STROMA-FREE T CELL DIFFERENTIATION FROM HUMAN PLURIPOTENT STEM CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 62/964,857 filed January 23, 2020, and U.S. Provisional Application No. 63/025,412 filed May 15, 2020, the contents of each of which are incorporated herein by reference in their entireties.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under Grant No. 2U01DK104218 awarded by the National Institutes of Health. The government has certain rights in the invention.

SEQUENCE LISTING

[0003] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on January 22, 2021, is named 701039-096580WOPT_SL.txt and is 108,672 bytes in size.

TECHNICAL FIELD

[0004] The technology described herein relates to immune cell differentiation methods.

BACKGROUND

[0005] There is a lack of supply of functional immune cells for the *in vivo* cellular replacement therapy, therapy for a host of diseases, disorders and conditions, and for the *in vitro* studies of disease modeling, drug screening, and hematological diseases. T cells are key components of human adaptive immune system and have great therapeutic potential. However, current T cell-mediated therapy relies on autologous T cells, which prevents its broad application. Human induced pluripotent stem cells (iPSCs) represent an ideal source for scalable manufacture of off-the-shelf products for cell therapy. However, the generation of mature and functional T cells from iPSCs has proven to be difficult. Additionally, the differentiation of iPSC requires co-culture with mouse stromal cells, which limits the translational potential of iPSC-derived T cells. As such there is a need for high-yield, clinically applicable T cell differentiation methods.

SUMMARY

[0006] The technology described herein is directed to methods of T cell differentiation. In one aspect, the method described herein is a stroma-free T cell differentiation method, i.e., a method that

does not comprise co-culturing with stromal cells or any other type of supporting cell. Co-culture with stromal cells such as mouse stromal cells limits the translational potential of iPSC-derived T cells; for example, there can be fears of transplantation rejection due to the presence of stromal cells. Furthermore, T cells differentiated using stromal cells exhibit an innate-like phenotype (e.g., as measured by TCRgd expression, which is a marker for gamma delta T cells). It is preferred that T cells exhibit an adaptive phenotype, for example characterized by expression of TCR α and β. Additionally, as described herein, stroma-free T cell differentiation methods result in increased numbers of CD3+ T cells (e.g., CD4+CD8+ cells) compared to differentiation methods comprising stromal co-culture.

[0007] Accordingly, T cells differentiated using stromal-free methods, and in one embodiment, in combination with inhibition of an epigenetic regulator (e.g., a histone methyl transferase (HMT); e.g., EZH1, G9a/GLP), exhibit at least the following unexpected benefits compared to stromal co-culture methods: (1) increased potential for transplantation in humans; (2) decreased number of innate-like T cells; (3) increased number and/or percentage of resultant T cells (e.g., CD5+CD7+ Pro-T cells; CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells; alpha-beta T cells); (4) gene expression profiles most similar to alpha beta T cells; (5) a more diverse TCR repertoire; and/or (6) increased TCR CDR length (see e.g., Example 1, Fig. 1C-1D, Fig. 3A-3B, Fig. 4, Fig. 5A-5D, Fig. 6-16).

[0008] In one aspect, described herein is a method comprising (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; (b) inhibiting a histone methyltransferase in the resultant population of $CD34^+$ hemogenic endothelium; and (c) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of $CD3^+$ T cells.

[0009] In another aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of CD34+ hemogenic endothelium; (b) inhibiting an epigenetic regulator in the resultant population of CD34+ hemogenic endothelium; and (c) differentiating the resultant population of CD34+ hemogenic endothelium in a CD3+-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of CD3+ T cells.

[0010] In another aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of CD34+ hemogenic endothelium; (b) inhibiting G9a and/or GLP in the resultant population of CD34+ hemogenic endothelium; and (c) differentiating the resultant

population of CD34+ hemogenic endothelium in a CD3+-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of CD3+ T cells.

[0011] In another aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and (b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of $CD3^+$ T cells.

[0012] In some embodiments of any of the aspects, the Notch ligand is attached to a solid substrate.

[0013] In some embodiments of any of the aspects, the Notch ligand is attached to a cell culture dish.

[0014] In some embodiments of any of the aspects, the Notch ligand is not derived from a stromal cell.

[0015] In some embodiments of any of the aspects, differentiating the hemogenic endothelium in the presence of a Notch ligand does not comprise co-culturing with a stromal cell expressing a Notch ligand.

[0016] In some embodiments of any of the aspects, differentiating the hemogenic endothelium in the presence of a Notch ligand does not comprise co-culturing with OP9-DL1 cells or OP9-DL4 cells.

[0017] In some embodiments of any of the aspects, the Notch ligand is selected from the group consisting of Delta-like-1 (DLL1), Delta-like-4 (DLL4), immobilized $Delta1^{ext\text{-}IgG}$, and immobilized $Delta4^{ext\text{-}IgG}$.

[0018] In some embodiments of any of the aspects, immobilized $Delta1^{ext\text{-}IgG}$ consists of an extracellular domain of human Delta-like-1 fused to the Fc domain of human IgG1.

[0019] In some embodiments of any of the aspects, the sufficient time to promote differentiation into a population of $CD3^+$ T cells is at least 4 weeks.

[0020] In some embodiments of any of the aspects, the $CD3^+$-T-cell-differentiation media is serum-free.

[0021] In some embodiments of any of the aspects, the $CD3^+$-T-cell-differentiation media comprises FLT3 and IL7.

[0022] In some embodiments of any of the aspects, the $CD3^+$-T-cell-differentiation media comprises 15 ng/ml FLT3 and 25 ng/ml IL7.

[0023] In some embodiments of any of the aspects, the $CD3^+$-T-cell-differentiation media further comprises 5 ng/mL thrombopoietin (TPO) and/or 30 ng/ml SCF for at least the first 2 weeks of differentiating in the $CD3^+$-T-cell-differentiation media.

[0024] In some embodiments of any of the aspects, $CD3^+$-T-cell-differentiation media comprising TPO promotes differentiation into a population of $CD5^+$ $CD7^+$ ProT cells.

[0025] In some embodiments of any of the aspects, the population of $CD3^+$ T cells comprises a population of $CD4^+CD8^+$ T cells.

[0026] In some embodiments of any of the aspects, the method further comprises differentiating the population of $CD4^+CD8^+$ T cells in a single-positive-T-cell-differentiation media for a sufficient time to promote differentiation into a population of $CD4^+$ cells and a population of $CD8^+$ cells.

[0027] In some embodiments of any of the aspects, the sufficient time to promote differentiation from the population of $CD4^+CD8^+$ T cells into a population of $CD4^+$ T cells and a population of $CD8^+$ cells is at least 1 week.

[0028] In some embodiments of any of the aspects, the sufficient time to promote differentiation from the population of $CD34^+$ hemogenic endothelium into a population of $CD4^+$ T cells and a population of $CD8^+$ cells is at least 5 weeks.

[0029] In some embodiments of any of the aspects, the single-positive-T-cell-differentiation media comprises 10 ng/mL IL-15 and a T cell activator.

[0030] In some embodiments of any of the aspects, the T cell activator comprises a 10ul/ml CD3/CD28 T cell activator.

[0031] In some embodiments of any of the aspects, the T cell activator comprises one bead of CD3/CD28 T cell activator dynabeads per cell.

[0032] In some embodiments of any of the aspects, the method further comprises, after at least 1 week, a step of $CD4^+$ cell enrichment and/or $CD8^+$ cell enrichment.

[0033] In some embodiments of any of the aspects, the population of pluripotent stem cells comprises induced pluripotent stem cells (iPS cells) or embryonic stem cells (ESC).

[0034] In some embodiments of any of the aspects, the induced pluripotent stem cells are produced by introducing only reprogramming factors *OCT4, SOX2, KLF4* and optionally *c-MYC* or nanog and LIN28 into mature cells.

[0035] In some embodiments of any of the aspects, the induced pluripotent stem cells are produced by introducing the reprogramming factors two or more times into the mature cells.

[0036] In some embodiments of any of the aspects, the population of pluripotent stem cells is differentiated into a population of $CD34^+$ hemogenic endothelium using embryoid bodies or 2D adherent cultures.

[0037] In some embodiments of any of the aspects, the sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium is at least 8 days.

[0038] In some embodiments of any of the aspects, the aggregation media comprises BMP4, SB-431542, CHIR99021, bFGF, VEGF, IL-6, IL-11, IGF-1, SCF, and EPO.

[0039] In some embodiments of any of the aspects, the aggregation media comprises 10 ng/ml BMP4, 6 mM SB-431542, 3 mM CHIR99021, 5 ng/ml bFGF, 15 ng/ml VEGF, 10 ng/ml IL-6, 5 ng/mL IL-11, 25 ng/mL IGF-1, 50 ng/mL SCF, and 2 U/ml EPO.

[0040] In some embodiments of any of the aspects, the method further comprises selecting or isolating the resultant population of $CD34^+$ hemogenic endothelium using expression of surface markers on the population of $CD34^+$ hemogenic endothelium.

[0041] In some embodiments of any of the aspects, the population of $CD34^+$ hemogenic endothelium is CD45 negative/low.

[0042] In some embodiments of any of the aspects, the population of $CD34^+$ hemogenic endothelium is CD38 negative/low.

[0043] In some embodiments of any of the aspects, the method further comprises the step of genetically modifying the resultant population of $CD34^+$ hemogenic endothelium or the resultant population of $CD3^+$ T cells.

[0044] In some embodiments of any of the aspects, the genetic modification is editing an endogenous HLA, removing an endogenous TCR, and/or expressing a chimeric antigen receptor (CAR).

[0045] In some embodiments of any of the aspects, the histone methyltransferase catalyzes the addition of methyl group to the histone 3 lysine residue 9 (H3K9) and/or histone 3 lysine residue 27 (H3K27).

[0046] In some embodiments of any of the aspects, the histone methyltransferase H3K9 and/or H3K27 is inhibited by a small molecule inhibitor or a nucleic acid inhibitor.

[0047] In some embodiments of any of the aspects, the histone methyltransferase H3K9 and/or H3K27 small molecule inhibitor is a heterorganic compound or an organometallic compound.

[0048] In some embodiments of any of the aspects, the histone methyltransferase H3K9 and/or H3K27 small molecule inhibitor is selected from the group consisting of BIX-01294, UNC0638, E72, BRD4770, A-366, chaetocin, UNC0224, UNC0631, UNC0646, EPZ005687, EPZ-6438 (E7438), 3-deazaneplanocin A (DZNep), EI1, GSK343, GSK126, and UNC1999.

[0049] In some embodiments of any of the aspects, the nucleic acid inhibitor is a nucleic acid targeting the expression of histone methyltransferase.

[0050] In some embodiments of any of the aspects, the nucleic acid inhibitor is a RNA interference inhibitor or agent.

[0051] In some embodiments of any of the aspects, the nucleic acid inhibitor is a EZH1 specific nucleic acid that is selected from the group consisting of an aptamer that binds EZH1, a EZH1 specific RNA interference agent, and a vector encoding a EZH1 specific RNA interference agent, wherein the RNA interference agent comprises one or more of the nucleotide sequences selected from SEQ ID NO: 11-19.

[0052] In some embodiments of any of the aspects, the epigenetic regulator is a DNA-methyltransferase (DNMT); a methyl-CpG-binding domain (MBD) protein; a DNA demethylase; a histone methyl transferase (HMT); a methyl-histone binding protein; a histone demethylase; a histone acetyl transferase (HAT); an acetyl-binding protein; or a histone deacetylase (HDAC).

[0053] In some embodiments of any of the aspects, the inhibitor of an epigenetic regulator is selected from the group consisting of: UNC0224; MC1568; and CAY10591.

[0054] In some embodiments of any of the aspects, the inhibitor of an epigenetic regulator is provided at a concentration of at least 500 nM.

[0055] In some embodiments of any of the aspects, the sufficient time to promote differentiation from the population of CD34+ cells into a population of CD5+CD7+ proT cells is about 14 days.

[0056] In some embodiments of any of the aspects, the G9a and/or GLP inhibitor is selected from the group consisting of: UNC0224; UNC0638; A366; BRD4770; BIX01294; UNC0642; UNC0631; UNC0646; UNC0321; E72; BIX-01338; BRD9539; Chaetocin; and DCG066.

[0057] In some embodiments of any of the aspects, the G9a and/or GLP inhibitor is UNC0224.

[0058] In some embodiments of any of the aspects, the G9a and/or GLP inhibitor is provided at a concentration of 300 nM - 5uM.

[0059] In some embodiments of any of the aspects, the sufficient time to promote differentiation from the population of CD34+ cells into a population of CD5+CD7+ proT cells is about 14 days.

[0060] In one aspect described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and (b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising 15 ng/ml FLT3 and 25 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells; wherein the $CD3^+$-T-cell-differentiation media further comprises 5 ng/mL TPO and 30 ng/ml SCF for at least the first two weeks.

[0061] In one aspect described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and (b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising 15 ng/ml FLT3 and 25 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells; wherein the $CD3^+$-T-cell-differentiation media further comprises 5 ng/mL TPO, 30 ng/ml SCF, and a G9a/GLP inhibitor for at least the first two weeks.

[0062] In some embodiments of any of the aspects, the population of $CD3^+$ T cells exhibits a gene expression profile that is most similar to alpha beta T cells.

[0063] In some embodiments of any of the aspects, the population of $CD3^+$ T cells exhibits a gene expression profile that is at least 10%, 20%, 30%, 40% or more similar to alpha beta T cells.

[0064] In some embodiments of any of the aspects, the population of CD3+ T cells exhibits a gene expression profile with a Pearson's correlation coefficient compared to peripheral blood alpha beta T cells that is at least 0.85.

[0065] In some embodiments of any of the aspects, the population of $CD3^+$ T cells exhibits a Productive Simpson Clonality value of about 0.025.

[0066] In some embodiments of any of the aspects, the population of $CD3^+$ T cells exhibits a T cell receptor (TCR) complementarity-determining region (CDR) that is at least 3 nucleotides longer than an immune cell differentiated without inhibition of a methyltransferase or using stromal cells.

[0067] In one aspect described herein is an immune cell produced by the method as described herein.

[0068] In some embodiments of any of the aspects, the immune cell exhibits a gene expression profile that is most similar to alpha beta T cells.

[0069] In some embodiments of any of the aspects, the immune cell exhibits a gene expression profile that is at least 10%, 20%, 30%, 40% or more similar to alpha beta T cells.

[0070] In some embodiments of any of the aspects, the immune cell exhibits a gene expression profile with a Pearson's correlation coefficient compared to peripheral blood alpha beta T cells that is at least 0.85.

[0071] In some embodiments of any of the aspects, the immune cell exhibits a Productive Simpson Clonality value of about 0.025.

[0072] In some embodiments of any of the aspects, the immune cell exhibits a T cell receptor (TCR) complementarity-determining region (CDR) that is at least 3 nucleotides longer than an immune cell differentiated without inhibition of methyltransferase, using stromal cells.

[0073] In another aspect described herein is a composition comprising an immune cell as described herein or population thereof.

[0074] In some embodiments of any of the aspects, the composition further comprises a pharmaceutically acceptable carrier.

[0075] In one aspect described herein is a pharmaceutical composition comprising an immune cell as described herein or population thereof, and a pharmaceutically acceptable carrier.

[0076] In another aspect described herein is a pharmaceutical composition as described herein for use in cellular replacement therapy in a subject.

[0077] In one aspect described herein is a method of cellular replacement therapy, the method comprising administering an immune cell as described herein or population thereof, or a composition as described herein, or a pharmaceutical composition as described herein to a recipient subject in need thereof.

[0078] In some embodiments of any of the aspects, the recipient subject has undergone chemotherapy and/or irradiation.

[0079] In some embodiments of any of the aspects, the recipient subject has deficiencies in immune function and/or lymphocyte reconstitution.

[0080] In some embodiments of any of the aspects, prior to transplanting, the immune cell or population thereof is treated *ex vivo* with prostaglandin E2 and/or antioxidant N-acetyl-L-cysteine (NAC) to promote subsequent engraftment in a recipient subject.

[0081] In some embodiments of any of the aspects, the immune cell or population thereof is autologous to the recipient subject.

[0082] In some embodiments of any of the aspects, the immune cell or population thereof is HLA type matched with the recipient subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0083] **Fig. 1A-1D** is a series of schematics and graphs showing the stroma-free differentiation of T cells from human pluripotent stem cells. **Fig. 1A** is a schematic showing the differentiation of $CD3^+$ T cells using a stroma-free method. Briefly, non-tissue culture treated plates are coated with recombinant human DL1/DL4-Fc proteins (10ug/ml in PBS, 3 hours in room temperature). Induced pluripotent stem cell (iPSC) derived hematopoietic stem and progenitor cells (HSPCs; e.g., $CD34^+$ hemogenic endothelium) are cultured on notch ligand (e.g., Delta Like Canonical Notch Ligand 4 (DLL4)) coated plates in media comprising IL-7, stem cell factor (SCF), Flit3, and thrombopoietin (TPO). After 2 weeks, $CD5^+CD7^+$ T cell progenitors (ProT) differentiate. The ProT cells continue differentiation in the DLL4-coated plates in media comprising IL-7 and Flit3; after approximately 3 more weeks, $CD3^+$ T cells have differentiated. **Fig. 1B** is a series of flow cytometry plots showing the expression of CD5 and CD7 (e.g., as markers for T cell progenitors) after 2 weeks of differentiation (top left, 28.1% $CD5^+CD7^+$) or 5 weeks of differentiation (top right, 59.2% $CD5^+CD7^+$), and the expression of CD3 (e.g., as a marker for T cells) after 2 weeks of differentiation (bottom left, 4.70% $CD3^+$) or 5 weeks of differentiation (bottom right, 58.8% $CD3^+$). Note the high proportion of $CD5^+CD7^+$ T cell progenitors at weeks 2 and 5, and the high proportion of $CD3^+$ T cells at week 5. **Fig. 1C** is a series of flow cytometry plots showing the expression of CD4 and CD8 before (left plot) and after (right plot) stimulation of $CD3^+$ cells with a CD3/CD28 antibody. Note the higher proportion of CD4 and CD8 single-positive cells after stimulation compared to before stimulation. **Fig. 1D** is a series of flow cytometry plots showing the expression of TCRgd (e.g., as a marker for innate-like T cells or gamma delta T cell) on T cells differentiated using OP9-DL1 stroma cells (left plot, 55.4% $TCRgd^+$) or the stroma-free method described herein (right plot, 5.71% $TCRgd^+$). Note the lower proportion of $TCRgd^+$ innate-like T cells using the stroma-free method compared to the OP9-DL1 stroma cell method.

[0084] **Fig. 2A-2D** is a series of schematics and plots showing the generation of iPSC-derived chimeric antigen receptor (CAR) T cells. **Fig. 2A** is a schematic showing the introduction of anti-CD19 CAR into iPSC HSPCs; T cell differentiation results in a population of CAR iPS-T cells. **Fig. 2B** is a series of flow cytometry plots showing the expression of mCherry (e.g., as a marker for the CD19 CAR) in: untransduced (UTD) control cells that had not undergone T cell differentiation (left plot, 0.95% $mCherry^+$); CD19 CAR transduced cells that had not undergone T cell differentiation (middle plot, 64.3% $mCherry^+$); and CD19 CAR transduced cells after T cell differentiation (right plot, 80.9% $mCherry^+$). Note that the expression of mCherry (e.g., as a marker for CD19 CAR) was maintained during differentiation. **Fig. 2C** is a line graph showing the T cell expansion of the UTD control and the CAR transduced cells for 1 week in culture. **Fig. 2D** is a series of flow cytometry plots showing the expression of CD8 and CD107a (e.g., with CD107a as a marker of immune cell activation and cytotoxic degranulation) in: CAR-iPSC T cells with no stimulation (left plot; 32.0% $CD8^-$ $CD107a^-$, 55.0% $CD8^+$ $CD107a^-$, 7.14% $CD8^-$ $CD107a^+$, 5.39% $CD8^+$ $CD107a^+$); UTD-iPSC T cells stimulated with CD19-K562 cells (middle plot; 28.0% $CD8^-$ $CD107a^-$, 50.2% $CD8^+$ $CD107a^-$, 11.7% $CD8^-$ $CD107a^+$, 10.1% $CD8^+$ $CD107a^+$); and CAR-iPSC T cells stimulated with CD19-K562 cells (right plot; 29.1% $CD8^-$ $CD107a^-$, 10.7 % $CD8^+$ $CD107a^-$, 29.8% $CD8^-$ $CD107a^+$, 30.4% $CD8^+$ $CD107a^+$). Note the increased expression of CD107a in the stimulated CAR-iPSC T cells compared to the unstimulated CAR-iPSC T cells and stimulated UTD-iPSC T cells.

[0085] **Fig. 3A-3B** is a series of heatmaps showing expression levels of **(Fig. 3A)** T cell signature genes involved in TCR function and activities, and **(Fig. 3B)** genes that distinguish αβ T cells from γδ T cells. abT, peripheral blood αβ T cells; gdT, peripheral blood γδ T cells; NK, peripheral blood NK cells; conT_OP9, ipsc-derived T cells using a OP9-DL4 co-culture system; conT_SF, stroma-free ipsc-T cells; CB_T, T cells differentiated from cord blood CD34+ HSPCs using the stroma free method described herein; EZ_T, stroma-free ipsc-T cells with EZH1 knockdown. Note that in both Fig. 3A and 3B, the EZ_T cells display a gene expression profile most similar to the alpha beta T cells from donor's peripheral blood (abT; see last 6 columns of each heatmap), while the other iPSC-derived T cells are more similar to innate-like cells (e.g., gamma delta T or NK cells). Based on the data in Figs. 3A-3B, Pearson's correlation coefficient was calculated between EZ-T and abT as a value of 0.8886. The value can range from -1 to 1, with 1 being perfect positive correlation. This result indicates that the EZ-T cells are highly similar to PBMC alpha beta T cells.

[0086] **Fig. 4** is a series of schematics showing that EZ-T cells exhibit a diverse TCR repertoire. EZ-T cells refer to T cells differentiated from CD34+ HE, including EZH1 inhibition and stromal-free T cell differentiation as described herein. TCR beta chain sequencing was performed on EZ-T cells and tens of thousands unique TCR rearrangements as a result of random TCR gene recombination during T cell differentiation were identified. Pie chart (left) shows the usage of T-cell receptor beta

chain variable (TCRBV) gene families in EZ-T cells. Each shade represents one TCRBV family. Productive Simpson Clonality value was 0.0233 indicating a highly diverse TCR repertoire.

[0087] **Fig. 5A-5D** is a series of schematics and graphs showing that EZ-T cells have longer CDR3 segments than control PSC-T cells. CDR3 is the most variable region of TCR and its length can be determined by the activity of TdT enzyme, which randomly adds nucleotides during TCR rearrangement. **Fig. 5A** is a schematic showing the activity of the TdT (see e.g., **SEQ ID NOs: 49-50**). It has been reported that CDR3 is shorter in immature T cells and iPSC-derived T cells compared to mature PBMC T cells. **Fig. 5B-5E** are a series of bar charts showing the percentage of productive TCR rearrangements (e.g., each productive TCR rearrangement can be translated into a unique TCR chain) with a certain length (**Fig. 5B** shows a CDR length of 6 to 27 nucleotides (nt); **Fig. 5C** shows a CDR length of 30 to 54 nt; **Fig. 5D** shows a CDR length of 57 to 78 nt). **Fig. 5B-5E** demonstrate that EZ-T cells (dark grey, left bars in each group) displayed an increased CDR3 length compared to control iPSC-derived T cells (light grey, right bars in each group; the control iPSC-T cells were differentiated using the stroma-free differentiation method without EZH1 knockdown), and were more similar to PBMC T cells (medium grey, middle bars in each group).

[0088] **Fig. 6** is a schematic showing a primary screen for small molecule inhibitors of epigenetic factors that promote T cell specification, using the stromal-free T cell differentiation methods as described herein. "5F cells" refer to cells expressing 5 transcription factors (HOXA9, ERG, RORA, SOX4, and MYB). The Cayman epigenetic library contains more than 140 small molecules that are known to modulate the activity of a variety of epigenetic 'writers and erasers' and 'reader' proteins. It may include compounds that modulate the activity of methyltransferases, demethylases, histone acetyltransferases, histone deacetylases, and acetylated histone binding proteins; see e.g., caymanchem.com/product/11076/epigenetics-screening-library-(96-well).

[0089] **Fig. 7** is a scatterplot showing identification of primary hits from the screen described in **Fig. 6**. Z scores were calculated for all the small molecules based on the number of T progenitors after treatment. Any small molecule with a Z score greater that 3 was considered as a primary hit. See e.g., **Table 2**.

[0090] **Fig. 8** is a bar chart showing fold change of the proT cells generated from 5F HSPCs after treatment with primary hits identified in **Fig. 7** (see e.g., **Table 2**). Three small molecules were confirmed to promote T cell specification: UNC0224, MC1568, and CAY10591.

[0091] **Fig. 9** is a schematic showing a second screen using wild type iPSC-derived CD34+ hemogenic endothelial (HE) cells (not 5F HSPCs, e.g., as used in **Fig. 6-9** and **Table 2**), to test small molecule inhibitors of epigenetic factors for promotion of T cell differentiation.

[0092] **Fig. 10A-10B** is a series of graphs showing the results of the screen from **Fig. 9**. **Fig. 10A** is a scatterplot of Z scores, which were calculated for all the small molecules based on the number of T progenitors after treatment. Any small molecule with a Z score greater that 3 was considered as a

primary hit. **Fig. 10B** is a bar graph showing verification of the primary hits. Primary hits were tested in triplicate. UNC0224 treatment led to a significant increase of proT cells generated from CD34+ HE cells.

[0093] **Fig. 11** is a schematic showing that two independent screens identified UNC0224 to enhance T cell specification. See e.g., **Fig. 6-10**, **Table 2**.

[0094] **Fig. 12A-12B** is a series of schematics and graphs showing that UNC0224 promotes T cell specification in a dose-dependent manner. **Fig. 12A** is a schematic summarizing the experiments (see e.g., **Fig. 6-11**, **Table 2**). **Fig. 12B** is a bar chart showing fold change of proT cells generated from CD34+ HE cells after treated with UNC0224 at different doses.

[0095] **Fig. 13A-13F** is a series of schematics and graphs showing that G9 inhibitors promote T cell differentiation using the stromal-free differentiation methods as described herein. **Fig. 13A** is a schematic summarizing the experiments (see e.g., **Fig. 6-12**, **Table 2**). **Figs. 13B-13E** are a bar charts showing fold change of proT cells generated from CD34+ HE cells after treatment with other G9a inhibitors at different doses. **Fig. 13B** shows T cell differentiation dose response to UNC0638. **Fig. 13C** shows T cell differentiation dose response to A366. **Fig. 13D** shows T cell differentiation dose response to BRD4770. **Fig. 13E** shows T cell differentiation dose response to BIX01294. **Fig. 13F** shows T cell differentiation dose response to UNC0642. At least four small molecules, in addition to UNC0224, are capable of promoting T cell differentiation: UNC0638; BRD4770; BIX01294; and UNC0642; see e.g., **Table 3**.

[0096] **Fig. 14A-14C** is a series of schematics and graphs showing that UNC0224 enhances T cell commitment at expense of erythroid/myeloid potential. **Fig. 14A** is a schematic showing a test of whether UNC0224 specifically affects T cell differentiation. iPSC-derived CD34+ HE cells were treated with UNC0224 and differentiated into CD34+CD45+ hematopoietic stem and progenitor cells (HSPC). These HSPCs were used to generate T cells, erythroid cells, and myeloid cells to determine their multipotency. **Fig. 14B** is a bar chart showing fold change of proT cells generated from CD34+CD45+ HSPC cells after treatment with UNC0224 (500nM). Note that UNC0224 treatment results in a significant increase in CD5+CD7+ ProT cells. **Fig. 14C** is a bar chart showing number of different types of colonies generated from CD34+CD45+ HSPCs in a colony-forming unit (CFU) assay. E, erythroid; M, macrophage; G, granulocyte; GM, granulocyte/macrophage; GEMM, granulocyte/erythroid/macrophage/megakaryocyte. Note that UNC0224 treatment results in a significant decrease in erythroid or myeloid lineage cells.

[0097] **Fig. 15A-15C** is a series of graphs showing that UNC0224 promotes T cell specification rather than cell proliferation. **Fig. 15A** is a bar chart showing fold change of proT cells generated from CD34+CD45+ HSPC cells after treatment with UNC0224 (500nM). Note that UNC0224 treatment results in a significant increase in CD5+CD7+ ProT cells. **Fig. 15B** is a bar chart showing fold change of total cell numbers after 14 days of T cell differentiation of CD34+CD45+

HSPCs treated with DMSO or UNC0224. Note that UNC0224 treatment results in a significant decrease in total cells. **Fig. 15C** is a bar chart showing the percentage of proT cells generated from CD34+CD45+ HSPCs treated with DMSO or UNC0224. Note that UNC0224 treatment results in a significant increase in the percentage of CD5+CD7+ ProT cells. N=3, **** P≤0.0001.

[0098] **Fig. 16** is a schematic showing an exemplary hypothesis concerning H3K9 methylation and T cell differentiation. Without wishing to be bound by theory, it is anticipated that H3K9 methylation mediates repression of lymphoid genes. As such, treatment with inhibitors of H3K9 methylation (see e.g., Fig. 6-15, Tables 2-3) promotes T cell differentiation, e.g., when using stromal-free T cell differentiation methods as described herein.

[0099] **Fig. 17** is a schematic showing the differentiation of $CD3^+$ T cells using a stroma-free method. Briefly, non-tissue culture treated plates are coated with recombinant human DL1/DL4-Fc proteins (10ug/ml in PBS, 3 hours in room temperature). Induced pluripotent stem cell (iPSC) derived hematopoietic stem and progenitor cells (HSPCs; e.g., $CD34^+$ hemogenic endothelium) are cultured on notch ligand (e.g., Delta Like Canonical Notch Ligand 4 (DLL4)) coated plates in media comprising IL-7, stem cell factor (SCF), Flit3, and thrombopoietin (TPO). After 2 weeks, $CD5^+CD7^+$ T cell progenitors (ProT) differentiate. The ProT cells continue differentiation in the DLL4-coated plates in media comprising IL-7, SCF, and Flit3; after approximately 3 more weeks, $CD3^+$ T cells have differentiated.

DETAILED DESCRIPTION

[00100] Embodiments of the technology described herein include methods of differentiating T cells. In one aspect, the method described herein is a stroma-free T cell differentiation method, i.e., a method that does not comprise co-culturing with stromal cells or any other type of supporting cell. Co-culture with stromal cells such as mouse stromal cells limits the translational potential of iPSC-derived T cells; for example, there can be fears of transplantation rejection due to the presence of stromal cells. Furthermore, T cells differentiated using stromal cells exhibit an innate-like phenotype (e.g., as measured by TCRgd expression, which is a marker for gamma delta T cells). It is preferred that T cells exhibit an adaptive phenotype, for example characterized by expression of TCR α and β.

[00101] Additionally, as described herein, stroma-free T cell differentiation methods result in increased numbers of CD3+ T cells (e.g., CD4+CD8+ cells) compared to differentiation methods comprising stromal co-culture. Accordingly, T cells differentiated without stromal cell methods, and in one embodiment, in combination with inhibition of an epigenetic regulator (e.g., an HMT; e.g., EZH1, G9a/GLP), exhibit at least the following unexpected benefits compared to stromal co-culture methods: (1) increased potential for transplantation in humans; (2) decreased number of innate-like T cells; (3) increased number and/or percentage of resultant T cells (e.g., CD5+CD7+ Pro-T cells; CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells; alpha-beta T cells); (4) gene

expression profiles most similar to alpha beta T cells; (5) a more diverse TCR repertoire; and/or (6) increased TCR CDR length (see e.g., Example 1, Fig. 1C-1D, Fig. 3A-3B, Fig. 4, Fig. 5A-5D, Fig. 6-16).

Differentiation Methods

[00102] In one aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in an aggregation media for a sufficient time to promote differentiation into a population of CD34+ hemogenic endothelium; and (b) differentiating the resultant population of CD34+ hemogenic endothelium in a CD3+-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of CD3+ T cells.

[00103] In some embodiments, the method further comprises inhibiting a histone methyltransferase in the resultant population of CD34+ hemogenic endothelium. Such an inhibition can increase the efficiency of differentiation into T cells. Accordingly, in one aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in an aggregation media for a sufficient time to promote differentiation into a population of CD34+ hemogenic endothelium; (b) inhibiting a histone methyltransferase in the resultant population of CD34+ hemogenic endothelium; and (c) differentiating the resultant population of CD34+ hemogenic endothelium in a CD3+-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of CD3+ T cells.

[00104] In one embodiment, the $CD34^+$ hemogenic endothelium population is cultured into a $CD3^+$-T-cell-differentiation media comprising 100 ng/ml SCF, 100 ng/ml FLT3, and 50 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells.

[00105] In one embodiment, the $CD34^+$ hemogenic endothelium population is cultured into a $CD3^+$-T-cell-differentiation media comprising 100 ng/ml FLT3 and 50 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells.

[00106] In one embodiment, the $CD34^+$ hemogenic endothelium population is cultured into a $CD3^+$-T-cell-differentiation media comprising 30 ng/ml SCF, 15 ng/ml FLT3, and 25 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells.

[00107] In one embodiment, the $CD34^+$ hemogenic endothelium population is cultured into a $CD3^+$-T-cell-differentiation media comprising 15 ng/ml FLT3 and 25 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells.

[00108] In one aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a

population of $CD34^+$ hemogenic endothelium; and (b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising, 15 ng/ml FLT3 and 25 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells; wherein the $CD3^+$-T-cell-differentiation media further comprises 5 ng/mL TPO and 30 ng/ml SCF for at least the first two weeks.

[00109] In another aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and (b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising, 15 ng/ml FLT3 and 25 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells; wherein the $CD3^+$-T-cell-differentiation media further comprises 5 ng/mL TPO, 30 ng/ml SCF, and a G9a inhibitor for at least the first two weeks.

[00110] In another aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and (b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising 100 ng/ml SCF, 100 ng/ml FLT3, and 50 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells; wherein the $CD3^+$-T-cell-differentiation media further comprises TPO (50 ng/mL) for at least the first two weeks.

[00111] In another aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and (b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising 100 ng/ml SCF, 100 ng/ml FLT3, and 50 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells; wherein the $CD3^+$-T-cell-differentiation media further comprises TPO (50 ng/mL) and a G9a/GLP inhibitor for at least the first two weeks.

[00112] In another aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and (b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising, 100 ng/ml FLT3 and 50 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells; wherein the $CD3^+$-T-cell-differentiation media further comprises 50 ng/mL TPO and 100 ng/ml SCF for at least the first two weeks.

[00113] In another aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and (b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising, 100 ng/ml FLT3 and 50 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells; wherein the $CD3^+$-T-cell-differentiation media further comprises 50 ng/mL TPO, 100 ng/ml SCF, and a G9a inhibitor for at least the first two weeks.

Pluripotent Stem Cells

[00114] In some embodiments, the stroma-free T cell differentiation method comprises differentiating a population of pluripotent stem cells. Pluripotent stem cells (PSCs) have the potential to give rise to all the somatic tissues. In one embodiment of any method, cells, or composition described herein, the population of pluripotent stem cells is induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESC). IPSC and ESC can be produced by any method known in the art. In some embodiments, the population of pluripotent stem cells comprises embryonic stem cells (ESC). Embryonic stem cells (ESCs) are stem cells derived from the undifferentiated inner mass cells of a human embryo.

[00115] Directed differentiation of PSCs aims to recapitulate embryonic development to generate patient-matched tissues by specifying the three germ layers. A common theme in directed differentiation across all germ layers is the propensity of PSCs to give rise to embryonic- and fetal-like cell types, which poses a problem for integration and function in an adult recipient. This distinction is particularly striking in the hematopoietic system, which emerges in temporally and spatially separated waves at during ontogeny. The earliest "primitive" progenitors emerge in the yolk sac at 8.5 dpc and give rise to a limited repertoire of macrophages, megakaryocytes and nucleated erythrocytes. These early embryonic-like progenitors are generally myeloid-based and cannot functionally repopulate the bone marrow of adult recipients. By contrast, "definitive" cells with hematopoietic stem cell (HSC) potential emerge later in arterial endothelium within the aorta-gonad-mesonephros (AGM) and other anatomical sites. Directed differentiation of PSCs gives rise to hematopoietic progenitors, which resemble those found in the yolk sac of the early embryo. These lack functional reconstitution potential, are biased to myeloid lineages, and express embryonic globins. Thus, understanding key fate determining mechanisms that promote development of either primitive or definitive lineages is critical for specifying HSCs, and other adult-like cell types (e.g., red blood cells) from PSCs.

[00116] In some embodiments, the population of pluripotent stem cells (PSCs) comprises induced pluripotent stem cells (iPS cells). In some embodiments, the induced pluripotent stem cells are

produced by introducing only reprogramming factors *OCT4, SOX2, KLF4* and optionally *c-MYC* or nanog and LIN28 into mature cells. In some embodiments, the induced pluripotent stem cells are produced by introducing the reprogramming factors two or more times into the mature cells.

[00117] In some embodiments, the pluripotent stem cells (PSCs) described herein are induced pluripotent stem cells (iPSCs). An advantage of using iPSCs is that the cells can be derived from the same subject to which the eventual immune cells would be reintroduced. That is, a somatic cell can be obtained from a subject, reprogrammed to an induced pluripotent stem cell, and then transfected and differentiated into a modified immune cell to be administered to the subject (*e.g.*, autologous cells). Since the progenitors are essentially derived from an autologous source, the risk of engraftment rejection or allergic responses is reduced compared to the use of cells from another subject or group of subjects. In some embodiments, the cells for generating iPSCs are derived from non-autologous sources. In addition, the use of iPSCs negates the need for cells obtained from an embryonic source. Thus, in one embodiment, the PSCs used in the disclosed methods are not embryonic stem cells.

[00118] Although differentiation is generally irreversible under physiological contexts, several methods have been recently developed to reprogram somatic cells to induced pluripotent stem cells. Exemplary methods are known to those of skill in the art and are described briefly herein below.

