

NATURAL KILLER CELL LINEAGES DERIVED FROM PLURIPOTENT CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of and priority to U.S. Provisional Patent
5 Application No. 63/413,383 filed October 5, 2022, the contents of which are hereby
incorporated by reference in their entirety.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted in
XML format via EFS-Web and is hereby incorporated by reference in its entirety. Said XML
10 copy, created on September 26, 2023, is named GRU-014PC_Sequence_Listing.xml and is
30,049 bytes in size.

BACKGROUND

Lymphocytic Natural killer (NK) cells hold promise for various applications,
including improving hematopoietic and solid organ transplantation, promoting antitumor
15 immunotherapy and controlling inflammatory, infectious, and autoimmune disorders.
However, clinical use of NK cells is hampered by the small numbers of functional NK cells
that can be isolated or otherwise obtained from regular leukapheresis products. Therefore,
development of large scale, off-the-shelf, NK cells would provide a powerful
immunotherapy tool.

20 SUMMARY OF THE DISCLOSURE

In various aspects and embodiments, the present disclosure provides methods for
generating immune compatible NK cell lineages for cell therapy, including adaptive NK
cells, cytotoxic NK cells, immature NK cells, unipotent NK cell precursors, lymphoid
precursors with the ability to generate NK cells, including lymphoid-primed multipotent
25 progenitor (LMPP) and common lymphoid progenitors (CLP), and CD34+CD7^{bright}
progenitors. In various embodiments, the invention provides for efficient *ex vivo* processes
for developing such hematopoietic lineages from human induced pluripotent stem cells

(iPSCs), including gene edited iPSCs. Cells generated according to the disclosure in various embodiments are functional and/or more closely resemble the corresponding lineage isolated from peripheral blood or lymphoid organs. The present invention also provides isolated cells and cell compositions produced by the methods disclosed herein, as well as methods for cell
5 therapy.

In one aspect, the disclosure provides a method for preparing a cell population comprising NK cells. The method comprises preparing a pluripotent stem cell (PSC) population, such as an induced pluripotent stem cell (iPSC) population differentiated to embryoid bodies, and enriching for CD34⁺ cells to thereby prepare a CD34⁺-enriched
10 population. Endothelial-to-hematopoietic transition (EHT) is induced in the CD34⁺-enriched population to thereby prepare a hematopoietic stem cell (HSC) population, optionally followed by a further enrichment of CD34⁺ cells. The resulting HSC population (or fraction thereof) can be differentiated to a NK cell lineage (including lymphoid-primed multipotent progenitor (LMPP) and common lymphoid progenitors (CLP)). In some
15 embodiments, the disclosure provides a method for generating various types of NK cells from the HSC population *ex vivo*.

In various embodiments, the iPSCs are prepared by reprogramming somatic cells. In some embodiments, iPSCs are generated from somatic cells such as (but not limited to) fibroblasts or PBMCs (or cells isolated therefrom). In some embodiments, iPSCs are derived
20 from CD34⁺ cells isolated from peripheral blood.

In various embodiments, the iPSCs are gene edited to assist in HLA matching, such as deletion of one or more HLA Class I and/or Class II alleles. For example, iPSCs can be gene edited to delete one or more of HLA-A, HLA-B, and HLA-C, and to delete one or more of HLA-DP, HLA-DQ, and HLA-DR. In certain embodiments, the iPSCs retain expression
25 of at least one HLA Class I and at least one HLA Class II complex. In certain embodiments, iPSCs are homozygous for at least one retained Class I and Class II loci. In some embodiments, the iPSCs are gene edited to be HLA-A^{neg}, homozygous for both HLA-B and HLA-C, and HLA-DPB1^{neg} and HLA-DQB1^{neg}. In some embodiments, the iPSCs are further homozygous for HLA-DRB1.

In various embodiments, iPSCs are prepared, and expanded using a culture system. Expanded iPSCs can be recovered from the culture for generating embryoid bodies (EBs). EBs, created by differentiation of the iPSCs, are three-dimensional aggregates of iPSCs and comprise the three (or alternatively two or one) embryonic germ layer(s) based on the differentiation method(s). In some embodiments, the process comprises harvesting CD34+ enriched cells from the EBs and inducing endothelial-to-hematopoietic differentiation.

In some embodiments, iPSC differentiation proceeds until cells are at least about 20% CD34+ or at least about 30% CD34+. In some embodiments, CD34 enrichment and EHT may be induced at Day 7 to Day 14 of iPSC differentiation. Differentiation of iPSCs can be according to known techniques. In some embodiments, iPSC differentiation involves factors such as, but not limited to, combinations of bFGF, Y27632, BMP4, VEGF, SCF, EPO, TPO, IL-6, IL-11, and/or IGF-1.

Induction of EHT can be with any known process. In some embodiments, induction of EHT generates a hematopoietic stem cell (HSC) population comprising LT-HSCs. In some embodiments, EHT generates HSCs through endothelial or hemogenic endothelial cell (HEC) precursors using mechanical, biochemical, pharmacological and/or genetic means (e.g., via stimulation, inhibition, and/or genetic modifications). In some embodiments, the EHT generates a stem cell population comprising one or more of long-term hematopoietic stem cells (LT-HSCs), short-term hematopoietic stem cells (ST-HSCs), and hematopoietic stem progenitor cells.

In various embodiments, CD34+ cells (e.g., the floater and/or adherent cells) are harvested from the culture undergoing endothelial-to-hematopoietic transition between Day 10 to Day 20 of iPSC differentiation, such as from Day 12 to Day 17 of iPSC differentiation. Hematopoietic stem cells (HSCs) which can give rise to innate myeloid, erythroid, and lymphoid lineages, can be identified based on the expression of CD34 and the absence of lineage specific markers (termed Lin-).

In various embodiments, the HSC population or fraction thereof is differentiated to a hematopoietic lineage, which can be selected from, including but not limited to therapeutic human NK cells, including adaptive NK cells, or their precursors (e.g., lineages giving rise

to natural killer cells (NK cells). NK cell lineages include adaptive NK cells, cytotoxic NK cells, immature NK cells, unipotent NK cell precursors, and lymphoid precursors with the ability to generate NK cells. Such precursors include lymphoid-primed multipotent progenitor (LMPP), common lymphoid progenitors (CLP), and CD34+CD7^{bright} progenitors, or a modified version thereof (e.g., genetically modified cells, such as NK-CAR cells). In some embodiments, the NK cell may express a chimeric antigen receptor (CAR).

In other aspects, the invention provides a cell population, or pharmaceutically acceptable composition thereof, comprising a NK lineage or precursor thereof, and which may be produced by the methods described herein. In some embodiments, the cell population is a progenitor NK lineage cell population capable of engraftment in a thymus, spleen, or secondary lymphoid organ upon administration to a subject in need. In various embodiments, the composition for cellular therapy is prepared that comprises the cell population and a pharmaceutically acceptable vehicle. In some embodiments, the cell population is HLA-A^{neg}, homozygous for both HLA-B and HLA-C, and HLA-DPB1^{neg} and HLA-DQB1^{neg}. In some embodiments, the cell population is further homozygous for HLA-DRB1.

In other aspects, the invention provides a method for cell therapy, comprising administering the cell population described herein, or pharmaceutically acceptable composition thereof, to a human subject in need thereof. In various embodiments, the methods described herein are used to treat blood (malignant and non-malignant), bone marrow, immune diseases, and infectious diseases. In various embodiments, the human subject has a condition comprising one or more of lymphopenia, a cancer (hematological malignancy or a solid tumor), an immune deficiency, an autoimmune disease, viral infection, a skeletal dysplasia, or a bone marrow failure syndrome.

Other aspects and embodiments of this disclosure will be apparent from the following detailed disclosure and working examples.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 shows that ETV2 over-expression (OE) does not affect pluripotency. **FIG. 1** shows FACS plots representative of transduction efficiency of iPSC with an adenoviral

vector to overexpress ETV2 and GFP sequences. ETV2 overexpression does not affect the iPSC stemness as shown by the expression of the TRA-1-60 stemness marker.

FIG. 2 shows that ETV2 over-expression (OE) increases the yield of hemogenic endothelial cells. Representative flow cytometric analysis of hemogenic endothelial cells (described as CD235a-CD34+CD31+) and relative quantification demonstrates that ETV2-OE enhances the formation of hemogenic endothelial cells.

FIG. 3 shows that ETV2 over-expression (OE) enhances CD34+ cell formation during iPSC differentiation. Representative flow cytometric analysis of CD34+ cells and relative quantification demonstrates that ETV2-OE enhances the CD34+ cell formation.

FIG. 4A and **FIG. 4B** show that iPSC-derived HSCs that are derived with Piezo1 activation undergo pro-T cell differentiation similar to bone marrow (BM)-HSCs. **FIG. 4A** is a FACS plot of differentiation efficiency to CD34+CD7+ pro T cells of Bone Marrow (BM) HSCs and iPSC-HSCs derived with Piezo1 activation. **FIG. 4B** is a quantification of CD34+CD7+ cells (%) derived with (1) BM-HSCs and (2) iPSC-HSCs (Piezo1 Activation). **FIG. 4B** shows the average of three experiments.

FIG. 5A and **FIG. 5B** show that iPSC-derived HSCs generated with Piezo1 activation undergo T cell differentiation and can be activated with CD3/CD28 beads similar to BM-HSCs. **FIG. 5A** is a FACS plot of activation efficiency (CD3+CD69+ expression) of T cells differentiated from BM-HSCs and iPSC-derived HSCs generated with Piezo1 activation. **FIG. 5B** is a quantification of CD3+CD69+ cells (%) derived with (1) BM-HSCs and (2) iPSC-HSCs (Piezo1 Activation). **FIG. 5B** shows the average of three experiments.

FIG. 6 shows that iPSC-derived HSCs (in this example with Piezo1 activation) can differentiate to functional T cells. IFN γ expression is a consequence of T cell activation after T cell receptor (TCR) stimulation via CD3/CD28 beads. IFN γ expression in T cells differentiated from iPSC-derived HSCs, generated upon Piezo1 activation, enhances HSC ability to further differentiate to functional T cells. **FIG 6** shows the average of three experiments.

FIG. 7 shows that HSCs derived from iPSC differentiation (D8+7 with Yoda1 (“Y”)) are activated by pharmacological stimulation better than NK cells derived from D8 iPSC-CD34+ cells, as measured by the activity of the functional marker CD107a.

FIG. 8A and **FIG. 8B** show HSC-derived NK cells (D8+7, +Y) outperform bone marrow derived NK cells in killing of tumor cells.

FIGS. 9A and **FIG. 9B** show the phenotype analysis of HLA edited (e.g., triple knockout cells performed by FACS and immunofluorescence. **FIG. 9A** shows the overall expression of HLA class-I molecules (HLA-A, HLA-B, and HLA-C) on the cell surface, where the HLA edited cells are positive for overall HLA class-I expression to a similar degree as wild-type cells. **FIG. 9B** shows cell expression of HLA-A via immunofluorescence, where HLA-A is not expressed in the HLA edited clone.

FIG. 10 shows that the HLA edited clones preserve their pluripotency (maintain trilineage differentiation), as illustrated by immunofluorescence, with ectoderm differentiation indicated by NESTIN-488 and PAX6-594 staining, mesoderm differentiation indicated by GATA-488 staining, and endoderm differentiation indicated by CXCR4-488 and FOX2A-594 staining.

FIG. 11 shows the immune compatibility of the HLA edited HSCs. HLA edited HSCs and control HSCs (WT, B2M KO, and HLA Class II null) were co-cultured with peripheral blood mononuclear cells (PBMCs) matching HLA-B and HLA-C, but with mismatched HLA-A, and PBMC-mediated cytotoxicity was measured by an annexin V staining assay.

FIG. 12 shows in vivo engrafting potential of HLA edited HSCs. Equal proportions of mCherry HLA edited HSCs and wild-type HSCs were mixed for a competitive transplant into mice, where bone marrow (BM) and peripheral blood samples were evaluated by FACS to compare the relative amounts of each cell type present in the samples.

FIG. 13 shows the capability of HSCs to effectively differentiation into NK cells as evidenced by fluorescence-activated cell sorting (FACS) experiments, gated based on CD56 expression.

FIGS. 14A-14C show HSC-derived NK cells effectively kill tumor cells. **FIG. 14A** shows an experimental schematic where the HSC-derived NK cells are co-cultured with K562 HLA-null cells, and the degree of NK cell degranulation is measured using AnnexinV staining and a cytotoxicity assay. **FIG. 14B** shows the results of NK cell degranulation as measured by fluorescence-activated cell sorting (FACS) using AnnexinV staining. **FIG. 14C** shows the results of the tumor cell cytotoxicity assay, with lactate dehydrogenase (LDH) as a measure for cell death.

The term “gHSC” is used herein to refer to the iPSC-derived hematopoietic stem cells of the present disclosure.

10 The terms “wild type” (WT), “unedited”, “non-HLA-edited” are used interchangeability herein to refer to the non-gene edited cells of the present disclosure.

EB34+ cells refer to Embryonic body derived CD34+ cells. These comprise hemogenic endothelial cells.

