem cells (PSCs) to express a chimeric antigen receptor (CAR), thereby producing CAR-PSCs;
 - (b) differentiating the CAR-PSCs to CD34⁺ hematopoietic progenitor cells (HPCs);
 - (c) further differentiating the CD34⁺ HPCs to T cells and/or NK cells; and
 - (d) expanding the T cells and/or NK cells, wherein expanding comprises co-culturing with antigen-specific target cells, thereby producing antigen-specific effector T cells and/or NK cells,wherein step (b) comprises performing directed differentiation comprising
 - (ba) generating embryoid bodies (EBs) in the presence of blebbistatin, a GSK-3 inhibitor, FGF2, and VEGF;
 - (bb) contacting the EBs with BMP-4, VEGF, and FGF2 to induce mesoderm induction; and
 - (bc) differentiating the EBs in the presence of Flt-3 ligand, IL3, SCF, and TPO, thereby producing HPCs.
2. The method of claim 1, wherein the PSCs of step (a) are induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs), preferably wherein the iPSCs are reprogrammed from T cells.
3. The method of claim 1 or 2, wherein differentiating of step (bc) is essentially free of BMP4, preferably wherein differentiating of step (bc) is free of BMP4; or wherein differentiating of step (bc) further comprises the presence of IL-11, cAMP, and/or VEGF, preferably wherein differentiating of step (bc) further comprises the presence of IL-11, cAMP, and VEGF.
4. The method of claim 1, wherein the GSK-3 inhibitor is CHIR99021.
5. The method of any one of claims 1-4, wherein the PSCs are transgene-free, preferably wherein the PSCs are human.
6. The method of claim 1 or claim 2, wherein the T cells are CD4⁺ T cells, CD8⁺ T cells, cytotoxic T cells, regulatory T cells, natural killer T cells, naïve T cells, memory T cells, or gamma delta T cells.
7. The method of claim 1, wherein the PSCs of step (a) are further engineered to express ERG/ETV2, GATA2, and HOXA9 under the control of a single inducible promoter,

preferably further comprising engineering the PSCs to express HMGA2, MYCN, NR4A2, SOX17, TFEC, MEIS1, and HOXA4.

8. The method of claim 1, wherein the method comprises culturing the cells under defined, feeder-free conditions.

9. The method of claim 1, wherein step (b) further comprises selecting for cells that express CD34 and CD43 prior to differentiating to antigen-specific T cells and/or NK cells,

preferably wherein selecting comprises performing magnetic-activated cell sorting (MACS); or

preferably wherein the cells that express CD34 and CD43 comprise at least 35 percent of the total cell population; or

preferably wherein the cells that express CD34 and CD43 comprise at least 65 percent of the total cell population.

10. The method of claim 1, wherein at least 5 percent of the HPCs express the CAR; or wherein at least 10 percent of the HPCs express the CAR; or wherein at least 15 percent of the HPCs express the CAR.

11. The method of claim 1, wherein differentiating of step (c) comprises culturing the HPCs on a retronectin and Notch DLL-4 coated surface in the presence of ascorbic acid and nicotinamide under hypoxic conditions,

preferably wherein the culture further comprises SCF, FLT-3 ligand, TPO, and IL7, more preferably wherein the culture further comprises a GSK-3 inhibitor, IL-2, and/or IL-12; or more preferably wherein the culture further comprises a GSK-3 inhibitor, IL-2, and IL-12; or

preferably wherein at least 1.5 percent of the differentiated CD34⁺ HPCs are CD3⁺CAR⁺ T cells; or

preferably wherein at least 2 percent of the differentiated CD34⁺ HPCs are CD3⁻CAR⁺ NK cells; or

preferably wherein at least 10 percent of the expanded CD34⁺ HPCs are CD3⁻CAR⁺ NK cells.

12. The method of claim 1, wherein expanding of step (d) further comprises culturing the antigen-specific T cells in the presence of anti-CD3 antibody, IL-2, and IL-15, preferably wherein at least 2 percent of the expanded CD34⁺ HPCs are CD3⁺CAR⁺ T cells; or wherein expanding of step (d) further comprises culturing the antigen-specific T cells in the presence of anti-CD3 antibody, IL-2, IL-15, and IL-21; or

wherein expanding of step (d) further comprises culturing the antigen-specific T cells in the presence of anti-CD3 antibody, FLT3-ligand, IL-7, IL-2, IL-15, and/or IL-21; or wherein expanding of step (d) further comprises one or two additional components selected from the group consisting of SCF and TPO.

13. The method of claim 1, wherein the CAR and the antigen-specific target cells are directed to the same antigen, preferably wherein the antigen is CD19; or wherein the antigen-specific target cells are tumor cells; or

wherein the antigen-specific target cells are human;

preferably wherein the antigen-specific target cells are HLA class I negative, more preferably wherein at least 5 percent of the antigen-specific effector T cells display cytotoxic activity against target cells or wherein at least 40 percent of the antigen-specific effector NK cells display cytotoxic activity against target cells.

14. The method of claim 1, wherein the CAR is encoded by DNA integrated into the genome of the PSCs; or

wherein the CAR comprises an intracellular signaling domain, a transmembrane domain, and an extracellular domain comprising an antigen binding region, preferably wherein the intracellular signaling domains comprise CD3 ζ and CD28 or wherein the antigen binding region is a F(ab')₂, Fab', Fab, Fv, or scFv.

15. The method of claim 1, wherein the PSCs are HLA homozygous, preferably wherein the HLA homozygous PSCs are homozygous for one or more of the loci alleles HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DP or HLA-DQ; or

preferably wherein the HLA homozygous PSCs are homozygous for two of the loci alleles HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DP or HLA-DQ, more preferably wherein the HLA homozygous PSCs are homozygous for HLA-A and HLA-B; or

preferably wherein the HLA homozygous PSCs are homozygous for HLA-A, HLA-B, and HLA-C.