[00119] As used herein, the term "reprogramming" refers to a process that alters or reverses the differentiation state of a differentiated cell (e.g., a somatic cell). Stated another way, reprogramming refers to a process of driving the differentiation of a cell backwards to a more undifferentiated or more primitive type of cell. It should be noted that placing many primary cells in culture can lead to some loss of fully differentiated characteristics. Thus, simply culturing such cells included in the term differentiated cells does not render these cells non-differentiated cells (e.g., undifferentiated cells) or pluripotent cells. The transition of a differentiated cell to pluripotency requires a reprogramming stimulus beyond the stimuli that lead to partial loss of differentiated character in culture. Reprogrammed cells also have the characteristic of the capacity of extended passaging without loss of growth potential, relative to primary cell parents, which generally have capacity for only a limited number of divisions in culture.

[00120] The cell to be reprogrammed can be either partially or terminally differentiated prior to reprogramming. In some embodiments, reprogramming encompasses complete reversion of the differentiation state of a differentiated cell (e.g., a somatic cell) to a pluripotent state or a multipotent state. In some embodiments, reprogramming encompasses complete or partial reversion of the differentiation state of a differentiated cell (e.g., a somatic cell) to an undifferentiated cell (e.g., an embryonic-like cell). Reprogramming can result in expression of particular genes by the cells, the expression of which further contributes to reprogramming. In certain embodiments described herein, reprogramming of a differentiated cell (e.g., a somatic cell) causes the differentiated cell to assume an

undifferentiated state (e.g., is an undifferentiated cell). The resulting cells are referred to as "reprogrammed cells," or "induced pluripotent stem cells (iPSCs or iPS cells)."

[00121] Reprogramming can involve alteration, e.g., reversal, of at least some of the heritable patterns of nucleic acid modification (e.g., methylation), chromatin condensation, epigenetic changes, genomic imprinting, etc., that occur during cellular differentiation. Reprogramming is distinct from simply maintaining the existing undifferentiated state of a cell that is already pluripotent or maintaining the existing less than fully differentiated state of a cell that is already a multipotent cell (e.g., a common myeloid stem cell). Reprogramming is also distinct from promoting the self-renewal or proliferation of cells that are already pluripotent or multipotent, although the compositions and methods described herein can also be of use for such purposes, in some embodiments.

[00122] The specific approach or method used to generate pluripotent stem cells from somatic cells (broadly referred to as "reprogramming") is not necessarily critical to the methods described. Thus, any method that re-programs a somatic cell to the pluripotent phenotype would be appropriate for use in the methods described herein.

[00123] Reprogramming methodologies for generating pluripotent cells using defined combinations of transcription factors have been described to induce pluripotent stem cells from somatic cells. Yamanaka and Takahashi converted mouse somatic cells to ES cell-like cells with expanded developmental potential by the direct transduction of Oct4, Sox2, Klf4, and optionally c-Myc. See US Patent Nos: 8058065 and 9045738 to Yamanaka and Takahashi. iPSCs resemble ES cells as they restore the pluripotency-associated transcriptional circuitry and much of the epigenetic landscape. In addition, mouse iPSCs satisfy all the standard assays for pluripotency: specifically, *in vitro* differentiation into cell types of the three germ layers, teratoma formation, contribution to chimeras, germline transmission, and tetraploid complementation.

[00124] Subsequent studies have shown that human iPS cells can be obtained using similar transduction methods, and the transcription factor trio, OCT4, SOX2, and NANOG, has been established as the core set of transcription factors that govern pluripotency. The production of iPS cells can be achieved by the introduction of nucleic acid sequences encoding stem cell-associated genes into an adult, somatic cell, using viral vectors.

[00125] OCT4, SOX2, KLF4 and c-MYC are the original four transcription factors identified to reprogram mouse fibroblasts into iPSCs. These same four factors were also sufficient to generate human iPSCs. OCT3/4 and SOX2 function as core transcription factors of the pluripotency network by regulating the expression of pluripotency-associated genes. Krüppel-like factor 4 (KLF4) is a downstream target of LIF-STAT3 signaling in mouse ES cells and regulates self-renewal. Human iPSCs can also be generated using four alternative factors; OCT4 and SOX2 are required but KLF4 and c-MYC could be replaced with NANOG, a homeobox protein important for the maintenance of pluripotency in both ES cells and early embryos, and LIN28, an RNA binding protein. The

combination of OCT4, SOX2, NANOG and LIN28 reprogramming factors have been reported to be also sufficient to generate human iPSCs.

[00126] In one embodiment of any method, cells, or composition described herein, the iPSCs are produced, for example, by introducing exogenous copies of only three reprogramming factors OCT4, SOX2, and KLF4 into mature or somatic cells. In one embodiment of any method, cells, or composition described herein, c-MYC, or nanog and/or LIN28 are further introduced to iPSCs having exogenous gene coding copies of OCT4, SOX2, and KLF4 to differentiate into mature or somatic cells. In one embodiment of any method, cells, or composition described herein, the iPSCs are produced by introducing exogenous copies of reprogramming factors OCT4, SOX2, and KLF4, and optionally with c-MYC or nanog and/or LIN28 to differentiate into mature or somatic cells.

[00127] In one embodiment of any method, cells, or composition described herein, the iPSCs are produced by contacting mature cells with at least one vector, wherein the at least one vector carries an exogenous gene coding copy of reprogramming factors OCT4, SOX2, and KLF4, and optionally with c-MYC, or nanog and/or LIN28 to differentiate into mature or somatic cells, and wherein the reprogramming factors are expressed *in vivo* in the contacted mature or somatic cells. The contacting is *in vitro* or *ex vivo*. The reprogramming factors needed for differentiation can all be expressed by one vector (e.g., a vector that carries an exogenous gene coding copy of OCT4, SOX2, KLF4, and c-MYC). Alternatively, the reprogramming factors can be expressed in more than one vector that is each used to contact the iPSCs. For example, an iPSCs can be contacted by a first vector that carries an exogenous gene coding copy of OCT4, SOX2, and a second vector that carries an exogenous gene coding copy KLF4 and c-MYC.

[00128] In one embodiment of any disclosed methods, the iPS cell comprises at least an exogenous copy of a nucleic acid sequence encoding a reprogramming factor selected from the group consisting of genes Oct4 (Pou5f1), Sox2, cMyc, Klf4, Nanog, Lin 28 and Glis1. In some embodiments, combinations of reprogramming factors are used. For example, a combination of four reprogramming factors consisting of Oct4, Sox2, cMyc, and Klf4, or a combination of four reprogramming factors consisting of Oct4, Sox2, Nanog, and Lin 28.

[00129] In one embodiment of any method, cells, or composition described herein, the iPSCs are produced by introducing the disclosed reprogramming factors, or any combination of the reprograming factors two or more times into the mature or somatic cells. In one embodiment, the combination of reprograming factors is different when a combination is introduced to the iPSC more than once, for example, the combination of Oct4 (Pou5f1), Sox2, cMyc, Klf4, Nanog is first introduced to the iPSCs, and the combination of Oct4 (Pou5f1), Sox2, cMyc is subsequently introduced to the iPSCs. In one embodiment of any method, cells, or composition described herein, the iPSCs are produced by contacting mature cells with the disclosed vector(s) factors two or more times into the mature/somatic cells.

[00130] In some embodiments, the population of pluripotent stem cells (e.g., iPSCs) are not differentiated in the presence of a Notch ligand. In some embodiments, the aggregation media used to promote the differentiation of the population of pluripotent stem cells (e.g., iPSCs) into a population of CD34+ hemogenic endothelium does not comprise a Notch ligand. In some embodiments, the cell culture vessel used during the differentiation of the population of pluripotent stem cells (e.g., iPSCs) into the population of CD34+ hemogenic endothelium does not comprise a Notch ligand.

[00131] iPS cells can be generated or derived from terminally differentiated somatic cells, as well as from adult stem cells, or somatic stem cells. That is, a non-pluripotent progenitor cell can be rendered pluripotent or multipotent by reprogramming. In such instances, it may not be necessary to include as many reprogramming factors as required to reprogram a terminally differentiated cell. Further, reprogramming can be induced by the non-viral introduction of reprogramming factors, e.g., by introducing the proteins themselves, or by introducing nucleic acids that encode the reprogramming factors, or by introducing messenger RNAs that upon translation produce the reprogramming factors (see e.g., Warren et al., Cell Stem Cell, 2010 Nov 5;7(5):618-30, this reference is incorporated herein by reference in its entirety). Reprogramming can be achieved by introducing a combination of nucleic acids encoding stem cell-associated genes including, for example Oct-4 (also known as Oct-3/4 or Pouf51), Sox1, Sox2, Sox3, Sox 15, Sox 18, NANOG, Klf1, Klf2, Klf4, Klf5, NR5A2, c-Myc, l-Myc, n-Myc, Rem2, Tert, and LIN28. In one embodiment, reprogramming using the methods and compositions described herein can further comprise introducing one or more of Oct-3/4, a member of the Sox family, a member of the Klf family, and a member of the Myc family to a somatic cell. In one embodiment, the methods and compositions described herein further comprise introducing one or more of each of Oct 4, Sox2, Nanog, c-MYC and Klf4 for reprogramming. As noted above, the exact method used for reprogramming is not necessarily critical to the methods and compositions described herein. However, where cells differentiated from the reprogrammed cells are to be used in, e.g., human therapy, in one embodiment the reprogramming is not effected by a method that alters the genome. Thus, in such embodiments, reprogramming is achieved, e.g., without the use of viral or plasmid vectors.

[00132] The efficiency of reprogramming (i.e., the number of reprogrammed cells) derived from a population of starting cells can be enhanced by the addition of various small molecules as shown by Shi, Y., et al (2008) Cell-Stem Cell 2:525-528, Huangfu, D., et al (2008) Nature Biotechnology 26(7):795-797, and Marson, A., et al (2008) Cell-Stem Cell 3:132-135, the contents of each of which are incorporated herein by reference in its entirety. Thus, an agent or combination of agents that enhance the efficiency or rate of induced pluripotent stem cell production can be used in the production of patient-specific or disease-specific iPSCs. Some non-limiting examples of agents that enhance reprogramming efficiency include soluble Wnt, Wnt conditioned media, BIX-01294 (a G9a histone methyltransferase), PD0325901 (a MEK inhibitor), DNA methyltransferase inhibitors, histone

deacetylase (HDAC) inhibitors, valproic acid, 5'-azacytidine, dexamethasone, suberoylanilide hydroxamic acid (SAHA), vitamin C, and trichostatin (TSA), among others.

[00133] Other non-limiting examples of reprogramming enhancing agents include: Suberoylanilide Hydroxamic Acid (SAHA (e.g., MK0683, vorinostat) and other hydroxamic acids), BML-210, Depudecin (e.g., (-)-Depudecin), HC Toxin, Nullscript (4-(1,3-Dioxo-1H,3H-benzo[de]isoquinolin-2-yl)-N-hydroxybutanamide), Phenylbutyrate (e.g., sodium phenylbutyrate) and Valproic Acid ((VPA) and other short chain fatty acids), Scriptaid, Suramin Sodium, Trichostatin A (TSA), APHA Compound 8, Apicidin, Sodium Butyrate, pivaloyloxymethyl butyrate (Pivanex, AN-9), Trapoxin B, Chlamydocin, Depsipeptide (also known as FR901228 or FK228), benzamides (e.g., CI-994 (e.g., N-acetyl dinaline) and MS-27–275), MGCD0103, NVP-LAQ-824, CBHA (m-carboxycinnaminic acid bishydroxamic acid), JNJ16241199, Tubacin, A-161906, proxamide, oxamflatin, 3-Cl-UCHA (e.g., 6-(3-chlorophenylureido)caproic hydroxamic acid), AOE (2-amino-8-oxo-9,10-epoxydecanoic acid), CHAP31 and CHAP 50. Other reprogramming enhancing agents include, for example, dominant negative forms of the HDACs (e.g., catalytically inactive forms), siRNA inhibitors of the HDACs, and antibodies that specifically bind to the HDACs. Such inhibitors are available, e.g., from BIOMOL International, Fukasawa, Merck Biosciences, Novartis, Gloucester Pharmaceuticals, Aton Pharma, Titan Pharmaceuticals, Schering AG, Pharmion, MethylGene, and Sigma Aldrich.

[00134] To confirm the induction of pluripotent stem cells for use with the methods described herein, isolated clones can be tested for the expression of a stem cell marker. Such expression in a cell derived from a somatic cell identifies the cells as induced pluripotent stem cells. Stem cell markers can be selected from the non-limiting group including SSEA3, SSEA4, CD9, Nanog, Fbx15, Ecat1, Esg1, Eras, Gdf3, Fgf4, Cripto, Dax1, Zpf296, Slc2a3, Rex1, Utf1, and Nat1. In one embodiment, a cell that expresses Oct4 or Nanog is identified as pluripotent. Methods for detecting the expression of such markers can include, for example, RT-PCR and immunological methods that detect the presence of the encoded polypeptides, such as Western blots or flow cytometric analyses. In some embodiments, detection does not involve only RT-PCR, but also includes detection of protein markers. Intracellular markers may be best identified via RT-PCR, while cell surface markers are readily identified, e.g., by immunocytochemistry.

[00135] The pluripotent stem cell character of isolated cells can be confirmed by tests evaluating the ability of the iPSCs to differentiate to cells of each of the three germ layers. As one example, teratoma formation in nude mice can be used to evaluate the pluripotent character of the isolated clones. The cells are introduced to nude mice and histology and/or immunohistochemistry is performed on a tumor arising from the cells. The growth of a tumor comprising cells from all three germ layers, for example, further indicates that the cells are pluripotent stem cells.

[00136] Many US Patents and Patent Application Publications teach and describe methods of generating iPSCs and related subject matter. For examples, US Patent Nos: 8058065, 9347044, 9347042 , 9347045, 9340775, 9341625, 9340772, 9250230, 9132152, 9045738, 9005975, 9005976, 8927277, 8993329, 8900871, 8852941, 8802438, 8691574, 8735150, 8765470, 8058065, 8048675, and US Patent Publication Nos: 20090227032, 20100210014, 20110250692, 20110201110, 20110200568, 20110223669, 20110306516, 20100021437, 20110256626, 20110044961, 20120276070, 20120214243, 20120263689, 20120128655, 20120100568, 20130295064, 20130029866, 20130059386, 20130183759, 20130189786, 20130295579, 20130130387, 20130157365, 20140234973, 20140227736, 20140093486, 20140301988, 20140170746, 20140178989, 20140349401, 20140065227, and 20150140662, all of which are incorporated herein by reference in their entireties.

[00137] In some embodiments, the iPSCs can be derived from somatic cells. Somatic cells, as that term is used herein, refer to any cells forming the body of an organism, excluding germline cells. Every cell type in the mammalian body—apart from the sperm and ova, the cells from which they are made (gametocytes) and undifferentiated stem cells—is a differentiated somatic cell. For example, internal organs, skin, bones, blood, and connective tissue are all made up of differentiated somatic cells. In one embodiment of any method, cells, or composition described herein, the mature cells from which iPS cells are made include any somatic cells such as B lymphocytes (B-cells), T lymphocytes, (T-cells), and fibroblasts and keratinocytes.

[00138] Additional somatic cell types for use with the compositions and methods described herein include: a fibroblast (e.g., a primary fibroblast), a muscle cell (e.g., a myocyte), a cumulus cell, a neural cell, a mammary cell, a hepatocyte and a pancreatic islet cell. In some embodiments, the somatic cell is a primary cell line or is the progeny of a primary or secondary cell line. In some embodiments, the somatic cell is obtained from a human sample, e.g., a hair follicle, a blood sample, a biopsy (e.g., a skin biopsy or an adipose biopsy), a swab sample (e.g., an oral swab sample), and is thus a human somatic cell.

[00139] Some non-limiting examples of differentiated somatic cells include, but are not limited to, epithelial, endothelial, neuronal, adipose, cardiac, skeletal muscle, skin, immune cells, hepatic, splenic, lung, peripheral circulating blood cells, gastrointestinal, renal, bone marrow, and pancreatic cells. In some embodiments, a somatic cell can be a primary cell isolated from any somatic tissue including, but not limited to brain, liver, gut, stomach, intestine, fat, muscle, uterus, skin, spleen, endocrine organ, bone, etc. Further, the somatic cell can be from any mammalian species, with non-limiting examples including a murine, bovine, simian, porcine, equine, ovine, or human cell. In some embodiments, the somatic cell is a human somatic cell.

[00140] When reprogrammed cells are used for generation of progenitor cells to be used in the therapeutic treatment of disease, it is desirable, but not required, to use somatic cells isolated from the

patient being treated. For example, somatic cells involved in diseases, and somatic cells participating in therapeutic treatment of diseases and the like can be used. In some embodiments, a method for selecting the reprogrammed cells from a heterogeneous population comprising reprogrammed cells and somatic cells they were derived or generated from can be performed by any known means. For example, a drug resistance gene or the like, such as a selectable marker gene can be used to isolate the reprogrammed cells using the selectable marker as an index.

[00141] Reprogrammed somatic cells as disclosed herein can express any number of pluripotent cell markers, including: alkaline phosphatase (AP); ABCG2; stage specific embryonic antigen-1 (SSEA-1); SSEA-3; SSEA-4; TRA-1-60; TRA-1-81; Tra-2-49/6E; ERas/ECAT5, E-cadherin; beta-III-tubulin; alpha-smooth muscle actin (α-SMA); fibroblast growth factor 4 (Fgf4), Cripto, Dax1; zinc finger protein 296 (Zfp296); N-acetyltransferase-1 (Nat1); ES cell associated transcript 1 (ECAT1); ESG1/DPPA5/ECAT2; ECAT3; ECAT6; ECAT7; ECAT8; ECAT9; ECAT10; ECAT15-1; ECAT15-2; Fthl17; Sal14; undifferentiated embryonic cell transcription factor (Utf1); Rex1; p53; G3PDH; telomerase, including TERT; silent X chromosome genes; Dnmt3a; Dnmt3b; TRIM28; F-box containing protein 15 (Fbx15); Nanog/ECAT4; Oct3/4; Sox2; Klf4; c-Myc; Esrrb; TDGF1; GABRB3; Zfp42, FoxD3; GDF3; CYP25A1; developmental pluripotency-associated 2 (DPPA2); T-cell lymphoma breakpoint 1 (Tcl1); DPPA3/Stella; DPPA4; other general markers for pluripotency, etc. Other markers can include Dnmt3L; Sox15; Stat3; Grb2; β-catenin, and Bmi1. Such cells can also be characterized by the down-regulation of markers characteristic of the somatic cell from which the induced pluripotent stem cell is derived. In one embodiment, the iPSCs are derived from mature, differentiated, somatic cells.

[00142] In some embodiments, the population of pluripotent stem cells used in the differentiation methods described herein does not comprise CD34+ HSPCs or multipotent lymphoid progenitors (MLPs) purified from a patient sample. In some embodiments, the population of pluripotent stem cells does not comprise stem cells purified or isolated from cord blood or bone marrow samples. In some embodiments, the population of pluripotent stem cells is not derived from stem cells isolated from a patient sample (e.g., cord blood or bone marrow). In a preferred embodiment, the population of pluripotent stem cells comprise iPSCs, such as those derived from a somatic cell sample from a patient. See e.g., Tabatabaei-Zavareh et al., J Immunol May 1, 2017, 198 (1 Supplement) 202.9.

Hemogenic Endothelium

[00143] In some embodiments, the methods described herein comprise differentiating a population of pluripotent stem cells (e.g., iPSCs) into a population of cells with hematopoietic potential. In some embodiments, the population of cells with hematopoietic potential comprises hemogenic endothelium and/or hematopoietic stem cells (HSCs). The cells with hematopoietic potential (e.g., hemogenic endothelium, HSCs) can be produced using any method known in the art.

[00144] One exemplary approach to generate HSCs from hPSCs is to specify HSCs from its ontogenetic precursors. It is now widely accepted that HSCs originate from hemogenic endothelium (HE) in the aorta-gonad-mesonephros (AGM) and arterial endothelium in other anatomical sites. Recent work on the directed differentiation of HE from hPSCs have provided valuable insights into some of the signaling pathways that control the emergence of primitive or definitive populations; however, the endothelial-to-hematopoietic transition (e.g., HE to HSC) remains incompletely understood in human hematopoietic development.

[00145] As used herein, the term "hemogenic endothelium" refers to a unique subset of endothelial cells scattered within blood vessels that can differentiate into haematopoietic cells. In the developing mouse, HSCs arise beginning embryonic day 10.5 from a small population of endothelial cells with hemogenic potential (hemogenic endothelium) located within the aorta-gonad-mesonephros region. In a process known as endothelial to hematopoietic transition (EHT), endothelial cells in the floor of the aorta round up and bud into the extravascular space followed by reentry into the circulation via the underlying vein. In some embodiments, a population of cells comprising the properties of hemogenic endothelium is differentiated *in vitro* from a population of pluripotent stem cells (e.g., iPSCs). Said "cells comprising the properties of hemogenic endothelium" can also be referred to herein as hemogenic endothelium.

[00146] Efforts to derive HSCs from pluripotent stem cells (PSCs) are complicated by the fact that embryonic hematopoiesis consists of two programs, primitive and definitive, but only definitive hematopoiesis generates HSCs and thus the lymphoid lineage. Definitive hematopoiesis, as measured by T-lymphoid potential, emerges after the establishment of the primitive hematopoietic program and develops from a progenitor population that displays characteristics of hemogenic endothelium.

[00147] In some embodiments, the stroma-free T cell differentiation method comprises differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of CD34+ hemogenic endothelium. In some embodiments, the resultant CD34+ hemogenic endothelium can undergo definitive hematopoiesis and/or exhibits lymphoid potential. In some embodiments, the hemogenic endothelium differentiates or is differentiated into hematopoietic stem cells (HSCs).

[00148] In some embodiments, the population of pluripotent stem cells (e.g., iPSCs) is differentiated into a population of CD34+ hemogenic endothelium using embryoid bodies (EBs) or 2D adherent cultures; see e.g., Pineda et al., Differentiation patterns of embryonic stem cells in two versus three dimensional culture, Cells Tissues Organs. 2013; 197(5): 399–410, which is incorporated herein by reference. EBs are three-dimensional aggregates of pluripotent stem cells produced and cultured *in vitro* in the presence of serum. The EBs can generate a mixture of primitive and definitive hematopoietic progenitor cell types. Primitive progenitors equate to those that arise *in vivo* naturally in the earliest stages of embryonic development, whereas at later stages of maturation the embryonic

populations give rise to definitive progenitor cells, which behave similarly to the cells typical of adult hematopoiesis.

[00149] In some embodiments, the sufficient time to promote differentiation into a population of CD34+ hemogenic endothelium is at least 8 days (e.g., at least 7, at least 8, at least 9, at least 10 days, or more). In some embodiments, the sufficient time to promote differentiation into a population of CD34+ hemogenic endothelium is at most 8 days, at most 9 days, at most 10 days or more.

[00150] In some embodiments, the aggregation media comprises BMP4, SB-431542, CHIR99021, bFGF, VEGF, IL-6, IL-11, IGF-1, SCF, and EPO or any combination of the same. In some embodiments, the aggregation media comprises 10 ng/ml BMP4, 6 mM SB-431542, 3 mM CHIR99021, 5 ng/ml bFGF, 15 ng/ml VEGF, 10 ng/ml IL-6, 5 ng/ml IL-11, 25 ng/ml IGF-1, 50 ng/ml SCF, and 2 U/ml EPO; see e.g., Example 2 and Table 1 presented herein.

[00151] In some embodiments, the components of the aggregation media are varied during the differentiation of pluripotent stem cells into hemogenic endothelium. As a non-limiting example, embryoid bodies are differentiated in the presence of BMP4, followed by stage-specific addition of bFGF, VEGF, and hematopoietic cytokines (e.g., IL-6, IL-11, IGF-1, SCF, and EPO). Activin-nodal signaling can be manipulated (e.g., using SB-431542 and CHIR99021) between days 2 and 3. See e.g., Example 2 herein below; and Sturgeon et al., Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells, Nat Biotechnol. 2014 Jun; 32(6): 554–561, which is incorporated herein by reference.

[00152] In some embodiments, the aggregation media comprises BMP (e.g., 10 ug/mL BMP) during days 0, 1, and/or 2 of differentiation. In some embodiments, the aggregation media does not comprise BMP during days 3, 4, 5, 6, 7, or 8 of differentiation.

[00153] In some embodiments, the aggregation media comprises SB-431542 (e.g., 6 mM SB-431542) and/or CHIR99021 (e.g., 3 mM CHIR99021) during day 2 of differentiation. SB-431542 is a small-molecule antagonist of activin-nodal signaling. CHIR99021 is a GSK-3 inhibitor and a Wnt agonist. Inhibition of activin-nodal signaling and activation of Wnt signaling has been shown to drive PSC differentiation into definitive progenitors ($KDR^{+}CD235a^{-}$) with lymphoid potential (see e.g., Sturgeon 2014, *supra*, which is incorporated herein by reference). In some embodiments, the aggregation media does not comprise SB-431542 and/or CHIR99021 during days 0, 1, 3, 4, 5, 6, 7, and/or 8 of differentiation.

[00154] In some embodiments, the aggregation media comprises bFGF (e.g., 5 ng/ml bFGF) during days 1, 2, 3, 4, 5, 6, 7, and/or 8 of differentiation. In some embodiments, the aggregation media does not comprise bFGF during day 0 of differentiation.

[00155] In some embodiments, the aggregation media comprises VEGF (e.g., 15 ng/ml VEGF) during days 3, 4, 5, 6, 7, and/or 8 of differentiation. In some embodiments, the aggregation media does not comprise VEGF during days 0, 1, or 2 of differentiation.

[00156] In some embodiments, the aggregation media comprises hematopoietic cytokine(s) during days 6, 7, and/or 8 of differentiation. In some embodiments, the aggregation media does not comprise hematopoietic cytokine(s) during days 0, 1, 2, 3, 4, or 5 of differentiation. In some embodiments, the hematopoietic cytokines are selected from the group consisting of: IL-6 (e.g., 10 ng/ml IL-6), IL-11 (e.g., 5 ng/ml IL-11), IGF-1 (e.g., 25 ng/ml IGF-1), SCF (e.g., 50 ng/ml SCF), and EPO (e.g., 2 U/ml EPO).

[00157] In some embodiments, the differentiation method further comprises selecting or isolating the resultant population of CD34+ hemogenic endothelium using expression of surface markers on the population of CD34+ hemogenic endothelium. Non-limiting examples of methods for selecting or isolating hemogenic endothelium include magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS). In some embodiments, the surface marker for hemogenic endothelium is CD34 (e.g., high CD34 surface expression).

[00158] In some embodiments, additional positive or negative markers for hemogenic endothelium can include, but are not limited to, CD45, CD38, KDR, CD235, and CD43. In some embodiments, the population of CD34+ hemogenic endothelium is CD45 negative/low. In some embodiments, the population of CD34+ hemogenic endothelium is CD38 negative/low. In some embodiments, the population of CD34+ hemogenic endothelium is KDR+. In some embodiments, the population of CD34+ hemogenic endothelium is CD235 negative/low. In some embodiments, the population of CD34+ hemogenic endothelium is CD43 negative/low.

[00159] In some embodiments, the hemogenic endothelium and/or HSCs are produced using any method known in the art. As a non-limiting example, the method of differentiating PSCs into hemogenic endothelium can comprise the introduction of transcription factors such as ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1, and/or SPI1; see e.g., International Application No. WO 2018/048828, US Patent Application No. 2019/0225940, Doulatov et al., Cell Stem Cell. 2013 October 3, 13(4); Vo et al., Nature 2018, 553(7689): 506–510; the contents of each of which are incorporated herein by reference in their entireties.

[00160] In some embodiments, the hemogenic endothelium is not derived from PSCs but is rather derived directly from endothelial cells. For example, endothelial cells (e.g., from lung, brain, and other tissues) can be directly reprogrammed into hemogenic endothelium by transduction of with transcription factors (e.g., Fosb, Gfi1, Runx1, and Spi1) and co-culture with an immortalized endothelial cell line; the endothelial cells can be further exposed to cell-extrinsic factors (e.g., serum, SB-431542, and/or endothelial mitogen). See, e.g., Lis et al., Nature. 2017 May 25, 545(7655):439-445; Blaser and Zon, Blood. 2018 Sep 27; 132(13): 1372–1378, which are incorporated herein by reference.

Inhibition of an Epigenetic Regulator

[00161] In some aspects described herein is a T-cell differentiation method comprising a step of inhibiting at least one epigenetic regulator. As used herein, the term "epigenetic regulator" refers to a factor, e.g., a polypeptide, e.g., an enzyme, that influences DNA methylation and/or histone modifications (e.g., histone acetylation, histone methylation), and as such affect the transcription levels of genes without an alteration (e.g., substitution or deletion) to the nucleotide sequence of the genome. Non-limiting examples of epigenetic regulators include: DNA-methyltransferase (DNMT; e.g., DNMT1; DNMT3a; DNMT3b); methyl-CpG-binding domain (MBD) protein (e.g., MeCP2; MBD1; MBD2; MCD4; KAISO; ZBTB4; ZBTB38; UHRHRF2); DNA demethylase (e.g., 5'-methylcytokine hydroxylase; TET1; TET2; TET3); histone methyl transferase (HMT; e.g., SUV39s; SET1s; EZH1; EZH2; Set2s; PRDMs; SMYDs; DOT1L; PRMTs; G9a; GLP); methyl-histone binding protein (e.g., HP1; Chd1; BPTF; L3MBTL1; ING2; BHC80; JMJD2A); histone demethylase (e.g., KDMs; e.g., LSDs; JHDMs; JMJDs; JARID; Uts; PHFs); histone acetyl transferase (HAT; e.g., HAT1; GCN5; PCAF; MYSTs; p300; CBP; SRC/p160); acetyl-binding proteins (e.g., BROMO-domain, DPF-domain, or YEATS-domain-containing proteins); histone deacetylase (HDAC; e.g., HDAC1; HDAC2; HDAC3; HDAC4; HDAC5; HDAC6; HDAC7; HDAC8; HDAC9; HDAC10; HDAC11; Sirt1; Sirt2; Sirt3; Sirt4; Sirt5; Sirt6; Sirt7). See e.g., Cheng et al., Signal Transduction and Targeted Therapy volume 4, Article number: 62 (2019); the content of which is incorporated herein by reference in its entirety.

[00162] In some embodiments, the method comprises the step of, after the step of differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of CD34+ hemogenic endothelium, inhibiting an epigenetic regulator in the resultant population of CD34+ hemogenic endothelium. In some embodiments, the method comprises the step of, prior to the step of differentiating a population of CD34+ hemogenic endothelium in a CD3+-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of CD3+ T cells, inhibiting an epigenetic regulator in the population of CD34+ hemogenic endothelium.

[00163] Accordingly, in one aspect, described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of CD34+ hemogenic endothelium; (b) inhibiting an epigenetic regulator in the resultant population of CD34+ hemogenic endothelium; and (c) differentiating the resultant population of CD34+ hemogenic endothelium in a CD3+-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of CD3+ T cells.

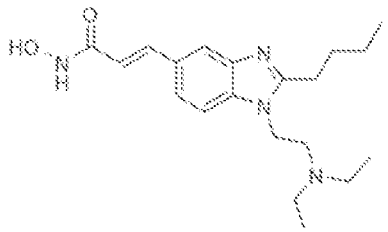
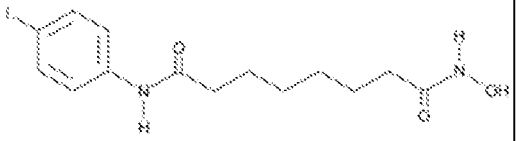
[00164] In some embodiment, CD34+ hemogenic endothelium is treated with an inhibitor of an epigenetic regulator. Exemplary inhibitors of an epigenetic regulator include an inhibitor of at least one of the following: DNMT; MBD; DNA demethylase; HMT; methyl-histone binding protein;

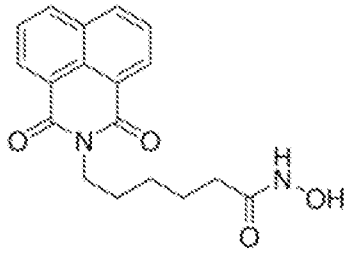
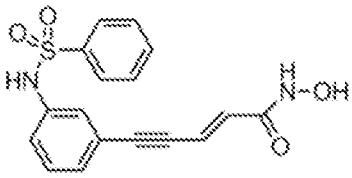
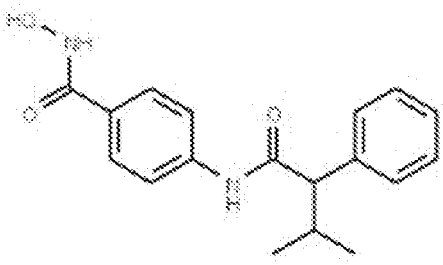
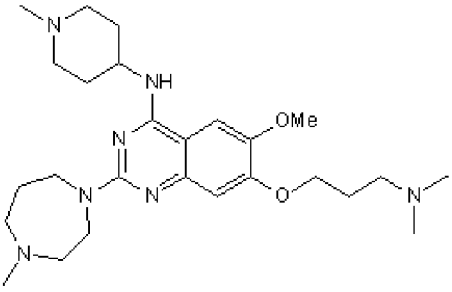
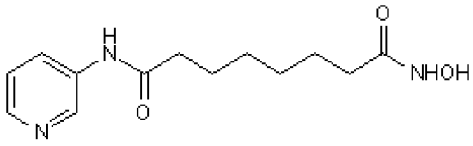
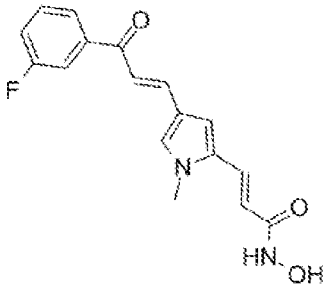
histone demethylase; HAT; acetyl-binding protein; or HDAC. In some embodiments, the epigenetic regulator is an H3K9 methyltransferase. Methylation of H3K9 in humans relies mostly on members of the Suv39 family, namely EHMT1/GLP, EHMT2/G9a, SUV39H1, SUV39H2, SETDB1 and SETDB2, as well as then non-Suv39 enzymes PRDM2 and ASH1L.

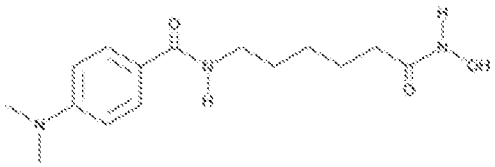
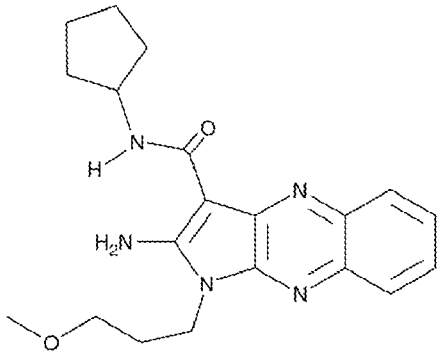
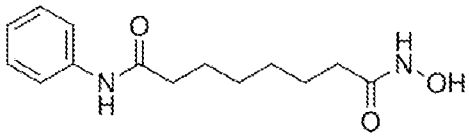
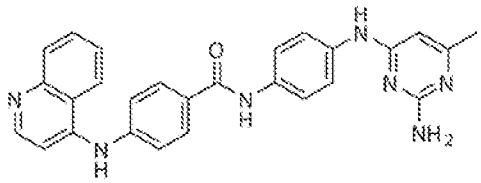
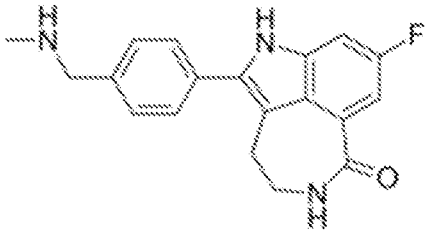
[00165] Non-limiting examples of DNMT inhibitors include azacitidine; decitabine; guadecitabine; hydralazine. Non-limiting examples of HMT inhibitors include pinometostat; tazemetostat; GSK2816126; CPI-1205; TCP; ORY-2001; GSK2879552; 4SC-202. Non-limiting examples of HDAC inhibitors include valproic acid, phenylbutyrate; vorinostat; trichostatin A; belinostat; entinostat; panobinostat; mocetinostat; CI-994; romidepsin; nicotinamide; suramin; PRI-724; GSK525762; CPI-0610; RO6870810; MK-8628.

[00166] In some embodiments, the inhibitor of an epigenetic regulator is selected from **Table 2**. In some embodiments, the inhibitor of an epigenetic regulator is selected from the group consisting of: SB939 (Pracinostat); 4-iodo-SAHA; Scriptaid; Oxaflatin (i.e., Oxamflatin); s-HDAC-42; UNC0224; Pyroxamide; MC1568; CAY10398; CAY10591; SAHA (Vorinostat) (SIH-359); SGI-1027; and Rucaparib (Rubraca™). In some embodiments, the inhibitor of an epigenetic regulator is selected from the group consisting of: SB939 (Pracinostat); 4-iodo-SAHA; Scriptaid; Oxaflatin (i.e., Oxamflatin); s-HDAC-42; UNC0224; Pyroxamide; MC1568; CAY10398; CAY10591; and SAHA (Vorinostat) (SIH-359); see e.g., **Fig. 7** and **Table 2**.

[00167] **Table 2: Small molecule inhibitors that can promote T cell differentiation** (e.g., at 500 nM; small molecules with a Z score greater than 3 are shown bolded; see e.g., **Fig. 6-7**).

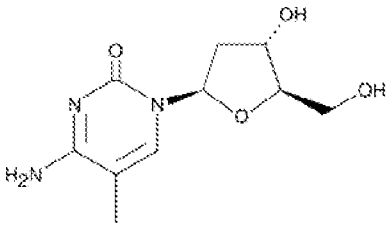
Well location	**$CD5^+$ $CD7^+$ %**	**Z score**	**Small molecule**	**Function of small molecule**	**Structure of small molecule**
1A4	39.4 %	**5.042400516**	**SB939 (Pracinostat)**	Pan-HDAC inhibitor (e.g., with IC50 of 40-140 nM with exception for HDAC6). It has no activity against the class III isoenzyme SIRT1.	
1A6	25.3 %	**5.894930088**	**4-iodo-SAHA**	4-iodo-SAHA is a hydrophobic derivative of the class I and class II HDAC (e.g., HDAC1 or HDAC6) inhibitor SAHA.	