DESCRIPTION OF THE INVENTION

15 The present disclosure, in various aspects and embodiments, provides methods for generating hematopoietic lineages for cell therapy, and particularly NK cell lineages, including their progenitors and progenies. In the various embodiments, the lineages include adaptive NK cells, cytotoxic NK cells, immature NK cells, unipotent NK cell precursors, lymphoid precursors with the ability to generate NK cells, including lymphoid-primed
20 multipotent progenitor (LMPP) and common lymphoid progenitors (CLP), and CD34+CD7^{bright} progenitors. In various embodiments, the invention provides for efficient *ex vivo* processes for developing such NK cell lineages from human induced pluripotent stem cells (iPSCs). Cells generated according to the disclosure in various embodiments are functional and/or more closely resemble the corresponding lineage isolated from peripheral
25 blood, bone marrow, or lymphoid organs. The present invention also provides isolated cells and cell compositions produced by the methods disclosed herein, as well as methods for cell therapy.

In accordance with aspects and embodiments of this disclosure, the ability of human induced pluripotent stem cells (hiPSCs) to produce essentially limitless pluripotent stem cells (PSCs) is leveraged to generate boundless supply of NK cell lineages, including but not limited to therapeutic human NK cells, including adaptive NK cells, or their precursors (e.g., lineages giving rise to natural killer cells (NK cells). NK cell lineages include adaptive NK cells, cytotoxic NK cells, immature NK cells, unipotent NK cell precursors, and lymphoid precursors with the ability to generate NK cells. Such precursors include lymphoid-primed multipotent progenitor (LMPP), common lymphoid progenitors (CLP), and CD34+CD7^{bright} progenitors, or a modified version thereof (e.g., genetically modified cells, such as NK-CAR cells). Use of NK cells as therapeutic lymphocytes have been limited by their restricted availability, cell numbers, limited expansion potential, and histocompatibility issues. Moreover, compared to primary cells, hiPSCs can more readily undergo genetic modifications *in vitro*, thereby offering opportunities to improve cell-target specificity, cell numbers, as well as bypassing HLA-matching issues for example. Additionally, fully engineered hiPSC clones, as compared to primary cells, can serve as a stable and safe source (Nianias and Themeli, 2019). Further, because hiPSCs, unlike human Embryonic Stem Cells (hESCs), are of non-embryonic origin, they are also free of ethical concerns and are consistent in quality. Accordingly, use of hiPSCs according to this disclosure confers several advantages over primary cells to generate therapeutic hematopoietic lineages, such as NK lymphocytes.

In one aspect, the disclosure provides a method for preparing a cell population of an NK cell lineage. The method comprises preparing a pluripotent stem cell (PSC) population, such as an induced pluripotent stem cell (iPSC) population differentiated to embryoid bodies and enriching for CD34⁺ cells to thereby prepare a CD34⁺-enriched population. Endothelial-to-hematopoietic transition (EHT) is induced in the CD34⁺-enriched population to thereby prepare a hematopoietic stem cell (HSC) population, optionally followed by a further enrichment of CD34⁺ cells. The resulting HSC population (or fraction thereof) can be differentiated to an NK cell lineage according to this disclosure.

In some embodiments, the method generates CD3⁺CD56⁺ NK cells developed from the CD34⁺ hematopoietic progenitors (i.e., the HSC population or a fraction thereof), or a

derivative of this population. For example, NK cells generated from the HSC population can be generated in cultures conditioned with a Notch ligand and/or cultured Serum-Free Expansion Medium (SFEM) supplemented with cytokines (e.g., SCF, TPO, Flt3L and IL-7). These conditions promote expansion of the HSC population and their differentiation into

5 CD7⁺CD5⁺ lymphoid progenitors. Cells can then be further cultured in the SFEM supplemented with cytokines including IL-15 (e.g., SCF, Flt3L, IL-7, IL-15) to promote differentiation into the NK cells. After culture, majority of the cells (for example, on average about 65% or 70% or 75% or 80% cells) express the NK cell marker CD56 and may also co-express NKp46 (a NK cell activating receptor or other NK cell lineage marker described

10 herein). In a more mature stage of development, mature CD56⁺CD16⁺ NK cell subset emerges in these cultures. Acquisition of CD94 marks commitment to the CD56^{bright}, and CD56^{bright} NK cells subsequently differentiate into CD56^{dim} NK cells that upregulate CD16 and killer immunoglobulin-like receptors (KIR). In some embodiments, the NK cells are cytotoxic NK cells capable of killing, among others, cancerous cells (e.g., leukemia cells) or

15 virus-infected cells. In embodiments, NK cells express CD107a as a functional marker for the identification of natural killer cell activity.

NK cells can be divided into CD56^{dim} or CD56^{bright} NK cell subsets. Around 90% of peripheral blood and spleen NK cells are CD56^{dim}CD16⁺ and express perforin. These CD56^{dim} NK cell are cytotoxic and produce IFN- γ upon interaction with, for example, tumor

20 cells in vitro. In contrast, most NK cells in lymph nodes and tonsils are CD56^{bright}CD16[–] and lack perforin. These cells readily produce cytokines such as IFN- γ in response to stimulation with interleukin (IL)-12, IL-15 and IL-18. Thus, in some embodiments, these NK cell properties can be manipulated to establish their functionalities, such as improved grafting or elimination of tumors (malignant or non-malignant, solid or otherwise) or in the

25 elimination of viral infections.

In some embodiments, this disclosure generates NK cell precursors. NK cell precursors are identified as Lin[–]CD34⁺CD38⁺CD123[–]CD45RA⁺CD7⁺CD10⁺CD127[–] cells and represent the unipotent NK cell precursor devoid of potential toward other lymphoid lineages. In some embodiments, NK cell precursors are identified as Lin[–]CD34⁺

30 CD38⁺CD123[–]CD45RA⁺CD7⁺CD10[–]CD127⁺. In some embodiments the NK cell

precursors are identified as Lin-CD34+CD38+CD123-CD45RA+CD7+CD10+CD127+ precursor cells (generating lymphoid lineage). In some embodiments, NK precursor cells are identified on the basis of their negative or positive expression of both IL-1 β and IL-2 β receptors. In some embodiments NK cells can be identified as CD34-CD117+/-
 5 CD94+HLADR-CD10-CD122+CD94+NKp44^{low}NKG2D+CD161+ i.e., mature NK cells that can then be further distinguished into the two final developmental stages according to the expression of CD56 and CD16, respectively. In embodiments, NK cells express CD107a as a functional marker for the identification of natural killer cell activity.

Several NK specific markers can be used to identify and isolate or enrich the NK
 10 cells. For example, NK cells that are typically defined as CD3-CD56+ cells may also be CD7+CD127-NKp46+T-bet+Eomes+. Different subtypes of human NK cells can be identified as either CD3-CD56^{dim}CD16+ or CD3-CD56^{bright}CD16-. The CD56^{dim}CD16+ subset of NK cells is predominantly found in the blood and is highly cytotoxic, while the CD56^{bright}CD16- subset is the main subtype found in the lymph nodes and has only weak
 15 cytotoxic potential. Other cell surface markers (or combinations thereof) can be used to characterize the NK cells of the invention. These include but are not limited to CD3-; CD56/NCAM-1+; CD94+; CD122/IL-2 R BETA+; CD127/IL-7 R ALPHA-; Fc gamma RIII/CD16+/-; KIR family receptors+; NKG2A+; NKG2D+; NKp30+; NKp44+; NKp46+; or NKp80+.

20 Conventionally, hematopoietic lineages (such as NK cells) are prepared by differentiation of iPSCs to embryoid bodies (e.g., Day 8) to harvest CD34+ cells. CD34 is commonly used as a marker of hemogenic endothelial cells, hematopoietic stem cells, and hematopoietic progenitor cells. In accordance with aspects and embodiments of this disclosure, it is discovered that inducing endothelial-to-hematopoietic transition (EHT) of a
 25 CD34+ cell population, and which can be derived from iPSCs-embryoid bodies, can be used for the *ex vivo* generation of superior hematopoietic lineages, including NK cell lineages.

In some embodiments, the CD34+ cells (i.e., from EB dissociation) are contacted with an effective amount of an agonist of a mechanosensitive receptor or a mechanosensitive channel that increases the activity or expression of Dnmt3b. In some embodiments, the

mechanosensitive receptor is Piezol. Exemplary Piezol agonists include Yoda1, Jedi1, single-stranded (ss) RNA (e.g., ssRNA40) and Jedi2. In some embodiments, the mechanosensitive receptor is Trpv4. An exemplary Trpv4 agonist is GSK1016790A. Other manners for inducing EHT can be used and are described herein. In some embodiments, after
5 inducing EHT, the cells (HSCs or progenies thereof) are differentiated to an NK cell lineage.

In some embodiments, the HSCs are differentiated to a CD7+ progenitor T cell population, which can be further differentiated to an NK cell lineage. For example, CD7+ progenitor T cell population can be generated from a hematopoietic stem cell (HSC) population comprising human long-term hematopoietic stem cells (LT-HSCs) generated
10 from iPSCs (e.g., hiPSCs). For example, the HSC population (or cells isolated therefrom) is cultured with a partial or full Notch ligand, sonic hedgehog (SHH), RetroNectin (or other extracellular matrix component(s)), and/or combinations thereof, to produce a population comprising CD7+ progenitor T cells or a derivative cell population.

The Notch signaling pathway regulates the formation, differentiation, and function
15 of NK cells. For example, Notch signaling induces CD34+ cells to give rise to CD7+ and cytoplasmic (cy) CD3+ cells that express CD56, progenitor T-cells, pre-T cells, and mature T lymphocytes. *In vivo*, NK and T cell development proceeds after lymphocyte progenitors differentiate from bone marrow hematopoietic stem cells and migrate to the thymus. Specialized thymic epithelial cells induce development of T cells and NK cells along a
20 controlled pathway. Notch signaling plays a critical role during T lineage commitment in the thymus. As lymphoid progenitors enter the thymus, they encounter dense expression of Notch ligands on thymic epithelium that drives thymopoiesis. The present disclosure provides HSC populations generated *ex vivo* from iPSCs and which respond to Notch ligand, SHH, and/or component(s) of extracellular matrix, by robust production of T progenitor cells
25 and T cell lineages *ex vivo*, including NK cells lineages.

In various embodiments, the iPSCs are prepared by reprogramming somatic cells. The term “induced pluripotent stem cell” or “iPSC” refers to cells derived from somatic cells, such as skin or blood cells that have been reprogrammed back into an embryonic-like pluripotent state. In some embodiments, iPSCs are generated from somatic cells such as (but

not limited to) fibroblasts or PBMCs (or cells isolated therefrom). In some embodiments, the iPSCs are derived from lymphocytes (e.g., NK-cells, T-cells, B-cells etc.), cord blood cells, PBMCs, CD34+ cells, or other human primary tissues. In some embodiments, iPSCs are derived from CD34+ cells isolated from peripheral blood. In various embodiments, the iPSCs are autologous or allogenic (e.g., HLA-matched at one or more loci) with respect to a recipient (a subject in need of treatment as described herein). In various embodiments, the iPSCs can be gene edited to assist in HLA matching (such as deletion of one or more HLA Class I and/or Class II alleles or their master regulators, including but not limited beta-2-microglobulin (B2M), CIITA, etc.), or gene edited to delete or express other functionalities.

For example, iPSCs can be gene edited to delete one or more of HLA-A, HLA-B, and HLA-C, and to delete one or more of HLA-DP, HLA-DQ, and HLA-DR. In certain embodiments, the iPSCs retain expression of at least one HLA Class I and at least one HLA Class II complex. In certain embodiments, iPSCs are homozygous for at least one retained Class I and Class II loci.

In various embodiments, the iPSCs are gene edited to be one of: (i) HLA-A-B+C+DP-DR+DQ+, (ii) HLA-A-B+C+DP+DR+DQ-, (iii) HLA-A-B+C+DP-DR+DQ-, (iv) HLA-A-B-C+DP-DR+DQ+, (v) HLA-A-B-C+DP+DR+DQ-, (vi) HLA-A-B-C+DP-DR+DQ-. For retained HLA (for example HLA-B, HLA-C, and HLA-DR), cells can be homozygous or retain only a single copy of the gene. For example, the modified cells are identified at least as (a) HLA-C+ and HLA-DR+, and optionally identified as one or more of (b) HLA-B-, (c) HLA-DP-, and (d) HLA-DQ-. In exemplary embodiments, the modified cells are HLA-B+, HLA-DP-, and HLA-DQ-.

In some embodiments, the iPSCs are gene edited to be HLA-A^{neg}, homozygous for both HLA-B and HLA-C, and HLA-DPB1^{neg} and HLA-DQB1^{neg}. In some embodiments, the iPSCs are further homozygous for HLA-DRB1.

As used herein, the term “neg,” (-), or “negative,” with respect to a particular HLA Class I or Class II molecule indicates that both copies of the gene have been disrupted in the cell line or population, and thus the cell line or population does not display significant functional expression of the gene. Such cells can be generated by full or partial gene

deletions or disruptions, or alternatively with other technologies such as siRNA. As used herein, the term “delete” in the context of a genetic modification of a target gene (i.e., gene edit) refers to abrogation of functional expression of the corresponding gene product (i.e., the corresponding polypeptide). Such gene edits include full or partial gene deletions or
5 disruptions of the coding sequence, or deletions of critical cis-acting expression control sequences.

Somatic cells may be reprogrammed by expression of reprogramming factors selected from Sox2, Oct3/4, c-Myc, Nanog, Lin28, and klf4. In some embodiments, the reprogramming factors are Sox2, Oct3/4, c-Myc, Nanog, Lin28, and klf4. In some
10 embodiments, the reprogramming factors are Sox2, Oct3/4, c-Myc, and klf4. Methods for preparing iPSCs are described, for example, in US Patent 10,676,165; US Patent 9,580,689; and US Patent 9,376,664, which are hereby incorporated by reference in their entireties. In various embodiments, reprogramming factors are expressed using well known viral vector systems, such as lentiviral, Sendai, or measles viral systems. Alternatively, reprogramming
15 factors can be expressed by introducing mRNA(s) encoding the reprogramming factors into the somatic cells. Further still, iPSCs may be created by introducing a non-integrating episomal plasmid expressing the reprogramming factors, i.e., for the creation of transgene-free and virus-free iPSCs. Known episomal plasmids can be employed with limited replication capabilities and which are therefore lost over several cell generations.