Well location	$CD5^+$ $CD7^+$ %	Z score	Small molecule	Function of small molecule	Structure of small molecule
1B3	19.7 %	**3.857176477**	**Scriptaid**	HDAC inhibitor (see e.g., US Patent 6,544,957).	
1H7	12.4 %	**6.539525618**	**Oxaflatin (i.e., Oxamflatin)**	HDAC inhibitor	
1H11	43.2 %	**4.262647859**	**s-HDAC**-42	HDAC inhibitor	
2A8	15.0 %	**3.475075737**	**UNC0224**	G9a and GLP inhibitor (H3K9 methyltransferase)	
2B2	11.6 %	**3.121963203**	**Pyroxamide**	HDAC (e.g., HDAC1) inhibitor	
2E5	14.0 %	**5.829159302**	**MC1568**	Class II HDAC (e.g., HDAC4 and HDAC5) inhibitor. Displays no inhibition of class I HDAC activity (e.g., HDAC1, HDAC2, HDAC3).	

Well location	$CD5^+$ $CD7^+$ %	Z score	Small molecule	Function of small molecule	Structure of small molecule
2F10	36.9 %	**4.181300807**	**CAY10398**	HDAC (e.g., HDAC1) inhibitor	
2G4	25.9 %	**8.575590126**	**CAY10591**	SIRT1 activator. Sirtuins (SIRTs) represent a distinct class of trichostatin A-insensitive lysyl-deacetylases (class III HDACs).	
2G6	34.7 %	**7.987069235**	**SAHA (Vorinostat) (SIH-359)**	Potent reversible pan-histone deacetylase HDAC inhibitor, including both class I and class II HDACs.	
1D11	19.9 %	0.093570319	SGI-1027	DNA Methyltransferase (DNMT; e.g., DNMT1, DNMT3A, or DNMT3B) inhibitor	
1E 8	37.2 %	0.467851594	Rucaparib (Rubraca™)	poly(ADP-ribose) polymerase (PARP; e.g., PARP1, PARP2, PARP3) inhibitor	
Control cells	11.60%				

[00168] In some embodiments, the inhibitor of an epigenetic regulator is selected from the group consisting of: UNC0224; MC1568; and CAY10591 (see e.g., **Fig. 8**). In some embodiments, the inhibitor of an epigenetic regulator is UNC0224. In some embodiments, the inhibitor of an epigenetic regulator is MC1568. In some embodiments, the inhibitor of an epigenetic regulator is CAY10591.

[00169] In some embodiments, the inhibitor of an epigenetic regulator is UNC0224 or 5-Methyl-2'-deoxycytidine (see e.g., **Fig. 10B**, and structure in Formula I below). In some embodiments, the inhibitor of an epigenetic regulator is 5-Methyl-2'-deoxycytidine. 5-Methyl-2'-deoxycytidine is a pyrimidine nucleoside that when incorporated into single-stranded DNA can act in cis to signal de novo DNA methylation; see e.g., Christman et al. Proceedings of the National Academy of Sciences of the United States of America 92(16), 7347-7351 (1995).



I: 5-Methyl-2'-deoxycytidine

[00170] In some embodiments, the inhibitor of an epigenetic regulator is provided at a concentration of at least 500 nM. In some embodiments, the inhibitor of an epigenetic regulator is provided at a concentration of at least 1 nM, at least 2 nM, at least 3 nM, at least 4 nM, at least 5 nM, at least 6 nM, at least 7 nM, at least 8 nM, at least 9 nM, at least 10 nM, at least 20 nM, at least 30 nM, at least 40 nM, at least 50 nM, at least 60 nM, at least 70 nM, at least 80 nM, at least 90 nM, at least 100 nM, at least 150 nM, at least 200 nM, at least 300 nM, at least 400 nM, at least 500 nM, at least 600 nM, at least 700 nM, at least 800 nM, at least 900 nM, at least 1.0 uM, at least 1.25 uM, at least 1.5 uM, at least 1.75 uM, at least 2.0 uM, at least 2.5 uM, at least 3 uM, at least 4 uM, at least 5 uM, at least 6 uM, at least 7 uM, at least 8 uM, at least 9 uM, or at least 10 uM. In some embodiments, the inhibitor of an epigenetic regulator is provided at a concentration of 1nM-10nM, 10nM-50nM, 50nM-100nM, 100nM-500nM, 500nM-1uM, 1uM-5uM, or 5uM-10uM.

[00171] In some embodiments, the cells (e.g., CD34+ hemogenic endothelium) are cultured exposed to an inhibitor of an epigenetic regulator until the development of CD5+CD7+ proT cells. In some embodiments, the cells (e.g., CD34+ hemogenic endothelium) are cultured exposed to an inhibitor of an epigenetic regulator for about 14 days. In some embodiments, the cells (e.g., CD34+ hemogenic endothelium) are cultured exposed to an inhibitor of an epigenetic regulator for at least 1 day, at least 2 days, at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days, at least 8 days, at least 9 days, at least 10 days, at least 10 days, at least 11 days, at least 12 days, at least 13 days, at least 14 days, at least 15 days, at least 16 days, at least 17 days, at least 18 days, at least 19 days, at least 20 days, at least 21 days, at least 22 days, at least 23 days, at least 24 days, at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, at least 30 days, at least 31 days, at least 32 days, at least 33 days, at least 34 days, at least 35 days, at least 36 days, at least 37 days, at least 38 days, at least 39 days, at least 40 days, at least 41 days, at least 42 days, at least 43 days, at

least 44 days, at least 45 days, at least 46 days, at least 47 days, at least 48 days, at least 49 days, at least 50 days, or more.

Inhibition of G9a and/or GLP

[00172] In some aspects described herein is a T-cell differentiation method comprising a step of inhibiting G9a and/or GLP. In some aspects described herein is a T-cell differentiation method comprising a step of inhibiting G9a. G9a can also be referred to interchangeably as Euchromatic Histone Lysine Methyltransferase 2 (EHMT2); Histone H3-K9 Methyltransferase 3; KMT1C; Lysine N-Methyltransferase 1C; BAT8; or NG36. G9a is a methyltransferase that methylates lysine residues of histone H3 (see e.g., NCBI Gene ID: 10919; SEQ ID NOs: 45-46 or a sequence that is at least 95% identical and maintains the same function, or a functional fragment thereof). In some aspects described herein is a T-cell differentiation method comprising a step of inhibiting G9a-like protein (GLP). GLP is also referred to interchangeably as Euchromatic Histone Lysine Methyltransferase 1 (EHMT1); KMT1D; Eu-HMTase1; or Histone-Lysine N-Methyltransferase, H3 Lysine-9 Specific 5 (see e.g., NCBI Gene ID: 79813; SEQ ID NOs: 47-48 or a sequence that is at least 95% identical and maintains the same function, or a functional fragment thereof).

[00173] G9a and GLP exist predominantly as a G9a–GLP heteromeric complex. G9a and GLP are the primary enzymes for mono- and dimethylation at Lys 9 of histone H3 (H3K9me1 and H3K9me2) in euchromatin. H3K9me represents a specific tag for epigenetic transcriptional repression by recruiting HP1 proteins to methylated histones. G9a/GLP also weakly methylates 'Lys-27' of histone H3 (H3K27me). G9a/GLP is also required for DNA methylation; the histone methyltransferase activity of G9a/GLP is not required for DNA methylation, suggesting that these two activities function independently. G9a/GLP is probably targeted to histone H3 by different DNA-binding proteins, e.g., E2F6, MGA, MAX and/or DP1. In addition to the histone methyltransferase activity, G9a/GLP also methylates non-histone proteins, e.g., dimethylation of 'Lys-373' of p53/TP53.

[00174] G9a also mediates monomethylation of 'Lys-56' of histone H3 (H3K56me1) in G1 phase, leading to promote interaction between histone H3 and PCNA and regulating DNA replication. G9a is also though to methylate histone H1. G9a also methylates CDYL, WIZ, ACIN1, DNMT1, HDAC1, ERCC6, KLF12, and itself. During G0 phase, GLP may contribute to silencing of MYC- and E2F-responsive genes, suggesting a role in G0/G1 transition in cell cycle. In addition to the histone methyltransferase activity, GLP also methylates non-histone proteins: mediates dimethylation of 'Lys-373' of p53/TP53.

[00175] **SEQ ID NO: 45**, Homo sapiens euchromatic histone lysine methyltransferase 2 (EHMT2), transcript variant 1, mRNA, NCBI Reference Sequence: NM_001289413.1 (region 5-3706), 3702 bp

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GGGACACCCCTCGTAGTGAAGAAACCCTGCCCAAGGCCACCCCCGACTCCCTGGAGCCT
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[00176] SEQ ID NO: 46, histone-lysine N-methyltransferase EHMT2 isoform c (Homo sapiens), NCBI Reference Sequence: NP_001276342.1, 1233 aa

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[00177] SEQ ID NO: 47, Homo sapiens euchromatic histone lysine methyltransferase 1 (EHMT1), transcript variant 2, mRNA, NCBI Reference Sequence: NM_001145527.2 (region 25-2451), 2427 bp

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CCTTTGGTGA

[00178] SEQ ID NO: 48, histone-lysine N-methyltransferase EHMT1 isoform 2 (Homo sapiens), NCBI Reference Sequence: NP_001138999.1, 808 aa

MAAADAEAVPARGEPQQDCCVKTELLGEETPMAADEGSAEKQAGEAHMAADGETNGSCE
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DCASRVNNASYCPHCGEESSKAKEVTIAKADTTSTVTPVPGQEKGSALEGRADTTTGSAAGP
PLSEDDKLQGAASHVPEGFDPTGPAGLGRPTPGLSQGPGKETLESALIALDSEKPKKLRFHPK
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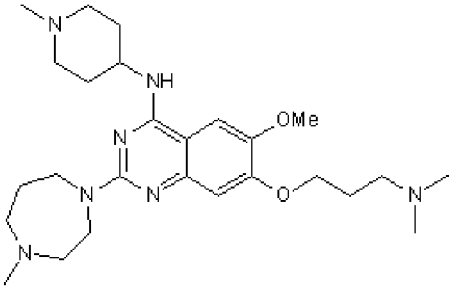
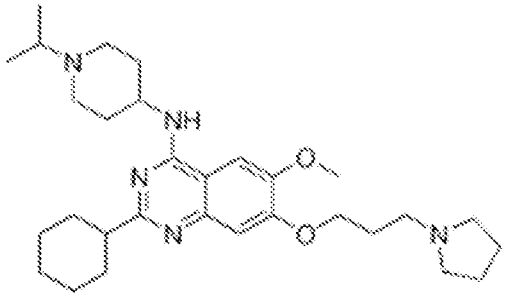
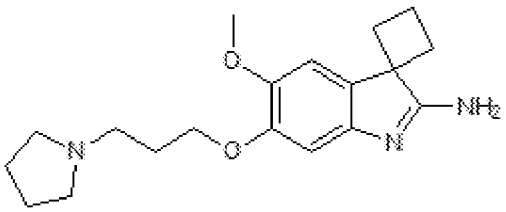
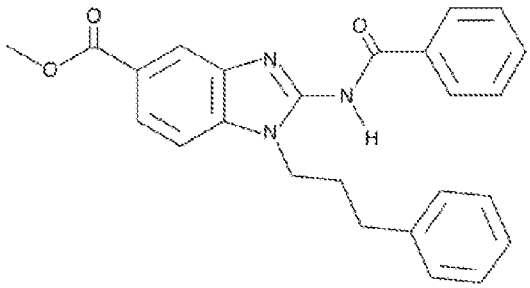
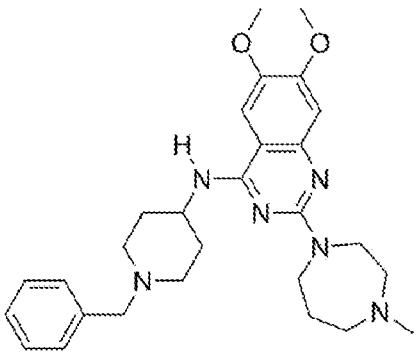
[00179] In some embodiments, the method comprises the step of, after the step of differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of CD34+ hemogenic endothelium, inhibiting G9a and/or GLP in the resultant population of CD34+ hemogenic endothelium. In some embodiments, the method comprises the step of, before the step of differentiating a population of CD34+ hemogenic endothelium in a CD3+-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of CD3+ T cells, inhibiting G9a and/or GLP in the population of CD34+ hemogenic endothelium.

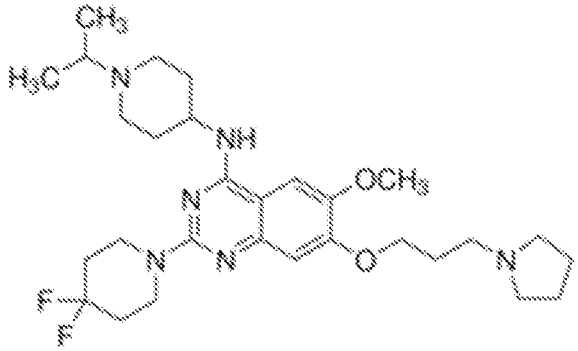
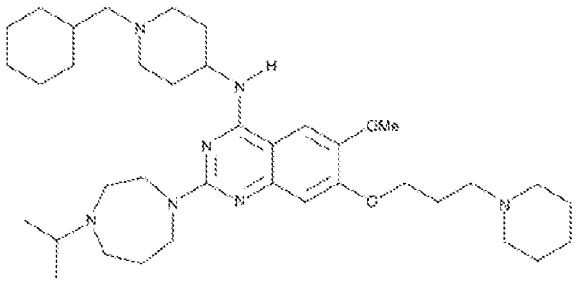
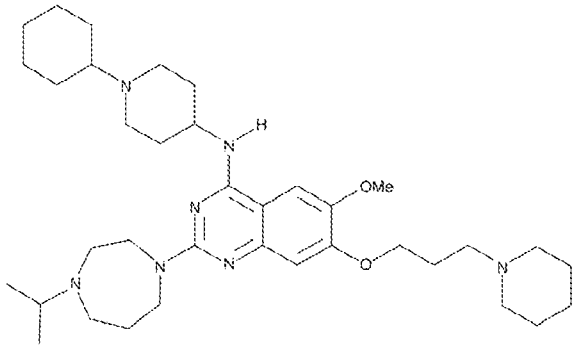
[00180] Accordingly, in one aspect described herein is a method comprising: (a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; (b) inhibiting G9a and/or GLP in the resultant population of $CD34^+$ hemogenic endothelium; and (c) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of $CD3^+$ T cells.

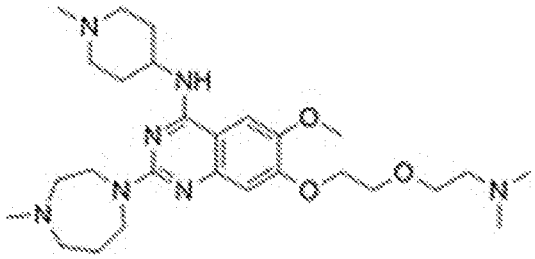
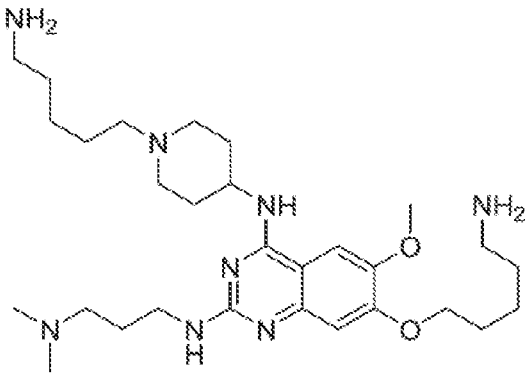
[00181] In one embodiment, the inhibitor is a G9a/GLP inhibitor. In one embodiment, the G9a/GLP inhibitor is selected from a compound listed in **Table 3**, or a derivative or analog thereof. In one embodiment, the G9a/GLP inhibitor is selected from the group consisting of: UNC0224; UNC0638; A366; BRD4770; BIX01294; UNC0642; UNC0631; UNC0646; UNC0321; E72; BIX-01338; BRD9539; Chaetocin; and DCG066. In one embodiment, the G9a/GLP inhibitor is selected from the group consisting of: UNC0224; UNC0638; A366; BRD4770; BIX01294; and UNC0642 (see e.g., **Fig. 8**, **10B**, **12B**, **13B-13F**). In some embodiments, the G9a/GLP inhibitor is selected from the group consisting of: UNC0224; UNC0638; BRD4770; BIX01294; and UNC0642 (see e.g., **Fig. 8**, **10B**, **12B**, **13B**, **13D-13F**).

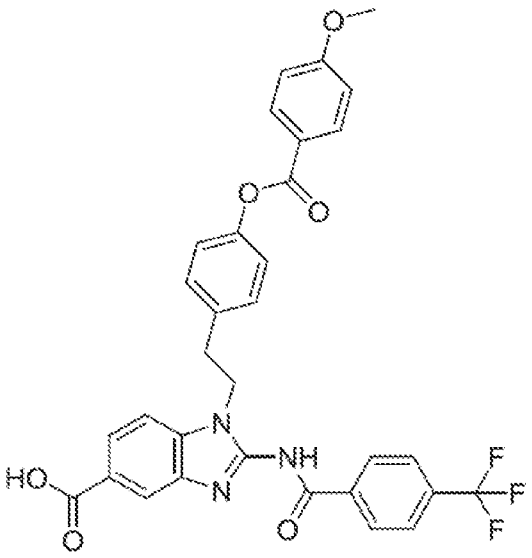
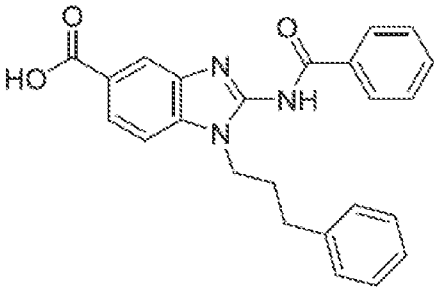
[00182] In some embodiments, the G9a/GLP inhibitor is a Type I G9a/GLP inhibitor (e.g., a BIX-01294 derivative) selected from the group consisting of: UNC0224; UNC0638; A366; BIX01294; UNC0642; UNC0631; UNC0646; UNC0321; and E72. In some embodiments, the G9a/GLP inhibitor is a Type II G9a/GLP inhibitor (e.g., a BIX-01338 derivative) selected from the group consisting of: BRD4770; BIX-01338; and BRD9539. In some embodiments, the G9a/GLP inhibitor is a Type III G9a/GLP inhibitor such as Chaetocin. In some embodiments, the G9a/GLP inhibitor is a Type IV G9a/GLP inhibitor selected from the group consisting of: DCG066.

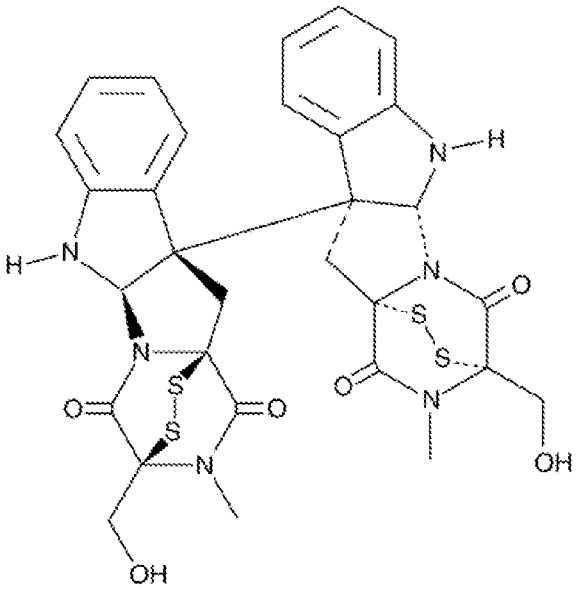
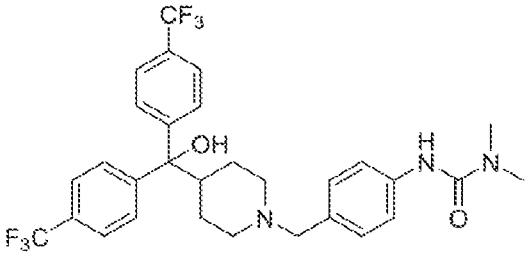
[00183] **Table 3: G9a/GLP inhibitors that can promote T cell differentiation** (see e.g., **Fig. 13A-13F**). All references cited in Table 3 are specifically incorporated herein by reference in their entireties.

G9a/GLP inhibitor	Exemplary results	Exemplary effective dose(s)	Small molecule structure
UNC0224 (e.g., Type I G9a inhibitor, BIX-01294 derivative)	See e.g., **Fig. 8**, **10B**, **12B**	312 nM, 625 nM, 1.25 uM, 2.5 uM, 5 uM	
UNC0638 (e.g., Type I G9a inhibitor, BIX-01294 derivative)	See e.g., **Fig. 13B**	8 nM	
A366 (e.g., Type I G9a inhibitor, BIX-01294 derivative)	See e.g., **Fig. 13C**	N/A	
BRD4770 (e.g., Type II G9a inhibitor, BIX-01338 derivative)	See e.g., **Fig. 13D**; see e.g., Yuan et al., ACS Chem Biol. 2012 Jul 20; 7(7): 1152–1157.	200 nM	
BIX01294 (e.g., Type I G9a inhibitor)	See e.g., **Fig. 13E**	200 nM	

G9a/GLP inhibitor	Exemplary results	Exemplary effective dose(s)	Small molecule structure
UNC0642 (e.g., Type I G9a inhibitor, BIX-01294 derivative)	See e.g., **Fig. 13F**	40 nM	
UNC0631 (e.g., Type I G9a inhibitor, BIX-01294 derivative)	See e.g., Liu et al. Journal of Medicinal Chemistry 54(17), 6139-6150 (2011).	E.g., in MDA-MB-231 cells, IC_{50} = 25 nM	
UNC0646 (e.g., Type I G9a inhibitor, BIX-01294 derivative)	See e.g., Liu et al. Journal of Medicinal Chemistry 54(17), 6139-6150 (2011).	E.g., in MDA-MB-231 cells, IC_{50} = 26 nM	

G9a/GLP inhibitor	Exemplary results	Exemplary effective dose(s)	Small molecule structure
UNC0321 (e.g., Type I G9a inhibitor, BIX-01294 derivative)	See e.g., Liu et al. J Med Chem. 2010 Aug 12; 53(15): 5844–5857; Liu et al. J Med Chem. 2009 Dec 24; 52(24): 7950–7953. A cell-permeable, quinazoline analog that potently and selectively inhibits PKMT G9a (IC_{50} = 6 nM and 9 nM in two biochemical assays for CLOT and ECSD, respectively, and Morrison Ki = 63 pM, which is approximately 250-fold more potent than a closely-related analog, BIX01294. It inhibits GLP with reduced potency (e.g., 15 nM) and is found to be inactive (IC50 > 40 μM) toward other protein lysine and arginine methyltransferases, such as SET7/9 (aH3K4 PKMT), SET8/PreSET7 (aH4K20 PKMT), and PRMT3, as well as JMJD2E (> 1000-fold selectivity) in ECSD enzymatic assays.	E.g., G9a (IC_{50} = 6 nM and 9 nM, and Morrison Ki = 63 pM; GLP (e.g., 15 nM)	
E72 (e.g., Type I G9a inhibitor, BIX-01294 derivative)	See e.g., Chang et al. J Mol Biol. 2010 Jul 2; 400(1): 1–7	E.g., G9a EC_{50} 100 nM	

G9a/GLP inhibitor	Exemplary results	Exemplary effective dose(s)	Small molecule structure
BIX-01338 (e.g., Type II G9a inhibitor)	See e.g., Greiner et al. Nature Chemical Biology 1(3), 143-145 (2005). A cell-permeable amino-benzimidazolo compound that is shown to inhibit a broad-spectrum of histone methyltransferases, including the PRMT1 H4R3 me2 activity, SET7/9 H3K4 me activity, G9a H3K9 me2 activity, as well as the H3K9 me3 activity of SUV39H1 (wild-type and H320R hyperactive mutant) and GLP (effective conc. = 15 μM) in a SAM-competitive manner.	G9a/GLP effective conc. = 15 μM	
BRD9539 (e.g., Type II G9a inhibitor, BIX-01338 derivative)	See e.g., Yuan et al., ACS Chem Biol. 2012 Jul 20; 7(7): 1152–1157. BRD9539 is an inhibitor of G9a, with an IC50 value of 6.3 μM. It inhibits polycomb repressive complex 2 (PRC2) to a similar extent with 54 and 43% activity remaining for G9a and PRC2, respectively, when used at a concentration of 10 μM. It is selective for G9a and PRC2 over SU39H1 and NDMT1 up to a concentration of 40 μM. It is more potent than BRD4770 in enzyme assays, but may require a higher concentration for cell-based assays.	E.g., G9a, IC50 value of 6.3 μM	

G9a/GLP inhibitor	Exemplary results	Exemplary effective dose(s)	Small molecule structure
Chaetocin (e.g., (+)-Chaetocin; Type III G9a inhibitor)	See e.g., Greiner et al. Nature Chemical Biology 1(3), 143-145 (2005). Chaetocin is a fungal mycotoxin that inhibits the HMT SU(VAR)3-9 (IC_{50} = 0.8 μM).1 It inhibits other Lys9-specific HMTs, including G9a (IC_{50} = 2.5 μM) and DIM5 (IC_{50} = 3 μM).1 Selectivity for Lys9-HMTs is indicated by higher IC50 values (>90 μM) for Lys20-specific PRSET7, Lys27-specific EZH2, and Lys4-specific SET7/9.	E.g., G9a IC_{50} = 2.5 μM	
DCG066 (e.g., Type IV G9a inhibitor)	See e.g., Kondengaden et al., Eur J Med Chem. 2016 Oct 21;122:382-393.	E.g., G9a EC_{50} 6.5 uM	

[00184] In some embodiments, the G9a/GLP inhibitor is provided at a concentration of at least 500 nM. In some embodiments, the G9a/GLP inhibitor is provided at a concentration of at least 1 nM, at least 2 nM, at least 3 nM, at least 4 nM, at least 5 nM, at least 6 nM, at least 7 nM, at least 8 nM, at least 9 nM, at least 10 nM, at least 20 nM, at least 30 nM, at least 40 nM, at least 50 nM, at least 60 nM, at least 70 nM, at least 80 nM, at least 90 nM, at least 100 nM, at least 150 nM, at least 200 nM, at least 300 nM, at least 400 nM, at least 500 nM, at least 600 nM, at least 700 nM, at least 800 nM, at least 900 nM, at least 1.0 uM, at least 1.25 uM, at least 1.5 uM, at least 1.75 uM, at least 2.0 uM, at least 2.5 uM, at least 3 uM, at least 4 uM, at least 5 uM, at least 6 uM, at least 7 uM, at least 8 uM, at least 9 uM, or at least 10 uM. In some embodiments, the G9a/GLP inhibitor is provided at a concentration of 1nM-10nM, 10nM-50nM, 50nM-100nM, 100nM-500nM, 500nM-1uM, 1uM-5uM, or 5uM-10uM.

[00185] In some embodiments, the G9a/GLP inhibitor (e.g., UNC0224; see e.g., **Fig. 8**, **10B**, **12B**) is provided at a concentration of at least 312 nM, at least 625 nM, at least 1.25 uM, at least 2.5 uM, or at least 5 uM. In some embodiments, the G9a/GLP inhibitor (e.g., UNC0638; see e.g., **Fig. 13B**) is

provided at a concentration of at least 8 nM. In some embodiments, the G9a/GLP inhibitor (e.g., BRD4770; see e.g., **Fig. 13D**) is provided at a concentration of at least 200 nM. In some embodiments, the G9a/GLP inhibitor (e.g., BIX01294; see e.g., **Fig. 13E**) is provided at a concentration of at least 200 nM. In some embodiments, the G9a/GLP inhibitor (e.g., UNC0642; see e.g., **Fig. 13F**) is provided at a concentration of at least 40 nM.

[00186] In some embodiments, the cells (e.g., CD34+ hemogenic endothelium) are cultured exposed to a G9a/GLP inhibitor until the development of CD5+CD7+ proT cells. In some embodiments, the cells (e.g., CD34+ hemogenic endothelium) are cultured exposed to a G9a/GLP inhibitor for about 14 days. In some embodiments, the cells (e.g., CD34+ hemogenic endothelium) are cultured exposed to a G9a/GLP inhibitor for at least 1 day, at least 2 days, at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days, at least 8 days, at least 9 days, at least 10 days, at least 10 days, at least 11 days, at least 12 days, at least 13 days, at least 14 days, at least 15 days, at least 16 days, at least 17 days, at least 18 days, at least 19 days, at least 20 days, at least 21 days, at least 22 days, at least 23 days, at least 24 days, at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, at least 30 days, at least 31 days, at least 32 days, at least 33 days, at least 34 days, at least 35 days, at least 36 days, at least 37 days, at least 38 days, at least 39 days, at least 40 days, at least 41 days, at least 42 days, at least 43 days, at least 44 days, at least 45 days, at least 46 days, at least 47 days, at least 48 days, at least 49 days, at least 50 days, or more.

[00187] In some embodiments, culturing cells (e.g., CD34+ hemogenic endothelium) in the presence of a G9a/GLP inhibitor increases the number of resultant cells (e.g., CD5+CD7+ Pro-T cells; CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells; alpha-beta T cells) by at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, at least 150%, at least 200%, at least 250%, at least 300%, at least 350%, at least 400%, at least 450%, at least 500%, at least 600%, at least 700%, at least 800%, at least 900%, or more, or at least 10x, 20x, 30x, 40x, 50x, 60x, 70x, 80x, 90x, 100x, 500x, 1,000x, or more higher compared to cells not cultured in the presence of a G9a/GLP inhibitor; see e.g., **Fig. 8**, **Fig. 10B**, **Fig. 12B**, **Fig. 13B-13F**, **Fig. 14B**, **Fig. 15A**.

[00188] In some embodiments, culturing cells (e.g., CD34+ hemogenic endothelium) in the presence of a G9a/GLP inhibitor decreases the number of erythroid or myeloid lineage cells (e.g., erythroid cell; macrophage; granulocyte; megakaryocyte) by at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, at least 150%, at least 200%, at least 250%, at least 300%, at least 350%, at least 400%, at

least 450%, at least 500%, at least 600%, at least 700%, at least 800%, at least 900%, or more, or at least 10x, 20x, 30x, 40x, 50x, 60x, 70x, 80x, 90x, 100x, 500x, 1,000x, or more higher compared to cells not cultured in the presence of a G9a/GLP inhibitor; see e.g., **Fig. 14C**.

[00189] In some embodiments, culturing cells (e.g., CD34+ hemogenic endothelium) in the presence of a G9a/GLP inhibitor decreases the total number of differentiated cells by at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, at least 150%, at least 200%, at least 250%, at least 300%, at least 350%, at least 400%, at least 450%, at least 500%, at least 600%, at least 700%, at least 800%, at least 900%, or more, or at least 10x, 20x, 30x, 40x, 50x, 60x, 70x, 80x, 90x, 100x, 500x, 1,000x, or more higher compared to cells not cultured in the presence of a G9a/GLP inhibitor; see e.g., **Fig. 15B**.

[00190] In some embodiments, culturing cells (e.g., CD34+ hemogenic endothelium) in the presence of a G9a/GLP inhibitor increases the percentage of resultant cells of interest (e.g., CD5+CD7+ Pro-T cells; CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells; alpha-beta T cells) amongst the total number of differentiated cells by at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, at least 150%, at least 200%, at least 250%, at least 300%, at least 350%, at least 400%, at least 450%, at least 500%, at least 600%, at least 700%, at least 800%, at least 900%, or more, or at least 10x, 20x, 30x, 40x, 50x, 60x, 70x, 80x, 90x, 100x, 500x, 1,000x, or more higher compared to cells not cultured in the presence of a G9a/GLP inhibitor; see e.g., **Fig. 15C**.

[00191] In some embodiments, a method for differentiating T cells as described herein (e.g., G9a/GLP inhibition and stromal-free T cell differentiation) produces a population that comprises at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99% of the cells of interest (e.g., CD5+CD7+ Pro-T cells; CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells; alpha-beta T cells). In some embodiments, a method for differentiating T cells as described herein (e.g., G9a/GLP inhibition and stromal-free T cell differentiation) produces a population that comprises at least 15% CD5+CD7+ ProT cells.

[00192] See e.g., Greiner et al. Nature Chemical Biology 1(3), 143-145 (2005); Liu et al. Journal of Medicinal Chemistry 54(17), 6139-6150 (2011); Liu et al. J Med Chem. 2010 Aug 12; 53(15): 5844–5857; Liu et al., J Med Chem. 2009 Dec 24; 52(24): 7950–7953; Kondengaden et al., Eur J Med Chem. 2016 Oct 21, 122:382-393; Yuan et al. ACS Chem Biol. 2012 Jul 20; 7(7): 1152–1157; Chang et al. J Mol Biol. 2010 Jul 2; 400(1): 1–7; Christman et al. Proceedings of the National Academy of

Sciences of the United States of America 92(16), 7347-7351 (1995); Cheng et al., Signal Transduction and Targeted Therapy volume 4, Article number: 62 (2019); the contents of each of which are incorporated herein by reference in their entireties.

Inhibition of a Histone Methyltransferase

[00193] In some embodiments, the differentiation method can comprise inhibiting a histone methyltransferase. The step of inhibiting a histone methyltransferase (e.g., EZH1 knockdown) can increase differentiation efficiency (e.g., of the T cells). Accordingly, in some embodiments, the differentiation method comprises inhibiting a histone methyltransferase, e.g., in the resultant population of CD34+ hemogenic endothelium. Methods of inhibiting a histone methyltransferase are known in the art; see e.g., International Application No. WO 2018/048828, US Application No. 2019/0225940, Doulatov et al., Cell Stem Cell. 2013 October 3, 13(4); Vo et al., Nature 2018, 553(7689): 506–510; the contents of each of which are incorporated herein by reference in their entireties.

[00194] However, the step of inhibiting a histone methyltransferase (e.g., EZH1 knockdown) is not required. Thus, in some embodiments, the differentiation method does not comprise inhibiting a histone methyltransferase, e.g., in the resultant population of CD34+ hemogenic endothelium.

[00195] In the course of these experiments, the inventors discovered that inhibition of specific histone modifying enzymes targeting H3K9 and H3K27 promotes lymphoid potential of hematopoietic progenitors derived from pluripotent stem cells. The histone modifying enzymes are histone lysine methyltransferases. Post-translational modifications of histone proteins regulate chromatin compaction, mediate epigenetic regulation of transcription, and control cellular differentiation in health and disease. Methylation of histone tails is one of the fundamental events of epigenetic signaling. Tri-methylation of lysine 9 of histone H3 (H3K9) mediates chromatin recruitment of HP1, heterochromatin condensation and gene silencing. Similarly, methylation of H3K27 and H4K20 are associated with a repressed state of chromatin, whereas expressed genes are methylated at H3K4, H3K36 and H3K79. Methylation of H3K9 in humans relies mostly on members of the Suv39 family, namely EHMT1/GLP, EHMT2/G9a, SUV39H1, SUV39H2, SETDB1 and SETDB2, as well as then non-Suv39 enzymes PRDM2 and ASH1L (see e.g., Hong Wu et al., Structural Biology of Human H3K9 Methyltransferases, 2010, PLoS ONE, 5(2): e8570, which is incorporated herein by reference). In contrast, the methylation of H3K27 is carry out by the polycomb repressive complex 2 (PRC2).

[00196] Di/trimethylation of H3K9 is mainly catalyzed by the conserved SUV39H1/2 histone methyltransferases, while the polycomb repressive complex 2 (PRC2) ensures di/trimethylation of H3K27 (see e.g., Rea S, 2000. Nature 406:593-599; Margueron R, and Reinberg D. 2011. Nature

469:343-349). PRC2 comprises the EZH1/2 catalytic subunit, SUZ12, EED, and RBBP7/4 (see e.g., Margueron R, and Reinberg D, 2011).

[00197] It is specifically contemplated herein that inhibiting the histone lysine methyltransferases that target H3K9 and H3K27 relieves transcriptional repression that results from methylation of histone H3, and thereby promotes gene expression which facilitates cell differentiation, specifically T cell specification.

[00198] In one embodiment, the histone methyltransferase catalyzes the addition of methyl group to the histone H3 lysine residue 9 (H3K9) and/or histone H3 lysine residue 27 (H3K27).

[00199] In one embodiment, the histone methyltransferase inhibitor inhibits the G9a/GLP heteromeric complex.

[00200] G9a (EC 2.1.1.43) (UniProtKB: Q96KQ7) is also known as EHMT2, (Euchromatic Histone-Lysine N-Methyltransferase 2), G9A Histone Methyltransferase and protein G9a.

[00201] GLP (EC 2.1.1.43) (UniProtKB: Q9H9B1) is also known as EHMT1 (Euchromatic Histone-Lysine N-Methyltransferase 1), G9a-Like Protein 1 and GLP1.

[00202] In one embodiment, the histone methyltransferase inhibitor inhibits EZH1 (Enhancer of Zeste 1 Polycomb Repressive Complex 2 Subunit).

[00203] In one embodiment, the H3K27 histone methyltransferase is EZH1 (EC:2.1.1.43) (UniproKB Q92800-1).

[00204] In one embodiment, the H3K27 histone methyltransferase is not EZH2 (EC:2.1.1.43) (Unipro Q15910-1).

[00205] In one embodiment, the inhibitor of histone methyltransferase inhibits the gene expression or protein catalytic activity of the histone methyltransferase.

[00206] In one embodiment, the histone methyltransferase H3K9 and/or H3K27 is inhibited by a small molecule or a nucleic acid or a CRISPR-mediated target genetic interference.

[00207] In some embodiments, the histone methyltransferase H3K9 and/or H3K27 is inhibited by a small molecule inhibitor or a nucleic acid inhibitor. In one embodiment of any method, cells, or composition described, the histone methyltransferase small molecule inhibitor is a chemical agent including, but not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, aptamers, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. In some embodiments, the small molecule is a heterorganic compound or an organometallic compound.