20 In some embodiments, the human pluripotent stem cells (e.g., iPSCs) are gene-edited. Gene-editing can include, but is not limited to, modification of HLA genes (e.g., deletion of one or more HLA Class I and/or Class II genes), deletion of $\beta 2$ microglobulin ($\beta 2M$), deletion of CIITA, deletion or addition of NK Cell Receptor genes, or addition of a chimeric antigen receptor (CAR) gene, for example. Exemplary CARs can target tumor
25 associated antigens. An exemplary CAR NK cell can target CD19, CD38, CD33, CD47, CD20 etc. For example, the iPSCs can be NK-cell receptor-transduced iPSCs. Such embodiments enable the production of large-scale regenerated lymphocytes with a desired antigen, tissue, or cell specificity. Alternatively, engineered iPSCs with one or more HLA knockouts can be placed in a bioreactor for a feeder-and-serum-free differentiation, under
30 GMP-grade conditions, to generate fully functional and histocompatible NK cells.

In some embodiments, the iPSCs are gene edited using gRNAs that are 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26 or more nucleotides in length. In some embodiments, the gRNAs comprise a modification at or near the 5' end (e.g., within 1-10, 1-5, or 1-2 nucleotides of the 5' end) and/or a modification at or near the 3' end (e.g., within 1-10, 1-5, or 1-2 nucleotides of the 3' end). In some embodiments, the modified gRNAs exhibit increased resistance to nucleases. In some embodiments, a gRNA comprises two separate RNA molecules (i.e., a "dual gRNA"). A dual gRNA comprises two separate RNA molecules: a "crispr RNA" (or "crRNA") and a "tracr RNA" and is well known to one of skill in the art.

10 Generally, various gene editing technologies are known, which can be applied according to various embodiments of this disclosure. Gene editing technologies include but are not limited to zinc fingers (ZFs), transcription activator-like effectors (TALEs), etc. Fusion proteins containing one or more of these DNA-binding domains and the cleavage domain of FokI endonuclease can be used to create a double-strand break in a desired region of DNA in a cell (See, e.g., US Patent Appl. Pub. No. US 2012/0064620, US Patent Appl. Pub. No. US 2011/0239315, U.S. Pat. No. 8,470,973, US Patent Appl. Pub. No. US 2013/0217119, U.S. Pat. No. 8,420,782, US Patent Appl. Pub. No. US 2011/0301073, US Patent Appl. Pub. No. US 2011/0145940, U.S. Pat. No. 8,450,471, U.S. Pat. No. 8,440,431, U.S. Pat. No. 8,440,432, and US Patent Appl. Pub. No. 2013/0122581, the contents of all of which are hereby incorporated by reference). In some embodiments, gene editing is conducted using CRISPR associated Cas system (e.g., CRISPR-Cas9), as known in the art. See, for example, US 8,697,359, US 8,906,616, and US 8,999,641, each of which is hereby incorporated by reference in its entirety. In various embodiments, the gene editing employs a Type II Cas endonuclease (such as Cas9) or employs a Type V Cas endonuclease (such as Cas12a). Type II and Type V Cas endonucleases are guide RNA directed. Design of gRNAs to guide the desired gene edit (while limiting or avoiding off target edits) is known in the art. See, for example, Mohr SE, et al., CRISPR guide RNA design for research applications, FEBS J. 2016 Sep; 283(17): 3232–3238. In still other embodiments, non-canonical Type II or Type V Cas endonucleases having homology (albeit low primary sequence homology) to *S. pyogenes* Cas9 or Prevotella and Francisella1 (Cpf1 or Cas12a) can be employed. Numerous such non-canonical Cas endonucleases are known in the art. Nidhi S, et al. Novel

CRISPR–Cas Systems: An Updated Review of the Current Achievements, Applications, and Future Research Perspectives, Int J Mol Sci. 2021 Apr; 22(7): 3327. In still other embodiments, the gene editing employs base editing or prime editing to incorporate mutations without instituting double strand breaks. See, for example, Antoniou P, et al., Base and Prime Editing Technologies for Blood Disorders, Front. Genome Ed., 28 January 2021; Matsuokas IG, Prime Editing: Genome Editing for Rare Genetic Diseases Without Double-Strand Breaks or Donor DNA, Front. Genet., 09 June 2020. Various other gene editing processes are known, including use of dead Cas (dCas) systems (e.g., Cas fusion proteins) to target DNA modifying enzymes to desired targets using the dCas as a guide RNA-directed system. Brezgin S, Dead Cas Systems: Types, Principles, and Applications, Int J Mol Sci. 2019 Dec; 20(23): 6041.

Base editors that can install precise genomic alterations without creating double-strand DNA breaks can also be used in gene editing (e.g., designing gene therapy vectors) in the cells (e.g., iPSCs). Base editors essentially comprise a catalytically disabled nuclease, such as Cas9 nickase (nCas9), which is incapable of making DSBs and is fused to a nucleobase deaminase enzyme and, in some cases, a DNA glycosylase inhibitor. Currently, there are 2 major categories of base editors, cytidine base editors (CBEs) and adenine base editors (ABEs), which catalyze C>T and A>G transitions. Base editors can be delivered, for example, via HDAd5/35++ vectors to efficiently edit promoters and enhancers to active or inactivate a gene. Exemplary methods are described in U.S. Patent Nos. 9,840,699; 10,167,457; 10,113,163; 11,306,324; 11,268,082; 11,319,532; and 11,155,803. Also contemplated are prime editors that comprise a reverse transcriptase conjugated to (e.g., fused with) a Cas endonuclease and a polynucleotide useful as a DNA synthesis template conjugated to (e.g., fused with) a guide RNA, as described in WO 2020/191153.

Exemplary vectors that can be used for the genome editing applications include, but are not limited to, plasmids, retroviral vectors, lentiviral vectors, adenovirus vectors (e.g., Ad5/35, Ad5, Ad26, Ad34, Ad35, Ad48, parvovirus (e.g., adeno-associated virus (AAV) vectors, herpes simplex virus vectors, baculoviral vectors, coronavirus, negative strand RNA viruses such as orthomyxovirus (e.g., influenza virus), rhabdovirus (e.g., rabies and vesicular stomatitis virus), paramyxovirus (e.g. measles and Sendai), positive strand RNA viruses,

such as picornavirus and alphavirus, and double stranded DNA viruses including herpes virus (e.g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e.g., canarypox, vaccinia or modified vaccinia virus. The vector comprising the nucleic acid molecule of interest may be delivered to the cell (e.g., iPS cells, endothelial cells, hemogenic endothelial cells, HSCs (ST-HSCs or LT-HSCs) via any method known in the art, including but not limited to transduction, transfection, infection, and electroporation. Any of these vectors may include transposable element (such as a piggyback transposon or sleeping beauty transposon). Transposons insert specific sequences of DNA into genomes of vertebrate animals. The gene of interest can be integrated into the genome of a mammalian cell by transposase-catalyzed cleavage of similar excision sites that exist within the nuclear genome of the cell.

For increased efficiency, in some embodiments, the Cas and the gRNA can be combined before being delivered into the cells. The Cas-gRNA complex is known as a ribonucleoprotein (RNP). A number of methods have been developed for direct delivery of RNPs to cells. For example, RNP can be delivered into cells in culture by lipofection or electroporation. Electroporation using a nucleofection protocol can be employed, and this procedure allows the RNP to enter the nucleus of cells quickly, so it can immediately start cutting the genome. See, for example, Zhang S, Shen J, Li D, Cheng Y. Strategies in the delivery of Cas9 ribonucleoprotein for CRISPR/Cas9 genome editing. *Theranostics*. 2021 Jan 1;11(2):614-648, hereby incorporated by reference in its entirety. In some embodiments, Cas9 and gRNA are electroporated as RNP into the donor iPSCs and/or HSCs.

Generally, a protospacer adjacent motif (PAM) is required for a Cas nuclease to cut and is generally found 3-4 nucleotides downstream from the cut site. The PAM is a short DNA sequence (usually 2-6 base pairs in length) that follows the DNA region targeted for cleavage by the CRISPR system, such as CRISPR-Cas9. In some embodiments, the PAM sequences, sgRNAs, or base editing tools targeting haplotypes or polymorphs of HLA loci does not include four Gs, four Cs, GC repeats, or combinations thereof.

In some embodiments, a CRISPR/Cas9 system specific to a unique HLA haplotype can be developed by designing singular gRNAs targeting each of the donor-specific HLA-

A, HLA-DPB1, and HLA-DQB1 genes (for example), using the gRNAs as described herein. To perform genetic knockout, the gRNA targets the Cas9 protein to the appropriate site to edit. Next, the Cas9 protein can perform a double strand break (DSB), where the DNA repairs through a non-homologous end joining (NHEJ) mechanism which generates indels
5 resulting in a frameshift mutation and terminates the resulting protein's function. However, off-target genetic modifications can occur and alter the function of otherwise intact genes. For example, the Cas9 endonuclease can create DSBs at undesired off-target locations, even in the presence of some degree of mismatch. This off-target activity can create genome instability events, such as point mutations and genomic structural variations. In various
10 embodiments, a sgRNA targeting HLA-A can target a region of chromosome 6 defined as 29942532-29942626. In various embodiments, a sgRNA targeting HLA-DQB1 can target a region of chromosome 6 defined as 32665067-32664798. In various embodiments, a sgRNA targeting HLA-DPB1 can target a region of chromosome 6 defined as 33080672-33080935.

gRNAs can be used to develop clonal iPSCs. Such iPSC lines can be evaluated for
15 (i) ON-target edits, (ii) OFF-target edits, and (iii) Translocation edits, for example using sequencing, as described herein. Specifically, such assays can be performed by multiplex PCR with primers designed to target and enrich regions of interest followed by next-generation sequencing (e.g., Amplicon sequencing, AMP-seq). The ON-target panel and the translocation panel can amplify the intended edited region, allowing for selection of iPSC
20 clones with the expected edits which are free from chromosomal translocation arising from unintended DSB cut-site fusion. The OFF-target panel can enrich any potential off-target regions identified via sequencing and allows for selection of iPSC clones with negligible off-target mutations. Together, these assays enable a screen of the iPSC clones to select the clones with the desired edits, while excluding potential CRISPR/Cas9-related genome
25 integrity issues.

In some embodiments, to further ensure the genomic stability and integrity of reprogrammed and edited iPSCs, genetic and genomic assays can be performed to select for clones which, for example, did not undergo translocation and mutation events, and that did not integrate the episomal vectors. For example, whole-genome sequencing (WGS) is
30 performed on CD34+ cells and on iPSC clones after reprogramming, where the genomes are

compared for differences arising from editing. These analyses provide an assessment of which iPSC clone genomes differ from the CD34+ starting material, enabling informed selection iPSC clones which did not accrue mutations during the reprogramming.

In some embodiments, karyotyping analyses using systems such as KARYOSTAT
5 assays is used to select iPSC clones which did not accrue indels and translocation during the reprogramming, for example as described in Ramme AP, et al, "Supporting dataset of two integration-free induced pluripotent stem cell lines from related human donors," Data Brief. 2021 May 15;37:107140, hereby incorporated by reference in its entirety. KARYOSTAT assays allow for visualization of chromosome aberrations with a resolution similar to G-
10 banding karyotyping. The size of structural aberration that can be detected is >2 Mb for chromosomal gains and >1 Mb for chromosomal losses. The KARYOSTAT array is functionalized for balanced whole-genome coverage with a low-resolution DNA copy number analysis, where the assay covers all 36,000 RefSeq genes, including 14,000 OMIM targets. The assay enables the detection of aneuploidies, submicroscopic aberrations, and
15 mosaic events.

In some embodiments, Array Comparative Genomic Hybridization (aCGH) analyses is used to select iPSC clones which did not accrue copy number aberrations (CNA) during reprogramming, for example as described in Wiesner et al. "Molecular Techniques," Editor(s): Klaus J. Busam, Pedram Gerami, Richard A. Scolyer, "Pathology of Melanocytic
20 Tumors," Elsevier, 2019, pp. 364-373, ISBN 9780323374576; and Hussein SM, et al. "Copy number variation and selection during reprogramming to pluripotency," Nature. 2011 Mar 3;471(7336):58-62, hereby incorporated by reference in its entirety. aCGH is a technique that analyzes the entire genome for CNA by comparing the sample DNA to reference DNA.

In some embodiments, targeted heme malignancy NGS panel analyses is used to
25 select iPSC clones which did not accrue hematologic malignancy mutations during reprogramming. For example, targeted heme malignancy NGS panels can focus on myeloid leukemia, lymphoma, and/or other hematologic malignancy-associated genes to generate a smaller, more manageable data set than broader methods. Targeted heme malignancy NGS

panel analysis includes the use of highly multiplexed PCR to amplify regions associated with hematologic malignancies followed by next-generation sequencing.

In some embodiments, Droplet Digital PCR (ddPCR) is used to select iPSC clones which did not integrate episomal vectors and that have been passaged enough for episomal vector clearance. As discussed herein, iPSC reprogramming of CD34+ cells can be achieved by delivering episomal vectors encoding reprogramming factors. However, episomal vectors can, albeit rarely, randomly integrate into the cellular genome, which could disrupt developmental processes, homeostasis, etc. Therefore, ddPCR methods can be used to detect residual episomal vector in the iPSC cultures and enable selection of iPSC clones which did not integrate episomal vectors.