[00208] In one embodiment, the histone methyltransferase small molecule inhibitor include but are not limited to AMI-1, A-366, BIX-01294, BIX01338, BRD4770, chaetocin, E72, UNC0224, UNC0631, UNC0638, UNC0642, UNC0646, EPZ5676, EPZ005687, GSK343, EPZ-6438 (E7438), 3-deazaneplanocin A (DZNeP) HCl, UNC1999, MM-102, SGC 0946, Entacapone, EPZ015666, UNC0379, EI1, MI-2 (Menin-MLL Inhibitor), MI-3 (Menin-MLL Inhibitor), PFI-2, GSK126, or EPZ004777.

[00209] In one embodiment, the histone methyltransferase small molecule inhibitor is selected from the group consisting of UNC0631, BRD4770, UNC1999, CPI-360, and BIX 01294.

[00210] In one embodiment, the nucleic acid inhibitor is a nucleic acid targeting the expression of histone methyltransferase. For example, targeting the mRNA or primary transcript of the histone methyltransferase, EZH1, thereby inhibiting protein expression of the enzyme. Histone-lysine N-methyltransferase aka Enhancer of Zeste 1 Polycomb Repressive Complex 2 Subunit (EZH1) or EC 2.1.1.43, is a component of a noncanonical Polycomb repressive complex-2 (PRC2) that mediates methylation of histone H3 (see MIM 602812) lys27 (H3K27) and functions in the maintenance of embryonic stem cell pluripotency and plasticity. The external identification for the human EZH1 gene are as follows: HGNC: 3526; Entrez Gene: 2145; Ensembl: ENSG00000108799; OMIM: 601674; UniProtKB: Q92800; EMBL: AB002386 mRNA and the corresponding mRNA translation: BAA20842.2; GENBANK: BT009782 mRNA and the corresponding mRNA translation: AAP88784.1.

[00211] In one embodiment, the nucleic acid inhibitor targets the human EZH1 mRNA.

[00212] In one embodiment, the nucleic acid inhibitor is a RNA interference inhibitor or CRISPR-mediated genetic interference inhibitor. The RNA interference inhibitor can be designed using the predictor RNAi softwares found at the Whitehead Institute, MIT, siRNA website, BLOCK-iT™ RNAi Designer at Invitrogen / ThermoFisher, and other online siRNA design tools at The RNAi Web using the mRNA of EZH1 as the target.

[00213] Similarly, Crisper guide RNA can be designed using the Broad Institute (MIT) CRISPR software (available on the world-wide web at, for example, portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design), dna20, Clontech, AddGene, e-crisp, and Innovative Genomic using the mRNA or genomic gene of EZH1 as the target.

[00214] CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Cas9-mediated gene disruption has been widely used in generating loss-of-function mutations in diverse organisms including mammals (Cong et al., 2013, Science, 339(6121):819-23; reviewed in Hsu et al., 2014, Cell, 157(6):1262-78)). Cas9-based knockout screens have been applied in identifying essential genes and genes involved in drug resistance in various cell lines. With respect to general information on CRISPR-Cas Systems, components thereof, and delivery of such components, including methods, materials, delivery vehicles, vectors, particles, AAV, and making and using thereof, including as to

amounts and formulations, all useful in the practice of the instant invention, reference is made to: US Patents Nos. 8,999,641, 8,993,233, 8,945,839, 8,932,814, 8,906,616, 8,895,308, 8,889,418, 8,889,356, 8,871,445, 8,865,406, 8,795,965, 8,771,945 and 8,697,359; US Patent Publications US 2014-0310830, US 2014-0287938, US 2014-0273234, US2014-0273232, US 2014-0273231, US 2014-0256046, US 2014-0248702, US 2014-0242700, US 2014-0242699, US 2014-0242664, US 2014-0234972, US 2014-0227787, US 2014-0189896, US 2014-0186958, US 2014-0186919, US 2014-0186843, US 2014-0179770 and US 2014-0179006, US 2014-0170753; European Patents EP 2 784 162 B1 and EP 2 771 468 B1; European Patent Applications EP 2 771 468 (EP13818570.7), EP 2 764 103 (EP13824232.6), and EP 2 784 162 (EP14170383.5); and International Application No. WO 2014/093661, all of which are incorporated herein by reference in their entirety.

[00215] The CRISPR/Cas system envisaged for use in the context of the invention can make use of any suitable CRISPR enzyme. In some embodiments, the CRISPR enzyme is a type II CRISPR system enzyme. In some embodiments, the CRISPR enzyme is a Cas9 enzyme. In some embodiments, the Cas9 enzyme is *S. pneumoniae, S. pyogenes*, or *S. thermophilus* Cas9, and may include mutated Cas9 derived from these organisms. The enzyme may be a Cas9 homolog or ortholog. In some embodiments, the CRISPR enzyme is codon-optimized for expression in a eukaryotic cell.

[00216] As described herein, the CRISPR/Cas system is used to specifically target a multitude of sequences within the continuous genomic region of interest. The targeting typically comprises introducing into each cell of a population of cells a vector system of one or more vectors comprising an engineered, non-naturally occurring CRISPR-Cas system comprising: at least one Cas protein, and one or more guide RNAs of the guide RNA library described herein.

[00217] In these methods, the Cas protein and the one or more guide RNAs may be on the same or on different vectors of the system and are integrated into each cell, whereby each guide sequence targets a sequence within the continuous genomic region in each cell in the population of cells. The Cas protein is operably linked to a regulatory element to ensure expression in said cell, more particularly a promoter suitable for expression in the cell of the cell population. In particular embodiments, the promoter is an inducible promoter, such as a doxycycline inducible promoter. When transcribed within the cells of the cell population, the guide RNA comprising the guide sequence directs sequence-specific binding of a CRISPR-Cas system to a target sequence in the continuous genomic region. Typically binding of the CRISPR-Cas system induces cleavage of the continuous genomic region by the Cas protein.

[00218] RNA interference (RNAi) mediated by short interfering RNAs (siRNA) or microRNAs (miRNA) is a powerful method for post-transcriptional regulation of gene expression. RNAi has been extensively used for the study of biological processes in mammalian cells and could constitute a therapeutic approach to human diseases in which selective modulation of gene expression would be

desirable. Depending on the degree of complementarity between miRNA and target mRNA sequences, loss of gene expression occurs by inducing degradation of the cognate mRNA or by translational attenuation. Endogenous miRNAs are transcribed as primary transcripts and subsequently processed by the RNAse III enzyme Drosha to create a stem loop structure. Nuclear export and cleavage by Dicer generates a mature short double stranded molecule (siRNA) that is separated into guide and passenger strands. The guide strand is loaded into the RNA induced silencing complex (RISC), the effector complex mediating cleavage of target mRNAs with the functional guide strand binding to RISC proteins while the passenger strand is degraded. The loading of guide versus passenger strands into RISC largely depends on the 5' end stability of the siRNA, with the less stable strand preferentially incorporated into RISC, although the exact regulation in mammalian cells is incompletely understood. The 5' end of the guide strand contains the "seed region," which is critical for target identification. Precise cleavage by Drosha and Dicer is critical for the generation of guide RNAs with defined seed regions that mediate efficient binding to the appropriate target mRNAs. Inaccurate processing results in binding to off-target molecules but a shift in cleavage sites also alters the nucleotide composition of duplex ends, which may have a profound effect on strand loading into RISC.

[00219] The inhibiting the expression of selected target polypeptides is through the use of RNA interference agents. RNA interference (RNAi) uses small interfering RNA (siRNA) duplexes that target the messenger RNA encoding the target polypeptide for selective degradation. siRNA-dependent post-transcriptional silencing of gene expression involves cleaving the target messenger RNA molecule at a site guided by the siRNA. RNAi is an evolutionally conserved process whereby the expression or introduction of RNA of a sequence that is identical or highly similar to a target gene results in the sequence specific degradation or specific post-transcriptional gene silencing (PTGS) of messenger RNA (mRNA) transcribed from that targeted gene (see e.g., Coburn, G. and Cullen, B. (2002) J. Virology 76(18):9225), thereby inhibiting expression of the target gene. In one embodiment, the RNA is double stranded RNA (dsRNA). This process has been described in plants, invertebrates, and mammalian cells. In nature, RNAi is initiated by the dsRNA-specific endonuclease Dicer, which promotes processive cleavage of long dsRNA into double-stranded fragments termed siRNAs. siRNAs are incorporated into a protein complex (termed "RNA induced silencing complex," or "RISC") that recognizes and cleaves target mRNAs. RNAi can also be initiated by introducing nucleic acid molecules, e.g., synthetic siRNAs or RNA interfering agents, to inhibit or silence the expression of target genes. As used herein, "inhibition of target gene expression" includes any decrease in expression or protein activity or level of the target gene or protein encoded by the target gene as compared to a situation wherein no RNA interference has been induced. The decrease will be of at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, or more as compared to the expression of a target

gene or the activity or level of the protein encoded by a target gene which has not been targeted by an RNA interfering agent.

[00220] The terms "RNA interference agent" and "RNA interference" as they are used herein are intended to encompass those forms of gene silencing mediated by double-stranded RNA, regardless of whether the RNA interfering agent comprises an siRNA, miRNA, shRNA or other double-stranded RNA molecule. siRNA is defined as an RNA agent which functions to inhibit expression of a target gene, e.g., by RNAi. An siRNA may be chemically synthesized, may be produced by *in vitro* transcription, or may be produced within a host cell. In one embodiment, siRNA is a double stranded RNA (dsRNA) molecule of about 15 to about 40 nucleotides in length, preferably about 15 to about 28 nucleotides, more preferably about 19 to about 25 nucleotides in length, and more preferably about 19, 20, 21, 22, or 23 nucleotides in length, and may contain a 3' and/or 5' overhang on each strand having a length of about 0, 1, 2, 3, 4, or 5 nucleotides. The length of the overhang is independent between the two strands, i.e., the length of the overhang on one strand is not dependent on the length of the overhang on the second strand. Preferably the siRNA is capable of promoting RNA interference through degradation or specific post-transcriptional gene silencing (PTGS) of the target messenger RNA (mRNA).

[00221] siRNAs also include small hairpin (also called stem loop) RNAs (shRNAs). In one embodiment, these shRNAs are composed of a short (e.g., about 19 to about 25 nucleotide) antisense strand, followed by a nucleotide loop of about 5 to about 9 nucleotides, and the analogous sense strand. Alternatively, the sense strand may precede the nucleotide loop structure and the antisense strand may follow. These shRNAs may be contained in plasmids, retroviruses, and lentiviruses and expressed from, for example, the pol III U6 promoter, or another promoter (see, e.g., Stewart, et al. (2003) RNA April; 9(4):493-501, incorporated by reference herein in its entirety). The target gene or sequence of the RNA interfering agent may be a cellular gene or genomic sequence, e.g., the G9a/GLP or EZH1 sequence. An siRNA may be substantially homologous to the target gene or genomic sequence, or a fragment thereof. As used in this context, the term "homologous" is defined as being substantially identical, sufficiently complementary, or similar to the target mRNA, or a fragment thereof, to effect RNA interference of the target. In addition to native RNA molecules, RNA suitable for inhibiting or interfering with the expression of a target sequence include RNA derivatives and analogs. Preferably, the siRNA is identical to its target. The siRNA preferably targets only one sequence. Each of the RNA interfering agents, such as siRNAs, can be screened for potential off-target effects by, for example, expression profiling. Such methods are known to one skilled in the art and are described, for example, in Jackson et al. Nature Biotechnology 6:635-637, 2003. In addition to expression profiling, one may also screen the potential target sequences for similar sequences in the sequence databases to identify potential sequences which may have off-target effects. For example, 15, or perhaps as few as 11 contiguous nucleotides, of sequence identity are

sufficient to direct silencing of non-targeted transcripts. Therefore, one may initially screen the proposed siRNAs to avoid potential off-target silencing using the sequence identity analysis by any known sequence comparison methods, such as BLAST. siRNA sequences are chosen to maximize the uptake of the antisense (guide) strand of the siRNA into RISC and thereby maximize the ability of RISC to target G9a/GLP or EZH1 mRNA for degradation. This can be accomplished by scanning for sequences that have the lowest free energy of binding at the 5'-terminus of the antisense strand. The lower free energy leads to an enhancement of the unwinding of the 5'-end of the antisense strand of the siRNA duplex, thereby ensuring that the antisense strand will be taken up by RISC and direct the sequence-specific cleavage of the human G9a/GLP or EZH1 mRNA. siRNA molecules need not be limited to those molecules containing only RNA, but, for example, further encompasses chemically modified nucleotides and non-nucleotides, and also include molecules wherein a ribose sugar molecule is substituted for another sugar molecule or a molecule which performs a similar function. Moreover, a non-natural linkage between nucleotide residues can be used, such as a phosphorothioate linkage. The RNA strand can be derivatized with a reactive functional group of a reporter group, such as a fluorophore. Particularly useful derivatives are modified at a terminus or termini of an RNA strand, typically the 3' terminus of the sense strand. For example, the 2'-hydroxyl at the 3' terminus can be readily and selectively derivatizes with a variety of groups. Other useful RNA derivatives incorporate nucleotides having modified carbohydrate moieties, such as 2'O-alkylated residues or 2'-O-methyl ribosyl derivatives and 2'-O-fluoro ribosyl derivatives. The RNA bases may also be modified. Any modified base useful for inhibiting or interfering with the expression of a target sequence may be used. For example, halogenated bases, such as 5-bromouracil and 5-iodouracil can be incorporated. The bases may also be alkylated, for example, 7-methylguanosine can be incorporated in place of a guanosine residue. Non-natural bases that yield successful inhibition can also be incorporated. Preferred siRNA modifications include 2'-deoxy-2'-fluorouridine or locked nucleic acid (LNA) nucleotides and RNA duplexes containing either phosphodiester or varying numbers of phosphorothioate linkages. Such modifications are known to one skilled in the art and are described, for example, in Braasch et al., Biochemistry, 42: 7967-7975, 2003. Most of the useful modifications to the siRNA molecules can be introduced using chemistries established for antisense oligonucleotide technology. Preferably, the modifications involve minimal 2'-O-methyl modification, preferably excluding such modification. Modifications also preferably exclude modifications of the free 5'-hydroxyl groups of the siRNA. The Examples herein provide specific examples of RNA interfering agents, such as shRNA molecules that effectively target mRNA.

[00222] In one embodiment, the nucleic acid is a G9a/GLP or EZH1 specific RNA interference agent or a vector encoding the RNA interference agent. In one embodiment, the RNA interference agent comprises one or more of the nucleotide sequences selected from the group consisting of CTATCTGGCAGTGCGAGAATG (**SEQ ID NO:** 11), AGACGTGCAAGCAGGTCTTTC (**SEQ ID**

NO: 12), TGGATGACTTATGCGTGATTT (**SEQ ID NO: 13**), CAACAGAACTTTATGGTAGAA (**SEQ ID NO: 14**), CCGCCGTGGTTTGTATTCATT (**SEQ ID NO: 15**), GCTTCCTCTTCAACCTCAATA (**SEQ ID NO: 16**), CCGCCGTGGTTTGTATTCATT (**SEQ ID NO: 17**), GCTCTTCTTTGATTACAGGTA (**SEQ ID NO: 18**), and GCTACTCGGAAAGGAAACAAA (**SEQ ID NO: 19**).

[00223] In some embodiments, the nucleic acid inhibitor is a EZH1 specific nucleic acid that is selected from the group consisting of an aptamer that binds EZH1, a EZH1 specific RNA interference agent, and a vector encoding a EZH1 specific RNA interference agent, wherein the RNA interference agent comprises one or more of the nucleotide sequences selected from SEQ ID NO: 11-19.

[00224] In one embodiment, the multilineage hematopoietic progenitor cells are contacted with the viral vector or vector carrying a nucleic acid molecule comprising a nucleic acid sequence selected from a group consisting of SEQ ID NO: 11-19.

[00225] In one embodiment, the contacting with the histone methyltransferase inhibitor occurs more than once. For example, after the initial first contacting of the multilineage hematopoietic progenitor cell with the virus or vector carrying a nucleic acid molecule comprising a nucleic acid sequence selected from a group consisting of SEQ ID NO: 11-19, or contacting with a small molecule inhibitor described herein, the contacted cell is washed to remove that virus or vector, and the washed cell is then contacted for a second time with the same virus or vector used in the first contact.

[00226] It is contemplated herein that the Cas9/CRISPR system of genome editing be employed with the methods, cells and compositions described herein. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) systems is useful for RNA-programmable genome editing (see e.g., Jinek, M. et al. Science (2012) 337(6096):816-821).

[00227] Trans-activating crRNA (tracrRNA) is a small trans-encoded RNA. It was first discovered in the human pathogen *Streptococcus pyogenes*. (See Deltcheva E, et al. (2011). Nature 471 (7340): 602-7). In bacteria and archaea, CRISPR/Cas (clustered, regularly interspaced short palindromic repeats/CRISPR-associated proteins) constitute an RNA-mediated defense system which protects against viruses and plasmids. This defensive pathway has three steps. First a copy of the invading nucleic acid is integrated into the CRISPR locus. Next, CRISPR RNAs (crRNAs) are transcribed from this CRISPR locus. The crRNAs are then incorporated into effector complexes, where the crRNA guides the complex to the invading nucleic acid and the Cas proteins degrade this nucleic acid. (See e.g., Terns MP and Terns RM (2011). Curr Opin Microbiol 14 (3): 321-7). There are several pathways of CRISPR activation, one of which requires a tracrRNA which plays a role in the maturation of crRNA. TracrRNA is complementary to and base pairs with a pre-crRNA forming an RNA duplex. This is cleaved by RNase III, an RNA-specific ribonuclease, to form a crRNA/tracrRNA hybrid. This hybrid acts as a guide for the endonuclease Cas9, which cleaves the

invading nucleic acid. (see e.g., Deltcheva E, et al. supra; Jinek M, et al. (2012), Science 337 (6096): 816-21; and Brouns SJ (2012), Science 337 (6096): 808-9).

[00228] In some embodiments, Cas9/CRISPR system guide RNAs are designed to target the exon 3 of EZH1 gene, which is present in all transcripts of EZH1 known. Exon 3 sequence is ATTACAGCAAGATGGAAATACCAAATCCCCCTACCTCCAAATGTATCACTTACTGGAAAA GAAAAGTGAAATCTGAATACATGCGACTTCGACAACTTAAACGGCTTCAGGCAAATATG GGTGCAAAG (**SEQ ID NO: 20**).

[00229] Non-limiting exemplary gRNAs that target exon 3 are TCGACAACTTAAACGGCTTC (**SEQ ID NO: 21**), TGCGACTTCGACAACTTAAA (**SEQ ID NO: 22**), CCTCCAAATGTATCACTTAC (**SEQ ID NO: 23**), TAAACGGCTTCAGGCAAATA (**SEQ ID NO: 24**) AAACGGCTTCAGGCAAATAT (**SEQ ID NO: 25**), CATTTGGAGGTAGGGGGATT (**SEQ ID NO: 26**), CCAGTAAGTGATACATTTGG (**SEQ ID NO: 27**), GTGATACATTTGGAGGTAGG (**SEQ ID NO: 28**), AAGTGATACATTTGGAGGTA (**SEQ ID NO: 29**), AGTGATACATTTGGAGGTAG (**SEQ ID NO: 30**), TTTCCAGTAAGTGATACATT (**SEQ ID NO: 31**), and TAAGTGATACATTTGGAGGT (**SEQ ID NO: 32**)

[00230] In other embodiments, Cas9/CRISPR system guide RNAs are designed to target the exon 4 of EZH1 gene, which is also present in all transcripts of EZH1 known. Exon 4 sequence is GCTTTGTATGTGGCAAATTTTGCAAAGGTTCAAGAAAAAACCCAGATCCTCAATGAAGA ATGGAAGAAGCTTCGTGTCCAACCTGTTCAGTCAATGAAGCCTGTGAGTGGACACCCTTT TCTCAAAAAG (**SEQ ID NO: 33**).

[00231] Non-limiting exemplary gRNAs that target exon 4 are GCTTCATTGACTGAACAGGT (**SEQ ID NO: 34**), ACAGGCTTCATTGACTGAAC (**SEQ ID NO: 35**), AGAAAAGGGTGTCCACTCAC (**SEQ ID NO: 36**), TCCATTCTTCATTGAGGATC (**SEQ ID NO: 37**), CCATTCTTCATTGAGGATCT (**SEQ ID NO: 38**), CCCAGATCCTCAATGAAGAA (**SEQ ID NO: 39**), GTATGTGGCAAATTTTGCAA (**SEQ ID NO: 40**), and CAGTCAATGAAGCCTGTGAG (**SEQ ID NO: 41**).

[00232] In one embodiment, a vector is used as a transport vehicle to introduce any of the herein described nucleic acid inhibitors of a histone methyltransferase into the target cells selected from the cell populations as described herein (e.g., ESCs; PSCs; iPSCs; hemogenic endothelium; HSCs). In one embodiment, a vector is used as a transport vehicle to introduce any of the herein described nucleic acid comprising the described nucleic acid inhibitors of a histone methyltransferase into the target cells selected from the cell populations as described herein (e.g., ESCs; PSCs; iPSCs; hemogenic endothelium; HSCs). The *in vivo* expression of the nucleic acid inhibitor is for degrading the mRNA of the targeted histone methyltransferase such as G9a/GLP or EZH1 so as to reduce and inhibit the expression of the respective histone methyltransferase, with the goal being to reduce methylation of the histone H3 in the transfected cells and relief repression of gene expression therein.

[00233] In one embodiment, the host cell is an embryonic stem cell, a somatic stem cell, a progenitor cell, a bone marrow cell, a hematopoietic stem cell, a hematopoietic progenitor cell, an immune cell such as a T cell or B cell, an erythrocyte, a fibroblast, a keratinocyte, or a myeloid progenitor cell. In one embodiment, the host cell is isolated from a subject. In one embodiment, the host cell is isolated from a subject who has been diagnosed with a hematological disease.

[00234] In one embodiment, the vector further comprises a spleen focus-forming virus promoter, a tetracycline-inducible promoter, a Doxycycline (Dox)-inducible, or a β-globin locus control region and a β-globin promoter. In one embodiment, the promoter provides for targeted expression of the nucleic acid molecule therein. Other examples of promoters include but are not limited to the CMV promoter and EF1-alpha promoters for the various transgenes, and U6 promoter for shRNAs targeting EZH1.

[00235] In one embodiment, the vector is a virus or a non-viral vector. Non-limiting examples of viral vectors for gene delivery and expressions in cells are retrovirus, adenovirus (types 2 and 5), adeno-associated virus (AAV), Helper-dependent adenoviral vector (HdAd), hybrid adenoviral vectors, herpes virus, pox virus, human foamy virus (HFV), and lentivirus. Exemplary vectors useful in the invention described herein include episomal vectors, integrating vectors, non-integrating vectors, and excisable vectors.

Stroma-Free T Cell Differentiation

[00236] In some embodiments, the differentiation method comprises differentiating the resultant population of CD34+ hemogenic endothelium in a CD3+-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of CD3+ T cells. The method described herein is a stroma-free T cell differentiation method. Compared to differentiation with stromal cells expressing a Notch ligand, stroma-free differentiation unexpectedly results in an increased number of differentiated T cells, with a smaller portion of these T cells being innate-like cells (see e.g., Example 1, Fig. 1D). Unexpectedly, the inventors found that the stroma-free protocol described herein requires starting with hemogenic endothelium (HE), not iPSC or HE-derived progenitors (e.g., lymphoid progenitor).

[00237] In nature, the haematopoietic stem cells (HSCs) in the bone marrow give rise to multipotent progenitors (MPPs) before differentiating into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CLPs migrate from the bone marrow to the thymus, where thymic epithelial cells that express Delta-like ligand 4 (DLL4) trigger canonical Notch 1 signaling in early thymic progenitors (ETPs). This Notch 1 signal is essential for T cell lineage commitment and is further required during early phases of thymocyte differentiation up to the double-negative 3 (DN3) stage. Active Notch signaling during these early stages of T cell development inhibits other lineage potentials, such as B cell and myeloid cell (including dendritic cell (DC)) potential. During β-

selection, Notch signaling is turned off as a consequence of pre-T cell receptor signaling. Thus subsequent stages of T cell development exhibit very low levels of Notch signaling. Notch was also suggested to influence the development of regulatory T (T_{Reg}) cells (specifically, thymic T_{Reg} cells). Notch signaling is mediated by the Notch 2 receptor. Notch signaling pathway is highly conserved in both vertebrate and invertebrate species and it regulates many different cell fate decisions. It is important for pattern formation during development such as neurogenesis, angiogenesis or myogenesis and regulates T cell development and stem cell maintenance. Notch signaling is also involved in cellular processes throughout adulthood. Signaling via Notch occurs between neighboring cells and both the receptor and its ligands are transmembrane proteins. See, e.g., Schmitt T.M., Zúñiga-Pflücker J.C. (2002) Induction of T cell development from hematopoietic progenitor cells by delta-like-1 *in vitro*. Immunity 17:749-756; Mohtashami M. (2010) Direct Comparison of Dll1- and Dll4-Mediated Notch Activation Levels Shows Differential Lymphomyeloid Lineage Commitment Outcomes. J Immunol. 185(2):867-76; Ohishi K et al. Delta-1 enhances marrow and thymus repopulating ability of human CD34($^{+}$) CD38($^{-}$) cord blood cells. J Clin Invest. 2002 Oct;110(8):1165-74; and Dallas MH et al. Density of the Notch ligand Delta1 determines generation of B and T cell precursors from hematopoietic stem cells J Exp Med. 2005 May 2; 201(9): 1361-1366, which are incorporated herein by reference.

Notch ligands

[00238] Accordingly, to initiate differentiation in the lymphoid lineage and T cell lineage commitment, the hemogenic endothelium is exposed to a Notch ligand to activate the Notch signaling pathway therein. Unexpectedly, the inventors found that the stroma-free protocol described herein, which comprising exposure to a Notch ligand requires starting with hemogenic endothelium (HE), not iPSC or HE-derived progenitors (e.g., lymphoid progenitor). Accordingly, in some embodiments, iPSC or HE-derived progenitors are not the initial population that is differentiated into T cells in the presence of a Notch ligand.

[00239] Notch ligands are single-pass transmembrane proteins with a DSL (Delta, Serrate, LAG-2)-domain and varying numbers of EGF-like repeats. There are two classes of canonical Notch ligands, the Delta/Delta-like and the Serrate/Jagged class. The later has an additional domain of cysteine rich repeats close to the transmembrane domain. There are 5 canonical Notch ligands in mammals: Jagged-1, Jagged-2, DLL1, DLL3 and DLL4. These can bind to the four Notch receptors Notch 1-4. DLL1, also known as Notch Delta ligand, Delta-like 1, is a protein which interacts with a NOTCH2 receptor. See e.g., Shimizu K, et al., 2001, J. Biol. Chem. 276 (28): 25753–8; Blaumueller CM, et al., 1997, Cell 90 (2): 281–91; Shimizu K, et al., 2000, Mol. Cell. Biol. 20 (18): 6913–22. DLL1 is a protein that in humans is encoded by the *DLL1* gene. DLL1 is a human homolog of the Notch Delta ligand.

[00240] In some embodiments, the Notch ligand is selected from the group consisting of Delta-like-1 (DLL1, also referred to as DL1), Delta-like-4 (DLL4, also referred to as DL4), immobilized Delta1ext-IgG, and immobilized Delta4ext-IgG. In some embodiments, immobilized Delta1ext-IgG consists of an extracellular domain of human Delta-like-1 fused to the Fc domain of human IgG1. "Immobilized Delta1ext-IgG" refers to recombinant Notch ligand made by fusing the extracellular domain of Delta-like 1 to the Fc domain of human IgG1 (see e.g., SEQ ID NO: 42). This is a synthetic way of providing a titratable dose of NOTCH ligand. See e.g., Varnum-Finney et al., J Cell Sci. 2000 Dec;113 Pt 23:4313-8, which is incorporated herein by reference in its entirety. Recombinant Notch ligands and Fc-fusions are commercially available at AdipoGen™. "Immobilized Delta4ext-IgG" refers to recombinant Notch ligand made by fusing the extracellular domain of Delta-like 4 to the Fc domain of human IgG1 (see, e.g., SEQ ID NO: 43).

[00241] In some embodiments, the IgG domain of Delta1ext-IgG or Delta4ext-IgG can comprise any known IgG domain in the art. In some embodiments, Delta1ext-IgG or Delta4ext-IgG can be immobilized to a solid substrate (e.g., tissue culture plate) by coating the solid substrate with a composition that binds IgG Fc, including but not limited to anti-human IgG antibody, Protein G, or Protein A.

[00242] In some embodiments, the nucleic acid sequence of the Notch ligand (e.g., DLL1) comprises SEQ ID NO: 1-3 or a sequence that is at least 85%, at least 87%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the sequence of SEQ ID NO: 1-3 that maintains the same functions as SEQ ID NO: 1-3 (e.g., binding and/or activating a Notch receptor).

[00243] **SEQ ID NO: 1**, DLL1 delta like canonical Notch ligand 1 [Homo sapiens (human)], Gene ID: 28514, NCBI Reference Sequence: NG_027940.1, 8873 bp

actgaccatttggcgatccattgagaggagggtttggaaaagtggctcctttgtgacagctctcgccagattgggggctgctgatttgcatctcatta
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[00244] SEQ ID NO: 3 Homo sapiens delta like canonical Notch ligand 1 (DLL1), CDS mRNA, NCBI Reference Sequence: NM_005618.4, 2172 bp

atgggcagtcggtgcgcgctggccctggcggtgctctcggccttgctgtgtcaggtctggagctctggggtgttcgaactgaagctgcaggagttc
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gtgtaa

[00245] In some embodiments, the amino acid sequence of the Notch ligand (e.g., DLL1) comprises SEQ ID NO: 4 or an amino acid sequence that is at least 85%, at least 87%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the sequence of SEQ ID NO: 4 that maintains the same functions as SEQ ID NO: 4 (e.g., binding and/or activating a Notch receptor).

[00246] **SEQ ID NO: 4** delta-like protein 1 precursor [Homo sapiens], NCBI Reference Sequence: NP_005609.3, 723 aa

MGSRCALALAVLSALLCQVWSSGVFELKLQEFVNKKGLLGNRNCCRGGAGPPPCACRTFFR
VCLKHYQASVSPEPPCTYGSAVTPVLGVDSFSLPDGGGADSAFSNPIRFPFGFTWPGTFSLIIE
ALHTDSPDDLATENPERLISRLATQRHLTVGEEWSQDLHSSGRTDLKYSYRFVCDEHYYGEG
CSVFCRPRDDAFGHFTCGERGEKVCNPGWKGPYCTEPICLPGCDEQHGFCDKPGECKCRVG
WQGRYCDECIRYPGCLHGTCQQPWQCNCQEGWGGLFCNQDLNYCTHHKPCKNGATCTNT
GQGSYTCSCRPGYTGATCELGIDECDPSPCKNGGSCTDLENSYSCTCPPGFYGKICELSAMTC
ADGPCFNGGRCSDSPDGGYSCRCPVGYSGFNCEKKIDYCSSSPCSNGAKCVDLGDAYLCRCQ
AGFSGRHCDDNVDDCASSPCANGGTCRDGVNDFSCTCPPGYTGRNCSAPVSRCEHAPCHNG
ATCHERGHRYVCECARGYGGPNCQFLLPELPPGPAVVDLTEKLEGQGGPFPWVAVCAGVIL
VLMLLLGCAAVVVCVRLRLQKHRPPADPCRGETETMNNLANCQREKDISVSIIGATQIKNTN
KKADFHGDHSADKNGFKARYPAVDYNLVQDLKGDDTAVRDAHSKRDTKCQPQGSSGEEK
GTPTTLRGGEASERKRPDSGCSTSKDTKYQSVYVISEEKDECVIATEV

[00247] In some embodiments, the Notch ligand (e.g., Delta1ext-IgG) comprises the extracellular domain of human DLL1, which corresponds to approximately amino acids 1-536, or amino acids 22-544, or amino acids 22-537 of DLL1 (see, e.g., SEQ ID NO: 4 for full-length sequence of DLL1). In some embodiments, the extracellular domain of human DLL1 comprises SEQ ID NO: 5, or an amino acid sequence that is at least 85%, at least 87%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the sequence of SEQ ID NO: 5, and that maintains the same functions as SEQ ID NO: 5 (e.g., binding and/or activating a Notch receptor).

[00248] **SEQ ID NO: 5**, human DLL1 extracellular domain, 536 amino acids

MGSRCALALAVLSALLCQVWSSGVFELKLQEFVNKKGLLGNRNCCRGGAGPPPCACRTFFR
VCLKHYQASVSPEPPCTYGSAVTPVLGVDSFSLPDGGGADSAFSNPIRFPFGFTWPGTFSLIIE
ALHTDSPDDLATENPERLISRLATQRHLTVGEEWSQDLHSSGRTDLKYSYRFVCDEHYYGEG
CSVFCRPRDDAFGHFTCGERGEKVCNPGWKGPYCTEPICLPGCDEQHGFCDKPGECKCRVG

WQGRYCDECIRYPGCLHGTCQQPWQCNCQEGWGGLFCNQDLNYCTHHKPCKNGATCTNT
GQGSYTCSCRPGYTGATCELGIDECDPSPCKNGGSCTDLENSYSCTCPPGFYGKICELSAMTC
ADGPCFNGGRCSDSPDGGYSCRCPVGYSGFNCEKKIDYCSSSPCSNGAKCVDLGDAYLCRCQ
AGFSGRHCDDNVDDCASSPCANGGTCRDGVNDFSCTCPPGYTGRNCSAPVSRCEHAPCHNG
ATCHERGHRYVCECARGYGGPNCQFLLPELPPGPAVVDLTEKL

[00249] In some embodiments, the nucleic acid sequence of the Notch ligand (e.g., DLL4) comprises SEQ ID NO: 6-9 or a sequence that is at least 85%, at least 87%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the sequence of SEQ ID NO: 6-9, and that maintains the same functions as SEQ ID NO: 6-9 (e.g., binding and/or activating a Notch receptor).

[00250] **SEQ ID NO: 6**, DLL4 delta like canonical Notch ligand 4 [Homo sapiens (human)], Gene ID: 54567, NCBI Reference Sequence: NG_046974.1, 9734 bp

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[00251] **SEQ ID NO: 7**, Homo sapiens delta like canonical Notch ligand 4 (DLL4), mRNA, NCBI Reference Sequence: NM_019074.4, 3426 bp

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[00252] **SEQ ID NO: 8**, Homo sapiens delta like canonical Notch ligand 4 (DLL4), CDS mRNA, NCBI Reference Sequence: NM_019074.4, 2058 bp

atggcggcagcgtcccggagcgcctctggctgggcgctactgctgctggtggcactttggcagcagcgcgcggccggctccggcgtcttccagc
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agtctgtgtgtttgatatcagaggagaggaatgaatgtgtcattgccacggaggtataa

[00253] In some embodiments, the amino acid sequence of the Notch ligand (e.g., DLL4) comprises SEQ ID NO: 4 or an amino acid sequence that is at least 85%, at least 87%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the sequence of SEQ ID NO: 4, and that maintains the same functions as SEQ ID NO: 4 (e.g., binding and/or activating a Notch receptor).

[00254] **SEQ ID NO: 9**, delta-like protein 4 precursor [Homo sapiens], NCBI Reference Sequence: NP_061947.1, 685 amino acids

MAAASRSASGWALLLLVALWQQRAAGSGVFQLQLQEFINERGVLASGRPCEPGCRTFFRVC
LKHFQAVVSPGPCTFGTVSTPVLGTNSFAVRDDSSGGGRNPLQLPFNFTWPGTFSLIIEAWHA
PGDDLRPEALPPDALISKIAIQGSLAVGQNWLLDEQTSTLTRLRYSYRVICSDNYYGDNCSRL
CKKRNDHFGHYVCQPDGNLSCLPGWTGEYCQQPICLSGCHEQNGYCSKPAECLCRPGWQG
RLCNECIPHNGCRHGTCSTPWQCTCDEGWGGLFCDQDLNYCTHHSPCKNGATCSNSGQRSY
TCTCRPGYTGVDCELELSECDSNPCRNGGSCKDQEDGYHCLCPPGYYGLHCEHSTLSCADSP
CFNGGSCRERNQGANYACECPPNFTGSNCEKKVDRCTSNPCANGGQCLNRGPSRMCRCRPG
FTGTYCELHVSDCARNPCAHGGTCHDLENGLMCTCPAGFSGRRCEVRTSIDACASSPCFNRA
TCYTDLSTDTFVCNCPYGFVGSRCEFPVGLPPSFPWVAVSLGVGLAVLLVLLGMVAVAVRQ
LRLRRPDDGSREAMNNLSDFQKDNLIPAAQLKNTNQKKELEVDCGLDKSNCGKQQNHTLD
YNLAPGPLGRGTMPGKFPHSDKSLGEKAPLRLHSEKPECRISAICSPRDSMYQSVCLISEERNE
CVIATEV

[00255] In some embodiments, the Notch ligand comprises the extracellular domain of human DLL4, which corresponds to amino acids 1-526 of DLL4, or amino acids 1-524 of DLL4, or amino acids 27-524 of DLL4, (see e.g., SEQ ID NO: 9 for full-length sequence of DLL4). In some embodiments, the extracellular domain of human DLL4 comprises SEQ ID NO: 10 or an amino acid sequence that is at least 85%, at least 87%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the sequence of SEQ ID NO: 10, and that maintains the same functions as SEQ ID NO: 10 (e.g., binding and/or activating a Notch receptor).

[00256] **SEQ ID NO: 10**, human DLL4 extracellular domain, 526 amino acids

MAAASRSASGWALLLLVALWQQRAAGSGVFQLQLQEFINERGVLASGRPCEPGCRTFFRVC
LKHFQAVVSPGPCTFGTVSTPVLGTNSFAVRDDSSGGGRNPLQLPFNFTWPGTFSLIIEAWHA
PGDDLRPEALPPDALISKIAIQGSLAVGQNWLLDEQTSTLTRLRYSYRVICSDNYYGDNCSRL
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TCTCRPGYTGVDCELELSECDSNPCRNGGSCKDQEDGYHCLCPPGYYGLHCEHSTLSCADSP
CFNGGSCRERNQGANYACECPPNFTGSNCEKKVDRCTSNPCANGGQCLNRGPSRMCRCRPG
FTGTYCELHVSDCARNPCAHGGTCHDLENGLMCTCPAGFSGRRCEVRTSIDACASSPCFNRA
TCYTDLSTDTFVCNCPYGFVGSRCEFPVGLPPS

[00257] In some embodiments, the Notch ligand (e.g., Delta1ext-IgG) comprises SEQ ID NO: 42 or an amino acid sequence that is at least 85%, at least 87%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical

to the sequence of SEQ ID NO: 42, and that maintains the same functions as SEQ ID NO: 42 (e.g., binding and/or activating a Notch receptor).

[00258] SEQ ID NO: 42, Recombinant Human DLL1 Fc Chimera Protein, R&D SYSTEMS 10184-DL: Human DLL1 (Ser22-Glu537) Accession # O00548 + IEGRMDP + Human IgG1 Fc (Pro100-Lys330)

SGVFELKLQEFVNKKGLLGNRNCCRGGAGPPPCACRTFFRVCLKHYQASVSPEPPCTYGSAV
TPVLGVDSFSLPDGGGADSAFSNPIRFPFGFTWPGTFSLIIEALHTDSPDDLATENPERLISRLA
TQRHLTVGEEWSQDLHSSGRTDLKYSYRFVCDEHYYGEGCSVFCRPRDDAFGHFTCGERGE
KVCNPGWKGPYCTEPICLPGCDEQHGFCDKPGECKCRVGWQGRYCDECIRYPGCLHGTCQQ
PWQCNCQEGWGGLFCNQDLNYCTHHKPCKNGATCTNTGQGSYTCSCRPGYTGATCELGID
ECDPSPCKNGGSCTDLENSYSCTCPPGFYGKICELSAMTCADGPCFNGGRCSDSPDGGYSCRC
PVGYSGFNCEKKIDYCSSSPCSNGAKCVDLGDAYLCRCQAGFSGRHCDDNVDDCASSPCAN
GGTCRDGVNDFSCTCPPGYTGRNCSAPVSRCEHAPCHNGATCHERGHRYVCECARGYGGPN
CQFLLPELPPGPAVVDLTEKLEIEGRMDPPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL
MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ
DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN
HYTQKSLSLSPGK

[00259] In some embodiments, the Notch ligand (e.g., Delta4ext-IgG) comprises SEQ ID NO: 43 or an amino acid sequence that is at least 85%, at least 87%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the sequence of SEQ ID NO: 43, and that maintains the same functions as SEQ ID NO: 43 (e.g., binding and/or activating a Notch receptor).

[00260] SEQ ID NO: 43, Human DLL4 Protein Fc Tag, ACRO BIOSYSTEMS DL4-H5259: Human DLL4 (Ser27-Pro524) + Human IgG1 Fc (Pro100-Lys330)

SGVFQLQLQEFINERGVLASGRPCEPGCRTFFRVCLKHFQAVVSPGPCTFGTVSTPVLGTNSF
AVRDDSSGGGRNPLQLPFNFTWPGTFSLIIEAWHAPGDDLRPEALPPDALISKIAIQGSLAVGQ
NWLLDEQTSTLTRLRYSYRVICSDNYYGDNCSRLCKKRNDHFGHYVCQPDGNLSCLPGWTG
EYCQQPICLSGCHEQNGYCSKPAECLCRPGWQGRLCNECIPHNGCRHGTCSTPWQCTCDEG
WGGLFCDQDLNYCTHHSPCKNGATCSNSGQRSYTCTCRPGYTGVDCELELSECDSNPCRNG
GSCKDQEDGYHCLCPPGYYGLHCEHSTLSCADSPCFNGGSCRERNQGANYACECPPNFTGSN
CEKKVDRCTSNPCANGGQCLNRGPSRMCRCRPGFTGTYCELHVSDCARNPCAHGGTCHDLE
NGLMCTCPAGFSGRRCEVRTSIDACASSPCFNRATCYTDLSTDTFVCNCPYGFVGSRCEFPVG
LPPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW
YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK

AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDS
DGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

[00261] In some embodiments, the Notch ligand comprises an extracellular domain of a Notch ligand as described herein linked (e.g., through an optional linker sequence) to the Fc domain of human IgG1. In some embodiments, the human IgG1 Fc domain comprises SEQ ID NO: 44 or an amino acid sequence that is at least 85%, at least 87%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to the sequence of SEQ ID NO: 44, and that maintains the same functions as SEQ ID NO: 44.

[00262] **SEQ ID NO: 44**, Pro100-Lys330 of P01857 (IGHG1_HUMAN)
PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK
GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGS
FFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

[00263] There are several ways to provide a Notch ligand, for example by providing a purified recombinant form of a Notch ligand or a Notch receptor-binding fragment, the receptor-binding fragment being sufficient to elicit cell signaling events *in vivo* upon contact and binding with the extracellular Notch receptors on these cells. In some embodiments, the Notch ligand is attached to a solid substrate, for example using a covalent or non-covalent bond or linkage. In some embodiments, the Notch ligand is attached to a cell culture dish.

[00264] In some embodiments, the Notch ligand further comprises a domain to immobilize the Notch ligand to a solid substrate. As a non-limiting example, the Notch ligand comprises a first member of an affinity pair, and the solid substrate comprises a second member of an affinity pair. In some embodiments, the first and second members of the affinity pair are selected from the group consisting of: a haptenic or antigenic compound in combination with a corresponding antibody or binding portion or fragment thereof (e.g., FLAG and anti-FLAG monoclonal antibody, the sequence of which are known in the art); digoxigenin and anti-digoxigenin; mouse immunoglobulin and goat anti-mouse immunoglobulin; a non-immunological binding pair; biotin and avidin; biotin and streptavidin; a hormone and a hormone-binding protein; thyroxine and cortisol-hormone binding protein; a receptor and a receptor agonist; a receptor and a receptor antagonist; acetylcholine receptor and acetylcholine or an analog thereof; IgG and protein A; lectin and carbohydrate; an enzyme and an enzyme cofactor; an enzyme and an enzyme inhibitor; complementary oligonucleotide pairs capable of forming nucleic acid duplexes; and a first molecule that is negatively charged and a second molecule that is positively charged.

[00265] In some embodiments, the population of hemogenic endothelium is differentiated into a population of CD3+ T cells by culturing in a non-tissue culture treated culture vessel; said another way, the culture vessel is not exposed to a plasma gas in order to modify the hydrophobic plastic

surface to make it more hydrophilic. As used herein, the term "culture vessel" includes dishes, flasks, plates, multi-well plates, and the like. In some embodiments, the culture vessel is coated with recombinant human DL1-Fc protein (e.g., commercially available via R&D SYSTEMS, item number 10184-DL), recombinant human DL4-Fc protein (e.g., commercially available via ACRO BIOSYSTEMS, item number DL4-H5259), or a mixture of both Notch ligands, or any Notch ligand as described herein. In some embodiments, the culture vessel is coated with Notch ligand for at least 0.5 hour, at least 1.0 hour, at least 1.5 hours, at least 2.0 hours, at least 2.5 hours, at least 3.0 hours, at least 3.5 hours, at least 4.0 hours, at least 4.5 hours, or at least 5.0 hours. In some embodiments, the culture vessel is coated with Notch ligand at room temperature.

[00266] In some embodiments, the non-stromal-derived Notch ligand (e.g., the Notch ligand immobilized on a tissue culture plate) is provided at a concentration of 1 μg/mL to 100 μg/mL or a concentration of 5 μg/mL to 15 μg/mL. In some embodiments, the non-stromal-derived Notch ligand is provided at a concentration of at least 1 μg/mL, at least 2 μg/mL, at least 3 μg/mL, at least 4 μg/mL, at least 5 μg/mL, at least 6 μg/mL, at least 7 μg/mL, at least 8 μg/mL, at least 9 μg/mL, at least 10 μg/mL, at least 11 μg/mL, at least 12 μg/mL, at least 13 μg/mL, at least 14 μg/mL, at least 15 μg/mL, at least 16 μg/mL, at least 17 μg/mL, at least 18 μg/mL, at least 19 μg/mL, at least 20 μg/mL, at least 25 μg/mL, at least 30 μg/mL, at least 35 μg/mL, at least 40 μg/mL, at least 45 μg/mL, at least 50 μg/mL, at least 55 μg/mL, at least 60 μg/mL, at least 65 μg/mL, at least 70 μg/mL, at least 75 μg/mL, at least 80 μg/mL, at least 85 μg/mL, at least 90 μg/mL, at least 95 μg/mL, or at least 100 μg/mL. In a preferred embodiment, the non-stromal-derived Notch ligand is provided at a concentration of 10 μg/mL.

[00267] In some embodiments, the cells are cultured exposed to a non-stromal-derived Notch ligand (e.g., a Notch ligand immobilized on a tissue culture plate) for at least 1 day, at least 2 days, at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days, at least 8 days, at least 9 days, at least 10 days, at least 10 days, at least 11 days, at least 12 days, at least 13 days, at least 14 days, at least 15 days, at least 16 days, at least 17 days, at least 18 days, at least 19 days, at least 20 days, at least 21 days, at least 22 days, at least 23 days, at least 24 days, at least 25 days, at least 26 days, at least 27 days, at least 28 days, at least 29 days, at least 30 days, at least 31 days, at least 32 days, at least 33 days, at least 34 days, at least 35 days, at least 36 days, at least 37 days, at least 38 days, at least 39 days, at least 40 days, at least 41 days, at least 42 days, at least 43 days, at least 44 days, at least 45 days, at least 46 days, at least 47 days, at least 48 days, at least 49 days, at least 50 days, or more.

Stroma-free differentiation

[00268] The method described herein is a stroma-free T cell differentiation method, i.e., a method that does not comprise co-culturing with stromal cells or any other type of supporting cell. Co-culture

with stromal cells such as mouse stromal cells limits the translational potential of iPSC-derived T cells; for example, there can be fears of transplantation rejection due to the presence of stromal cells. Furthermore, T cells differentiated using stromal cells exhibit an innate-like phenotype (e.g., as measured by TCRgd expression, which is a marker for gamma delta T cells). It is preferred that T cells exhibit an adaptive phenotype, for example characterized by expression of TCR α and β. Additionally, as described herein, stroma-free T cell differentiation methods result in increased numbers of CD3+ T cells (e.g., CD4+CD8+ cells) compared to differentiation methods comprising stromal co-culture.

[00269] Accordingly, T cells differentiated using stromal-free methods, and in one embodiment, in combination with inhibition of an epigenetic regulator (e.g., an HMT; e.g., EZH1, G9a/GLP), exhibit at least the following unexpected benefits compared to stromal co-culture methods: (1) increased potential for transplantation in humans; (2) decreased number of innate-like T cells; (3) increased number and/or percentage of resultant T cells (e.g., CD5+CD7+ Pro-T cells; CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells; alpha-beta T cells); (4) gene expression profiles most similar to alpha beta T cells; (5) a more diverse TCR repertoire; and/or (6) increased TCR CDR length (see e.g., Example 1, Fig. 1C-1D, Fig. 3A-3B, Fig. 4, Fig. 5A-5D, Fig. 6-16).

[00270] As used herein, the term "supporting cell or stromal cell" when used in the context of cell differentiation refers to any cells that are capable of creating, promoting, or supporting a microenvironment for the growth, proliferation, differentiation, or expansion of multipotent hematopoietic progenitor cells or T cells or B cells. Non-limiting examples of supporting cells that are not comprised by the differentiation methods described herein include, but are not limited to, stromal cells and fibroblast cells.

[00271] Supporting cells used previously in co-cultures for cell differentiation purposes are typically stromal cells. However, the methods described herein do not comprise co-cultures comprising stromal cells. Examples of stromal cell lines that are not comprised by the differentiation methods described herein include, but are not limited to, murine MS5 stromal cell line; murine bone marrow-derived stromal cell lines, such as S10, S17, OP9 (e.g., OP9-DL1 cells or OP9-DL4 cells) and BMS2 cell lines; human marrow stromal cell lines such as those described in U.S. Patent No. 5,879,940, which is incorporated herein by reference in its entirety; or any other similar cells that express and display extracellular or secretes a Notch ligand. OP9-DL1 cells are a bone-marrow-derived stromal cell line that ectopically expresses the Notch ligand, Delta-like 1 (DLL1). Method of differentiating pluripotent stem cells to T-cells using OP9-Notch ligand expressing cells are known in the art. See, e.g., US Patent Nos: 7575925, 8772028, 8871510, and 9206394 and US Patent Publication Nos: 20090217403, 20110123502, 20110052554 20110027881, 20110236363, 20120149100, 20130281304, 20140322808, 20140248248, and 20140037599. These references are incorporated herein by reference in their entirety.

[00272] Described herein are methods of differentiating T cells from pluripotent stem cells, wherein the methods do not comprise a step of co-culturing the cells with supporting cells or stromal cells. In some embodiments, the Notch ligand used herein is not derived from a stromal cell. In some embodiments, differentiating the hemogenic endothelium in the presence of a Notch ligand does not comprise co-culturing with a stromal cell expressing a Notch ligand. In some embodiments, differentiating the hemogenic endothelium in the presence of a Notch ligand does not comprise co-culturing with OP9-DL1 cells or OP9-DL4 cells.

T cell differentiation medias

[00273] In some embodiments, the differentiation method comprises differentiating the resultant population of CD34+ hemogenic endothelium in a CD3+-T-cell differentiation media for a sufficient time to promote differentiation into a population of CD3+ T cells. In some embodiments, the sufficient time to promote differentiation into a population of CD3+ T cells is at least 3 weeks, at least 3.5 weeks, at least 4 weeks, at least 4.5 weeks, at least 5 weeks, at least 5.5 weeks, at least 6 weeks, or more. In some embodiments, the sufficient time to promote differentiation into a population of CD3+ T cells is at most 6 weeks.

[00274] In some embodiments, a polypeptide (e.g., growth or differentiation factors) that can be expressed by the supporting cell or stromal cell can be provided in the cell culture medium. Non limiting examples of polypeptides that support the differentiation of T cells that can be included in the cell culture medium include IL-7, SCF, Flt3, and TPO. Interleukin-7 (IL-7) is a hematopoietic growth factor secreted by stromal cells in the bone marrow and thymus, and it is involved in B and T cell development. Stem cell factor (also known as SCF, KIT-ligand, KL, or steel factor) is a cytokine that binds to the c-KIT receptor (CD117) and is involved in T cell differentiation. FLT3 (also referred to as Flit3 or Fms-Like Tyrosine Kinase 3) is a class III receptor tyrosine kinase that regulates hematopoiesis. Thrombopoietin (TPO or THPO) is a cytokine that is chiefly responsible for megakaryocyte production but also has a role in maintaining hematopoietic stem cells (HSCs). See, e.g., Wang et al., Distinct roles of IL-7 and stem cell factor in the OP9-DL1 T cell differentiation culture system. Exp Hematol. 2006 Dec;34(12):1730-40.

[00275] In some embodiments, the CD3+-T-cell-differentiation media is serum-free. In some embodiments, the CD3+-T-cell-differentiation media comprises at least one of SCF, FLT3, and/or IL7. In some embodiments, the CD3+-T-cell-differentiation media comprises SCF, FLT3, and IL7. In some embodiments, the CD3+-T-cell-differentiation media comprises 30 ng/ml SCF, 15 ng/ml FLT3, and 25 ng/ml IL7. In some embodiments, the CD3+-T-cell-differentiation media comprises 100 ng/ml SCF, 100 ng/ml FLT3, and 50 ng/ml IL7. In some embodiments, the CD3+-T-cell-differentiation media comprises FLT3 and IL7. In some embodiments, the CD3+-T-cell-differentiation media

comprises 15 ng/ml FLT3 and 25 ng/ml IL7. In some embodiments, the CD3+-T-cell-differentiation media comprises 100 ng/ml FLT3 and 50 ng/ml IL7.

[00276] The concentrations of SCF, FLT3, and/or IL7 should be used such that they promote the differentiation of hemogenic endothelium into a population of CD3+ T cells. The concentration of SCF can range from 1 ng/mL to 200 ng/mL. In some embodiments, the concentration of SCF (e.g., in the CD3+-T-cell-differentiation media) is 30 ng/mL. In some embodiments, the concentration of SCF (e.g., in the CD3+-T-cell-differentiation media) is 100 ng/ml. The concentration of FLT3 can range from 1 ng/mL to 200 ng/mL. In some embodiments, the concentration of FLT3 (e.g., in the CD3+-T-cell-differentiation media) is 15 ng/ml. In some embodiments, the concentration of FLT3 (e.g., in the CD3+-T-cell-differentiation media) is 100 ng/ml. The concentration of IL7 can range from 1 ng/mL to 200 ng/mL. In some embodiments, the concentration of IL7 (e.g., in the CD3+-T-cell-differentiation media) is 25 ng/ml. In some embodiments, the concentration of IL7 (e.g., in the CD3+-T-cell-differentiation media) is 50 ng/ml.

[00277] In some embodiments, the CD3+-T-cell-differentiation media further comprises thrombopoietin (TPO) for at least the first 2 weeks of differentiating in the CD3+-T-cell-differentiation media. As a non-limiting example, the CD3+-T-cell-differentiation media further comprises thrombopoietin (TPO) for at least the first 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, or 21 days of differentiating in the CD3+-T-cell-differentiation media. In some embodiments, CD3+-T-cell-differentiation media comprising TPO promotes differentiation into a population of CD5+ CD7+ ProT cells. Such CD5+ CD7+ ProT cells can be detected after at least 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days of differentiating in the CD3+-T-cell-differentiation media. In some embodiments, CD5+ CD7+ ProT cells can be detected after at least 2 weeks of differentiating in the CD3+-T-cell-differentiation media.

[00278] In some embodiments, the concentration of TPO should be used such that it promotes the differentiation of hemogenic endothelium into a population of CD3+ T cells. In some embodiments, the concentration of TPO can range from 1 ng/mL to 200 ng/mL. In some embodiments, the concetration of TPO (e.g., in the CD3+-T-cell-differentiation media) is 5 ng/mL. In some embodiments, the concentration of TPO (e.g., in the CD3+-T-cell-differentiation media) is 50 ng/ml.

[00279] In some embodiments, the CD3+-T-cell-differentiation media (e.g., comprising IL-7 and/or FLT3) further comprises SCF for at least the first 2 weeks of differentiating in the CD3+-T-cell-differentiation media. As a non-limiting example, the CD3+-T-cell-differentiation media further comprises SCF for at least the first 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, or 21 days of differentiating in the CD3+-T-cell-differentiation media. In some embodiments,

CD3+-T-cell-differentiation media comprising SCF promotes differentiation into a population of CD5+ CD7+ ProT cells.

[00280] In some embodiments, SCF, FLT3, IL7, and/or TPO are provided in the CD3+-T-cell-differentiation media at a concentration of at least 1 ng/mL, at least 2 ng/mL, at least 3 ng/mL, at least 4 ng/mL, at least 5 ng/mL, at least 6 ng/mL, at least 7 ng/mL, at least 8 ng/mL, at least 9 ng/mL, at least 10 ng/mL, at least 11 ng/mL, at least 12 ng/mL, at least 13 ng/mL, at least 14 ng/mL, at least 15 ng/mL, at least 16 ng/mL, at least 17 ng/mL, at least 18 ng/mL, at least 19 ng/mL, at least 20 ng/mL, at least 25 ng/mL, at least 30 ng/mL, at least 35 ng/mL, at least 40 ng/mL, at least 45 ng/mL, at least 50 ng/mL, at least 55 ng/mL, at least 60 ng/mL, at least 65 ng/mL, at least 70 ng/mL, at least 75 ng/mL, at least 80 ng/mL, at least 85 ng/mL, at least 90 ng/mL, at least 95 ng/mL, at least 100 ng/mL, at least 105 ng/mL, at least 110 ng/mL, at least 115 ng/mL, at least 120 ng/mL, at least 125 ng/mL, at least 130 ng/mL, at least 135 ng/mL, at least 140 ng/mL, at least 145 ng/mL, at least 150 ng/mL, at least 155 ng/mL, at least 160 ng/mL, at least 165 ng/mL, at least 170 ng/mL, at least 175 ng/mL, at least 180 ng/mL, at least 185 ng/mL, at least 190 ng/mL, at least 195 ng/mL, or at least 200 ng/mL. The concentration of SCF, FLT3, IL7, and/or TPO can be the same or different.

[00281] In some embodiments, CD3+ T cells can be detected after at least 5.0 weeks of differentiating in the CD3+-T-cell-differentiation media. In some embodiments, CD3+ T cells can be detected after at least 1.5 weeks, 2 weeks, 2.5 weeks, 3.0 weeks, 3.5 weeks, 4.0 weeks, 4.5 weeks, or 5.0 weeks of differentiating in the CD3+-T-cell-differentiation media. In some embodiments, the population of CD3+ T cells comprises a population of CD4+CD8+ T cells, also referred to herein as double-positive or DP T cells. Such CD4+CD8+ CD3+ T cells can be detected after at least 1.5 weeks, 2 weeks, 2.5 weeks, 3.0 weeks, 3.5 weeks, 4.0 weeks, 4.5 weeks, or 5.0 weeks of differentiating in the CD3+-T-cell-differentiation media.

[00282] In some embodiments, the method further comprises differentiating the population of CD4+CD8+ T cells in a single-positive-T-cell-differentiation media for a sufficient time to promote differentiation into a population of CD4+ cells and a population of CD8+ cells. In some embodiments, the sufficient time to promote differentiation from the population of CD4+CD8+ T cells into a population of CD4+ T cells and a population of CD8+ cells is at least 1 day, at least 2 days, at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days, at least 8 days, at least 9 days, or at least 10 days. In some embodiments, the sufficient time to promote differentiation from the population of CD34+ hemogenic endothelium into a population of CD4+ T cells and a population of CD8+ cells is at least 4.0 weeks, 4.5 weeks, 5.0 weeks, 5.5 weeks, or 6.0 weeks.

[00283] In some embodiments, the single-positive-T-cell-differentiation media comprises 10 ng/ml IL-15 and a T cell activator. Interleukin-15 (IL-15), like IL-7, is a member of the interleukin 2 (IL-2) superfamily, and shares many activities with IL-2, including the ability to stimulate lymphocytes. In some embodiments, a variety of concentrations of IL-15 can be used as long as it still promotes the

differentiation of CD4+CD8+ T cells into single positive CD4+ cells and CD8+ cells. In some embodiments, the concentration of IL15 can range from 1 ng/mL to 200 ng/mL, with a preferred concentration of 10 ng/ml.

[00284] In some embodiments, the T cell activator comprises components (e.g., soluble tetrameric antibody complexes) that bind CD3 and CD28 (and optionally CD2) cell surface ligands. Binding of the T cell activator results in the cross-linking of CD3 and CD28 (and optionally CD2) cell surface ligands, thereby providing the required primary and co-stimulatory signals for T cell activation.

[00285] In some embodiments, the T cell activator comprises a CD3/CD28 T cell activator (e.g., at a concentration of 10ul/ml). Such a CD3/CD28 T cell activator is available commercially (e.g., via StemCell Technology™, item #10970). In some embodiments, the concentration of the CD3/CD28 T cell activator should be used such that it promotes the differentiation of CD4+CD8+ T cells into single positive CD4+ cells and CD8+ cells. In some embodiments, the concentration can range from 1 ul/mL to 200 ul/mL, with a preferred concentration of 10 ul/ml.

[00286] In some embodiments, the T cell activator comprises CD3/CD28 T cell activator Dynabeads (e.g., used at one bead per cell). Such CD3/CD28 T cell activator Dynabeads are available commercially (e.g., via ThermoFisher™ #11132D). In some embodiments, the concentrations of CD3/CD28 T cell activator Dynabeads should be used such that it promotes the differentiation of CD4+CD8+ T cells into single positive CD4+ cells and CD8+ cells. In some embodiments, the concentration can range from 1 bead/cell to 20 beads/cell, with a preferred concentration of 1 bead/cell.

[00287] In some embodiments, the method further comprises, after at least 1 week (e.g., in the single-positive-T-cell-differentiation media), a step of CD4+ cell enrichment and/or CD8+ cell enrichment. In some embodiments, a step of CD4+ cell enrichment and/or CD8+ cell enrichment can occur at least 1 day, at least 2 days, at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days, at least 8 days, at least 9 days, at least 10 days, at least 11 days, at least 12 days, at least 13 days, or at least 14 days of culturing in the single-positive-T-cell-differentiation media.

[00288] Methods of enriching for CD4+ or CD8+ cells are known in the art. As non-limiting examples, the CD4+ or CD8+ cells can be enriched using magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) with anti-CD4 or anti-CD8 antibodies accordingly.

[00289] In some embodiments, the entire T cell differentiation protocol described herein occurs in a stromal-free environment, e.g., the cells are cultured exposed to a non-stromal-derived Notch ligand (e.g., Notch ligand immobilized on a tissue culture plate). In some embodiments, at least a portion of the T cell differentiation protocol (e.g., comprising culturing in the $CD3^+$-T-cell-differentiation media and in the single-positive-T-cell-differentiation media) occurs in a stromal-free environment, e.g., the cells are cultured exposed to a non-stromal-derived Notch ligand (e.g., Notch ligand immobilized on a tissue culture plate).

Derived T Cell Population

[00290] As described herein, the population of T cells derived using stromal-free methods as described herein, and in one embodiment, in combination with inhibition of an epigenetic regulator (e.g., an HMT; e.g., EZH1, G9a/GLP), exhibits at least the following unexpected benefits compared to stromal co-culture methods: (1) increased potential for transplantation in humans; (2) decreased number of innate-like T cells; (3) increased number and/or percentage of resultant T cells (e.g., CD5+CD7+ Pro-T cells; CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells; alpha-beta T cells); (4) gene expression profiles most similar to alpha beta T cells; (5) a more diverse TCR repertoire; and/or (6) increased TCR CDR length (see e.g., Example 1, Fig. 1C-1D, Fig. 3A-3B, Fig. 4, Fig. 5A-5D, Fig. 6-16).

[00291] In some embodiments, the population of T cells (e.g., CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells) derived using stromal-free methods and/or inhibition of an epigenetic regulator (e.g., an HMT; e.g., EZH1, G9a/GLP) as described herein exhibits at least a 10% higher transplantation or engraftment rate than a population of T cells derived using a stromal method. In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, at least 150%, at least 200%, at least 250%, at least 300%, at least 350%, at least 400%, at least 450%, or at least 500% or more, or at least 10x, 20x, 30x, 40x, 50x, 60x, 70x, 80x, 90x, 100x, 500x, 1,000x, or more higher transplantation or engraftment rate than a population of T cells derived using a stromal method or without inhibition of an epigenetic regulator.

[00292] In some embodiments, a minority of the population of T cells (e.g., CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells) derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein are TCRgd^{+} (i.e., innate-like gamma delta T cells). Gamma delta T cells (γδ T cells) are T cells that have a distinctive T-cell receptor (TCR) on their surface. Most T cells are αβ (alpha beta) T cells with a TCR composed of two glycoprotein chains called α (alpha) and β (beta) TCR chains. In contrast, gamma delta (γδ) T cells have a TCR that is made up of one γ (gamma) chain and one δ (delta) chain. Like other 'unconventional' T cell subsets bearing invariant TCRs, such as CD1d-restricted Natural Killer T cells, gamma delta T cells exhibit several characteristics that place them at the border between the more evolutionarily primitive innate immune system that permits a rapid beneficial response to a variety of foreign agents and the adaptive immune system, where B and T cells coordinate a slower but highly antigen-specific immune response leading to long-lasting memory against subsequent challenges by the same antigen. Gamma delta T cells may be considered a component of adaptive immunity in that they rearrange TCR genes

to produce junctional diversity and can develop a memory phenotype. However, the various subsets may also be considered part of the innate immunity in which a specific TCR can function as a pattern recognition receptor. See, e.g., Born WK, Reardon CL, O'Brien RL (February 2006). "The function of gammadelta T cells in innate immunity". Current Opinion in Immunology. 18 (1): 31–8.

[00293] In some embodiments, at most 10% of the population of T cells (e.g., CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells) derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein are $TCRgd^{+}$. In some embodiments, at most 1%, at most 2%, at most 3%, at most 4%, at most 5%, at most 6%, at most 7%, at most 8%, at most 9%, at most 10%, at most 11%, at most 12%, at most 13%, at most 14%, at most 15%, at most 16%, at most 17%, at most 18%, at most 19%, at most 20%, at most 21%, at most 22%, at most 23%, at most 24%, at most 25%, at most 26%, at most 27%, at most 28%, at most 29%, at most 30%, at most 31%, at most 32%, at most 33%, at most 34%, at most 35%, at most 36%, at most 37%, at most 38%, at most 39%, at most 40%, at most 41%, at most 42%, at most 43%, at most 44%, at most 45%, at most 46%, at most 47%, at most 48%, or at most 49% of the population of T cells (e.g., CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells) are $TCRgd^{+}$.

[00294] In some embodiments, the population of T cells (e.g., CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells) derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein comprises at least 10% more T cells than a population of T cells derived using a stromal method or without inhibition of an epigenetic regulator. In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein comprises at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 100%, at least 150%, at least 200%, at least 250%, at least 300%, at least 350%, at least 400%, at least 450%, or at least 500% or more, or at least 10x, 20x, 30x, 40x, 50x, 60x, 70x, 80x, 90x, 100x, 500x, 1,000x, or more T cells than a population of T cells derived using a stromal method or without inhibition of an epigenetic regulator.

[00295] In some embodiments, the population of T cells (e.g., CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells) derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits a gene expression profile that is more similar to αβ T cells, than to other cells (e.g., γδ T cells; NK cells; iPSCs derived T cells using a OP9-DL4 co-culture system; T cells differentiated from cord blood CD34+ HSPCs), e.g., the gene profile of the derived T cells is at least 0.5% more similar to a αβ T cells as compared to another cell type. In one embodiment, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits a gene expression profile of T cell signature genes and/or αβ T cell

signature genes that is at most 10% divergent from the gene expression profile of αβ T cells. In one embodiment, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits a gene expression profile of T cell signature genes and/or αβ T signature cell genes that is at most 20% (e.g., at most 1%, at most 2%, at most 3%, at most 4%, at most 5%, at most 6%, at most 7%, at most 8%, at most 9%, at most 10%, at most 11%, at most 12%, at most 13%, at most 14%, at most 15%, at most 16%, at most 17%, at most 18%, at most 19%, or more) divergent from the gene expression profile of αβ T cells. In one embodiment, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits a gene expression profile of T cell signature genes and/or αβ T cell signature genes that is 1%-5%, 2%-6%, 3%-7%, 4%-8%, 5%-9%, 5%-10%, 5%-15%, 10%-15%, or 15%-20% divergent from the gene expression profile of αβ T cells.

[00296] In one embodiment, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits a gene expression profile that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more similar to the gene expression profile of αβ T cells compared to a population of T cells derived using a stromal method or without inhibition of an epigenetic regulator. In one embodiment, the derived T cell has a greater percentage of similarity to the gene expression profile of an αβ T cell than the gene profile of another cell type. One skilled in the art can determine the similarity of gene expression in a T cell derived from stromal-free methods described herein and an αβ T cell using standard methods, e.g., transcriptome sequencing of specific cell types (FACS-sorted cells).

[00297] In one embodiment, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits a gene expression profile with a Pearson's correlation coefficient compared to peripheral blood alpha beta T cells that is at least 0.75, 0.755, 0.76, 0.765, 0.77, 0.775, 0.78, 0.785, 0.79, 0.795, 0.8, 0.805, 0.81, 0.815, 0.82, 0.825, 0.83, 0.835, 0.84, 0.845, 0.85, 0.855, 0.86, 0.865, 0.87, 0.875, 0.88, 0.885, 0.89, 0.895, 0.9, 0.905, 0.91, 0.915, 0.92, 0.925, 0.93, 0.935, 0.94, 0.945, 0.95, 0.955, 0.96, 0.965, 0.97, 0.975, 0.98, 0.985, 0.99, 0.995, or 1.0.

[00298] In some embodiments, the population of CD3+ T cells exhibits a gene expression profile that is most similar to alpha beta T cells. In some embodiments, the population of CD3+ T cells exhibits a gene expression profile that is similar or substantially similar to alpha beta T cells. In some embodiments, the population of $CD3^+$ T cells exhibits a gene expression profile that is at least 10%,

20%, 30%, 40% or more similar to alpha beta T cells. In some embodiments, the population of CD3+ T cells exhibits a gene expression profile with a Pearson's correlation coefficient compared to peripheral blood alpha beta T cells that is at least 0.85.

[00299] In some embodiments, the immune cell, e.g., derived using stromal-free and/or inhibition of an epigenetic regulator as described herein, exhibits a gene expression profile that is most similar to alpha beta T cells. In some embodiments, the immune cell exhibits a gene expression profile that is similar or substantially similar to alpha beta T cells. In some embodiments, the immune cell exhibits a gene expression profile that is at least 10%, 20%, 30%, 40% or more similar to alpha beta T cells. In some embodiments, the immune cell exhibits a gene expression profile with a Pearson's correlation coefficient compared to peripheral blood alpha beta T cells that is at least 0.85.

[00300] In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein expresses at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, at least 41, at least 42, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, at least 50, at least 51, at least 52, at least 53, at least 54, at least 55, at least 56, at least 57, at least 58, at least 59, at least 60, at least 61, at least 62, at least 63, at least 64, at least 65, at least 66, at least 67, at least 68, at least 69, at least 70, at least 71, at least 72, at least 73, at least 74, at least 75, at least 76, at least 77, at least 78, at least 79, at least 80, at least 81, at least 82, at least 83, at least 84, at least 85, at least 86, at least 87, at least 88, at least 89, at least 90, at least 91, at least 92, at least 93, at least 94, at least 95, at least 96, at least 97, at least 98, at least 99, at least 100, at least 125, at least 150 or more signature genes from an αβ T cell. In one embodiment, the derived T cell expresses a greater number of signature genes from an αβ T cell than signature genes from another cell type. As used herein, the term "signature gene" refers to a gene that exhibits a characteristic expression pattern in a specific cell type (e.g., T cell, αβ T cell); a signature gene can be required for the function of a specific cell type. Non-limiting examples of T cell signature genes and αβ T cell signature genes are described further herein. A specific cell type (e.g., T cell, αβ T cell) exhibits a gene signature or gene expression signature, which comprises a single or combined group of genes in a cell with a uniquely characteristic pattern of gene expression (i.e., signature genes).

[00301] In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein expresses at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at

least 31, at least 32, at least 33, at least 34, at least 35, at least 36, at least 37, at least 38, at least 39, at least 40, at least 41, at least 42, at least 43, at least 44, at least 45, at least 46, at least 47, at least 48, at least 49, at least 50, at least 51, at least 52, at least 53, at least 54, at least 55, at least 56, at least 57, at least 58, at least 59, at least 60, at least 61, at least 62, at least 63, at least 64, at least 65, at least 66, at least 67, at least 68, at least 69, at least 70, at least 71, at least 72, at least 73, at least 74, at least 75, at least 76, at least 77, at least 78, at least 79, at least 80, at least 81, at least 82, at least 83, at least 84, at least 85, at least 86, at least 87, at least 88, at least 89, at least 90, at least 91, at least 92, at least 93, at least 94, at least 95, at least 96, at least 97, at least 98, at least 99, at least 100, at least 125, at least 150 or more genes from an αβ T cell. In one embodiment, the derived T cell expresses a greater number of genes from a αβ T cells than signature genes from another cell type.

[00302] Non-limiting examples of T cell signature genes include GRB2 (Growth Factor Receptor Bound Protein 2); NFATC3 (Nuclear Factor Of Activated T Cells 3); ZAP70 (Zeta Chain Of T Cell Receptor Associated Protein Kinase 70); RAF1 (Raf-1 Proto-Oncogene, Serine/Threonine Kinase); PIK3CG (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Gamma); PIK3R1 (Phosphoinositide-3-Kinase Regulatory Subunit 1); CALM3 (Calmodulin 3); PTPN7 (Protein Tyrosine Phosphatase Non-Receptor Type 7); LAT (Linker For Activation Of T Cells); NFKBIA (NFKB Inhibitor Alpha); VAV1 (Vav Guanine Nucleotide Exchange Factor 1); SHC1 (SHC (Src Homology 2 Domain Containing) Adaptor Protein 1); PRKCB (Protein Kinase C Beta); MAP2K4 (Mitogen-Activated Protein Kinase Kinase 4); MAP2K1 (Mitogen-Activated Protein Kinase Kinase 1); RAC1 (Rac Family Small GTPase 1); FYN (Fyn Proto-Oncogene, Src Family Tyrosine Kinase); RELA (RELA Proto-Oncogene, NF-KB Subunit, v-rel avian reticuloendotheliosis viral oncogene homolog A); LCK (Lck Proto-Oncogene, Src Family Tyrosine Kinase); CALM2 (Calmodulin 2); CD3D (CD3 Antigen, Delta Subunit); CALM1 (Calmodulin 1); CD247 (T-Cell Surface Glycoprotein CD3 Zeta Chain); CD3E (T-Cell Surface Glycoprotein CD3 Epsilon Chain); CD3G (T-Cell Surface Glycoprotein CD3 Gamma Chain); FOS (Fos Proto-Oncogene, AP-1 Transcription Factor Subunit); PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha); PLCG1 (Phospholipase C Gamma 1); SOS1 (Son Of Sevenless Homolog 1, SOS Ras/Rac Guanine Nucleotide Exchange Factor 1); ELK1 (ETS Transcription Factor ELK1); PPP3CC (Protein Phosphatase 3 Catalytic Subunit Gamma); MAP3K1 (Mitogen-Activated Protein Kinase Kinase Kinase 1); PPP3CA (Protein Phosphatase 3 Catalytic Subunit Alpha); NFKB1 (Nuclear Factor Kappa B Subunit 1); NFATC2 (Nuclear Factor Of Activated T Cells 2); NFATC1 (Nuclear Factor Of Activated T Cells 1, AP-1 Transcription Factor Subunit); JUN (Jun Proto-Oncogene); MAPK8 (Mitogen-Activated Protein Kinase 8); RASA1 (RAS P21 Protein Activator 1); PPP3CB (Protein Phosphatase 3 Catalytic Subunit Beta); PRKCA (Protein Kinase C Alpha); MAPK3 (Mitogen-Activated Protein Kinase 3); and NFATC4 (Nuclear Factor Of Activated T Cells 4) (see e.g., **Fig. 3A**).