In some embodiments, after assessing that the selected clones are free from genomic aberrations related to editing, the clones can be additionally tested for spontaneous mutations that might arise during expansion. For example, mutations affecting hematologic malignancy genes, indel, translocations, number aberrations, e.g., as described for the pre-edited reprogrammed clones. Analyses for spontaneous mutations can include whole-genome sequencing (WGS), KARYOSTAT analysis, Array Comparative Genomic Hybridization (aCGH) analysis, targeted heme malignancy NGS panel AMP-Seq analysis, and/or Droplet Digital PCR (ddPCR).

In various embodiments, iPSCs are prepared, and expanded using a culture system. Expanded iPSCs can be recovered from the culture for generating embryoid bodies (EBs). EBs, created by differentiation of iPSCs, are three-dimensional aggregates of iPSCs and comprise the three (or alternatively two or one) embryonic germ layer(s) based on the differentiation method(s). Preparation of EBs is described, for example, in US 2019/0177695, which is hereby incorporated by reference in its entirety. In some embodiments, EBs prepared by differentiation of the iPSCs, are expanded in a bioreactor as described, for example, in Abecasis B. et al., Expansion of 3D human induced pluripotent stem cell aggregates in bioreactors: Bioprocess intensification and scaling-up approaches. *J. of Biotechnol.* 246 (2017) 81-93. EBs can be used to generate any desired cell type. Other

methods, including a 3D suspension culture, for expansion or differentiation of EBs is described in WO 2020/086889, which is hereby incorporated by reference in its entirety.

5 In some embodiments, the process according to each aspect can comprise generating CD34⁺-enriched cells from the pluripotent stem cells (e.g., EBs) and inducing endothelial-to-hematopoietic differentiation. HSCs comprising relatively high frequency of LT-HSCs can be generated from the cell populations using various stimuli or factors, including mechanical, biochemical, metabolic, and/or topographical stimuli, as well as factors such as extracellular matrix, niche factors, cell-extrinsic factors, induction of cell-intrinsic properties; and including pharmacological and/or genetic means.

10 In some embodiments, the method comprises preparing endothelial cells with hemogenic potential from pluripotent stem cells, prior to induction of EHT. In some embodiments, the combined over-expression of GATA2/ETV2, GATA2/TAL1, or ER71/GATA2/SCL can lead to the formation of endothelial cells with hemogenic potential from PSC sources. In some embodiments, the method comprises overexpression of E26
15 transformation-specific variant 2 (ETV2) transcription factor in the iPSCs. ETV2 can be expressed by introduction of an encoding non-integrating episomal plasmid, for constitutive or inducible expression of ETV2, and for production of transgene-free hemogenic ECs. In some embodiments, ETV2 is expressed from an mRNA introduced into the iPSCs. mRNA can be introduced using any available method, including electroporation or lipofection.
20 Differentiation of cells expressing ETV2 can comprise addition of VEGF-A. See, Wang K, et al., Robust differentiation of human pluripotent stem cells into endothelial cells via temporal modulation of ETV2 with mRNA, *Sci. Adv.* Vol. 6 (2020). Cells generated in this manner may be used for producing CD34⁺ cells and inducing EHT according to embodiments of this disclosure.

25 Following CD34⁺ enrichment, HSCs are then generated from the endothelial cells using mechanical, biochemical, pharmacological and/or genetic stimulation or modification.

In some embodiments, iPSC differentiation proceeds until cells are at least about 10% CD34⁺, or at least about 20% CD34⁺, or at least about 25% CD34⁺, or at least about 30% CD34⁺. In some embodiments, CD34 enrichment and EHT may be induced at Day 7

to Day 14 of iPSC differentiation, such as for example, Day 8, Day 9, Day 10, Day 11, Day 12, Day 13, or Day 14. Differentiation of iPSCs can be according to known techniques. In some embodiments, iPSC differentiation involves factors such as, but not limited to, combinations of bFGF, Y27632 (or other ROCK inhibitor), WNT agonist (e.g., CHIR99021), BMP4, VEGF, SCF, EPO, TPO, IL-6, IL-11, and/or IGF-1. In some
5 embodiments, hPSCs are differentiated using feeder-free, serum-free, and/or GMP-compatible materials, such as pomalidomide or lenalidomide. In some embodiments, hPSCs are co-cultured with murine bone marrow-derived feeder cells such as OP9 or MS5 cell line in serum-containing medium. The culture can contain growth factors and cytokines to
10 support differentiation of embryoid bodies or monolayer system. The OP9 co-culture system can be used to generate multipotent HSPCs, which can be differentiated further to several hematopoietic lineages including T lymphocytes, B lymphocytes, megakaryocytes, monocytes or macrophages, and erythrocytes. See Netsrithong R. et al., Multilineage differentiation potential of hematoendothelial progenitors derived from human induced pluripotent stem cells, *Stem Cell Research & Therapy* Vol. 11 Art. 481 (2020). Alternatively,
15 a step-wise process using defined conditions with specific signals can be used. For example, the expression of HOXA9, ERG, RORA, SOX4, and MYB in human PSCs favors the direct differentiation into CD34+/CD45+ progenitors with multilineage potential. Further, expression of factors such as HOXB4, CDX4, SCL/TAL1, or RUNX1a support the
20 hematopoietic program in human PSCs. See Doulatov S. et al., Induction of multipotential hematopoietic progenitors from human pluripotent stem cells via re-specification of lineage-restricted precursors, *Cell Stem Cell*. 2013 Oct 3; 13(4).

Exemplary ROCK inhibitors that find use for establishing and differentiation iPSCs include but are not limited to: thiazovivin, Y27632, Fasudil, AR122-86, RevitaCell.TM.
25 Supplement, H-1152, Y-30141, Wf-536, HA-1077, hydroxyl-HA-1077, GSK269962A, SB-772077-B, N-(4-Pyridyl)-N'-(2,4,6-trichlorophenyl)urea, 3-(4-Pyridyl)-1H-indole, and (R)-(+)-trans-N-(4-Pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, H-100, and ROCK inhibitors disclosed in U.S. Pat. No. 8,044,201, which is hereby incorporated by reference in its entirety.

Differentiation of iPSCs (e.g., to EBs) may employ a WNT agonist, such as CHIR99021. A WNT agonist is a molecule that mimics or increases WNT signaling. Non-limiting examples of WNT agonists include small molecules CHIR-99021 (CAS 252917-06-9), a 2-amino-4,6-disubstituted pyrimidine, e.g. BML 284 (CAS 853220-52-7), SKL 2001 (CAS 909089-13-0), WAY 262611 (CAS 1123231-07-1), WAY 316606 (CAS 915759-45-4), SB 216763 (CAS 280744-09-4), IQ 1 (CAS 331001-62-8), QS 11 (CAS 944328-88-5), deoxycholic acid (CAS 83-44-3), BIO (CAS 667463-62-9), kenpaullone (CAS 142273-20-9), or a (hetero) arylpyrimidine. In some embodiments, a WNT agonist is an agonist antibody or functional fragment thereof or an antibody-like polypeptide.

10 Induction of EHT can be with any known process. In some embodiments, induction of EHT generates a hematopoietic stem cell (HSC) population comprising LT-HSCs. In some embodiments, EHT generates HSCs through endothelial or hemogenic endothelial cell (HEC) precursors using mechanical, biochemical, pharmacological and/or genetic means (e.g., via stimulation, inhibition, and/or genetic modifications). In some embodiments, the
15 EHT generates a stem cell population comprising one or more of long-term hematopoietic stem cells (LT-HSCs), short-term hematopoietic stem cells (ST-HSCs), and hematopoietic stem progenitor cells. In some embodiments, the EHT culture includes one or more (e.g., combinations of) Y-27632, TPO, IL-3, SCF, IL-6, IL-11, IGF-1, VEGF, bFGF, BMP4, and FLT3.

20 In some embodiments, the method comprises increasing the expression or activity of dnmt3b in PSCs, embryoid bodies, CD34-enriched cells, ECs, HECs or HSCs, which can be by mechanical, genetic, biochemical, or pharmacological means. In some embodiments, the method comprises increasing activity or expression of DNA (cytosine-5-)-methyltransferase 3 beta (Dnmt3b) and/or GTPase IMAP Family Member 6 (Gimap6) in the cells. See WO
25 2019/236943 and WO 2021/119061, which is hereby incorporated by reference in its entirety. In some embodiments, the induction of EHT comprises increasing the expression or activity of dnmt3b.

In some embodiments, cells are contacted with an effective amount of an agonist of a mechanosensitive receptor or a mechanosensitive channel that increases the activity or

expression of Dnmt3b. In some embodiments, the mechanosensitive receptor is Piezo1. An exemplary Piezo1 agonist is Yoda1. In some embodiments, the mechanosensitive receptor is Trpv4. An exemplary Trpv4 agonist is GSK1016790A. Yoda1 (2-[5-[(2,6-Dichlorophenyl)methyl]thio]-1,3,4-thiadiazol-2-yl]-pyrazine) is a small molecule agonist developed for the mechanosensitive ion channel Piezo1. Syeda R, Chemical activation of the mechanotransduction channel Piezo1. *eLife* (2015).

Derivatives of Yoda1 can be employed in various embodiments. For example, derivatives comprising a 2,6-dichlorophenyl core are employed in some embodiments. Exemplary agonists are disclosed in Evans EL, et al., Yoda1 analogue (Dooku1) which antagonizes Yoda1-evoked activation of Piezo1 and aortic relaxation, *British J. of Pharmacology* 175(1744-1759): 2018. Still other Piezo1 agonist include Jedi1, Jedi2, single-stranded (ss) RNA (e.g., ssRNA40) and derivatives and analogues thereof. See Wang Y., et al., A lever-like transduction pathway for long-distance chemical- and mechano-gating of the mechanosensitive Piezo1 channel. *Nature Communications* (2018) 9:1300; Sugisawa, et al., RNA Sensing by Gut Piezo1 Is Essential for Systemic Serotonin Synthesis, *Cell*, Volume 182, Issue 3, 2020, Pages 609-624, hereby incorporated by reference in their entireties. These Piezo1 agonists are commercially available. In various embodiments, the effective amount of the Piezo1 agonist or derivative is in the range of about 1 μ M to about 500 μ M, or about 5 μ M to about 200 μ M, or about 5 μ M to about 100 μ M, or in some embodiments, in the range of about 25 μ M to about 150 μ M, or about 25 μ M to about 100 μ M, or about 25 μ M to about 50 μ M.

In various embodiments, pharmacological Piezo1 activation is applied to CD34+ cells (i.e., CD34-enriched cells). In certain embodiments, pharmacological Piezo1 activation may further be applied to iPSCs, embryoid bodies, ECs, hemogenic endothelial cells (HECs), HSCs, hematopoietic progenitors, as well as hematopoietic lineage(s). In certain embodiments, Piezo1 activation is applied at least to EBs generated from iPSCs, CD34+ cells isolated from EBs, and/or combinations thereof, which in accordance with various embodiments, allows for superior generation of T progenitor cells as compared to other methods for inducing EHT.

Alternatively or in addition, the activity or expression of Dnmt3b can be increased directly in the cells, e.g., in CD34-enriched cells. For example, mRNA expression of Dnmt3b can be increased by delivering Dnmt3b-encoding transcripts to the cells, or by introducing a Dnmt3b-encoding transgene, or a transgene-free method, not limited to introducing a non-integrating episome to the cells. In some embodiments, gene editing is employed to introduce a genetic modification to Dnmt3b expression elements in the cells, such as, but not limited to, to increase promoter strength, ribosome binding, RNA stability, and/or impact RNA splicing.

In some embodiments, the method comprises increasing the activity or expression of Gimap6 in the cells, alone or in combination with Dnmt3b and/or other genes that are up- or down regulated upon cyclic strain or Piezo1 activation. To increase activity or expression of Gimap6, Gimap6-encoding mRNA transcripts can be introduced to the cells, transgene-free approaches can also be employed, including but not limited, to introducing an episome to the cells; or alternatively a Gimap6-encoding transgene. In some embodiments, gene editing is employed to introduce a genetic modification to Gimap6 expression elements in the cells (such as one or more modifications to increase promoter strength, ribosome binding, RNA stability, or to impact RNA splicing).

In embodiments of this disclosure employing mRNA delivery to cells, known chemical modifications can be used to avoid the innate-immune response in the cells. For example, synthetic RNA comprising only canonical nucleotides can bind to pattern recognition receptors, and can trigger a potent immune response in cells. This response can result in translation block, the secretion of inflammatory cytokines, and cell death. RNA comprising certain non-canonical nucleotides can evade detection by the innate immune system, and can be translated at high efficiency into protein. See US 9,181,319, which is hereby incorporated by reference, particularly with regard to nucleotide modification to avoid an innate immune response.

In some embodiments, expression of Dnmt3b and/or Gimap6 is increased by introducing a transgene into the cells, which can direct a desired level of overexpression (with various promoter strengths or other selection of expression control elements).

Transgenes can be introduced using various viral vectors or transfection reagents (including Lipid Nanoparticles) as are known in the art. In some embodiments, expression of Dnmt3b and/or Gimap6 is increased by a transgene-free method (e.g., episome delivery). In some embodiments, expression or activity of Dnmt3b and/or Gimap6 or other genes disclosed
5 herein are increased using a gene editing technology, for example, to introduce one or more modifications to increase promoter strength, ribosome binding, or RNA stability.