[00303] Non-limiting examples of αβ T cell signature genes include ATP11B (ATPase Phospholipid Transporting 11B); PPP4R3A (Protein Phosphatase 4 Regulatory Subunit 3A); CAB39 (Calcium Binding Protein 39); GLS (Glutaminase); UBE2Z (Ubiquitin Conjugating Enzyme E2 Z); INPP4A (Inositol Polyphosphate-4-Phosphatase Type I A); RAB22A (Ras-Related Protein Rab-22A, Member Ras Oncogene Family); SMARCD2 (SWI/SNF (SWItch/Sucrose Non-Fermentable) Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily D, Member 2); VPS26B (VPS26, Retromer Complex Component B, Vacuolar Protein Sorting-Associated Protein 26B); CERK (Ceramide Kinase); ESYT2 (Extended Synaptotagmin 2); RAC1 (Rac Family Small GTPase 1); EIF3B (Eukaryotic Translation Initiation Factor 3 Subunit B); NEK7 (NIMA (Never In Mitosis Gene A)-Related Kinase 7); MDFIC (MyoD (myoblast determination protein 1) Family Inhibitor Domain Containing); YWHAH (Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Eta); MCMBP (Minichromosome Maintenance Complex Binding Protein); GOLPH3 (Golgi Phosphoprotein 3); PTGER4 (Prostaglandin E Receptor 4); B3GNT2 (UDP-GlcNAc:BetaGal Beta-1,3-N-Acetylglucosaminyltransferase 2, Galactosyltransferase 7); PITPNC1 (Phosphatidylinositol Transfer Protein Cytoplasmic 1); ARAP2 (ArfGAP With RhoGAP Domain, Ankyrin Repeat And PH Domain 2; Arf And Rho GAP Adapter Protein 2); ZFP36L2 (Zinc Finger Protein 36, C3H1 Type-Like 2); EFHD2 (EF-Hand Domain Family Member D2, Swiprosin-1); CPD (Carboxypeptidase D); KLRB1 (Killer Cell Lectin Like Receptor B1); DUSP1 (Dual Specificity Phosphatase 1); CMPK1 (Cytidine/Uridine Monophosphate Kinase 1); RASGRP1 (Ras Guanyl Releasing Protein 1); TM9SF3 (Transmembrane 9 Superfamily Member 3); MAPK1 (Mitogen-Activated Protein Kinase 1); GSPT1 (G1 To S Phase Transition 1); PNRC1 (Proline Rich Nuclear Receptor Coactivator 1); TMEM248 (Transmembrane Protein 248); STT3B (STT3 (STaurosporine and Temperature sensitive) Oligosaccharyltransferase Complex Catalytic Subunit B); KHDRBS1 (KH (K Homology) RNA Binding Domain Containing, Signal Transduction Associated 1); GNPTAB (N-Acetylglucosamine-1-Phosphate Transferase Subunits Alpha And Beta); GRSF1 (G-Rich RNA Sequence Binding Factor 1); TARP (TCR Gamma Alternate Reading Frame Protein, T-Cell Receptor Gamma-Chain); ZBTB16 (Zinc Finger And BTB (for BR-C, ttk and bab) Domain Containing 16, Zinc Finger Protein 145 (Kruppel-Like, Expressed In Promyelocytic Leukemia)); TGFBR1 (Transforming Growth Factor Beta Receptor 1); LGALS3BP (Galectin 3 Binding Protein); CD5 (T-Cell Surface Glycoprotein CD5); CD4 (T-Cell Surface Glycoprotein CD4); LRRN3 (Leucine Rich Repeat Neuronal 3); SLC40A1 (Solute Carrier Family 40 Member 1); CYSLTR1 (Cysteinyl Leukotriene Receptor 1); H4C3 (H4 Clustered Histone 3); CISH (Cytokine Inducible SH2 (Src Homology 2) Containing Protein); CD8B (T-Cell Surface Glycoprotein CD8 Beta Chain); MAL (Mal, T Cell Differentiation Protein, Myelin And Lymphocyte Protein); SUN2 (Sad1 And Unc84 Domain Containing 2, Rab5-Interacting Protein); CCR7 (C-C Motif Chemokine Receptor 7); GNLY (Granulysin); ANKLE2 (Ankyrin Repeat And LEM (LAP2, emerin, MAN1) Domain Containing 2); PSIP1 (PC4 (Positive Cofactor 4) And SFRS1

(Serine And Arginine Rich Splicing Factor 1) Interacting Protein 1, Lens Epithelium-Derived Growth Factor); PITPNA (Phosphatidylinositol Transfer Protein Alpha); RBM15B (RNA Binding Motif Protein 15B); PTPRA (Protein Tyrosine Phosphatase Receptor Type A); MARK2 (Microtubule Affinity Regulating Kinase 2); BLOC1S4 (Biogenesis Of Lysosomal Organelles Complex 1 Subunit 4); SIAH2 (Siah E3 Ubiquitin Protein Ligase 2); MXD4 (Max Dimerization Protein 4); SRM (Spermidine Synthase); SESN1 (Sestrin 1); SSBP4 (Single Stranded DNA Binding Protein 4); TAF10 (TATA-Box Binding Protein Associated Factor 10); DUSP2 (Dual Specificity Phosphatase 2); LPCAT1 (Lysophosphatidylcholine Acyltransferase 1); RASAL3 (Ras Protein Activator Like 3); TRIM65 (Tripartite Motif Containing 65); FAM50A (Family With Sequence Similarity 50 Member A); PIM3 (Pim-3 Proto-Oncogene, Serine/Threonine Kinase); SIPA1 (Signal-Induced Proliferation-Associated 1); FAM89B (Family With Sequence Similarity 89 Member B); ZBTB7A (Zinc Finger And BTB (for BR-C, ttk and bab) Domain Containing 7A, Factor That Binds To Inducer Of Short Transcripts Protein 1); NIN (Ninein); NR1D2 (Nuclear Receptor Subfamily 1 Group D Member 2); SIK3 (Salt-Inducible Kinase 3); ARHGAP26 (Rho GTPase Activating Protein 26); IL18RAP (Interleukin 18 Receptor Accessory Protein); CNR2 (Cannabinoid Receptor 2); EOMES (Eomesodermin); KLRC1 (Killer Cell Lectin Like Receptor C1); SEL1L3 (Suppressor Of Lin-12-Like Protein 3); IL12RB2 (Interleukin 12 Receptor Subunit Beta 2); COTL1 (Coactosin Like F-Actin Binding Protein 1); PIK3AP1 (Phosphoinositide-3-Kinase Adaptor Protein 1); TBX21 (T-Box Transcription Factor 21); FAM43A (Family With Sequence Similarity 43 Member A); KLRD1 (Killer Cell Lectin Like Receptor D1); SLAMF7 (signaling lymphocytic activation molecule (SLAM) family member 7); S1PR5 (Sphingosine-1-Phosphate Receptor 5); LAG3 (Lymphocyte Activating 3); ABCG1 (ATP Binding Cassette Subfamily G Member 1); S100B (S100 Calcium-Binding Protein, Beta); CCL22 (C-C Motif Chemokine Ligand 22); CEBPD (CCAAT box Enhancer Binding Protein Delta); IL17F (Interleukin 17F); and CEACAM1 (CEA Cell Adhesion Molecule 1); (see e.g., **Fig. 3B**).

[00304] In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits a more diverse TCR repertoire compared to T cells not derived using such stromal-free methods or without inhibition of an epigenetic regulator. In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits a Productive Simpson Clonality value of about 0.000-0.025. A value closer to 0 represents a higher level of diversity compared to clonality. A value closer to 1 represents a higher level of clonality compared to diversity. In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits a Productive Simpson Clonality value of at most 0.01, at most 0.015, at most 0.02, at most 0.025, at most 0.03, at most 0.035, at most 0.04, at most 0.045, at most 0.05, at most 0.055, at most 0.06, at most 0.065, at most 0.07, at most

0.075, at most 0.08, at most 0.085, at most 0.09, at most 0.095, or at most 0.1. In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits a Productive Simpson Clonality value of about 0.025; (see e.g., **Fig. 4**).

[00305] The variable domain of both the T-cell receptor (TCR) α-chain and β-chain each have three hypervariable or complementarity-determining regions (CDRs; e.g., CDR1, CDR2, CDR3). In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits an increased CDR (e.g., CDR1, CDR2, CDR3) length compared to T cells derived using stromal methods or without inhibition of an epigenetic regulator. In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits CDR (e.g., CDR1, CDR2, CDR3) length that is, on average, about 3 nucleotides (nt), 6 nt, 9 nt, or 12 nt or more longer than the CDRs of T cells derived using stromal methods or without inhibition of an epigenetic regulator. In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits CDR (e.g., CDR1, CDR2, CDR3) length that is, on average, about 27 nt, 30 nt, 33 nt, 36 nt, 39 nt, 42 nt, 45 nt, 48 nt, 51 nt, 54 nt, 57 nt, or 60 nt or longer (see e.g., **Fig. 5A-5D**). In some embodiments, the population of T cells derived using stromal-free methods and/or inhibition of an epigenetic regulator as described herein exhibits a CDR3 length that is, on average, about 42 nt long, compared to 39 nt on average for control iPSC-derived T cells, or 45 on average for peripheral blood mononuclear cell (PBMC)-derived T cells (see e.g., **Fig. 5C**).

Genetic Modifications of T Cells

[00306] In some embodiments, the resultant population of CD34+ hemogenic endothelium or another population as described herein (e.g., ESCs; iPSCs; HSCs; CD5+CD7+ ProT cells; CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells) are genetically modified. In some embodiments, the native T cell receptor locus can be removed and/or replaced to enhance targeted specificity. In some embodiments, an endogenous HLA (e.g., class I and/or class II major histocompatibility complexes) can be edited or removed. In some embodiments, the genetic modification can comprise introduction and expression of non-canonical HLA-G and HLA-E to prevent NK cell-mediated lysis (see e.g., Riolobos L et al. 2013), which can provide a source of universal T cells for immunotherapy, e.g., cancer immune therapy.

[00307] In some embodiments, the genetic modification comprises expressing a chimeric antigen receptor (CAR). Chimeric antigen receptors (CARs, also known as chimeric immunoreceptors, chimeric T cell receptors or artificial T cell receptors) are receptor proteins that have been engineered to give T cells the new ability to target a specific protein. The receptors are chimeric because they combine both antigen-binding and T-cell activating functions into a single receptor. Methods of

engineering chimeric antigen receptor T cells (also known as CAR T cells) are known in the art. See e.g., US Patents US7446190, US8399645, US8822647, US9212229, US9273283, US9447194, US9587020, US9932405, US10125193, US10221245, US10273300, US10287354; US patent publication US20160152723; PCT publication WO2009091826, WO2012079000, WO2014165707, WO2015164740, WO2016168595A1, WO2017040945, WO2017100428, WO2017117112, WO2017149515, WO2018067992, WO2018102787, WO2018102786, WO2018165228, WO2019084288; the contents of each of which are incorporated herein by reference in their entireties.

[00308] In some embodiments, methods of genetically modifying a cell to express a CAR can comprise but are not limited to: transfection or electroporation of a cell with a vector encoding a CAR; transduction with a viral vector (e.g., retrovirus, lentivirus) encoding a CAR; gene editing using zin finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganuclease-TALENs, or CRISPR-Cas; or any other methods known in the art of genetically modifying a cell to express a CAR.

[00309] Preferably, a population of cells at an early stage of differentiation (e.g., ESCs; PSCs; iPSCs; hemogenic endothelium; HSCs) is genetically modified with the CAR.

[00310] In some embodiments, the antigen-binding region of the CAR is directed against an antigen involved in a disease or disorder, such as but not limited to cancer, autoimmune disease, or heart disease (e.g., cardiac fibrosis). As used herein, the term "cancer" relates generally to a class of diseases or conditions in which abnormal cells divide without control and can invade nearby tissues. Cancer cells can also spread to other parts of the body through the blood and lymph systems. There are several main types of cancer. Carcinoma is a cancer that begins in the skin or in tissues that line or cover internal organs. Sarcoma is a cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Leukemia is a cancer that starts in blood-forming tissue such as the bone marrow, and causes large numbers of abnormal blood cells to be produced and enter the blood. Lymphoma and multiple myeloma are cancers that begin in the cells of the immune system. Central nervous system cancers are cancers that begin in the tissues of the brain and spinal cord.

[00311] In some embodiments, the cancer is a primary cancer. In some embodiments, the cancer is a malignant cancer. As used herein, the term "malignant" refers to a cancer in which a group of tumor cells display one or more of uncontrolled growth (*i.e.*, division beyond normal limits), invasion (*i.e.*, intrusion on and destruction of adjacent tissues), and metastasis (*i.e.*, spread to other locations in the body via lymph or blood). As used herein, the term "metastasize" refers to the spread of cancer from one part of the body to another. A tumor formed by cells that have spread is called a "metastatic tumor" or a "metastasis." The metastatic tumor contains cells that are like those in the original (primary) tumor. As used herein, the term "benign" or "non-malignant" refers to tumors that may grow larger but do not spread to other parts of the body. Benign tumors are self-limited and typically do not invade or metastasize.

[00312] A "cancer cell" or "tumor cell" refers to an individual cell of a cancerous growth or tissue. A tumor refers generally to a swelling or lesion formed by an abnormal growth of cells, which may be benign, pre-malignant, or malignant. Most cancer cells form tumors, but some, *e.g.*, leukemia, do not necessarily form tumors. For those cancer cells that form tumors, the terms cancer (cell) and tumor (cell) are used interchangeably.

[00313] As used herein the term "neoplasm" refers to any new and abnormal growth of tissue, e.g., an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues. Thus, a neoplasm can be a benign neoplasm, premalignant neoplasm, or a malignant neoplasm.

[00314] A subject that has a cancer or a tumor is a subject having objectively measurable cancer cells present in the subject's body. Included in this definition are malignant, actively proliferative cancers, as well as potentially dormant tumors or micrometastases. Cancers which migrate from their original location and seed other vital organs can eventually lead to the death of the subject through the functional deterioration of the affected organs.

[00315] Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, leukemia, basal cell carcinoma, biliary tract cancer; bladder cancer; bone cancer; brain and CNS cancer; breast cancer; cancer of the peritoneum; cervical cancer; choriocarcinoma; colon and rectum cancer; connective tissue cancer; cancer of the digestive system; endometrial cancer; esophageal cancer; eye cancer; cancer of the head and neck; gastric cancer (including gastrointestinal cancer); glioblastoma (GBM); hepatic carcinoma; hepatoma; intra-epithelial neoplasm.; kidney or renal cancer; larynx cancer; leukemia; liver cancer; lung cancer (*e.g.*, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, and squamous carcinoma of the lung); lymphoma including Hodgkin's and non-Hodgkin's lymphoma; melanoma; myeloma; neuroblastoma; oral cavity cancer (*e.g.*, lip, tongue, mouth, and pharynx); ovarian cancer; pancreatic cancer; prostate cancer; retinoblastoma; rhabdomyosarcoma; rectal cancer; cancer of the respiratory system; salivary gland carcinoma; sarcoma; skin cancer; squamous cell cancer; stomach cancer; testicular cancer; thyroid cancer; uterine or endometrial cancer; cancer of the urinary system; vulval cancer; as well as other carcinomas and sarcomas; as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia); chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); Hairy cell leukemia; chronic myeloblastic leukemia; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome. Preferably, in the case of CAR T therapy, the cancer is a blood cancer such as a leukemia or lymphoma.

[00316] Immunotherapy with chimeric antigen receptor (CAR) T cells offers a promising method to improve cure rates and decrease morbidities for patients with cancer. In this regard, CD19-specific CAR T cell therapies have achieved dramatic objective responses for a high percent of patients with CD19-positive leukemia or lymphoma. Accordingly, in some embodiments, the antigen-binding region of the CAR is directed against CD19; see e.g., US patents US10221245, US10357514; US patent publication US20160152723; PCT publication WO2016033570; the contents of each of which are incorporated herein by reference in their entireties.

[00317] Tumor antigens are proteins that are produced by tumor cells that elicit an immune response, particularly T-cell mediated immune responses. The selection of the antigen binding domain of the invention will depend on the particular type of cancer to be treated. Tumor antigens are well known in the art and include, for example, a glioma-associated antigen, carcinoembryonic antigen (CEA), EGFRvIII, IL-11Ra, IL-13Ra, EGFR, B7H3, Kit, CA-IX, CS-1, MUC1, BCMA, bcr-abl, HER2, β-human chorionic gonadotropin, alphafetoprotein (AFP), ALK, CD19, CD123, cyclin B 1, lectin-reactive AFP, Fos-related antigen 1, ADRB3, thyroglobulin, EphA2, RAGE-1, RU1, RU2, SSX2, AKAP-4, LCK, OY-TES1, PAX5, SART3, CLL-1, fucosyl GM1, GloboH, MN-CA IX, EPCAM, EVT6-AML, TGS5, human telomerase reverse transcriptase, polysialic acid, PLAC1, RU1, RU2 (AS), intestinal carboxyl esterase, lewisY, sLe, LY6K, mut hsp70-2, M-CSF, MYCN, RhoC, TRP-2, CYP1B1, BORIS, prostase, prostate-specific antigen (PSA), PAX3, PAP, NY-ESO-1, LAGE-1a, LMP2, NCAM, p53, p53 mutant, Ras mutant, gp100, prostein, OR51E2, PANX3, PSMA, PSCA, Her2/neu, hTERT, HMWMAA, HAVCR1, VEGFR2, PDGFR-beta, legumain, HPV E6,E7, survivin and telomerase, sperm protein 17, SSEA-4, tyrosinase, TARP, WT1, prostate-carcinoma tumor antigen-1 (PCTA-1), ML-IAP, MAGE, MAGE-A1, MAD-CT-1, MAD-CT-2, MelanA/MART1, XAGE1, ELF2M, ERG (TMPRSS2 ETS fusion gene), NA17, neutrophil elastase, sarcoma translocation breakpoints, NY-BR-1, ephrinB2, CD20, CD22, CD24, CD30, CD33, CD38, CD44v6, CD97, CD171, CD179a, androgen receptor, insulin growth factor (IGF)-I, IGF-II, IGF-I receptor, GD2, o-acetyl-GD2, GD3, GM3, GPRC5D, GPR20, CXORF61, folate receptor (FRa), folate receptor beta, ROR1, Flt3, TAG72, TN Ag, Tie 2, TEM1, TEM7R, CLDN6, TSHR, UPK2, and mesothelin. In a preferred embodiment, the tumor antigen is selected from the group consisting of folate receptor (FRa), mesothelin, EGFRvIII, IL-13Ra, CD123, CD19, CD33, BCMA, GD2, CLL-1, CA-IX, MUC1, HER2, and any combination thereof; see e.g., US Patent publications 20170209492 and 20180022795, the contents of each of which are incorporated herein by reference in their entireties.

Cellular Replacement Therapy

[00318] In one embodiment, provided herein a population of engineered immune cells produced by a method described herein, where in the T cell population is produced using a stroma-free differentiation method as described herein. In some embodiments, the population of engineered

immune cells comprises an immune cell differentiated using methods described herein, including but not limited to: PSCs; iPSCs; hemogenic endothelium; HSCs; CD5+CD7+ ProT cells; CD3+ T cells; CD4+CD8+ T cells; CD4+ T cells; CD8+ T cells. In some embodiments, the immune cell exhibits a gene expression profile that is most similar to alpha beta T cells.

[00319] In one embodiment, the population of cells further comprises a pharmaceutically acceptable carrier. These engineered immune cells can be culture expanded to increase the number of cells for use.

[00320] The engineered immune cells described herein are useful in the laboratory for biological studies. For examples, these cells can be derived from an individual having a genetic disease or defect, and used in the laboratory to study the biological aspects of the disease or defect, and to screen and test for potential remedy for that disease or defect.

[00321] Alternatively, the engineered immune cells described herein are useful in cellular replacement therapy and other medical treatment in subjects having the need. For example, patients who have undergone chemotherapy or irradiation or both, and manifest deficiencies in immune function and/or lymphocyte reconstitution, or in cancer immune therapy.

[00322] In various embodiments, the engineered immune cells described herein are administered (i.e., implanted or transplanted) to a subject in need of cellular replacement therapy.

[00323] In one embodiment, provided herein is a method of cellular replacement therapy, or for the treatment of cancer, autoimmune disorders, hematological diseases, or other genetic diseases and disorders in a subject, comprising (a) providing a somatic cell from a donor subject, (b) generating multilineage hematopoietic progenitor cells (e.g., hemogenic endothelium, HSPCs) from pluripotent stem cells derived from the somatic cell as described in any of the preceding paragraphs; (c) optionally inhibiting a histone methyltransferase in the resultant population of multilineage hematopoietic progenitor cells as described in any of the preceding paragraphs; (d) differentiating the resultant population of multilineage hematopoietic progenitor cells in the presence of a notch ligand to promote differentiation into the lymphoid lineage (e.g., T cells) as described in any of the preceding paragraphs, and (e) implanting or administering the resultant differentiated lymphoid cells into a recipient subject.

[00324] In one embodiment, the host subject and the recipient subject are the same individual. Alternatively, the host subject and the recipient subject are not the same individual, but are at least HLA compatible.

[00325] Hematological diseases are disorders which primarily affect the blood. Non-limiting examples of such diseases or disorders include myeloid derived disorders such as hemoglobinopathies (congenital abnormality of the hemoglobin molecule or of the rate of hemoglobin synthesis), sickle-cell disease, thalassemia, and methemoglobinemia; Anemias (lack of red blood cells or hemoglobin), Pernicious anemia; disorders resulting in decreased numbers of cells, such as myelodysplastic

syndrome, neutropenia (decrease in the number of neutrophils), and thrombotic thrombocytopenic purpura (TTP), thrombocytosis, hematological malignancies such as lymphomas, myelomas, and leukemia. Lymphomas such as Hodgkin's disease, Non-Hodgkin's lymphoma, Burkitt's lymphoma, Anaplastic large cell lymphoma, Splenic marginal zone lymphoma, Hepatosplenic T-cell lymphoma, and Angioimmunoblastic T-cell lymphoma (AILT); myelomas such as Multiple myeloma, Waldenström macroglobulinemia, Plasmacytoma; leukemias that increases defect WBC such as Acute lymphocytic leukemia (ALL), Chronic lymphocytic leukemia (CLL), Acute myelogenous leukemia (AML), Chronic Idiopathic Myelofibrosis (MF), Chronic myelogenous leukemia (CML), T-cell prolymphocytic leukemia (T-PLL), B-cell prolymphocytic leukemia (B-PLL), Chronic neutrophilic leukemia (CNL), Hairy cell leukemia (HCL), T-cell large granular lymphocyte leukemia (T-LGL), and Aggressive NK-cell leukemia.

[00326] Provided herein is a method of treating an autoimmune disease, which comprises administering an effective amount of an immune cell or population thereof, or a composition, or a pharmaceutical composition as described herein to a patient in need thereof. "Autoimmune disease" refers to a class of diseases in which a subject's own antibodies react with host tissue or in which immune effector T cells are autoreactive to endogenous self-peptides and cause destruction of tissue. Thus an immune response is mounted against a subject's own antigens, referred to as self-antigens. A "self-antigen" as used herein refers to an antigen of a normal host tissue. Normal host tissue does not include neoplastic cells.

[00327] Non-limiting examples of autoimmune diseases that can be treated include pemphigus (pemphigus vulgaris, pemphigus foliaceus or paraneoplastic pemphigus), Crohn's disease, idiopathic thrombocytopenic purpura (ITP), heparin induced thrombocytopenia (HIT), thrombotic thrombocytopenic purpura (TTP), Myasthenia Gravis (MG), and Chronic Inflammatory Demyelinating Polyneuropathy (CIDP). Additional non-limiting autoimmune diseases include autoimmune thrombocytopenia, immune neutropenia, antihemophilic FVIII inhibitor, antiphospholipid syndrome, Kawasaki Syndrome, ANCA-associated disease, polymyositis, bullous pemphigoid, multiple sclerosis (MS), Guillain-Barre Syndrome, chronic polyneuropathy, ulcerative colitis, diabetes mellitus, autoimmune thyroiditis, Graves' opthalmopathy, rheumatoid arthritis, ulcerative colitis, primary sclerosing cholangitis, systemic lupus erythematosus (SLE), autoimmune encephalomyelitis, Hashimoto's thyroiditis, Goodpasture's syndrome, autoimmune hemolytic anemia, scleroderma with anticollagen antibodies, mixed connective tissue disease, pernicious anemia, idiopathic Addison's disease, autoimmune-associated infertility, glomerulonephritis (e.g., crescentic glomerulonephritis, proliferative glomerulonephritis), insulin resistance, and autoimmune diabetes mellitus (type 1 diabetes mellitus; insulin dependent diabetes mellitus). Autoimmune disease has been recognized also to encompass atherosclerosis and Alzheimer's disease. In another embodiment, the autoimmune diseases include hepatitis, autoimmune hemophilia, autoimmune lymphoproliferative

syndrome (ALPS), autoimmune uveoretinitis, glomerulonephritis, agammaglobulinemia, alopecia areata, amyloidosis, ankylosing spondylitis, autoimmune angioedema, autoimmune aplastic anemia, autoimmune dysautonomia, autoimmune hyperlipidemia, autoimmune immunodeficiency, autoimmune inner ear disease (AIED), autoimmune myocarditis, autoimmune pancreatitis, autoimmune retinopathy, autoimmune urticaria, autoimmune urticarial neuropathy, autoimmune axonal neuropathy, Balo disease, Behçet's disease, Castleman disease, celiac disease, Chagas disease, chronic recurrent multifocal osteomyelitis (CRMO), Churg-Strauss syndrome, cicatricial pemphigoid, benign mucosal pemphigoid, Cogan's syndrome, cold agglutinin disease, coxsackie myocarditis, CREST disease, essential mixed cryoglobulinemia, dermatitis herpetiformis, dermatomyositis, Devic's disease (neuromyelitis optica), dilated cardiomyopathy, discoid lupus, Dressler's syndrome, endometriosis, eosinophilic angiocentric fibrosis, Eosinophilic fasciitis, Erythema nodosum, Evans syndrome, Fibrosing alveolitis, Giant cell arteritis (temporal arteritis), Hashimoto's encephalitis, Henoch-Schonlein purpura, Herpes gestationis, Idiopathic hypocomplementemic tubulointestitial nephritis, multiple myeloma, multifocal motor neuropathy, NMDA receptor antibody encephalitis, IgG4-related disease, IgG4-related sclerosing disease, inflammatory aortic aneurysm, inflammatory pseudotumour, inclusion body myositis, interstitial cystitis, juvenile arthritis, Kuttner's tumour, Lambert-Eaton syndrome, leukocytoclastic vasculitis, lichen planus, lichen sclerosus, Ligneous conjunctivitis, Linear IgA disease (LAD), Lyme disease, chronic, mediastinal fibrosis, Meniere's disease, Microscopic polyangiitis, Mikulicz's syndrome, Mooren's ulcer, Mucha-Habermann disease, multifocal fibrosclerosis, narcolepsy, optic neuritis, Ormond's disease (retroperitoneal fibrosis), palindromic rheumatism, PANDAS (pediatric autoimmune neuropsychiatric disorders associated with Streptococcus), paraneoplastic cerebellar degeneration, paraproteinemic polyneuropathies, paroxysmal nocturnal hemoglobinuria (PNH), Parry Romberg syndrome, Parsonnage-Turner syndrome, periaortitis, periarteritis, peripheral neuropathy, perivenous encephalomyelitis, POEMS syndrome, polyarteritis nodosa, Type I, II, & III autoimmune polyglandular syndromes, polymyalgia rheumatic, postpericardiotomy syndrome, progesterone dermatitis, primary biliary cirrhosis, psoriasis, psoriatic arthritis, idiopathic pulmonary fibrosis, pyoderma gangrenosum, pure red cell aplasia, Raynaud's phenomenon, reflex sympathetic dystrophy, Reiter's syndrome, relapsing polychondritis, restless legs syndrome, rheumatic fever, Riede's thyroiditis, sarcoidosis, Schmidt syndrome, scleritis, Sjogren's syndrome, sperm and testicular autoimmunity, stiff person syndrome, subacute bacterial endocarditis (SBE), Susac's syndrome, sympathetic ophthalmia, Takayasu's arteritis, Tolosa-Hunt syndrome, transverse myelitis, undifferentiated connective tissue disease (UCTD), vesiculobullous dermatosis, vitiligo, Rasmussen's encephalitis, Waldenstrom's macroglobulinaemia.

[00328] As used herein, the terms "administering," "introducing" and "transplanting" are used interchangeably in the context of the placement of described cells, e.g. hematopoietic progenitor cells, into a subject, by a method or route which results in at least partial localization of the introduced cells

at a desired site, such as a site of injury or repair, such that a desired effect(s) is produced. The cells e.g. hematopoietic progenitor cells, or their differentiated progeny (e.g., T cells) can be administered by any appropriate route which results in delivery to a desired location in the subject where at least a portion of the implanted cells or components of the cells remain viable.

[00329] In various embodiments, the engineered immune cells described herein are optionally expanded *ex vivo* prior to administration to a subject. In other embodiments, the engineered immune cells are optionally cryopreserved for a period, then thawed prior to administration to a subject.

[00330] The engineered immune cells used for cellular replacement therapy can be autologous/autogenic ("self") or non-autologous ("non-self," e.g., allogeneic, syngeneic or xenogeneic) in relation to the recipient of the cells. "Autologous," as used herein, refers to cells from the same subject. "Allogeneic," as used herein, refers to cells of the same species that differ genetically to the cell in comparison. "Syngeneic," as used herein, refers to cells of a different subject that are genetically identical to the cell in comparison. "Xenogeneic," as used herein, refers to cells of a different species to the cell in comparison. In preferred embodiments, the cells of the invention are allogeneic.

[00331] In various embodiments, the engineered immune cell described herein that is to be implanted into a subject in need thereof is autologous or allogeneic to the subject.

[00332] In various embodiments, the engineered immune cell described herein can be derived from one or more donors, or can be obtained from an autologous source. In some embodiments, the engineered immune cells are expanded in culture prior to administration to a subject in need thereof.

[00333] In various embodiments, the engineered immune cell described herein can be derived from one or more donors, or can be obtained from an autologous source.

[00334] In various embodiments, prior to implantation, the recipient subject is treated with chemotherapy and/or radiation.

[00335] In one embodiment, the chemotherapy and/or radiation is to reduce endogenous stem cells to facilitate engraftment of the implanted cells.

[00336] In various embodiments, prior to implantation, the engineered immune cells or the histone methyltransferase inhibited, multilineage hematopoietic progenitor cells or T cells differentiated using a stroma-free method as described herein are treated *ex vivo* with prostaglandin E2 and/or antioxidant N-acetyl-L-cysteine (NAC) to promote subsequent engraftment in a recipient subject.

[00337] In various embodiments, the recipient subject is a human.

[00338] In various embodiments, the subject has been previously diagnosed with HIV or other viral disease, a hematological disease, or undergoing a cancer treatment.

[00339] In one embodiment, a subject is selected to donate a somatic cell which would be used to produce iPSCs and an engineered immune cell described herein. In one embodiment, the selected subject has a genetic disease or defect.

[00340] In various embodiments, the donor subject is a human, non-human animal, rodent or non-rodent. For example, the subject can be any mammal, e.g., a human, other primate, pig, rodent such as mouse or rat, rabbit, guinea pig, hamster, cow, horse, cat, dog, sheep or goat, or a non-mammal such as a bird.

[00341] In various embodiments, the donor has been previously diagnosed with HIV, a hematological disease or cancer.

[00342] In one embodiment, a biological sample, a population of embryonic stem cells, somatic stem cells, progenitor cells, bone marrow cells, hematopoietic stem cells, or hematopoietic progenitor cells is obtained from the donor subject.

[00343] In various embodiments, the biological sample, a population of embryonic stem cells, somatic stem cells, progenitor cells, bone marrow cells, hematopoietic stem cells, or hematopoietic progenitor cells described herein can be derived from one or more donors, or can be obtained from an autologous source.

[00344] In one embodiment, the embryonic stem cells, somatic stem cells, progenitor cells, bone marrow cells, hematopoietic stem cells, hematopoietic progenitor cells are isolated from the donor subject, transfected, cultured (optional), and transplanted back into the same subject, i.e. an autologous cell transplant. Here, the donor and the recipient subject is the same individual. In another embodiment, the embryonic stem cells, somatic stem cells, progenitor cells, bone marrow cells, hematopoietic stem cells, or hematopoietic progenitor cells are isolated from a donor who is an HLA-type match with a subject (recipient). Donor-recipient antigen type-matching is well known in the art. The HLA-types include HLA-A, HLA-B, HLA-C, and HLA-D. These represent the minimum number of cell surface antigen matching required for transplantation. That is the transfected cells are transplanted into a different subject, i.e., allogeneic to the recipient host subject. The donor's or subject's embryonic stem cells, somatic stem cells, progenitor cells, bone marrow cells, hematopoietic stem cells, or hematopoietic progenitor cells can be transfected with a vector or nucleic acid comprising the nucleic acid molecule(s) described herein, the transfected cells are cultured, inhibited, and differentiated as disclosed, optionally expanded, and then transplanted into the recipient subject. In one embodiment, the transplanted engineered immune cells engraft in the recipient subject. In one embodiment, the transplanted engineered immune cells reconstitute the immune system in the recipient subject. The transfected cells can also be cryopreserved after transfected and stored, or cryopreserved after cell expansion and stored.

[00345] The engineered immune cells or the histone methyltransferase inhibited, multilineage hematopoietic progenitor cells or T cells differentiated using a stroma-free method as described herein may be administered as part of a bone marrow or cord blood transplant in an individual that has or has not undergone bone marrow ablative therapy. In one embodiment, genetically modified cells

contemplated herein are administered in a bone marrow transplant to an individual that has undergone chemoablative or radioablative bone marrow therapy.

[00346] In one embodiment, a dose of cells is delivered to a subject intravenously. In one embodiment, the cells are intravenously administered to a subject.

[00347] In particular embodiments, patients receive a dose of the modified cells described herein, e.g., engineered immune cells or the histone methyltransferase inhibited, multilineage hematopoietic progenitor cells or T cells differentiated using a stroma-free method as described herein, of about 1 x 10^5 cells/kg, about 5 x 10^5 cells/kg, about 1 x 10^6 cells/kg, about 2 x 10^6 cells/kg, about 3 x 10^6 cells/kg, about 4 x 10^6 cells/kg, about 5 x 10^6 cells/kg, about 6 x 10^6 cells/kg, about 7 x 10^6 cells/kg, about 8 x 10^6 cells/kg, about 9 x 10^6 cells/kg, about 1 x 10^7 cells/kg, about 5 x 10^7 cells/kg, about 1 x 10^8 cells/kg, or more in one single intravenous dose.

[00348] In certain embodiments, patients receive a dose of the modified cells described herein, e.g., engineered immune cells or the histone methyltransferase inhibited, multilineage hematopoietic progenitor cells or T cells differentiated using a stroma-free method as described herein, of at least 1 x 10^5 cells/kg, at least 5 x 10^5 cells/kg, at least 1 x 10^6 cells/kg, at least 2 x 10^6 cells/kg, at least 3 x 10^6 cells/kg, at least 4 x 10^6 cells/kg, at least 5 x 10^6 cells/kg, at least 6 x 10^6 cells/kg, at least 7 x 10^6 cells/kg, at least 8 x 10^6 cells/kg, at least 9 x 10^6 cells/kg, at least 1 x 10^7 cells/kg, at least 5 x 10^7 cells/kg, at least 1 x 10^8 cells/kg, or more in one single intravenous dose.

[00349] In an additional embodiment, patients receive a dose of the modified cells described herein, e.g., engineered immune cells or the histone methyltransferase inhibited, multilineage hematopoietic progenitor cells or T cells differentiated using a stroma-free method as described herein, of about 1 x 10^5 cells/kg to about 1 x 10^8 cells/kg, about 1 x 10^6 cells/kg to about 1 x 10^8 cells/kg, about 1 x 10^6 cells/kg to about 9 x 10^6 cells/kg, about 2 x 10^6 cells/kg to about 8 x 10^6 cells/kg, about 2 x 10^6 cells/kg to about 8 x 10^6 cells/kg, about 2 x 10^6 cells/kg to about 5 x 10^6 cells/kg, about 3 x 10^6 cells/kg to about 5 x 10^6 cells/kg, about 3 x 10^6 cells/kg to about 4 x 10^8 cells/kg, or any intervening dose of cells/kg.

[00350] In general, the engineered immune cells or the histone methyltransferase inhibited, multilineage hematopoietic progenitor cell described herein or T cells differentiated using a stroma-free method as described herein are administered as a suspension with a pharmaceutically acceptable carrier. For example, as therapeutic compositions. Therapeutic compositions contain a physiologically tolerable carrier together with the cell composition and optionally at least one additional bioactive agent as described herein, dissolved or dispersed therein as an active ingredient. In a preferred embodiment, the therapeutic composition is not substantially immunogenic when administered to a mammal or human patient for therapeutic purposes, unless so desired. One of skill in the art will recognize that a pharmaceutically acceptable carrier to be used in a cell composition will not include buffers, compounds, cryopreservation agents, preservatives, or other agents in amounts that

substantially interfere with the viability of the cells to be delivered to the subject. A formulation comprising cells can include e.g., osmotic buffers that permit cell membrane integrity to be maintained, and optionally, nutrients to maintain cell viability or enhance engraftment upon administration. Such formulations and suspensions are known to those of skill in the art and/or can be adapted for use with the cells as described herein using routine experimentation.

[00351] As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like. A pharmaceutically acceptable carrier will not promote the raising of an immune response to an agent with which it is admixed, unless so desired. The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically, such compositions are prepared as injectable either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified or presented as a liposome composition. The active ingredient can be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients include, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents and the like which enhance the effectiveness of the active ingredient. The therapeutic composition of the present invention can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and the like. Physiologically tolerable carriers are well known in the art. Exemplary liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, polyethylene glycol and other solutes. Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, and water-oil emulsions. The amount of an active agent used in the methods described herein that will be

effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, A. Osol, a standard reference text in this field of art. For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution.

[00352] In one embodiment, the "pharmaceutically acceptable" carrier does not include *in vitro* cell culture media.

[00353] In some embodiments, the composition of engineered immune cells described further comprises a pharmaceutically acceptable carrier.