In some embodiments, the method comprises applying cyclic 2D, 3D, or 4D stretch to cells. In various embodiments, the cells subjected to cyclic 2D, 3D, or 4D stretch are selected from one or more of CD34-enriched cells, iPSCs, ECs, and HECs. For example, a
10 cell population is introduced to a bioreactor that provides a cyclic-strain biomechanical stretching, as described in WO 2017/096215, which is hereby incorporated by reference in its entirety. The cyclic-strain biomechanical stretching can increase the activity or expression of Dnmt3b and/or Gimap6. In these embodiments, mechanical means apply stretching forces to the cells, or to a cell culture surface having the cells (e.g., ECs or HECs) cultured thereon.
15 For example, a computer controlled vacuum pump system or other means for providing a stretching force (e.g., the FlexCell™ Tension System, the Cytostretcher System) attached to flexible biocompatible and/or biomimetic surface can be used to apply cyclic 2D, 3D, or 4D stretch *ex vivo* to cells under defined and controlled cyclic strain conditions. For example, the applied cyclic stretch can be from about 1% to about 20% cyclic strain (e.g., about 6%
20 cyclic strain) for several hours or days (e.g., about 7 days). In various embodiments, cyclic strain is applied for at least about one hour, at least about two hours, at least about six hours, at least about eight hours, at least about 12 hours, at least about 24 hours, at least about 48 hrs, at least about 72 hrs, at least about 96 hrs, at least about 120 hrs, at least about 144 hrs, or at least about 168 hrs.

25 Alternatively or in addition, EHT is stimulated by Trpv4 activation. The Trpv4 activation can be by contacting cells (e.g., CD34-enriched cells, ECs, or HECs) with one or more Trpv4 agonists, which are optionally selected from GSK1016790A, 4alpha-PDD, or analogues and/or derivatives thereof.

Where cell populations are described herein as having a certain phenotype it is understood that the phenotype represents a significant portion of the cell population, such as at least 25%, at least 40%, or at least about 50%, or at least about 60%, or at least about 75%, or at least about 80%, or at least about 90% of the cell population. Further, at various steps, cell populations can be enriched for cells of a desired phenotype, and/or depleted of cells of an undesired phenotype, such that cell population comprise at least about 75%, or at least about 80%, or at least about 90% of the desired phenotype. Such positive and negative selection methods are known in the art. For example, cells can be sorted based on cell surface antigens (including those described herein) using a fluorescence activated cell sorter, or magnetic beads which bind cells with certain cell surface antigens. Negative selection columns can be used to remove cells expressing undesired cell-surface markers. In some embodiments, cells are enriched for CD34+ cells (prior to and/or after undergoing EHT). In some embodiments, the cell population is cultured under conditions that promote expansion of CD34+ cells to thereby produce an expanded population of stem cells. In some embodiments, the cell population is cultured under conditions that promote expansion of CD34+ cells to thereby produce an expanded population of stem cells. Further, NK cells produced according to this disclosure can be enriched for desired markers, such as the expression or expression level of CD56.

In various embodiments, CD34+ cells (e.g., the floater and/or adherent cells) are harvested from the culture undergoing endothelial-to-hematopoietic transition, such as between Day 8 to Day 20 of iPSC differentiation (e.g., Day 10 to Day 17).

In various embodiments, the HSCs or CD34+-enriched cells are further expanded. For example, the HSCs or CD34-enriched cells can be expanded according to methods disclosed in US 8,168,428; US 9,028,811; US 10,272,110; and US 10,278,990, which are hereby incorporated by reference in their entireties. In some embodiments, *ex vivo* expansion of HSCs or CD34-enriched cells employs prostaglandin E₂ (PGE₂) or a PGE₂ derivative. In some embodiments of this disclosure, the HSCs comprise at least about 0.01% LT-HSCs, or at least about 0.05% LT-HSCs, or at least about 0.1% LT-HSCs, or at least about 0.5% LT-HSCs, or at least about 1% LT-HSCs.

Hematopoietic stem cells (HSCs) which give rise to erythroid, myeloid, and lymphoid lineages, can be identified based on the expression of CD34 and the absence of lineage specific markers (termed Lin⁻). In some embodiments, a population of stem cells comprising HSCs are enriched, for example, as described in US 9,834,754, which is hereby incorporated by reference in its entirety. For example, this process can comprise sorting a cell population based on expression of one or more of CD34, CD90, CD38, and CD43. A fraction can be selected for further differentiation that is one or more of CD34⁺, CD90⁺, CD38⁻, and CD43⁻. In some embodiments, the stem cell population for differentiation to a hematopoietic lineage is at least about 80% CD34⁻, or at least about 90% CD34⁺, or at least about 95% CD34⁺.

In some embodiments, the stem cell population, or CD34⁺-enriched cells or fraction thereof, or derivative population are expanded as described in US 2020/0308540, which is hereby incorporated by reference in its entirety. For example, the cells are expanded by exposing the cells to an aryl hydrocarbon receptor antagonist including, for example, SR1 or an SR1-derivative. See also, Wagner et al., *Cell Stem Cell* 2016;18(1):144-55 and Boitano A., et al., Aryl Hydrocarbon Receptor Antagonists Promote the Expansion of Human Hematopoietic Stem Cells. *Science* 2010 Sep 10; 329(5997): 1345–1348.

In some embodiments, the compound that promotes expansion of CD34⁺ cells includes a pyrimidoindole derivative including, for example, UM171 or UM729 (see US 2020/0308540, which is hereby incorporated by reference).

In some embodiments, the stem cell population or CD34⁺-enriched cells are further enriched for cells that express Periostin and/or Platelet Derived Growth Factor Receptor Alpha (pdgfra) or are modified to express Periostin and/or pdgfra, as described in WO 2020/205969 (which is hereby incorporated by reference in its entirety). Such expression can be by delivering encoding transcripts to the cells, or by introducing an encoding transgene, or a transgene-free method, not limited to introducing a non-integrating episome to the cells. In some embodiments, gene editing is employed to introduce a genetic modification to expression elements in the cells, such as to modify promoter activity or strength, ribosome binding, RNA stability, or impact RNA splicing.

In still other embodiments, the stem cell population or CD34-enriched cells are cultured with an inhibitor of histone methyltransferase EZH1. Alternatively, EZH1 is partially or completely deleted or inactivated or is transiently silenced in the stem cell population. Inhibition of EZH1 can direct myeloid progenitor cells (e.g., CD34+CD45+) to lymphoid lineages. See WO 2018/048828, which is hereby incorporated by reference in its entirety. In still other embodiments, EZH1 is overexpressed in the stem cell population.

The HSC population or fraction thereof is differentiated to an NK cell lineage.

In vivo, NK cell development can be demarcated into about six stages based on bone marrow (BM) and lymph node (LN) development. See Abel AM et al: Natural Killer Cells: Development, Maturation, and Clinical Utilization. Front Immunol. 2018 Aug 13;9:1869. NK cells consistent with these stages can be generated according to the present disclosure. CD3ε-CD7+CD127+ cells mark the earliest stage of committed NKPs (Stage 2a). CD7 expression persists throughout development. Expression of IL-1R, a receptor for IL-1β defines Stage 2b. Expression of activation receptors including NKG2D, CD335 (Natural cytotoxicity receptor, NCR1, NKp46), and CD337 (NCR3, NKp30) marks the transition of NK cells from Stage 2b to Stage 3. Stage 4 of human NK cell development is sub-divided into two parts based on the expression of the activating receptor NKP80 (KLRF1, type II transmembrane protein). The primary distinction of NK cells in the Stage 4a is that they express abundant amounts of CD56 (CD56^{bright}). These NK cells are NKP80- and express the maximal levels of NKG2D, CD335, CD337, inhibitory NKG2A and CD161 (NK1.1, KLRB1, NKR-P1A). At Stage 4b, human NK cells become positive for NKP80 and maintain their CD56^{bright} status. Downregulation of CD56^{bright} expression to become CD56^{dim}, and the expression of immunoglobulin superfamily member CD16 (FcγRIII) in a subset of NK cells, defines Stage 5. Expression levels of CD56 provides a functional classification of human NK cells. Most human NK cells in the peripheral blood are CD56^{dim}. CD56^{bright} NK cells are considered less mature and reside primarily in SLTs while the CD56^{dim} subset represents the majority of NK cells in circulation. Most of the immature NK cells (iNKs) cells transition into a minor CD56^{bright} population (~5%) that convert into major CD56^{dim} (>90%) population. The downregulation of CD56 during human NK cell maturation is strongly associated with the acquisition of anti-tumor cytotoxicity, since CD56^{bright} NK cells are

potent producers of inflammatory cytokines, while the cytolytic function of human NK cells resides primarily in the CD56^{dim} population. Terminal maturation (Stage 6) of CD56^{dim} NK cells is defined by the expression of CD57 (HNK-1, Leu-7). Additional classification such as “antigen-experienced” or “adaptive” CD2⁺ NK cells is defined by a higher expression of
5 NKG2C (KLRC2, CD159c).

Thus in some embodiments, generating Natural Killer (NK) cells from pluripotent stem cells (e.g., iPSCs) may comprise the steps of: (i) preparing an HSC population comprising CD34⁺ cells as described; (ii) culturing the HSC population under conditions sufficient for differentiating the cells to NK cells, which can optionally be a culture medium
10 comprising factors such as FGF2, VEGF, TPO, SCF, IL-3 and FLT3L; (iii) during differentiation, factors such as IL-7 and IL-15 can be included in the culture medium. NK cells can be identified by NK cell markers such as CD3-CD56, and can be further characterized as CD56^{bright} NK cells or CD56^{dim} NK cells based on their level of differentiation.

15 In some embodiments, the HSC population or fraction thereof is differentiated to NK cells or progenitors or derivatives thereof independent of the use of an agonist of a mechanosensitive receptor or a mechanosensitive channel such as Yoda1. In some embodiments, the use of an agonist of a mechanosensitive receptor or a mechanosensitive channel such as Yoda1 is optional. Thus, in some embodiments, CD34⁺ cells are enriched
20 from a differentiated pluripotent stem cell population to prepare a CD34⁺-enriched population. Endothelial-to-hematopoietic transition of the CD34⁺-enriched cell population is induced for at least two days, but no more than 12 days in which the use of an agonist of a mechanosensitive receptor or a mechanosensitive channel such as Yoda1, jedi1, jedi2, ssRNA40 is optional. The HSCs and/or HSPCs are differentiated to a progenitor NK cell
25 population or a NK cell population.

In some embodiments, the endothelial-to-hematopoietic transition of the CD34⁺-enriched cell population is induced for at least for two days and further for 4 hours, or 8 hours, or 12 hours, or 16 hours, or 20 hours, or 24 hours, or 2 days, or 3 days, or 4 days, or 5 days, or 6 days, or 7 days, or 8 days, or 9 days, or 10 days, or 11 days or 12 days but no
30 more than 12 days.

In some embodiments, the NK cell lineage generated according to this disclosure is predominately (e.g., at least about 50% or at least about 75%) CD56^{bright}. In some embodiments, the NK cell lineage generated according to this disclosure is predominately (e.g., at least about 50% or at least about 75%) CD56^{dim}. In some embodiments, the NK cells are cytotoxic innate lymphoid natural killers (NK). Once activated, cytolytic NK cells share similar effector functions with T cells including production of cytotoxic granules and inflammatory cytokines. NK cells can also shape the adaptive immune system by influencing T cells in different stages of their lifespan. For example, during T cell priming, NK cells indirectly alter T cell responses by affecting dendritic cells (DCs).

10 In some embodiments, the NK cells produced according to this disclosure secrete comparable levels of cytokines as endogenous NK cells. Such cytokines include but are not limited to IFN- γ , TNF- α , GM-CSF, IL-10, IL-5, and IL-13 and chemokines such as MIP-1 α , MIP-1 β , IL-8, and RANTES. As an example, IFN- γ , secreted by the NK cells is a potent effector cytokine that plays a crucial role in antiviral, antibacterial, and antitumor responses.

15 In some embodiments, the NK cells prepared according to this disclosure secrete chemokines, such as but not limited to XCL1, CCL2, CCL3, CCL4, CCL5, CCL22, CXCL8, MIP-1 alpha, MIP-1beta, IL-8, and RANTES, or any combination thereof. Such chemokines secreted by NK cells may recruit other effector cells during immune responses. Cytokines and chemokines can be routinely measured by known methods such as quantitative polymerase chain reaction (q-PCR), enzyme-linked immunosorbent assay (ELISA), or flow cytometry analysis.

20 During differentiation, the HSC population and progenies thereof are cultured with a Notch ligand, partial or full, SHH, extracellular matrix component(s), and/or combinations thereof, *ex vivo*, to differentiate HSCs to CD7⁺ progenitor T cells, and then to an NK cell lineage. Further, according to known processes, xenogenic OP9-DL1 or irradiated K562-mbIL21-41BBL cells can be employed for differentiation of hematopoietic cells to T cells and NK cells. The OP9-DL1 co-culture system uses a bone marrow stromal cell line (OP9) transduced with the Notch ligand delta-like-1 (DLL1) or DLL-4 to support T cell development from stem cell sources. The OP9-DL1 system limits the potential of the cells

for clinical application. There is a need for feeder-cell-free systems that can generate cells such as NK cells from the hiPSCs for clinical use, and in some embodiments the present invention meets this objective. In a non-limiting example, to generate mature NK cells utilizing the notch ligands, iPSC expansion is performed for 6 days, followed by embryoid
5 body formation, which takes about 8 days. The cells are further cultured for about 5 days to enable the development of CD34+hemogenic endothelial cells, from which HSCs are derived. The HSCs are then cultured in a T cell or NK-cell specific media supplemented with retroNectin and DLL-4 for the generation of Tpro cells, identified as CD34+CD7+CD5+/- . Tpro cells can then be differentiated to generate the NK cells.