[00354] In various embodiments, at least a second or subsequent dose of cells is administered to the recipient subject. For example, a second administration can be given between about one day to 30 weeks from the previous administration. Two, three, four or more total subsequent administrations can be delivered to the individual, as needed, e.g., determined by a skilled clinician.

[00355] A cell composition can be administered by any appropriate route which results in effective cellular replacement treatment in the subject, i.e. administration results in delivery to a desired location in the subject where at least a portion of the composition delivered, i.e. at least 1 x 10^4 cells are delivered to the desired site for a period of time. Modes of administration include injection, infusion, or instillation, "Injection" includes, without limitation, intravenous, intra-arterial, intraventricular, intracardiac injection and infusion. For the delivery of cells, administration by injection or infusion is generally preferred.

[00356] Efficacy testing can be performed during the course of treatment using the methods described herein. Measurements of the degree of severity of a number of symptoms associated with a particular ailment are noted prior to the start of a treatment and then at later specific time period after the start of the treatment. In some embodiments, a pharmaceutical composition comprising an immune cell as described herein or a population thereof can be used for cellular replacement therapy in a subject.

[00357] Accordingly, it is also the objective of this the present disclosure to provide compositions of modified (also referred to as engineered) cells for use in *in vivo* cellular replacement therapy, medical therapy such as cancer immune therapy, and for the *in vitro* studies of disease modeling, drug screening, and hematological diseases.

[00358] The advantage of the disclosure protocols is the methods permit semi-permanent bulk production of desired immune cells or other types of hematopoietic cells (i.e. cells differentiated from multipotent HSCs,) from a variety of types of cell source, from stem cells, hematopoietic progenitor cells, and mature and differentiated somatic cells, all of which can be readily collected from the patient's body.

[00359] The produced engineered immune cells or engineered histone methyltransferase-inhibited, $CD34^{+}/CD\ 38^{lo/-}$ hematopoietic progenitor cells (e.g., hemogenic endothelium) or T cells differentiated using a stroma-free method as described herein can be transplanted into a patient for various medical treatments such as immune system reconstruction therapy (e.g., after bone marrow ablation) or immunotherapy (e.g., in cancer therapy or autoimmune diseases). One added advantage is that if the donor of the source cells and recipient of the engineered immune cells are the same person, the produced engineered immune cells have HLA that are identical to the recipient and this avoids host-graft immune rejection after the transplantation. For recipient patients that are HLA allogeneic to the donor person of the source cells, host-graft immune rejection is greatly reduced.

[00360] The produced engineered immune cells or engineered histone methyltransferase-inhibited, CD34+/CD 38- hematopoietic progenitor cells or T cells differentiated using a stroma-free method as described herein can also be cryopreserved till needed in the future.

[00361] Currently, bone marrow transplantation is the most established cellular replacement therapy for a variety of hematological disorders. The functional unit of a bone marrow transplant is the hematopoietic stem cell (HSC), which resides at the apex of a complex cellular hierarchy and replenishes blood development throughout life. The scarcity of HLA-matched HSCs severely limits the ability to carry out transplantation, disease modeling and drug screening. As such, many studies have aimed to generate HSCs from alternative sources. Advances in reprogramming to induced pluripotent stem cells (iPSCs) has provided access to a wide array of patient-specific pluripotent cells, a promising source for disease modeling, drug screens and cellular therapies. However, the inability to derive engraftable hematopoietic stem and progenitor cells from human pluripotent stem cells (hPSCs) has limited the characterization of hematological diseases to *in vitro* assays. Generation of HSCs by directed differentiation has remained elusive, and there is a need for novel approaches to this problem.

[00362] Accordingly, in one aspect described herein is a method of cellular replacement therapy, the method comprising administering an immune cell as described herein or population thereof, or a composition comprising said immune cell or population thereof, or a pharmaceutical composition comprising said immune cell or population thereof to a recipient subject in need thereof.

[00363] In some embodiments, the recipient subject has undergone chemotherapy and/or irradiation. In some embodiments, the recipient subject has deficiencies in immune function and/or lymphocyte reconstitution. In some embodiments, prior to transplanting, the immune cell or population thereof is treated *ex vivo* with prostaglandin E2 and/or antioxidant N-acetyl-L-cysteine (NAC) to promote subsequent engraftment in a recipient subject.

Kits

[00364] Another aspect of the technology described herein relates to kits for differentiating T cells using a stroma-free method as described herein, among others. Described herein are kit components that can be included in one or more of the kits described herein.

[00365] In some embodiments, the kit comprises an effective amount of CD3+ T-cell differentiation factors (e.g., IL-7, SCF, FLT3, and/or TPO); or an effective amount of iPSC differentiation factors (e.g., OCT4, SOX2, KLF4, c-MYC, nanog, and/or LIN28); or an effective amount of hemogenic endothelium differentiation factors (e.g., BMP4, SB-431542, CHIR99021, bFGF, VEGF, IL-6, IL-11, IGF-1, SCF, and EPO); or an effective amount of single-positive T-cell differentiation factors (e.g., IL-15 and/or a T cell activator such as a CD3/CD28 T cell activator); or an effective amount of an inhibitor of an epigenetic regulator (e.g., MC1568; CAY10591; UNC0224; UNC0638; A366; BRD4770; BIX01294; UNC0642; UNC0631; UNC0646; UNC0321; E72; BIX-01338; BRD9539; Chaetocin; or DCG066; e.g., an EZH1 RNA interference agent). As will be appreciated by one of skill in the art, such cell differentiation factors can be supplied in a lyophilized form or a concentrated form that can diluted prior to use with cultured cells. Preferred formulations include those that are non-toxic to the cells and/or does not affect growth rate or viability etc. T-cell differentiation factors can be supplied in aliquots or in unit doses.

[00366] In some embodiments, the kit comprises a cell culture vessel comprising an immobilized Notch ligand. In some embodiments, the kit comprises a cell culture vessel and a Notch ligand that can be immobilized to the cell culture vessel using reagents and/or instructions provided therein. In some embodiments, the kit does not comprise stromal cells as described herein.

[00367] In some embodiments, the kit further comprises a vector comprising a nucleic acid encoding a CAR.

[00368] In some embodiments, the components described herein can be provided singularly or in any combination as a kit. The kit includes the components described herein, e.g., a composition comprising Notch ligand that does not comprise stromal cells, a composition(s) comprising differentiation factor(s), a composition(s) that includes a vector comprising e.g., CAR as described throughout the specification. Such kits can optionally include one or more agents that permit the detection of markers for T cell maturation (e.g., CD5, CD7, CD3, CD4, CD8, TCRgd, TCR alpha or beta, etc.) or a set thereof. Such kits can optionally include one or more agents that permit the detection of markers for T cell activation (e.g., CD107a, CD69, CD25, HLA-DR, IFNg, TNFa, etc.) or a set thereof. Such kits can optionally include one or more agents that permit the detection of markers for hemogenic endothelium (e.g., CD34, CD38, CD45, KDR, CD235, CD43, etc.). In addition, the kit optionally comprises informational material. The kit can also contain a substrate for coating culture dishes, such as laminin, fibronectin, Poly-L-Lysine, or methylcellulose.

[00369] In some embodiments, the compositions in the kit can be provided in a watertight or gas tight container which in some embodiments is substantially free of other components of the kit. For

example, a cell differentiation reagent can be supplied in more than one container, e.g., it can be supplied in a container having sufficient reagent for a predetermined number of differentiation assays, e.g., 1, 2, 3 or greater. One or more components as described herein can be provided in any form, e.g., liquid, dried or lyophilized form. It is preferred that the components described herein are substantially pure and/or sterile. When the components described herein are provided in a liquid solution, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being preferred.

[00370] The informational material can be descriptive, instructional, marketing or other material that relates to the methods described herein. The informational material of the kits is not limited in its form. In one embodiment, the informational material can include information about production of a cell culture vessel comprising immobilized Notch ligand; or the production of T cells differentiated using a stroma-free method as described herein; or the concentration, date of expiration, batch or production site information, and so forth of reagents used herein such as cell differentiation factors. In one embodiment, the informational material relates to methods for using or administering the components of the kit.

[00371] The kit can include a component for the detection of a marker for cell differentiation. In addition, the kit can include one or more antibodies that bind a cell marker, or primers for an RT-PCR or PCR reaction, e.g., a semi-quantitative or quantitative RT-PCR or PCR reaction. Such components can be used to assess the activation of cell maturation markers or the loss of undifferentiated or immature cell markers. If the detection reagent is an antibody, it can be supplied in dry preparation, e.g., lyophilized, or in a solution. The antibody or other detection reagent can be linked to a label, e.g., a radiological, fluorescent (e.g., GFP) or colorimetric label for use in detection. If the detection reagent is a primer, it can be supplied in dry preparation, e.g., lyophilized, or in a solution.

[00372] The kit will typically be provided with its various elements included in one package, e.g., a fiber-based, e.g., a cardboard, or polymeric, e.g., a Styrofoam box. The enclosure can be configured so as to maintain a temperature differential between the interior and the exterior, e.g., it can provide insulating properties to keep the reagents at a preselected temperature for a preselected time.

Definitions

[00373] For convenience, the meaning of some terms and phrases used in the specification, examples, and appended claims, are provided below. Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. The definitions are provided to aid in describing particular embodiments, and are not intended to limit the claimed invention, because the scope of the invention is limited only by the claims. Unless otherwise defined, 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 invention belongs. If there is an apparent discrepancy

between the usage of a term in the art and its definition provided herein, the definition provided within the specification shall prevail.

[00374] For convenience, certain terms employed herein, in the specification, examples and appended claims are collected here.

[00375] As used herein, the term "cell" refers to a single cell as well as to a population of (i.e., more than one) cells. The population may be a pure population comprising one cell type, such as a population of pluripotent stem cells or a population of differentiated T cells. As used herein, the term "population" refers to a pure population or to a population comprising a majority (e.g., at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%) of one cell type. Alternatively, the population may comprise more than one cell type, for example a mixed cell population. It is not meant to limit the number of cells in a population; for example, a mixed population of cells may comprise at least one differentiated cell. In the present invention, there is no limit on the number of cell types that a mixed cell population may comprise.

[00376] As used herein, in one embodiment, the term "hematopoietic stem cell" or "HSC" refers to a stem cell that has self-renewal capacity and also give rise to all the blood cell types of the three hematopoietic lineages, erythroid, lymphoid, and myeloid. These cell types include the myeloid lineages (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and the lymphoid lineages (T-cells, B-cells, NK-cells). Human HSCs are determined as $CD34^{+}$, $CD59^{+}$, $CD90/Thy1^{+}$, $CD38^{low/-}$, $c\text{-}kit/CD117^{-/low}$, and Lin^{-}. Mouse HSC- are considered $CD34^{low/-}$, $SCA\text{-}1^{+}$, $CD90/Thy1^{+/low}$, $CD38^{+}$, $c\text{-}Kit/CD117^{+}$, and Lin^{-}. Detecting the expression of these marker panels allows separation of specific cell populations via techniques like fluorescence-activated cell sorting (FACS). In one embodiment, the term "hematopoietic stem cell" or "HSC" refers to a stem cell that has self-renewal capacity and that have the following cell surface markers: CD34+, CD59+, $Thy1/CD90^{+}$, $CD38^{lo/-}$, CD133+, $c\text{-}Kit/CD117^{-/lo}$, and Lin^{-}. In one embodiment, the term "hematopoietic stem cell" or "HSC" refers to a stem cell that is at least CD34+. In one embodiment, the term "hematopoietic stem cell" or "HSC" refers to a stem cell that has self-renewal capacity and that is at least $CD34^{+}$ and $c\text{-}kit/CD117^{lo/-}$. In one embodiment, the term "hematopoietic stem cell" or "HSC" refers to a stem cell that has self-renewal capacity and that is at least $CD38^{low/-}$, $c\text{-}kit/CD117^{-/low}$. The term HSC can be used interchangeably with the term "hematopoietic stem and progenitor cell" (HSPC).

[00377] As used herein, the terms "iPS cell", "iPSC", and "induced pluripotent stem cell" are used interchangeably and refers to a pluripotent cell artificially derived by the transfection of the following reprogramming factors OCT4, SOX2, KLF4, and optionally c-MYC or nanog and LIN28, from a differentiated cell, e.g., a somatic cell. Alternative combinations of reprogramming factors include OCT4, SOX2, NANOG, and LIN28. The term hPSC refers to a human pluripotent stem cell.

[00378] As used herein, the term "lineage" when used in the context of stem and progenitor cell differentiation and development refers to the cell differentiation and development pathway, which the cell can take to becoming a fully differentiated cell. For example, a HSC has three hematopoietic lineages, erythroid, lymphoid, and myeloid; the HSC has the potential, i.e., the ability, to differentiate and develop into those terminally differentiated cell types known for all these three lineages. When the term "multilineage" used, it means the cell is able to, in the future, differentiate and develop into those terminally differentiated cell types known for more than one lineage. For example, the HSC has multilineage potential. When the term "limited lineage" used, it means the cell can differentiate and develop into those terminally differentiated cell types known for one lineage. For example, a common myeloid progenitor cell (CMP) or a megakaryocyte-erythroid progenitor (MEP) has a limited lineage because the cell can only differentiate and develop into those terminally differentiated cell types of the myeloid lineage and not that of the lymphoid lineage. Terminally differentiated cells of the myeloid lineage include erythrocytes, monocytes, macrophages, megakaryocytes, myeloblasts, dendritic cells, and granulocytes (basophils, neutrophils, eosinophils, and mast cells); and terminally differentiated cells of the lymphoid lineage include T lymphocytes/ T cells, B lymphocytes/B cells, dendritic cells, and natural killer cells.

[00379] As used herein, the term "a progenitor cell" refers to an immature or undifferentiated cell that has the potential later on to mature (differentiate) into a specific cell type (a fully differentiated or terminally differentiated cell), for example, a blood cell, a skin cell, a bone cell, or hair cells. Progenitor cells have a cellular phenotype that is more primitive (e.g., is at an earlier step along a developmental pathway or progression than is a fully differentiated cell) relative to a cell, which it can give rise to by differentiation. Often, progenitor cells also have significant or very high proliferative potential. Progenitor cells can give rise to multiple distinct differentiated cell types or to a single differentiated cell type, depending on the developmental pathway and on the environment in which the cells develop and differentiate. A progenitor cell also can proliferate to make more progenitor cells that are similarly immature or undifferentiated.

[00380] The term "differentiated cell" is meant any primary cell that is not, in its native form, pluripotent as that term is defined herein. The term a "differentiated cell" also encompasses cells that are partially differentiated, such as multipotent cells (e.g. adult somatic stem cells). In some embodiments, the term "differentiated cell" also refers to a cell of a more specialized cell type derived from a cell of a less specialized cell type (e.g., from an undifferentiated cell or a reprogrammed cell) where the cell has undergone a cellular differentiation process.

[00381] In the context of cell ontogeny, the term "differentiate", or "differentiating" is a relative term meaning a "differentiated cell" is a cell that has progressed further down the developmental pathway than its precursor cell. Thus in some embodiments, a reprogrammed cell as this term is defined herein, can differentiate to lineage-restricted precursor cells (such as a mesodermal stem cell

or a endodermal stem cell), which in turn can differentiate into other types of precursor cells further down the pathway (such as an tissue specific precursor, for example, a cardiomyocyte precursor, or a pancreatic precursor), and then to an end-stage differentiated cell, which plays a characteristic role in a certain tissue type, and may or may not retain the capacity to proliferate further.

[00382] The term "multipotent" when used in reference to a "multipotent cell" refers to a cell that is able to differentiate into some but not all of the cells derived from all three germ layers. Thus, a multipotent cell is a partially differentiated cell. Multipotent cells are well known in the art, and examples of multipotent cells include adult somatic stem cells, such as for example, hematopoietic stem cells and neural stem cells, hair follicle stem cells, liver stem cells etc. Multipotent means a stem cell may form many types of cells in a given lineage, but not cells of other lineages. For example, a multipotent blood stem cell can form the many different types of blood cells (red, white, platelets, etc.), but it cannot form neurons; cardiovascular progenitor cell (MICP) differentiation into specific mature cardiac, pacemaker, smooth muscle, and endothelial cell types; pancreas-derived multipotent progenitor (PMP) colonies produce cell types of pancreatic lineage (cells that produces insulin, glucagon, amylase or somatostatin) and neural lineage (cells that are morphologically neuron-like, astrocytes-like or oligodendrocyte-like).

[00383] The term a "reprogramming gene", as used herein, refers to a gene whose expression, contributes to the reprogramming of a differentiated cell, e.g. a somatic cell to an undifferentiated cell (e.g. a cell of a pluripotent state or partially pluripotent state, multipotent state). A reprogramming gene can be, for example, genes encoding master transcription factors Sox2, Oct3/4, Klf4, Nanog, Lin-28, c-myc and the like. The term "reprogramming factor" refers to the protein encoded by the reprogramming gene.

[00384] The term "exogenous" refers to a substance present in a cell other than its native source. The terms "exogenous" when used herein refers to a nucleic acid (e.g. a nucleic acid encoding a reprogramming transcription factor, e.g. Sox2, Oct3/4, Klf4, Nanog, Lin-28, c-myc and the like) or a protein (e.g., a transcription factor polypeptide) that has been introduced by a process involving the hand of man into a biological system such as a cell or organism in which it is not normally found or in which it is found in lower amounts. A substance (e.g. a nucleic acid encoding a sox2 transcription factor, or a protein, e.g., a SOX2 polypeptide) will be considered exogenous if it is introduced into a cell or an ancestor of the cell that inherits the substance.

[00385] The term "isolated" as used herein signifies that the cells are placed into conditions other than their natural environment. The term "isolated" does not preclude the later use of these cells thereafter in combinations or mixtures with other cells.

[00386] As used herein, the term “expanding” refers to increasing the number of like cells through cell division (mitosis). The term “proliferating” and “expanding” are used interchangeably.

[00387] As used herein, a “cell-surface marker” refers to any molecule that is expressed on the surface of a cell. Cell-surface expression usually requires that a molecule possesses a transmembrane domain. Some molecules that are normally not found on the cell-surface can be engineered by recombinant techniques to be expressed on the surface of a cell. Many naturally occurring cell-surface markers are termed “CD” or “cluster of differentiation” molecules. Cell-surface markers often provide antigenic determinants to which antibodies can bind to. A cell-surface marker of particular relevance to the methods described herein is CD34. The useful hematopoietic progenitor cells (e.g., hemogenic endothelium) according to the present disclosure preferably express CD34 or in other words, they are CD34 positive.

[00388] A cell can be designated “positive” or “negative” for any cell-surface marker, and both such designations are useful for the practice of the methods described herein. A cell is considered “positive” for a cell-surface marker if it expresses the marker on its cell-surface in amounts sufficient to be detected using methods known to those of skill in the art, such as contacting a cell with an antibody that binds specifically to that marker, and subsequently performing flow cytometric analysis of such a contacted cell to determine whether the antibody is bound the cell. It is to be understood that while a cell may express messenger RNA for a cell-surface marker, in order to be considered positive for the methods described herein, the cell must express it on its surface. Similarly, a cell is considered “negative” or “negative/low” (abbreviated as “-/lo” or “lo/-”) for a cell-surface marker if the cell does not express the marker on its cell surface in amounts sufficient to be detected using methods known to those of skill in the art, such as contacting a cell with an antibody that binds specifically to that marker and subsequently performing flow cytometric analysis of such a contacted cell to determine whether the antibody is bound the cell. In some embodiments, where agents specific for cell-surface lineage markers used, the agents can all comprise the same label or tag, such as fluorescent tag, and thus all cells positive for that label or tag can be excluded or removed, to leave uncontacted hematopoietic stem or progenitor cells for use in the methods described herein.

[00389] As used herein, the term "a histone methyltransferase inhibitor" or “inhibitor” is any molecule that inhibits of expression of a histone methyltransferase (e.g., G9a, GLP, EZH1), or inhibits the catalytic activity of the enzyme to methylate lysine resides on the substrate histone protein. For example, a histone methyltransferase inhibitor can be an siRNA or dsRNA that inhibits of expression of G9a, GLP, or EZH1 in the inhibited cell, or a gRNA that promotes the degradation of the mRNA of G9a, GLP, or EZH1 in the inhibited cell. For example, a histone methyltransferase inhibitor is a small molecule that antagonizes the enzyme activity. Examples include but are not limited to small molecules AMI-1, A-366, BIX-01294, BIX01338, BRD4770, chaetocin, UNC0224, UNC0631, UNC0638, UNC0642, UNC0646, EPZ5676, EPZ005687, GSK343, EPZ-6438, 3-deazaneplanocin A (DZNeP) HCl, UNC1999, MM-102, SGC 0946, Entacapone, EPZ015666, UNC0379, EI1, MI-2

(Menin-MLL Inhibitor), MI-3 (Menin-MLL Inhibitor), PFI-2, GSK126, EPZ004777, BRD4770, and EPZ-6438 as described herein.

[00390] As used herein, the term "small molecule" refers to a chemical agent including, but not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, aptamers, nucleotides, nucleotide analogs, organic or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. In some embodiments, the small molecule is a heterorganic compound or an organometallic compound.

[00391] The term "inhibitory RNA" is meant to include a nucleic acid molecule that contains a sequence that is complementary to a target nucleic acid (e.g., a target microRNA) that mediates a decrease in the level or activity of the target nucleic acid. Non-limiting examples of inhibitory RNAs include interfering RNA, shRNA, siRNA, ribozymes, antagomirs, and antisense oligonucleotides. Methods of making inhibitory RNAs are described herein. Additional methods of making inhibitory RNAs are known in the art. In one embodiment, the G9a/GLP or EZH1 microRNA described herein is an inhibitory RNA that causes a decrease in the activity of G9a/GLP or EZH1 mRNA.

[00392] As used herein, "an interfering RNA" refers to any double stranded or single stranded RNA sequence, capable - either directly or indirectly (i.e., upon conversion) of inhibiting or down-regulating gene expression by mediating RNA interference. Interfering RNA includes, but is not limited to, small interfering RNA ("siRNA") and small hairpin RNA ("shRNA"). "RNA interference" refers to the selective degradation of a sequence-compatible messenger RNA transcript.

[00393] As used herein "an shRNA" (small hairpin RNA) refers to an RNA molecule comprising an antisense region, a loop portion and a sense region, wherein the sense region has complementary nucleotides that base pair with the antisense region to form a duplex stem. Following post-transcriptional processing, the small hairpin RNA is converted into a small interfering RNA by a cleavage event mediated by the enzyme Dicer, which is a member of the RNase III family. As used herein, the phrase "post-transcriptional processing" refers to mRNA processing that occurs after transcription and is mediated, for example, by the enzymes Dicer and/or Drosha.

[00394] A "small interfering RNA" or "siRNA" as used herein refers to any small RNA molecule capable of inhibiting or down regulating gene expression by mediating RNA interference in a sequence specific manner. The small RNA can be, for example, about 18 to 21 nucleotides long. Each siRNA duplex is formed by a guide strand and a passenger strand. The endonuclease Argonaute 2 (Ago 2) catalyzes the unwinding of the siRNA duplex. Once unwound, the guide strand is

incorporated into the RNA Interference Specificity Complex (RISC), while the passenger strand is released. RISC uses the guide strand to find the mRNA that has a complementary sequence leading to the endonucleolytic cleavage of the target mRNA.

[00395] Retroviruses are RNA viruses that utilize reverse transcriptase during their replication cycle. The term "retrovirus" refers to any known retrovirus (e.g., type c retroviruses, such as Moloney murine sarcoma virus (MoMSV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), gibbon ape leukemia virus (GaLV), feline leukemia virus (FLV), spumavirus).

[00396] The retroviral genomic RNA is converted into double-stranded DNA by reverse transcriptase. This double-stranded DNA form of the virus is capable of being integrated into the chromosome of the infected cell; once integrated, it is referred to as a "provirus." The provirus serves as a template for RNA polymerase II and directs the expression of RNA molecules, which encode the structural proteins and enzymes needed to produce new viral particles.

[00397] At each end of the provirus are structures called "long terminal repeats" or "LTRs." The term "long terminal repeat (LTR)" refers to domains of base pairs located at the ends of retroviral DNAs which, in their natural sequence context, are direct repeats and contain U3, R, and U5 regions. LTRs generally provide functions fundamental to the expression of retroviral genes (e.g., promotion, initiation and polyadenylation of gene transcripts) and to viral replication. The LTR contains numerous regulatory signals including transcriptional control elements, polyadenylation signals and sequences needed for replication and integration of the viral genome. The viral LTR is divided into three regions called U3, R and U5. The U3 region contains the enhancer and promoter elements. The U5 region is the sequence between the primer binding site and the R region and contains the polyadenylation sequence. The R (repeat) region is flanked by the U3 and U5 regions. The LTR composed of U3, R, and U5 regions, appears at both the 5' and 3' ends of the viral genome. In one embodiment of the invention, the promoter within the LTR, including the 5' LTR, is replaced with a heterologous promoter. Examples of heterologous promoters that can be used include, for example, a spleen focus-forming virus (SFFV) promoter, a tetracycline-inducible (TET) promoter, a β-globin locus control region and a β-globin promoter (LCR), and a cytomegalovirus (CMV) promoter.

[00398] The term "lentivirus" refers to a group (or genus) of retroviruses that give rise to slowly developing disease. Viruses included within this group include HIV (human immunodeficiency virus; including HIV type 1, and HIV type 2), the etiologic agent of the human acquired immunodeficiency syndrome (AIDS); visna-maedi, which causes encephalitis (visna) or pneumonia (maedi) in sheep, the caprine arthritis-encephalitis virus, which causes immune deficiency, arthritis, and encephalopathy in goats; equine infectious anemia virus, which causes autoimmune hemolytic anemia, and encephalopathy in horses; feline immunodeficiency virus (FIV), which causes immune deficiency in cats; bovine immune deficiency virus (BIV), which causes lymphadenopathy, lymphocytosis, and

possibly central nervous system infection in cattle; and simian immunodeficiency virus (SIV), which cause immune deficiency and encephalopathy in sub-human primates. Diseases caused by these viruses are characterized by a long incubation period and protracted course. Usually, the viruses latently infect monocytes and macrophages, from which they spread to other cells. HIV, FIV, and SIV also readily infect T lymphocytes, i.e., T-cells.

[00399] The term "R region" refers to the region within retroviral LTRs beginning at the start of the capping group (i.e., the start of transcription) and ending immediately prior to the start of the poly A tract. The R region is also defined as being flanked by the U3 and U5 regions. The R region plays an important role during reverse transcription in permitting the transfer of nascent DNA from one end of the genome to the other.

[00400] The term "promoter/enhancer" refers to a segment of DNA which contains sequences capable of providing both promoter and enhancer functions. For example, the long terminal repeats of retroviruses contain both promoter and enhancer functions. The enhancer/promoter may be "endogenous," "exogenous," or "heterologous." An "endogenous" enhancer/promoter is one which is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one which is placed in juxtaposition to a gene by means of genetic manipulation (i.e., molecular biological techniques) such that transcription of that gene is directed by the linked enhancer/promoter.

[00401] As used herein, the term "nucleic acid" or "nucleic acid sequence" refers to any molecule, preferably a polymeric molecule, incorporating units of ribonucleic acid, deoxyribonucleic acid or an analog thereof. The nucleic acid can be either single-stranded or double-stranded. A single-stranded nucleic acid can be one nucleic acid strand of a denatured double- stranded DNA. Alternatively, it can be a single-stranded nucleic acid not derived from any double-stranded DNA. In one aspect, the nucleic acid can be DNA. In another aspect, the nucleic acid can be RNA. Suitable DNA can include, e.g., genomic DNA or cDNA. Suitable RNA can include, e.g., mRNA, iRNA, miRNA, siRNA, etc.

[00402] The nucleic acid can be selected, for example, from a group including: nucleic acid encoding a protein of interest, oligonucleotides, nucleic acid analogues, for example peptide-nucleic acid (PNA), pseudo-complementary PNA (pc-PNA), and locked nucleic acid (LNA). Such nucleic acid sequences include, for example, but are not limited to, nucleic acid sequence encoding proteins, for example that act as transcriptional repressors, antisense molecules, ribozymes, small inhibitory nucleic acid sequences, for example but are not limited to RNAi, shRNAi, siRNA, microRNAi (miRNA), and antisense oligonucleotides.

[00403] As used herein, the term "engraftment" in reference to a recipient host is when the new blood-forming cells start to grow and which are derived from the implanted cells and make healthy blood stem cells that show up in recipient's blood after a minimum period of 10 days after

implantation. Engraftment can occur as early as 10 days after transplant but is more common around 14-20 days.

[00404] As used herein, the term "reconstitution" with respect to the immune system or the blood system in a recipient host refers to the rebuilding the innate reservoir or working system, or part thereof within the body of recipient host to a natural or a functionally state. For example, such as bone marrow after chemotherapy had obliterated the bone marrow stem cells.

[00405] The terms "decrease", "reduced", "reduction", or "inhibit" are all used herein to mean a decrease by a statistically significant amount. In some embodiments, "reduce," "reduction" or "decrease" or "inhibit" typically means a decrease by at least 10% as compared to a reference level (e.g. the absence of a given treatment or agent) and can include, for example, a decrease by at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 98%, at least about 99% , or more. As used herein, "reduction" or "inhibition" does not encompass a complete inhibition or reduction as compared to a reference level. "Complete inhibition" is a 100% inhibition as compared to a reference level. A decrease can be preferably down to a level accepted as within the range of normal for an individual without a given disorder.

[00406] The terms "increased", "increase", "enhance", or "activate" are all used herein to mean an increase by a statically significant amount. In some embodiments, the terms "increased", "increase", "enhance", or "activate" can mean an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level. In the context of a marker or symptom, a "increase" is a statistically significant increase in such level.

[00407] As used herein, a "subject" means a human or animal. Usually the animal is a vertebrate such as a primate, rodent, domestic animal or game animal. Primates include chimpanzees, cynomolgus monkeys, spider monkeys, and macaques, e.g., Rhesus. Rodents include mice, rats, woodchucks, ferrets, rabbits and hamsters. Domestic and game animals include cows, horses, pigs, deer, bison, buffalo, feline species, e.g., domestic cat, canine species, e.g., dog, fox, wolf, avian species, e.g., chicken, emu, ostrich, and fish, e.g., trout, catfish and salmon. In some embodiments, the subject is a mammal, e.g., a primate, e.g., a human. The terms, "individual," "patient" and "subject" are used interchangeably herein.

[00408] Preferably, the subject is a mammal. The mammal can be a human, non-human primate, mouse, rat, dog, cat, horse, or cow, but is not limited to these examples. Mammals other than humans can be advantageously used as subjects that represent animal models of cellular replacement therapy. A subject can be male or female.

[00409] A subject can be one who has been previously diagnosed with or identified as suffering from or having a condition in need of treatment (e.g. hematologic disease, cancer, etc.) or one or more complications related to such a condition, and optionally, have already undergone treatment for a hematologic disease or the one or more complications related to a hematologic disease. Alternatively, a subject can also be one who has not been previously diagnosed as having a hematologic disease or one or more complications related to a hematologic disease. For example, a subject can be one who exhibits one or more risk factors for a hematologic disease or one or more complications related to a hematologic disease or a subject who does not exhibit risk factors.

[00410] A "subject in need" of treatment for a particular condition can be a subject having that condition, diagnosed as having that condition, or at risk of developing that condition.

[00411] A variant amino acid or DNA sequence can be at least 85%, at least 87%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or more, identical to a native or reference sequence. The degree of homology (percent identity) between a native and a mutant sequence can be determined, for example, by comparing the two sequences using freely available computer programs commonly employed for this purpose on the world wide web (e.g. BLASTp or BLASTn with default settings).

[00412] Alterations of the native amino acid sequence can be accomplished by any of a number of techniques known to one of skill in the art. Mutations can be introduced, for example, at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion. Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered nucleotide sequence having particular codons altered according to the substitution, deletion, or insertion required. Techniques for making such alterations are very well established and include, for example, those disclosed by Walder et al. (Gene 42:133, 1986); Bauer et al. (Gene 37:73, 1985); Craik (BioTechniques, January 1985, 12-19); Smith et al. (Genetic Engineering: Principles and Methods, Plenum Press, 1981); and U.S. Pat. Nos. 4,518,584 and 4,737,462, which are herein incorporated by reference in their entireties. Any cysteine residue not involved in maintaining the proper conformation of the polypeptide also can be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bond(s) can be added to the polypeptide to improve its stability or facilitate oligomerization.

[00413] The term "expression" refers to the cellular processes involved in producing RNA and proteins and as appropriate, secreting proteins, including where applicable, but not limited to, for example, transcription, transcript processing, translation and protein folding, modification and processing. Expression can refer to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from a nucleic acid fragment or fragments of the invention and/or to the translation of mRNA into a polypeptide.

[00414] In some embodiments, the expression of a biomarker(s), target(s), or gene/polypeptide described herein is/are tissue-specific. In some embodiments, the expression of a biomarker(s), target(s), or gene/polypeptide described herein is/are global. In some embodiments, the expression of a biomarker(s), target(s), or gene/polypeptide described herein is systemic.

[00415] "Expression products" include RNA transcribed from a gene, and polypeptides obtained by translation of mRNA transcribed from a gene. The term "gene" means the nucleic acid sequence which is transcribed (DNA) to RNA *in vitro* or *in vivo* when operably linked to appropriate regulatory sequences. The gene may or may not include regions preceding and following the coding region, e.g. 5' untranslated (5'UTR) or "leader" sequences and 3' UTR or "trailer" sequences, as well as intervening sequences (introns) between individual coding segments (exons).

[00416] In some embodiments, a polypeptide, nucleic acid, or cell as described herein can be engineered. As used herein, "engineered" refers to the aspect of having been manipulated by the hand of man. For example, a polypeptide is considered to be "engineered" when at least one aspect of the polypeptide, e.g., its sequence, has been manipulated by the hand of man to differ from the aspect as it exists in nature. As is common practice and is understood by those in the art, progeny of an engineered cell are typically still referred to as "engineered" even though the actual manipulation was performed on a prior entity.

[00417] In some embodiments, the differentiated and/or engineered T cell described herein is exogenous. In some embodiments, the differentiated and/or engineered T cell described herein is ectopic. In some embodiments, the differentiated and/or engineered T cell described herein is not endogenous.

[00418] The term "exogenous" refers to a substance present in a cell other than its native source. The term "exogenous" when used herein can refer to a nucleic acid (e.g. a nucleic acid encoding a polypeptide) or a polypeptide that has been introduced by a process involving the hand of man into a biological system such as a cell or organism in which it is not normally found and one wishes to introduce the nucleic acid or polypeptide into such a cell or organism. Alternatively, "exogenous" can refer to a nucleic acid or a polypeptide that has been introduced by a process involving the hand of man into a biological system such as a cell or organism in which it is found in relatively low amounts and one wishes to increase the amount of the nucleic acid or polypeptide in the cell or organism, e.g., to create ectopic expression or levels. In contrast, the term "endogenous" refers to a substance that is

native to the biological system or cell. As used herein, "ectopic" refers to a substance that is found in an unusual location and/or amount. An ectopic substance can be one that is normally found in a given cell, but at a much lower amount and/or at a different time. Ectopic also includes substance, such as a polypeptide or nucleic acid that is not naturally found or expressed in a given cell in its natural environment.

[00419] Nucleic acids encoding a polypeptide as described herein (e.g. a CAR polypeptide) can be comprised by a vector. The term "vector", as used herein, refers to a nucleic acid construct designed for delivery to a host cell or for transfer between different host cells. As used herein, a vector can be viral or non-viral. The term "vector" encompasses any genetic element that is capable of replication when associated with the proper control elements and that can transfer gene sequences to cells. A vector can include, but is not limited to, a cloning vector, an expression vector, a plasmid, phage, transposon, cosmid, chromosome, virus, virion, etc.

[00420] The vector can be recombinant, e.g., it comprises sequences originating from at least two different sources. In some embodiments, the vector comprises sequences originating from at least two different species. In some embodiments, the vector comprises sequences originating from at least two different genes, e.g., it comprises a fusion protein or a nucleic acid encoding an expression product which is operably linked to at least one non-native (e.g., heterologous) genetic control element (e.g., a promoter, suppressor, activator, enhancer, response element, or the like).

[00421] In some embodiments, the vector or nucleic acid described herein is codon-optimized, e.g., the native or wild-type sequence of the nucleic acid sequence has been altered or engineered to include alternative codons such that altered or engineered nucleic acid encodes the same polypeptide expression product as the native/wild-type sequence, but will be transcribed and/or translated at an improved efficiency in a desired expression system. In some embodiments, the expression system is an organism other than the source of the native/wild-type sequence (or a cell obtained from such organism). In some embodiments, the vector and/or nucleic acid sequence described herein is codon-optimized for expression in a mammal or mammalian cell, e.g., a mouse, a murine cell, or a human cell. In some embodiments, the vector and/or nucleic acid sequence described herein is codon-optimized for expression in a human cell. In some embodiments, the vector and/or nucleic acid sequence described herein is codon-optimized for expression in a yeast or yeast cell. In some embodiments, the vector and/or nucleic acid sequence described herein is codon-optimized for expression in a bacterial cell. In some embodiments, the vector and/or nucleic acid sequence described herein is codon-optimized for expression in an *E. coli* cell.

[00422] As used herein, the term "expression vector" refers to a vector that directs expression of an RNA or polypeptide from sequences linked to transcriptional regulatory sequences on the vector. The sequences expressed will often, but not necessarily, be heterologous to the cell. An expression vector may comprise additional elements, for example, the expression vector may have two

replication systems, thus allowing it to be maintained in two organisms, for example in human cells for expression and in a prokaryotic host for cloning and amplification.

[00423] As used herein, the term “viral vector" refers to a nucleic acid vector construct that includes at least one element of viral origin and has the capacity to be packaged into a viral vector particle. The viral vector can contain the nucleic acid encoding a polypeptide as described herein in place of non-essential viral genes. The vector and/or particle may be utilized for the purpose of transferring any nucleic acids into cells either *in vitro* or *in vivo*. Numerous forms of viral vectors are known in the art. Non-limiting examples of a viral vector of this invention include an AAV vector, an adenovirus vector, a lentivirus vector, a retrovirus vector, a herpesvirus vector, an alphavirus vector, a poxvirus vector, a baculovirus vector, and a chimeric virus vector.