10 The term "Notch ligand" as used herein refers to a ligand capable of binding to a Notch receptor polypeptide present in the membrane of a hematopoietic stem cell or progenitor T cell. The Notch receptors include Notch-1, Notch-2, Notch-3, and Notch-4. Notch ligands typically have a DSL domain (D-Delta, S-Serrate, and L-Lag2) comprising 20-22 amino acids at the amino terminus, and from 3 to 8 EGF repeats on the extracellular
15 surface. In various embodiments, the Notch ligand comprises at least one of Delta-Like-1 (DLL1), Delta-Like-4 (DLL4), SFIP3, Delta^{Max} (disclosed in PCT/US2020/041765 and PCT/US2020/030977, which are incorporated herein in their entirety by reference) or a functional portion thereof, Jagged 1 (JAG1), Jagged 2 (JAG2), Delta-like ligand 3 (DLL3), and X-delta 2. A key signal that is delivered to incoming lymphocyte progenitors by the
20 thymus stromal cells *in vivo* is mediated by DL4, which is expressed by cortical thymic epithelial cells.

"Notch ligand" as used herein also includes intact (full-length), partial (a truncated form), or modified (comprising one or more mutations, such as conservative mutations) notch ligands as well as Notch ligands from any species or fragments thereof that retain at
25 least one activity or function of a full-length Notch ligand. Also included are peptides that mimic notch ligands. Notch ligands can be "canonical notch ligands" or "non-canonical notch ligands." Canonical notch ligands are characterized by extracellular domains typically comprising an N-terminal (NT) domain followed by a Delta/Serrate/LAG-2 (DSL) domain and multiple tandemly arranged Epidermal Growth Factor (EGF)-like repeats. The DSL
30 domain together with the flanking NT domain and the first two EGF repeats containing the

Delta and OSM-11-like proteins (DOS) motif are typically required for canonical ligands to bind Notch. The intracellular domains of some canonical ligands contain a carboxy-terminal PSD-95/Dlg/ZO-1-ligand (PDZL) motif that plays a role independent of Notch signaling. *C. elegans* DSL ligands lack a DOS motif but have been proposed to cooperate with DOS-only
5 containing ligands to activate Notch signaling.

In various embodiments, the HSC/HSPC population is cultured in an artificial thymic organoid (ATO). See, Hagen, M. et al. (2019). The ATO will include culture of HSCs (or aggregates of HSCs) with a Notch ligand-expressing stromal cell line in serum-free conditions. The artificial thymic organoid is a 3D system, inducing differentiation of
10 hematopoietic precursors to naive $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells. In some embodiments, an artificial thymic organoid comprises DLL4 and BMP2, or functional fragments thereof.

In some embodiments, progenitor NK cells or T cells are isolated by enrichment for CD7 expression. In some embodiments, progenitor T cells are expanded as described in US
15 2020/0308540, which is hereby incorporated by reference in its entirety. For example, the cells may be expanded by exposing the cells to an aryl hydrocarbon receptor antagonist including, for example, SR1 or an SR1-derivative. See also, Wagner et al., *Cell Stem Cell* 2016;18(1):144-55. In some embodiments, the compound that promotes expansion includes a pyrimidoindole derivative including, for example, UM171 or UM729 (see US
20 2020/0308540, which is hereby incorporated by reference).

Differentiation to progenitor NK cells or progenitor of T cells from which NK cells can be generated can further include in some embodiments the presence of stem cell factor (SCF), Flt3L and interleukin (IL)-7. In various embodiments, $CD7^+$ progenitor T cells created express CD1a. The $CD7^+$ progenitor NK cells or T cells do not express CD34 or
25 express a diminished level of CD34 compared to the HSC population. In some embodiments, the $CD7^+$ progenitor T cells (or a portion thereof) further express CD5. Accordingly, the phenotype of the progenitor T cells may be $CD7^+CD1a^+$. In some embodiments, the phenotype of the progenitor T cells is $CD7^+CD5^+$. In some embodiments, the progenitor T cells are $CD7^+CD1a^+CD5^+$, and optionally $CD34^+$. In some embodiments, the progenitor T

cells exhibit a diminished level of CD34 expression, minimal CD34 expression (compared to the HSC population), or no CD34 expression. In some embodiments, CD34 expression is diminished in the population by at least about 50%, or at least about 75%, relative to the HSC population.

5 In some embodiments, the Notch ligand is an anti-Notch (agonistic) antibody that can bind and engage Notch signaling. In some embodiments, the antibody is a monoclonal antibody (including a human or humanized antibody), a single chain antibody (scFv), a nanobody, or other antibody fragment or antigen-binding molecule capable of activating the Notch signaling pathway.

10 In some embodiments, the Notch ligand is a Delta family Notch ligand. The Delta family ligand in some embodiments is Delta-1 (Genbank Accession No. AF003522, *Homo sapiens*), Delta-like 1 (DLL1, Genbank Accession No. NM_005618 and NP_005609, *Homo sapiens*; Genbank Accession No. X80903, 148324, *M. musculus*), Delta-4 (Genbank Accession No. AF273454, BAB18580, *Mus musculus*; Genbank Accession No. AF279305, 15 AAF81912, *Homo sapiens*), and/or Delta-like 4 (DLL4; Genbank Accession. No. Q9NR61, AAF76427, AF253468, NM_019074, *Homo sapiens*; Genbank Accession No. NM 019454, *Mus musculus*). Notch ligands are commercially available or can be produced, for example, by recombinant DNA techniques.

20 In some embodiments, the Notch ligand is a DLL4 having one or more affinity enhancing mutations, such as one or more (or all) of: G28S, F107L, I143F, H194Y, L206P, N257P, T271L, F280Y, S301R and Q305P, with respect to hDLL4. See Gonzalez-Perez, et al., Affinity-matured DLL4 ligands as broad-spectrum modulators of Notch signaling, *Nature Chemical Biology* (2022).

25 In some embodiments, the Notch ligand comprises an amino acid sequence that is at least about 70%, or at least about 80%, or at least about 90%, or at least about 95%, or at least about 97% identical (e.g., about 100% identical) to human DLL1 or DLL4 Notch ligand. Functional derivatives of Notch ligands (including fragments or portions thereof) will be capable of binding to and activating a Notch receptor. Binding to a Notch receptor

may be determined by a variety of methods known in the art including in vitro binding assays and receptor activation/cell signaling assays.

In various embodiments, the Notch ligands are soluble, and are optionally immobilized on microparticles or nanoparticles, which are optionally paramagnetic to allow
5 for magnetic enrichment or concentration processes. In still other embodiments, the Notch ligands are immobilized on a 2D or 3D culture surface, optionally with other adhesion molecules such as VCAM-1. See US 2020/0399599, which is hereby incorporated by reference in its entirety. In other embodiments, the beads or particles are polymeric (e.g., polystyrene or PLGA), gold, iron dextran, or constructed of biological materials, such as
10 particles formed from lipids and/or proteins. In various embodiments, the particle has a diameter or largest dimension of from about 0.01 μm (10 nm) to about 500 μm (e.g., from about 1 μm to about 7 μm). In still other embodiments, polymeric scaffolds with conjugated ligands can be employed, as described in WO 2020/131582, which is hereby incorporated by reference in its entirety. For example, scaffold can be constructed of polylactic acid,
15 polyglycolic acid, PLGA, alginate or an alginate derivative, gelatin, collagen, agarose, hyaluronic acid, poly(lysine), polyhydroxybutyrate, poly-epsilon-caprolactone, polyphosphazines, poly(vinyl alcohol), poly(alkylene oxide), poly(ethylene oxide), poly(allylamine), poly(acrylate), poly(4- aminomethylstyrene), pluronic polyol, polyoxamer, poly(uronic acid), poly(anhydride), poly(vinylpyrrolidone), and any
20 combination thereof. In some embodiments, the scaffold comprises pores having a diameter between about 1 μm and 100 μm .

In some embodiments, the C-terminus of the Notch ligand is conjugated to the selected support. In some embodiments, this can include adding a sequence at the C-terminal end of the Notch ligand that can be enzymatically conjugated to the support, for example,
25 through a biotin molecule. In another embodiment, a Notch ligand-Fc fusion is prepared, such that the Fc segment can be immobilized by binding to protein A or protein G that is conjugated to the support. Of course, any of the known protein conjugation methods can be employed.

Thus, in various embodiments, the Notch ligand is immobilized, functionalized, and/or embedded in 2D or 3D culture system. The Notch ligand may be incorporated along with a component of extracellular matrix, such as one or more selected from fibronectin, RetroNectin, and laminin. In some embodiments, the Notch ligand and/or component of
5 extracellular matrix are embedded in inert materials providing 3D culture conditions. Exemplary materials include, but are not limited to, cellulose, alginate, and combinations thereof. In some embodiments, the Notch ligand, a component of extracellular matrix, or combinations thereof, are in contact with culture conditions providing topographical patterns and/or textures (e.g., roughness) to cells conducive to differentiation and/or expansion.

10 In some embodiments, HSCs are differentiated to progenitor T cells by culture in medium comprising TNF- α and/or antagonist of aryl hydrocarbon / dioxin receptor (SR1), and in the presence of Notch ligand. See US 2020/0390817, US 2021/0169934, and US 2021/0169935, which are hereby incorporated by reference in its entirety. In some
15 embodiments the HSCs are cultured in a medium comprising TNF- α , IL-7, thrombopoietin (TPO), Flt3L, and stem cell factor (SCF), and optionally SR1, in the presence of an immobilized Delta-Like-4 ligand and a fibronectin fragment. In some embodiments, the cells are cultured with RetroNectin, which is a recombinant human fibronectin containing three functional domains: the human fibronectin cell-binding domain (C-domain), heparin-binding domain (H-domain), and CS-1 sequence domain. In some embodiments, cells are
20 cultured in the presence of an immobilized Delta-Like-4 ligand and a RetroNectin. In some embodiments, cells are cultured in the presence of an immobilized Delta-Like-4 ligand, TNF-alpha, and a RetroNectin. In some embodiments, cells are cultured in the presence of an immobilized Delta-Like-1 ligand and a RetroNectin. In some embodiments, cells are cultured in the presence of SFIP3 and RetroNectin. In some embodiments, cells are cultured
25 in the presence of an immobilized Delta-Like-4 ligand and SHH molecules and/or functional derivatives thereof. Exemplary fibronectin fragments include one or more RGDS, CS-1, and heparin-binding motifs. Fibronectin fragments can be free in solution or immobilized to the culture surface or on particles. In some embodiments, cells are cultured for 5 to 7 days to prepare CD7+ progenitor T cells, which can then be utilized to generate the NK cells from
30 the T cell progenitors that retain the potential to generate NK cells.

In some embodiments, presence of cytokines and/or growth factors are desired for differentiation of the HSCs or cells isolated therefrom, and these can be selected from or comprise (or consist essentially of): TPO, SCF, Flt3L, IL3, IL7, and SDF-1a. In addition, T cells or progenitor T cells that arise in the culture are further cultured in the presence of, for example, IL-3 and/or IL-15. IL-15 is added to support NK cell differentiation, and IL-3 is optionally excluded after formation of early NK cells. In some embodiments, the medium is free of one or more of VEGF, bFGF, TPO, BMP activators and ROCK inhibitors, to initiate differentiation of the pre-NK cell progenitors to NK cell progenitors or NK cells. In some embodiments, the pluripotent stem cell-derived NK progenitors are CD3-CD45+CD56+CD7+. In some embodiments, the pluripotent stem cell-derived NK cells are CD3-CD45+CD56+, and optionally further defined by NKp46+, CD57+ and CD16+.

In some embodiments, NK cells are generated from progenitor T cells as described in US 10,266,805, which is hereby incorporated by reference in its entirety. For example, the progenitor T cells can give rise to NK cells when cultured with IL-15. In some embodiments, the NK cell expresses a CAR, based on gene editing of iPSC, embryonic bodies, hCD34+ cells, or NK cells, or via mRNA expression in NK cells. Additionally, or optionally, the NK CAR may be engineered to express cytokines (e.g., IL-15) to make to make the NK-CAR more potent in targeting tumors.

In a non-limiting example, NK cells can be efficiently transduced by a vector, such as but not limited to retroviral or nonintegrating viral vectors (e.g., adenoviral, adeno-associated viral, integration-deficient retro-lentiviral, poxviral) or nonviral vectors (e.g., plasmid vectors, artificial chromosomes) or episomal or episomal hybrid vectors) carrying a second-generation CAR targeting tumor antigen (e.g., CD19, CD38, CD33, CD47, CD20 etc.). CAR expression can be demonstrated across the different NK-cell subsets following routine protocols. NK CAR cell (e.g., CAR.CD19-NK cells, CAR.CD38-NK cells, CAR.CD33-NK cells, CAR.CD47-NK cells, CAR.CD20-NK cells etc.) may display higher tumor activity (e.g., antileukemic activity) toward CD19, CD38, CD33, CD47, CD20 cell lines and primary blasts obtained from patients, for example, with B-cell precursor ALL compared with unmodified NK cells.