[00424] It should be understood that the vectors described herein can, in some embodiments, be combined with other suitable compositions and therapies. For example, the use of a suitable episomal vector provides a means of maintaining the nucleotide of interest in the subject in high copy number extra chromosomal DNA thereby eliminating potential effects of chromosomal integration.

[00425] As used herein, the terms "treat,” "treatment," "treating,” or “amelioration” refer to therapeutic treatments, wherein the object is to reverse, alleviate, ameliorate, inhibit, slow down or stop the progression or severity of a condition associated with a disease or disorder, e.g. a hematological disease or cancer. The term “treating" includes reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder associated with a hematological disease or cancer. Treatment is generally “effective" if one or more symptoms or clinical markers are reduced. Alternatively, treatment is “effective" if the progression of a disease is reduced or halted. That is, “treatment" includes not just the improvement of symptoms or markers, but also a cessation of, or at least slowing of, progress or worsening of symptoms compared to what would be expected in the absence of treatment. Beneficial or desired clinical results include, but are not limited to, alleviation of one or more symptom(s), diminishment of extent of disease, stabilized (*i.e.*, not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, remission (whether partial or total), and/or decreased mortality, whether detectable or undetectable. The term "treatment" of a disease also includes providing relief from the symptoms or side-effects of the disease (including palliative treatment).

[00426] As used herein, the term "administering," refers to the placement of a compound as disclosed herein into a subject by a method or route which results in at least partial delivery of the agent at a desired site. Pharmaceutical compositions comprising the compounds disclosed herein can be administered by any appropriate route which results in an effective treatment in the subject. In some embodiments, administration comprises physical human activity, e.g., an injection, act of ingestion, an act of application, and/or manipulation of a delivery device or machine. Such activity can be performed, e.g., by a medical professional and/or the subject being treated.

[00427] As used herein, “contacting" refers to any suitable means for delivering, or exposing, an agent to at least one cell. Exemplary delivery methods include, but are not limited to, direct delivery to cell culture medium, perfusion, injection, or other delivery method well known to one skilled in the art. In some embodiments, contacting comprises physical human activity, e.g., an injection; an act of dispensing, mixing, and/or decanting; and/or manipulation of a delivery device or machine.

[00428] The term “statistically significant" or “significantly" refers to statistical significance and generally means a two standard deviation (2SD) or greater difference.

[00429] Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term “about.” The term “about” when used in connection with percentages can mean ±1%.

[00430] As used herein, the term “comprising” means that other elements can also be present in addition to the defined elements presented. The use of “comprising” indicates inclusion rather than limitation.

[00431] The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

[00432] As used herein the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of additional elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment of the invention.

[00433] As used herein, the term “corresponding to” refers to an amino acid or nucleotide at the enumerated position in a first polypeptide or nucleic acid, or an amino acid or nucleotide that is equivalent to an enumerated amino acid or nucleotide in a second polypeptide or nucleic acid. Equivalent enumerated amino acids or nucleotides can be determined by alignment of candidate sequences using degree of homology programs known in the art, e.g., BLAST.

[00434] The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. The abbreviation, "e.g." is derived from the Latin exempli gratia, and is used herein to indicate a non-limiting example. Thus, the abbreviation "e.g." is synonymous with the term "for example."

[00435] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member can be referred to and claimed individually or in any combination with other members of the group or other elements found herein. One or more members of a group can be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is herein deemed to

contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[00436] Unless otherwise defined herein, scientific and technical terms used in connection with the present application shall have the meanings that are commonly understood by those of ordinary skill in the art to which this disclosure belongs. It should be understood that this invention is not limited to the particular methodology, protocols, and reagents, etc., described herein and as such can vary. The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention, which is defined solely by the claims. Definitions of common terms in immunology and molecular biology can be found in The Merck Manual of Diagnosis and Therapy, 20th Edition, published by Merck Sharp & Dohme Corp., 2018 (ISBN 0911910190, 978-0911910421); Robert S. Porter et al. (eds.), The Encyclopedia of Molecular Cell Biology and Molecular Medicine, published by Blackwell Science Ltd., 1999-2012 (ISBN 9783527600908); and Robert A. Meyers (ed.), Molecular Biology and Biotechnology: a Comprehensive Desk Reference, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8); Immunology by Werner Luttmann, published by Elsevier, 2006; Janeway's Immunobiology, Kenneth Murphy, Allan Mowat, Casey Weaver (eds.), W. W. Norton & Company, 2016 (ISBN 0815345054, 978-0815345053); Lewin's Genes XI, published by Jones & Bartlett Publishers, 2014 (ISBN-1449659055); Michael Richard Green and Joseph Sambrook, Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA (2012) (ISBN 1936113414); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (2012) (ISBN 044460149X); Laboratory Methods in Enzymology: DNA, Jon Lorsch (ed.) Elsevier, 2013 (ISBN 0124199542); Current Protocols in Molecular Biology (CPMB), Frederick M. Ausubel (ed.), John Wiley and Sons, 2014 (ISBN 047150338X, 9780471503385), Current Protocols in Protein Science (CPPS), John E. Coligan (ed.), John Wiley and Sons, Inc., 2005; and Current Protocols in Immunology (CPI) (John E. Coligan, ADA M Kruisbeek, David H Margulies, Ethan M Shevach, Warren Strobe, (eds.) John Wiley and Sons, Inc., 2003 (ISBN 0471142735, 9780471142737), the contents of which are all incorporated by reference herein in their entireties.

[00437] In some embodiments, the disclosure described herein does not concern a process for cloning human beings, processes for modifying the germ line genetic identity of human beings, uses of human embryos for industrial or commercial purposes or processes for modifying the genetic identity of animals which are likely to cause them suffering without any substantial medical benefit to man or animal, and also animals resulting from such processes.

[00438] Other terms are defined herein within the description of the various aspects of the invention.

[00439] All patents and other publications; including literature references, issued patents, published patent applications, and co-pending patent applications; cited throughout this application

are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the technology described herein. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents is based on the information available to the applicants and does not constitute any admission as to the correctness of the dates or contents of these documents.

[00440] The description of embodiments of the disclosure is not intended to be exhaustive or to limit the disclosure to the precise form disclosed. While specific embodiments of, and examples for, the disclosure are described herein for illustrative purposes, various equivalent modifications are possible within the scope of the disclosure, as those skilled in the relevant art will recognize. For example, while method steps or functions are presented in a given order, alternative embodiments may perform functions in a different order, or functions may be performed substantially concurrently. The teachings of the disclosure provided herein can be applied to other procedures or methods as appropriate. The various embodiments described herein can be combined to provide further embodiments. Aspects of the disclosure can be modified, if necessary, to employ the compositions, functions and concepts of the above references and application to provide yet further embodiments of the disclosure. These and other changes can be made to the disclosure in light of the detailed description. All such modifications are intended to be included within the scope of the appended claims.

[00441] Specific elements of any of the foregoing embodiments can be combined or substituted for elements in other embodiments. Furthermore, while advantages associated with certain embodiments of the disclosure have been described in the context of these embodiments, other embodiments may also exhibit such advantages, and not all embodiments need necessarily exhibit such advantages to fall within the scope of the disclosure.

[00442] Some embodiments of the technology described herein can be defined according to any of the following numbered paragraphs:

1. A method comprising:
    a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium;
    b) inhibiting a histone methyltransferase in the resultant population of $CD34^+$ hemogenic endothelium; and

c) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of $CD3^+$ T cells.

2. A method comprising:
    a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium;
    b) inhibiting an epigenetic regulator in the resultant population of $CD34^+$ hemogenic endothelium; and
    c) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of $CD3^+$ T cells.
3. A method comprising:
    a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium;
    b) inhibiting G9a and/or GLP in the resultant population of $CD34^+$ hemogenic endothelium; and
    c) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of $CD3^+$ T cells.
4. A method comprising:
    a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and
    b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of $CD3^+$ T cells.
5. The method of any one of paragraphs 1-4, wherein the Notch ligand is attached to a solid substrate.
6. The method of any one of paragraphs 1-5, wherein the Notch ligand is attached to a cell culture dish.
7. The method of any one of paragraphs 1-6, wherein the Notch ligand is not derived from a stromal cell.

8. The method of any one of paragraphs 1-7, wherein differentiating the hemogenic endothelium in the presence of a Notch ligand does not comprise co-culturing with a stromal cell expressing a Notch ligand.
9. The method of any one of paragraphs 1-8, wherein differentiating the hemogenic endothelium in the presence of a Notch ligand does not comprise co-culturing with OP9-DL1 cells or OP9-DL4 cells.
10. The method of any one of paragraphs 1-9, wherein the Notch ligand is selected from the group consisting of Delta-like-1 (DLL1), Delta-like-4 (DLL4), immobilized $Delta1^{ext\text{-}IgG}$, and immobilized $Delta4^{ext\text{-}IgG}$.
11. The method of paragraph 10, wherein immobilized $Delta1^{ext\text{-}IgG}$ consists of an extracellular domain of human Delta-like-1 fused to the Fc domain of human IgG1.
12. The method of any one of paragraphs 1-11, wherein the sufficient time to promote differentiation into a population of $CD3^+$ T cells is at least 4 weeks.
13. The method of any one of paragraphs 1-12, wherein the $CD3^+$-T-cell-differentiation media is serum-free.
14. The method of any one of paragraphs 1-13, wherein the $CD3^+$-T-cell-differentiation media comprises FLT3 and IL7.
15. The method of any one of paragraphs 1-14, wherein the $CD3^+$-T-cell-differentiation media comprises 15 ng/ml FLT3 and 25 ng/ml IL7.
16. The method of any one of paragraphs 1-15, wherein the $CD3^+$-T-cell-differentiation media further comprises 5 ng/mL thrombopoietin (TPO) and/or 30 ng/ml SCF for at least the first 2 weeks of differentiating in the $CD3^+$-T-cell-differentiation media.
17. The method of any one of paragraphs 1-16, wherein $CD3^+$-T-cell-differentiation media comprising TPO promotes differentiation into a population of $CD5^+$ $CD7^+$ ProT cells.
18. The method of any one of paragraphs 1-4, wherein the population of $CD3^+$ T cells comprises a population of $CD4^+CD8^+$ T cells.
19. The method of paragraph 18, further comprising differentiating the population of $CD4^+CD8^+$ T cells in a single-positive-T-cell-differentiation media for a sufficient time to promote differentiation into a population of $CD4^+$ cells and a population of $CD8^+$ cells.
20. The method of paragraph 19, wherein the sufficient time to promote differentiation from the population of $CD4^+CD8^+$ T cells into a population of $CD4^+$ T cells and a population of $CD8^+$ cells is at least 1 week.
21. The method of paragraph 19, wherein the sufficient time to promote differentiation from the population of $CD34^+$ hemogenic endothelium into a population of $CD4^+$ T cells and a population of $CD8^+$ cells is at least 5 weeks.

22. The method of paragraph 19, wherein the single-positive-T-cell-differentiation media comprises 10 ng/mL IL-15 and a T cell activator.
23. The method of paragraph 22, wherein the T cell activator comprises a 10ul/ml CD3/CD28 T cell activator.
24. The method of paragraph 22, wherein the T cell activator comprises one bead of CD3/CD28 T cell activator dynabeads per cell.
25. The method of any one of paragraphs 18-24, further comprising, after at least 1 week, a step of $CD4^+$ cell enrichment and/or $CD8^+$ cell enrichment.
26. The method of any one of paragraphs 1-4, wherein the population of pluripotent stem cells comprises induced pluripotent stem cells (iPS cells) or embryonic stem cells (ESC).
27. The method of paragraph 26, wherein the induced pluripotent stem cells are produced by introducing only reprogramming factors *OCT4, SOX2, KLF4* and optionally *c-MYC* or nanog and LIN28 into mature cells.
28. The method of paragraph 26, wherein the induced pluripotent stem cells are produced by introducing the reprogramming factors two or more times into the mature cells.
29. The method of any one of paragraphs 1-4, wherein the population of pluripotent stem cells is differentiated into a population of $CD34^+$ hemogenic endothelium using embryoid bodies or 2D adherent cultures.
30. The method of any one of paragraphs 1-4, wherein the sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium is at least 8 days.
31. The method of any one of paragraphs 1-4, wherein the aggregation media comprises BMP4, SB-431542, CHIR99021, bFGF, VEGF, IL-6, IL-11, IGF-1, SCF, and EPO.
32. The method of any one of paragraphs 29-31, wherein the aggregation media comprises 10 ng/ml BMP4, 6 mM SB-431542, 3 mM CHIR99021, 5 ng/ml bFGF, 15 ng/ml VEGF, 10 ng/ml IL-6, 5 ng/mL IL-11, 25 ng/mL IGF-1, 50 ng/mL SCF, and 2 U/ml EPO.
33. The method of any one of paragraphs 29-32, further comprising selecting or isolating the resultant population of $CD34^+$ hemogenic endothelium using expression of surface markers on the population of $CD34^+$ hemogenic endothelium.
34. The method of any one of paragraphs 29-33, wherein the population of $CD34^+$ hemogenic endothelium is CD45 negative/low.
35. The method of any one of paragraphs 29-34, wherein the population of $CD34^+$ hemogenic endothelium is CD38 negative/low.
36. The method of any one of paragraphs 1-4, further comprising the step of genetically modifying the resultant population of $CD34^+$ hemogenic endothelium or the resultant population of $CD3^+$ T cells.

37. The method of paragraph 36, wherein the genetic modification is editing an endogenous HLA, removing an endogenous TCR, and/or expressing a chimeric antigen receptor (CAR).
38. The method of paragraph 1, wherein the histone methyltransferase catalyzes the addition of methyl group to the histone 3 lysine residue 9 (H3K9) and/or histone 3 lysine residue 27 (H3K27).
39. The method of paragraph 1, wherein the histone methyltransferase H3K9 and/or H3K27 is inhibited by a small molecule inhibitor or a nucleic acid inhibitor.
40. The method of paragraph 39, wherein the histone methyltransferase H3K9 and/or H3K27 small molecule inhibitor is a heterorganic compound or an organometallic compound.
41. The method of paragraph 39, wherein the histone methyltransferase H3K9 and/or H3K27 small molecule inhibitor is selected from the group consisting of BIX-01294, UNC0638, E72, BRD4770, A-366, chaetocin, UNC0224, UNC0631, UNC0646, EPZ005687, EPZ-6438 (E7438), 3-deazaneplanocin A (DZNep), EI1, GSK343, GSK126, and UNC1999.
42. The method of paragraph 39, wherein the nucleic acid inhibitor is a nucleic acid targeting the expression of histone methyltransferase.
43. The method of paragraph 39, wherein the nucleic acid inhibitor is a RNA interference inhibitor or agent.
44. The method of paragraph 39, wherein the nucleic acid inhibitor is a EZH1 specific nucleic acid that is selected from the group consisting of an aptamer that binds EZH1, a EZH1 specific RNA interference agent, and a vector encoding a EZH1 specific RNA interference agent, wherein the RNA interference agent comprises one or more of the nucleotide sequences selected from SEQ ID NO: 11-19.
45. The method of paragraph 2, wherein the epigenetic regulator is a DNA-methyltransferase (DNMT); a methyl-CpG-binding domain (MBD) protein; a DNA demethylase; a histone methyl transferase (HMT); a methyl-histone binding protein; a histone demethylase; a histone acetyl transferase (HAT); an acetyl-binding protein; or a histone deacetylase (HDAC).
46. The method of paragraph 45, wherein the inhibitor of an epigenetic regulator is selected from the group consisting of: UNC0224; MC1568; and CAY10591.
47. The method of any one of paragraphs 45-46, wherein the inhibitor of an epigenetic regulator is provided at a concentration of at least 500 nM.
48. The method of any one of paragraphs 45-46, wherein the sufficient time to promote differentiation from the population of CD34+ cells into a population of CD5+CD7+ proT cells is about 14 days.
49. The method of paragraph 3, wherein the G9a and/or GLP inhibitor is selected from the group consisting of: UNC0224; UNC0638; A366; BRD4770; BIX01294; UNC0642; UNC0631; UNC0646; UNC0321; E72; BIX-01338; BRD9539; Chaetocin; and DCG066.

50. The method of paragraph 49, wherein the G9a and/or GLP inhibitor is UNC0224.
51. The method of any one of paragraphs 49-50, wherein the G9a and/or GLP inhibitor is provided at a concentration of 300 nM - 5uM.
52. The method of any one of paragraphs 49-51, wherein the sufficient time to promote differentiation from the population of CD34+ cells into a population of CD5+CD7+ proT cells is about 14 days.
53. A method comprising:
    a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and
    b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising 15 ng/ml FLT3 and 25 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells;
    wherein the $CD3^+$-T-cell-differentiation media further comprises 5 ng/mL TPO and 30 ng/ml SCF for at least the first two weeks.
54. A method comprising:
    a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and
    b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising 15 ng/ml FLT3 and 25 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells;
    wherein the $CD3^+$-T-cell-differentiation media further comprises 5 ng/mL TPO, 30 ng/ml SCF, and a G9a/GLP inhibitor for at least the first two weeks.
55. The method of any one of paragraphs 1-54, wherein the population of $CD3^+$ T cells exhibits a gene expression profile that is most similar to alpha beta T cells.
56. The method of any one of paragraphs 1-55, wherein the population of $CD3^+$ T cells exhibits a gene expression profile that is at least 10%, 20%, 30%, 40% or more similar to alpha beta T cells.
57. The method of any one of paragraphs 1-56, wherein the population of CD3+ T cells exhibits a gene expression profile with a Pearson's correlation coefficient compared to peripheral blood alpha beta T cells that is at least 0.85.
58. The method of any one of paragraphs 1-57, wherein the population of $CD3^+$ T cells exhibits a Productive Simpson Clonality value of about 0.025.

59. The method of any one of paragraphs 1-58, wherein the population of $CD3^+$ T cells exhibits a T cell receptor (TCR) complementarity-determining region (CDR) that is at least 3 nucleotides longer than an immune cell differentiated without inhibition of a methyltransferase or using stromal cells.
60. An immune cell produced by the method of any one of paragraphs 1-59.
61. The immune cell of paragraph 60, wherein the immune cell exhibits a gene expression profile that is most similar to alpha beta T cells.
62. The immune cell of any one of paragraphs 60-61, wherein the immune cell exhibits a gene expression profile that is at least 10%, 20%, 30%, 40% or more similar to alpha beta T cells.
63. The immune cell of any one of paragraphs 60-62, wherein the immune cell exhibits a gene expression profile with a Pearson's correlation coefficient compared to peripheral blood alpha beta T cells that is at least 0.85.
64. The immune cell of any one of paragraphs 60-63, wherein the immune cell exhibits a Productive Simpson Clonality value of about 0.025.
65. The immune cell of any one of paragraphs 60-64, wherein the immune cell exhibits a T cell receptor (TCR) complementarity-determining region (CDR) that is at least 3 nucleotides longer than an immune cell differentiated without inhibition of methyltransferase, using stromal cells.
66. A composition comprising an immune cell of any one of paragraphs 60-65 or population thereof.
67. The composition of paragraph 66, further comprising a pharmaceutically acceptable carrier.
68. A pharmaceutical composition comprising an immune cell of any one of paragraphs 60-65 or population thereof, and a pharmaceutically acceptable carrier.
69. The pharmaceutical composition of paragraph 68 for use in cellular replacement therapy in a subject.
70. A method of cellular replacement therapy, the method comprising administering an immune cell of any one of paragraphs 60-65 or population thereof, or a composition of paragraphs 66-67, or a pharmaceutical composition of paragraphs 68-69 to a recipient subject in need thereof.
71. The method of cellular replacement therapy of paragraph 70, wherein the recipient subject has undergone chemotherapy and/or irradiation.
72. The method of cellular replacement therapy of paragraph 70, wherein the recipient subject has deficiencies in immune function and/or lymphocyte reconstitution.
73. The method of cellular replacement therapy of any one of paragraphs 70-72, wherein prior to transplanting, the immune cell or population thereof is treated *ex vivo* with prostaglandin E2 and/or antioxidant N-acetyl-L-cysteine (NAC) to promote subsequent engraftment in a recipient subject.
74. The method of cellular replacement therapy of any one of paragraphs 70-73, wherein the immune cell or population thereof is autologous to the recipient subject.

75. The method of cellular replacement therapy of any one of paragraphs 70-74, wherein the immune cell or population thereof is HLA type matched with the recipient subject.

EXAMPLES

Example 1: Stroma-free T cell differentiation from human pluripotent stem cells

[00443] T cells are key components of human adaptive immune system and have great therapeutic potential. However, current T cell-mediated therapy relies on autologous T cells, which prevents its broad application. Human induced pluripotent stem cells (iPSCs) represent an ideal source for scalable manufacture of off-the-shelf products for cell therapy. However, the generation of mature and functional T cells from iPSCs has proven to be difficult. Additionally, the differentiation of iPSC requires co-culture with mouse stromal cells, which limits the translational potential of iPSC-derived T cells.

[00444] Described herein is a serum-free, stromal-free differentiation protocol for T cell differentiation. Hemogenic $CD34^+$ endothelial cells were first derived from iPS (see e.g., Example 2). Non-tissue culture treated plates were coated with recombinant human DL1/DL4-Fc proteins (10ug/ml in PBS, 3 hours in room temperature). The iPSC-derived hemogenic endothelial cells were cultured on the tissue culture plates coated with Notch ligands and growth factors (Flt3, SCF, Il7, TPO) that are essential for T cells development were added to the media sequentially (see e.g., Fig. 1A or Fig. 17). Using this protocol, $CD5^+CD7^+$ T cell progenitors can be generated after 2 weeks of differentiation, and $CD3^+$ T cells are observed after 5 weeks of differentiation (see e.g., Fig. 1B). These $CD3^+$ are can be further stimulated by CD3/CD28 antibody, which results in enhanced proliferation and induction of CD4 or CD8 single positive T cells (see e.g., Fig. 1C). Additionally, traditional T cell differentiation protocol using OP9-DL4 cells produces innate-like T cells that express gamma delta TCR. The stroma-free protocol described herein generates an increased number of T cells and only a small portion of these cells are innate-like cells (see e.g., Fig. 1D), indicating that this method produces T cells that exhibit a more mature phenotype. In sum, described herein is a new platform for the generation of more clinically relevant iPSC-derived T cells.

[00445] One application of the stroma-free T cell differentiation method is the generation of CAR iPSC-T cells. Anti-CD19 chimeric antigen receptor (CAR) was introduced into iPSC HSPCs, and the cells were differentiated into T cells using the stroma-free T cell differentiation method described herein (see e.g., Fig. 2A). The expression of CAR was maintained during differentiation (see e.g., Fig. 2B). The CAR T cells expanded similarly to the untransduced (UTD) control (see e.g., Fig. 2C). Stimulation with CD19-K562 cells resulted in the activation of CAR-iPSC T cells but not the untransduced (UTD) control or unstimulated CAR-iPSC T cells (see e.g., Fig. 2D).

[00446] RNA-seq analysis was performed on primary T cells and iPSC-derived T cells. The expression of T cell signature genes was examined to compare the similarity of iPSC-T cells to

PBMC αβT, γδT, and NK cells (see e.g., **Fig. 3A**). The expression of genes that distinguish alpha beta T cells and gamma delta T cells was examined to compare the similarity of iPSC-T cells to TCRαβ and TCRγδ T cells (see e.g., **Fig. 3B**). The results show that the differentiation methods described herein allow the generation of iPSC-T cell (EZ-T) that display a gene expression profile similar to the alpha beta T cells from donor's peripheral blood (abT). In comparison, the traditional iPSC-T cells (conT_OP9) had a phenotype of innate-like T cells. Accordingly, the methods described herein (e.g., stroma-free and EZH1 knock down) generate T cells with expression profiles most similar to natural T cells, compared to stromal methods.

[00447] EZ-T cells also exhibit a diverse TCR repertoire. EZ-T cells refer to T cells differentiated from CD34+ HE including EZH1 inhibition and stromal-free T cell differentiation as described herein. TCR beta chain sequencing was performed on EZ-T cells and tens of thousands unique TCR rearrangements as a result of random TCR gene recombination during T cell differentiation were identified. The usage of TCRBV gene families in EZ-T cells was examined. Each shade represents one TCRBV family. Productive Simpson Clonality value was 0.0233 indicating a highly diverse TCR repertoire. See e.g., **Fig. 4**.

[00448] EZ-T cells also have longer CDR3 segments than control PSC-T cells. CDR3 is the most variable region of TCR and its length can be determined by the activity of TdT enzyme, which randomly adds nucleotides during TCR rearrangement. It has been reported that CDR3 is shorter in immature T cells and iPSC-derived T cells compared to mature PBMC T cells. EZ-T cells displayed an increased CDR3 length compared to control iPSC-derived T cells, and were more similar to PBMC T cells (see e.g., Fig., **5A-5D**). As EZ-T cells show longer regions added by TdT, such CDRs show more sequence variability and thus a more diverse TCR repertoire.

Example 2: Method for producing hemogenic endothelium

[00449] Described herein is an 8-day protocol for producing hemogenic ($CD34^+$) endothelium from induced pluripotent stem (iPS) cells; see e.g., Sturgeon et al., Wnt Signaling Controls the Specification of Definitive and Primitive Hematopoiesis From Human Pluripotent Stem Cells, Nat Biotechnol. 2014 Jun; 32(6): 554–561, the content of which is incorporated herein by reference in its entirety.

Day 0: Formation of EBs from iPSCs on MEFs

[00450] Generally, after three days to one week of culture on murine embryonic fibroblasts (MEFs), iPS cells are ready to make embryoid bodies (EBs). The following protocol is followed for D0.

[00451] 1. Wash the iPS cells grown on MEFs with 5 mL of DMEM/F12 media.

[00452] 2. Aspirate the media from each dish. Replace with 5 mL of 1X collagenase IV diluted in 0.22 μM filtered DMEM/F12.

[00453] 3. Incubate at 37°C for 5-10 minutes and check periodically on the microscope that the iPS colonies are detaching.

[00454] 4. Aspirate collagenase IV and replace with 5 mL of filtered DMEM/F12.

[00455] 5. Using a sterile cell scrapper, scrape colonies first around the edges of the dish then left to right then top to bottom.

[00456] 6. Gently and slowly transfer the cells to a conical tube using a 10 mL serological pipet. If there are residual colonies, wash plates with additional 5 mL and add to same tube.

[00457] 7. Spin down 1100 rpm for 1 min

[00458] 8. While cells are spinning, add 9 mL of Aggregation media with BMP4 (see e.g., Table 1) to Corning Ultra Low Adherent 10 cm dishes.

[00459] 9. Aspirate the media from the pelleted iPS colonies and resuspend in 1 mL of Aggregation media with BMP4.

[00460] 10. Gently transfer the 1 mL of cells to each Ultra Low Adherent 10 cm containing the Aggregation media. Using the same pipette, pipet up 1 mL from an area of the plate without any cells and wash the conical. Add back the 1 mL to the conical; final volume of each plate now containing 3-4 starting plates of iPS cells is 10 mL.

[00461] 11. Transfer to hypoxic incubator (5% O_2) at 37°C. 4-5 plates can be stacked on top of one another, with one plate filled with PBS at the bottom of the stack to prevent evaporation. This is Day 0 of EB culture.

Day 1: Add bFGF

[00462] On Day 1 the following protocol is followed: 1. Directly add bFGF to each 10 cm dish of EBs for a final concentration of 5ng/mL. Shake plate to distribute in media.

Day 2: Complete media change with D2 Media

[00463] On Day 2, the D2 media is introduced. The following protocol is followed for D2.

[00464] 1. The D2 Aggregation Media comprises the following (see e.g., Table 1): BMP4, bFGF, CHIR99021 (StemCell Technologies Inc. # 72054), and SB431542 (StemCell Technologies Inc. # 72234). Once SB and CHIR are thaw it is not recommendable to freeze them again.

[00465] 2. Collect EBs using a 10 mL serological pipet and place into a conical tube.

[00466] 3. Let EBs settle for ~15 minutes.

[00467] 4. Aspirate the media and resuspend in D2 media (10 mL/10-cm dish) then gently transfer back to the Ultra-Low Adherent dishes.

Day 3: Complete media change with D3 Media

[00468] On Day 3, the D3 media is introduced. The following protocol is followed for D3.

[00469] 1. The D3 Aggregation Media comprises the following (see e.g., Table 1): VEGF and bFGF.

[00470] 2. Collect EBs using a 10 mL serological pipet and place into a conical tube.

[00471] 3. Let EBs settle for ~15 minutes.

[00472] 4. Aspirate the media and resuspend in D3 media (10 mL/10-cm dish) then gently transfer back to the Ultra-Low Adherent dishes.

Days 4-5: No media change

[00473] No media change is made on Days 4-5.

Day 6: Complete media change with D6 Media

[00474] On Day 6, the D6 media is introduced. The following protocol is followed for D6.

[00475] 1. The D6 Aggregation Media comprises the following (see e.g., Table 1): VEGF Recombinant Human VEGF 165 (VEGF-A) (R & D Systems (R&D) # 293-VE-500), bFGF, SCF, EPO, IL-6 Recombinant Human IL-6 (20 ug) (Peprotech™ # 200-06), IL-11, and IGF-1.

[00476] 2. Collect EBs using a 10 mL serological pipet and place into a conical tube

[00477] 3. Let EBs settle for ~15 minutes

[00478] 4. Aspirate the media and resuspend in D6 media (10 mL/10-cm dish) then gently transfer back to the Ultra-Low Adherent dishes

Day 7: No media change

[00479] No media change is made on Day 7.

Day 8: Isolation of hemogenic endothelium by MAC sorting for CD34+ cells

[00480] On Day 8, the hemogenic endothelium is isolated using magnetic-activated cell sorting (MACS) for $CD34^+$ cells. The population of $CD34^+$ hemogenic endothelium can then be used to differentiate T cells using the stroma-free T cell differentiation method as described herein (see e.g., Example 1).

[00481] Table 1: Cytokines for EB culture

Product	[Final]	[Stock]	Dilution	Days	For 10 mL media
BMP4	10 ng/ml	100 ug/ml	10000	0,2	1
SB-431542	6 mM	100 mM	16666	2	0.6
CHIR99021	3 mM	50 mM	16666	2	0.6
bFGF	5 ng/ml	50 ug/ml	2000	1,2,3,6	1
VEGF	15 ng/ml	150 ug/ml	10000	3,6	1
IL-6	10 ng/ml	100 ug/ml	10000	6	1
IL-11	5 ng/ml	50 ug/ml	10000	6	1
IGF-1	25 ng/ml	250 ug/ml	10000	6	1
SCF	50 ng/ml	100 ug/ml	2000	6	5
EPO	2 U/ml	10000 U/ml	5000	6	2

Example 3: Inhibition of Epigenetic Regulators (e.g., G9a/GLP)

[00482] A group of epigenetic regulators were tested for their ability to promote T cell differentiation. In a first screen in 5F cells, UNC0224, MC1568, or CAY10591 significantly increased the number of resultant proT cells (see e.g., **Fig. 6-8**). In a second screen in EB-derived CD34+ cells (e.g., CD34+ hemogenic endothelium), e.g., using stromal-free T cell differentiation methods as described herein, UNC0224 significantly increased the number of resultant proT cells (see e.g., **Fig. 9-11**). A dose response showed that a UNC0224 concentration of 312nM to 5uM worked best to promote T cell differentiation (see e.g., **Fig. 12A-12B**). UNC0224 is an inhibitor of G9a/GLP, so a variety of other G9a/GLP inhibitors were tested. UNC0638, BRD4770, BIX01294, and UNC0642 each significantly increased the number of resultant proT cells (see e.g., **Fig. 13B**, **13D-13F**).

[00483] UNC0224 enhanced T cell commitment at expense of erythroid/myeloid potential. While UNC0224 treatment resulted in a significant increase in CD5+CD7+ ProT cells, it also led to a significant decrease in erythroid or myeloid lineage cells (see e.g., **Fig. 14A-14C**). UNC0224 also promoted T cell specification rather than cell proliferation. While UNC0224 treatment resulted in a significant increase in the number or percentage of CD5+CD7+ ProT cells, it also led to a significant decrease in total cells (see e.g., **Fig. 15A-15C**). Without wishing to be bound by theory, it is anticipated that H3K9 methylation mediates repression of lymphoid genes. As such, treatment with inhibitors of H3K9 methylation (see e.g., **Fig. 6-16**, **Tables 2-3**) promotes T cell differentiation, e.g.,

when using stromal-free T cell differentiation methods as described herein. Such H3K9 methylation inhibitors can be used in place of, or in combination with, inhibition of histone methyltransferases (e.g., EZH1 knockdown).

CLAIMS

1. A method comprising:

(a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium;

(b) inhibiting a histone methyltransferase in the resultant population of $CD34^+$ hemogenic endothelium; and

(c) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of $CD3^+$ T cells.

2. A method comprising:

(a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium;

(b) inhibiting an epigenetic regulator in the resultant population of $CD34^+$ hemogenic endothelium; and

(c) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of $CD3^+$ T cells.

3. A method comprising:

(a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium;

(b) inhibiting G9a and/or GLP in the resultant population of $CD34^+$ hemogenic endothelium; and(c) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of $CD3^+$ T cells.

4. A method comprising:

(a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and

(b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media in the presence of a Notch ligand for a sufficient time to promote differentiation into a population of $CD3^+$ T cells.

5. A method according to any preceding claim, in which the Notch ligand is attached to a solid substrate.

6. A method according to any of Claims 1 to 4, in which the population of pluripotent stem cells comprises induced pluripotent stem cells (iPS cells) or embryonic stem cells (ESC).

7. A method according to Claim 6, in which the induced pluripotent stem cells are produced by introducing only reprogramming factors *OCT4, SOX2, KLF4* and optionally *c-MYC* or nanog and LIN28 into mature cells.

8. A method according to Claim 6, in which the induced pluripotent stem cells are produced by introducing the reprogramming factors two or more times into the mature cells.

9. A method according to any of Claims 1 to 4, in which the population of pluripotent stem cells is differentiated into a population of $CD34^+$ hemogenic endothelium using embryoid bodies or 2D adherent cultures.

10. A method according to any of Claims 1 to 4, in which the sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium is at least 8 days.

11. A method according to any of Claims 1 to 4, in which the aggregation media comprises BMP4, SB-431542, CHIR99021, bFGF, VEGF, IL-6, IL-11, IGF-1, SCF, and EPO.

12. A method according to any of Claims 9, 10 or 11, in which the aggregation media comprises 10 ng/ml BMP4, 6 mM SB-431542, 3 mM CHIR99021, 5 ng/ml bFGF, 15 ng/ml VEGF, 10 ng/ml IL-6, 5 ng/mL IL-11, 25 ng/mL IGF-1, 50 ng/mL SCF, and 2 U/ml EPO.

13. A method according to any of Claims 1 to 4, further comprising the step of genetically modifying the resultant population of $CD34^+$ hemogenic endothelium or the resultant population of $CD3^+$ T cells.

14. A method according to Claim 13, in which the genetic modification is editing an endogenous HLA, removing an endogenous TCR, and/or expressing a chimeric antigen receptor (CAR).

15. A method according to Claim 1, in which the histone methyltransferase catalyzes the addition of methyl group to the histone 3 lysine residue 9 (H3K9) and/or histone 3 lysine residue 27 (H3K27).

16. A method according to Claim 1, in which the histone methyltransferase H3K9 and/or H3K27 is inhibited by a small molecule inhibitor or a nucleic acid inhibitor.

17. A method according to Claim 16, in which:

(a) the small molecule inhibitor is selected from the group consisting of BIX-01294, UNC0638, E72, BRD4770, A-366, chaetocin, UNC0224, UNC0631, UNC0646, EPZ005687, EPZ-6438 (E7438), 3-deazaneplanocin A (DZNep), EI1, GSK343, GSK126, and UNC1999; and/or

(b) the nucleic acid inhibitor is a nucleic acid targeting the expression of histone methyltransferase, a RNA interference inhibitor or agent or a EZH1 specific nucleic acid that is selected from the group consisting of an aptamer that binds EZH1, a EZH1 specific RNA interference agent, and a vector encoding a EZH1 specific RNA interference agent, in which the RNA interference agent comprises one or more of the nucleotide sequences selected from SEQ ID NO: 11-19.

18. A method according to Claim 2, in which the epigenetic regulator is a DNA-methyltransferase (DNMT); a methyl-CpG-binding domain (MBD) protein; a DNA demethylase; a histone methyl transferase (HMT); a methyl-histone binding protein; a histone demethylase; a histone acetyl transferase (HAT); an acetyl-binding protein; or a histone deacetylase (HDAC).

19. A method according to Claim 18, in which the inhibitor of an epigenetic regulator is selected from the group consisting of: UNC0224; MC1568; and CAY10591.

20. A method according to Claim 18 or 19, in which the sufficient time to promote differentiation from the population of CD34+ cells into a population of CD5+CD7+ proT cells is about 14 days.

21. A method according to Claim 3, in which the G9a and/or GLP inhibitor is selected from the group consisting of: UNC0224; UNC0638; A366; BRD4770; BIX01294; UNC0642; UNC0631; UNC0646; UNC0321; E72; BIX-01338; BRD9539; Chaetocin; and DCG066.

22. A method according to Claim 21, in which the G9a and/or GLP inhibitor is UNC0224.

23. A method according to Claim 21 or 22, in which the G9a and/or GLP inhibitor is provided at a concentration of 300 nM - 5μM.

24. A method comprising:

(a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and

(b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising 15 ng/ml FLT3 and 25 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells;

in which the $CD3^+$-T-cell-differentiation media further comprises 5 ng/mL TPO and 30 ng/ml SCF for at least the first two weeks.

25. A method comprising:

(a) differentiating a population of pluripotent stem cells in aggregation media for a sufficient time to promote differentiation into a population of $CD34^+$ hemogenic endothelium; and

(b) differentiating the resultant population of $CD34^+$ hemogenic endothelium in a $CD3^+$-T-cell-differentiation media comprising 15 ng/ml FLT3 and 25 ng/ml IL7 in the presence of 10 μg/mL Notch ligand for at least 4 weeks to promote differentiation into a population of $CD3^+$ T cells;

in which the $CD3^+$-T-cell-differentiation media further comprises 5 ng/mL TPO, 30 ng/ml SCF, and a G9a/GLP inhibitor for at least the first two weeks.