- CARs are designed to enhance a cells ability to recognize, bind to, and kill tumor cells. In some embodiments, the CAR enhances the NK cell's ability to recognize tumor cells. In some embodiments, the CAR enhances the NK cells anti-tumor activity. In some embodiments, and without limitation, the CAR is a G protein coupled receptor 87 (GPR87)
- 5 CAR and solute carrier family 7 member 11 (SLC7A11 (xCT)) CAR, TNF receptor superfamily member 17 (BCMA) CAR, CD30 CAR, CD19 CAR, CD22-CAR, CD33 CAR, CD133-CAR, NKG2D CAR (or a CAR or receptor comprising an NKG2D ectodomain), mesothelin-CAR, CD70 CAR, NKp30 CAR, CD73 CAR, or CAR-NK cells targeting the following tumors or tumor antigens:
- 10 (i) Human epidermal growth factor receptor 2(HER2) - ovarian cancer, breast cancer, glioblastoma, colon cancer, osteosarcoma, and medulloblastoma;
- (ii) Epidermal growth factor receptor(EGFR) - non-small cell lung cancer, epithelial carcinoma, and glioma;
- (iii) Mesothelin - mesothelioma, ovarian cancer, and pancreatic adenocarcinoma;
- 15 (iv) Prostate-specific membrane antigen(PSMA) - prostate cancer;
- (v) Carcinoembryonic antigen(CEA) - pancreatic adenocarcinoma, breast cancer, and colorectal carcinoma;
- (vi) Glypican-3 - hepatocellular carcinoma;
- (vii) Variant III of the epidermal growth factor receptor (EGFRvIII) – glioblastoma;
- 20 (viii) Disialoganglioside 2(GD2) - neuroblastoma and melanoma;
- (ix) Carbonic anhydrase IX(CAIX) - renal cell carcinoma;
- (x) Interleukin-13Ra2 – glioma;
- (xi) Fibroblast activation protein(FAP) - malignant pleural mesothelioma;
- (xii) L1 cell adhesion molecule(L1-CAM) - neuroblastoma, melanoma, and ovarian;

(xiii) Cancer antigen 125 (CA 125) - epithelial ovarian cancer;

(xiv) Cluster of differentiation 133 (CD 133) - glioblastoma and cholangiocarcinoma, adenocarcinoma;

(xv) Cancer/testis antigen 1B(CTAG1B) - melanoma and ovarian cancer;

5 (xvi) Mucin 1 - seminal vesicle cancer;

(xvii) Folate receptor-a(FR-a) - ovarian cancer;

(xviii) Growth factor receptor selected from one or more of ErbB1, ErbB2, ErbB3, or ErbB4, IGF1R, IGF2R, T β R I-II, VEGFR1, VEGFR2, VEGFR3, PDGFR (α/β) or FGFR1 through 4.

10 The cell populations, or cells derived therefrom (e.g., progeny) can be used with FDA approved CAR-T therapy, such as, Tisagenlecleucel, also known as tisa-cel (Kymriah), Axicabtagene ciloleucel, also known as axi-cel (Yescarta), Brexucabtagene autoleucel, also known as brexu-cel (Tecartus), Lisocabtagene maraleucel, also known as liso-cel (Breyanzi), Idecabtagene vicleucel, also known as ide-cel (Abecma), Ciltacabtagene
15 autoleucel, also known as cilta-cel (Carvykti) or any other CAR-based therapy which damage the normal cells during their therapeutic applications.

Therefore, in some aspects and embodiments of the invention, a genetically modified NK cell line or a precursor or progeny thereof is engineered to express a chimeric antigen receptor (CAR) on a cell surface, and particularly a CAR that specifically binds to a growth
20 factor receptor. Most typically, the CAR comprises an intracellular domain from the Fc epsilon receptor gamma (Fc epsilon RI gamma). However, in further contemplated embodiments the CAR may also comprise a T cell receptor (TCR) CD3 zeta (CD3zeta) intracellular domain, alone or in combination with additional components from the second or third generation CAR constructs (e.g., CD28, CD134, CD137, and/or ICOS).

25 In an aspect, the NK cell or precursor or progenies thereof is modified to express an autocrine growth stimulating cytokine or variant thereof. Additionally, or optionally, the

genetically modified NK cells will also express a recombinant CD16 or high-affinity variant thereof to impart to the cells target specific ADCC.

In an aspect, the NK cell or precursor or progenies thereof is modified to express a homing receptor. "Homing receptor" refers to a receptor that activates a cellular pathway that results directly or indirectly in the cell migrating toward a target cell or tissue. For example, homing receptors expressed by leukocytes are used by leukocytes and lymphocytes to enter secondary lymphoid tissues via high endothelial venules. Homing receptors can also be used by cells to migrate toward the source of a chemical gradient, such as a chemokine gradient. Examples of homing receptors include G-protein coupled receptors such as chemokine receptors, including CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, CXCR7, CX3CR1, XCR1, CCXCR, D6, and DARC; cytokine receptors; cell adhesion molecules such as selectins, including L-selectin (CD62L), integrins such as alpha 4 beta 7 integrin, LPAM-1, and LFA-1. Homing receptors generally bind to cognate ligands on the target tissues or cell. In some embodiments, homing receptors bind to addressins on the endothelium of venules, such as mucosal vascular addressing cell adhesion molecule 1 (MAdCAM-1).

In other aspects, the invention provides a cell population, or pharmaceutically acceptable composition thereof, produced by the method described herein. In some embodiments, the cell population is a progenitor NK cell population capable of engraftment in a thymus, spleen, or secondary lymphoid organ upon administration to a subject in need. In various embodiments, the composition for cellular therapy is prepared that comprises the cell population and a pharmaceutically acceptable vehicle. The pharmaceutical composition may comprise at least about 10^2 cells, or at least about 10^3 , or at least about 10^4 , or at least about 10^5 , or at least about 10^6 , or at least about 10^7 , or at least about 10^8 cells, or at least about 10^9 cells, or at least about 10^{10} cells, or at least about 10^{11} cells, or at least about 10^{12} cells, or at least about 10^{13} cells, or at least about 10^{14} cells. For example, in some embodiments, the pharmaceutical composition is administered, comprising from about 100,000 to about 400,000 cells per kilogram (e.g., about 200,000 cells /kg). In other embodiments, cells are administered at from about 10^5 to about 5×10^5 cells per kilogram of body weight (e.g., about 2.5×10^5 cells /kg), or from about 10^6 to about 5×10^6 cells per

kilogram (e.g., about 2.5×10^6 cells /kg), or from about 5×10^6 to about 10^7 cells per kilogram (e.g., about 5×10^6 cells /kg) or from about 10^7 to about 10^8 cells per kilogram (e.g., about 5×10^7 cells /kg) or from about 10^8 to about 10^9 cells per kilogram (e.g., about 5×10^8 cells /kg) or from about 10^9 to about 10^{10} cells per kilogram or from about 10^{10} to about 10^{11} cells
5 or from about 10^{11} to about 10^{12} cells per kilogram or from about 10^{12} to about 10^{13} cells per kilogram or from about 10^{13} to about 10^{14} cells per kilogram of a recipient's body weight.

In some embodiments, the NK cells are derived from HLA-edited iPSCs as described. For example, in some embodiments the NK cells are HLA-A^{neg}, homozygous for both HLA-B and HLA-C, and HLA-DPB1^{neg} and HLA-DQB1^{neg}. In some embodiments, the
10 NK cells are further homozygous for HLA-DRB1.

The cell composition of this disclosure (e.g., prepared according to this disclosure) may further comprise a pharmaceutically acceptable excipient or a carrier. Such excipients or carrier solutions also can contain buffers, diluents, and other suitable additives. A buffer refers to a solution or liquid whose chemical makeup neutralizes acids or bases without a
15 significant change in pH. Examples of buffers envisioned by the invention include, but are not limited to, normal/physiologic saline (0.9% NaCl), 5% dextrose in water (D5W), Dulbecco's phosphate buffered saline (PBS), Ringer's solution. The composition may comprise a vehicle suitable for intravenous infusion or other administration route, and the composition may include a suitable cryoprotectant. An exemplary carrier is DMSO (e.g.,
20 about 10% DMSO). Other carriers may include dimethoxy ethane (DME), N,N-dimethylformamide (DMF), or dimethylacetamide, including mixtures or combinations thereof. Cell compositions may be provided in implantable devices (e.g., scaffolds) or in bags or in vials, tubes or a container in an appropriate volume and stored frozen until use.

The pharmaceutical compositions for use in the disclosed methods may also contain
25 additional therapeutic agents for treatment of the particular targeted disorder. For example, a pharmaceutical composition may also include cytokines and growth factors (interleukins, interferons, FGF, VEGF, PDGF, PIGF, STAT etc.). Such additional factors and/or agents may be included in the pharmaceutical composition to produce advantages of the therapeutic

approaches disclosed herein, i.e., provide improved therapeutic efficacy with reduced systemic toxicity.

The NK cells or the CAR-NK cells can be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disease or disorder being treated, the particular mammal being treated (e.g., human), the clinical condition of the individual patient, the cause of the disease or disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The therapeutically effective amount of the cells to be administered will be governed by such considerations.

The formulation herein may also contain more than one active agent as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide a cytotoxic agent, cytokine, or immunosuppressive agent. The effective amount of such other agents depends on the amount of acceptable carriers, excipients, or stabilizers, present in the formulation, the type of disease or disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

In other aspects, the disclosure provides a method for cell therapy, comprising administering the cell population described herein, or pharmaceutically acceptable composition thereof, to a human subject in need thereof. In various embodiments, the subject has a cancer or infectious disease, such as a viral infection. In various embodiments, the methods described herein are used to treat blood (malignant and non-malignant), bone marrow, immune diseases, and infectious diseases. In various embodiments, the human subject has a condition comprising one or more of lymphopenia, a cancer, an immune deficiency, an autoimmune disease.

Examples of diseases include various autoimmune disorders, including but not limited to, alopecia areata, autoimmune hemolytic anemia, autoimmune hepatitis, dermatomyositis, diabetes (type 1), some forms of juvenile idiopathic arthritis, glomerulonephritis, Graves' disease, Guillain-Barre syndrome, idiopathic thrombocytopenic

purpura, myasthenia gravis, some forms of myocarditis, multiple sclerosis, pemphigus/pemphigoid, pernicious anemia, polyarteritis nodosa, polymyositis, primary biliary cirrhosis, psoriasis, rheumatoid arthritis, scleroderma/systemic sclerosis, Sjogren's syndrome, systemic lupus, erythematosus, some forms of thyroiditis, some forms of uveitis, vitiligo, granulomatosis with polyangiitis (Wegener's). Hematological malignancies that can be treated include but are not limited to acute and chronic leukemias, lymphomas, multiple myeloma and myelodysplastic syndromes. Infectious diseases that can be treated include but are not limited to HIV--(human immunodeficiency virus), RSV--(Respiratory Syncytial Virus), EBV--(Epstein-Barr virus), CMV--(cytomegalovirus), adenovirus- and BK polyomavirus-associated disorders. Other conditions include: skeletal dysplasia, hemoglobinopathies; anemias, including but not limited to, iron-deficiency anemia, pernicious anemia, aplastic anemia, sickle cell anemia, Vitamin deficiency anemia, and hemolytic anemia; a bone marrow failure syndrome, and certain genetic disorders (e.g., a genetic disorder impacting the immune system).

15 In some embodiments, the subject has cancer, such as a hematological malignancy, including but not limited to leukemia, lymphoma and multiple myeloma; or a solid tumor, including but not limited to, tumor of the brain, prostate, breast, lung, colon, uterus, skin, liver, bone, pancreas, ovary, testes, bladder, kidney, head, neck, stomach, cervix, rectum, larynx, or esophagus.

20 In some embodiments, the subject has a condition selected from acute myeloid leukemia; acute lymphoblastic leukemia; chronic myeloid leukemia; chronic lymphocytic leukemia; myeloproliferative disorders; myelodysplastic syndromes; multiple myeloma; Non-Hodgkin lymphoma; Hodgkin disease; aplastic anemia; pure red-cell aplasia; paroxysmal nocturnal hemoglobinuria; Fanconi anemia; thalassemia major; sickle cell anemia; severe combined immunodeficiency (SCID); Wiskott-Aldrich syndrome; hemophagocytic lymphohistiocytosis; inborn errors of metabolism; severe congenital neutropenia; Shwachman-Diamond syndrome; Diamond-Blackfan anemia; and leukocyte adhesion deficiency.

With respect to the use of HLA-edited NK cells (e.g., derived from gene-edited iPSCs), the subject can be matched at retained HLA loci, such as one or more (or all) of HLA-B, HLA-C, and HLA-DRB1.

Agents such as hormones, growth factors and cytokines antibodies that may be co-administered with the derivatives of cell line(s) or banks of expanded primary cells (e.g., iPSC-derived HSCs or progenitors or progenies thereof) of the present invention include molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor vmc, factor IX, tissue factor (TF), and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin, such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT4, NT-5, or NT-6), or a nerve growth factor such as NGF-beta; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; fibroblast growth factor receptor 2 (FGFR2), epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta4, or TGF-beta5; bone morphogenetic protein (BMP), including BMP1, BMP6, BMP7, and BMP-receptor 2; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins, hepatocyte growth factor (HGF), EpCAM, GD3, FLT3, PSMA, PSCA, MUC1, MUC16, STEAP, CEA, TENB2, EphA receptors, EphB receptors, folate receptor, FOLR1, mesothelin, cripto, alphavbeta6,

integrins, VEGF, VEGFR, EGFR, transferrin receptor, IRTA1, IRTA2, IRTA3, IRTA4, IRTA5; CD proteins such as CD2, CD3, CD4, CD5, CD6, CD8, CD11, CD14, CD19, CD20, CD21, CD22, CD25, CD26, CD28, CD30, CD33, CD36, CD37, CD38, CD40, CD44, CD52, CD55, CD56, CD59, CD70, CD79, CD80, CD81, CD103, CD105, CD134, CD137, CD138, CD152, TNF alpha, IFN alpha, GM-CSF, IL-3 or an antibody which binds to one or more tumor-associated antigens or cell-surface receptors; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon, such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-2, IL-6, IL-12, IL-23, IL-12/23 p40, IL-17, IL-15, IL-21, IL-1a, IL-1b, IL-18, IL-8, IL-4, IL-3, and IL-5; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the HIV envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins, such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; endoglin, c-Met, c-kit, IGF1R, PSGR, NGEP, PSMA, PSCA, LGR5, B7H4, TAG72 (tumor-associated glycoprotein 72) and fragments of any of the above-listed polypeptides.

Examples of antibodies or fragments thereof that may be administered include, but are not limited to, anti-PD-L1 antibodies, abciximab (Reopro), adalimumab (Humira, Amjevita), alefacept (Amevive), alemtuzumab (Campath), basiliximab (Simulect), belimumab (Benlysta), bezlotoxumab (Zinplava), canakinumab (Ilaris), certolizumab pegol (Cimzia), cetuximab (Erbix), daclizumab (Zenapax, Zinbryta), denosumab (Prolia, Xgeva), efalizumab (Raptiva), golimumab (Simponi, Simponi Aria), inflectra (Remicade), ipilimumab (Yervoy), ixekizumab (Taltz), natalizumab (Tysabri), nivolumab (Opdivo), olaratumab (Lartruvo), omalizumab (Xolair), palivizumab (Synagis), panitumumab (Vectibix), pembrolizumab (Keytruda), rituximab (Rituxan), tocilizumab (Actemra), trastuzumab (Herceptin), secukinumab (Cosentyx), ranibizumab, abciximab, raxibacumab, caplacizumab, infliximab, bevacizumab, dabigatran, Idarucizumab, or ustekinumab (Stelara) or a combination thereof. Furthermore, the antibodies may be selected from anti-estrogen receptor antibody, anti-progesterone receptor antibody, anti-p53 antibody, anti-EGFR antibody, anti-cathepsin D antibody, anti-Bcl-2 antibody, anti-E-cadherin antibody, anti-CA125 antibody, anti-CA15-3 antibody, anti-CA19-9 antibody, anti-c-erbB-2 antibody,

anti-P-glycoprotein antibody, anti-CEA antibody, anti-retinoblastoma protein antibody, anti-ras oncoprotein antibody, anti-Lewis X antibody, anti-Ki-67 antibody, anti-PCNA antibody, anti-CD3 antibody, anti-CD4 antibody, anti-CD5 antibody, anti-CD7 antibody, anti-CD8 antibody, anti-CD9/p24 antibody, anti-CD1-antibody, anti-CD11c antibody, anti-
 5 CD13 antibody, anti-CD14 antibody, anti-CD15 antibody, anti-CD19 antibody, anti-CD20 antibody, anti-CD22 antibody, anti-CD23 antibody, anti-CD30 antibody, anti-CD31 antibody, anti-CD33 antibody, anti-CD34 antibody, anti-CD35 antibody, anti-CD38 antibody, anti-CD39 antibody, anti-CD41 antibody, anti-LCA/CD45 antibody, anti-CD45RO antibody, anti-CD45RA antibody, anti-CD71 antibody, anti-CD95/Fas antibody,
 10 anti-CD99 antibody, anti-CD100 antibody, anti-S-100 antibody, anti-CD106 antibody, anti-ubiquitin antibody, anti-c-myc antibody, anti-cytokeratin antibody, anti-lambda light chains antibody, anti-melanosomes antibody, anti-prostate specific antigen antibody, anti-tau antigen antibody, anti-fibrin antibody, anti-keratins antibody, and anti-Tn-antigen antibody.

Co-administration does not require the therapeutic agents to be administered
 15 simultaneously, if the timing of their administration is such that the pharmacological activities of the additional therapeutic agent and the active ingredient(s) in the pharmaceutical composition overlap in time, thereby exerting a combined therapeutic effect. In general, each agent will be administered at a dose and on a time, schedule determined for that agent.

20 The present pharmaceutical compositions may be administered in any dose appropriate to achieve a desired outcome. In some embodiments, the desired outcome is a reduction in the intensity, severity, frequency, and/or delay of onset of one or more symptoms of infection. In some embodiments, the desired outcome is the inhibition or prevention of infection. The dose required will vary from subject to subject depending on
 25 the species, age, weight, and general condition of the subject, the severity of the infection being prevented or treated, the particular composition being used, and its mode of administration.

In some embodiments, pharmaceutical compositions in accordance with the present disclosure are administered in single or multiple doses. In some embodiments, the

pharmaceutical compositions are administered in multiple doses administered on different days.

As used herein, the term “about” means $\pm 10\%$ of the associated numerical value.

Certain aspects and embodiments of this disclosure are further described with
5 reference to the following examples.

EXAMPLES

Example 1 – ETV2 over-expression increases the yield of hemogenic endothelial cells and enhances the CD34⁺ cell formulation during iPSC differentiation but does not affect pluripotency.

10 Methods

iPSCs were developed from hCD34⁺ cells by episomal reprogramming as known in the art and essentially as described in Yu, et al. Induced pluripotent stem cell lines derived from human somatic cells, *Science* 318, 1917-1920, (2007); and J. Yu, et al. Human induced pluripotent stem cells free of vector and transgene sequences. *Science* 324, 797-801, (2009).
15 Embryoid Bodies and hemogenic endothelium differentiation was performed essentially as described in: R. Sugimura, et al., Haematopoietic stem and progenitor cells from human pluripotent stem cells. *Nature* 545, 432-438, (2017); C. M. Sturgeon, et al, Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells. *Nat Biotechnol* 32, 554-561, (2014); J. Yu, et al. Induced pluripotent stem cell
20 lines derived from human somatic cells. *Science* 318, 1917-1920, (2007); and J. Yu, et al. Human induced pluripotent stem cells free of vector and transgene sequences. *Science* 324, 797-801, (2009).

Briefly, hiPSC were dissociated and resuspended in media supplemented with L-glutamine, penicillin/streptomycin, ascorbic acid, human holo-Transferrin,
25 monothioglycerol, BMP4, and Y-27632. Next, cells were seeded in 10 cm dishes (EZSPHERE or low attachment plate) for the EB formation. On Day 1, bFGF and BMP4 were added to the medium. On Day 2, the media was replaced with a media containing SB431542, CHIR99021, bFGF, and BMP4. On Day 4, the cell media was replaced with a

media supplemented with VEGF and bFGF. On day 6, the cell media was replaced with a media supplemented with bFGF, VEGF, interleukin (IL)-6, IGF-1, IL-11, SCF, and EPO. Cells were maintained in a 5% CO₂, 5% O₂, and 95% humidity incubator. To harvest the CD34⁺ cells, the EBs were dissociated on day 8, cells were filtered through a 70 µm strainer, and CD34⁺ cells were isolated by CD34 magnetic bead staining.

Results

An adenoviral vector containing both ETV2 and GFP sequences under the control of the EF1A promoter was used to transduce induced pluripotent stem cells (iPSCs). After the transduction, about 45% of the iPSC culture was observed to be GFP positive, thus confirming ETV2 overexpression (ETV2-OE). It was further observed that ETV2-OE in iPSC cells preserves the pluripotency properties of iPSCs as shown by the stemness marker expression TRA-1-60 (**FIG. 1**). **FIG. 1** shows FACS plots representative of transduction efficiency of iPSC with an adenoviral vector to overexpress the ETV2 and the GFP sequences.

Next, the ETV2-OE-iPSCs were differentiated (along with control iPSCs transduced with a vector bearing the GFP sequence without ETV2) to embryoid bodies and subsequently to hemogenic endothelial cells (Strugeon *et al.*, 2014). The results suggest that the overexpression of ETV2 boosts the formation of hemogenic endothelial cells as demonstrated by the expression of the CD34⁺ and CD31⁺ markers within the CD235a⁻ population (**FIG. 2**). Specifically, **FIG. 2** shows representative flow cytometric analysis of hemogenic endothelial cells (defined here as CD235a⁻CD34⁺CD31⁺) and relative quantification demonstrates that ETV2-OE enhances the formation of hemogenic endothelial cells as compared to controls.

Moreover, the results suggest that ETV2-OE enhances the formation of the CD34⁺ cells (**FIG. 3**). **FIG. 3** shows representative flow cytometric analysis of CD34⁺ cells and relative quantification demonstrates that ETV2-OE enhances the CD34⁺ cell formation.

Overall, these data indicate that ETV2 overexpression in iPSCs does not affect their pluripotency properties and facilitates their ability to undergo the hemogenic endothelial and hematopoietic differentiations.

Example 2 – iPSC-derived HSCs generated with Piezo1 activation undergo T cell differentiation similar to Bone Marrow-derived HSCs.

Methods

To analyze the EHT, EB-derived CD34+ cells were suspended in medium containing Y-27632, TPO, IL-3, SCF, IL-6, IL-11, IGF-1, VEGF, bFGF, BMP4, and FLT3. After the cells had adhered to the bottom of the wells for approximately 4-18 hours (by visual inspection), Yoda1 was added to the cultures for some experiments. After 4-7 days, the cells were collected for analysis.

iPSCs were differentiated to embryoid bodies for 8 days. At day 8, CD34+ cells from iPSC-derived embryoid bodies were harvested and cultured for additional 5 to 7 days to induce endothelial-to-hematopoietic (EHT) transition. Then, CD34+ cells were harvested from the EHT culture between day 5 to day 7 for further hematopoietic lineage differentiation.

CD34+ cells, harvested from the EHT culture between day 5-7 (or total of day 13-21 differentiation from iPSCs), were seeded in 48-well plates pre-coated with rhDL4 and RetroNectin. T lineage differentiation was induced in media containing aMEM, FBS, ITS-G, 2BME, ascorbic acid-2-phosphate, Glutamax, rhSCF, rhTPO, rhIL7, FLT3L, rhSDF-1a, and SB203580.

Between day 2 to day 6, 80% of the media was changed every other day. At D7, cells were transferred into new coated plates and analyzed for the presence of pro-T cells (CD34+ CD7+ CD5+/-).

Between day 8 to day 13, 80% of the media was changed every other day. At D14, 100,000 cells/wells were transferred to a new coated plate and the cells analyzed for the presence of pre-T cells (CD34- CD7+ CD5+/-).

Between day 15 to day 20, 80% of the media was changed every other day. Cells were harvested at D21, and the cells were analyzed for CD3, CD8, CD5, CD7, TCRab expression, as surrogates for T cells, via FACS, and/or activated using CD3/CD28 beads to evaluate their functional properties.

5 After 21 days of differentiation, cells were collected and re-seeded at approximately 80,000 cells into new 96-well culture plates in RPMI 1640 (no L-glutamine; no phenol red) plus FBS, L-glutamine, IL-2, and then activated with 1:1 CD3/CD28 beads. After 72 hours of activation with CD3/CD28 beads, cells were analyzed for CD3, CD69, CD25 expression by FACS and IFN- γ expression using RT-qPCR. The supernatant was analyzed by ELISA.

10 Results

FIG. 4A and **FIG. 4B** show that iPSC-derived HSCs that are derived with Piezo1 activation undergo pro-T cell differentiation similar to bone marrow (BM)-HSCs. Further, **FIG. 5A** and **FIG. 5B** show that iPSC-derived HSCs generated with Piezo1 activation undergo T cell differentiation and can be activated with CD3/CD28 beads similar to BM-
15 HSCs. **FIG. 6** shows that iPSC-derived HSCs generated with Piezo1 activation can differentiate to functional T cells, as demonstrated by INF γ expression upon stimulation with CD3/CD28 beads. Together, these results demonstrate that Piezo1 activation during HSC formation enhances HSC ability to further differentiate to progenitor T cells and functional T cells *ex vivo*, as well as related lineages such as NK cells.

20 **FIG. 7** shows HSCs derived from iPSC differentiation (D8+7) differentiate to NK cells and are activated by pharmacological stimulation. **FIG. 7** shows that HSCs derived from iPSC differentiation (D8+7, with Yoda1 or “Y”) are activated pharmacologically (measured by the activity of the functional marker CD107a).

FIG. 8A and **FIG. 8B** show HSC-derived NK cells (D8+7) outperform the bone
25 marrow derived NK cells in killing of tumor cells. **FIGS. 8A** and **8B** shows superior killing of tumor cells by the HSC-derived NK cells as compared to the BM-derived NK cells. The ratio of NK cells to tumor cells is 5:1.

Example 3 – Evaluating Off-Target Editing in HLA Knockout HSCs

HLA typing of the HLA edited HSC clones was performed to check for unwanted editing and to ensure that no major editing events, e.g., deletion(s), occurred within other regions of chromosome 6. Sequencing methods and analyses were performed to evaluate the degree of gRNA off-target activity and to select gRNAs that represent a low risk of affecting non-target HLA genes.

Sequencing was performed by using in situ break labelling in fixed and permeabilized cells by ligating a full-length P5 sequencing adapter to end-prepared DSBs. Genomic DNA was extracted, fragmented, end-prepared, and ligated using a chemically modified half-functional P7 adapter. The resulting DNA libraries contained a mixture of functional DSB-labelled fragments (P5:P7) and non-functional genomic DNA fragments (P7:P7). Subsequent DNA sequencing of the DNA libraries enriched for DNA-labelled fragments, eliminating all extraneous, non-functional DNA. As the library preparation is PCR-free, each sequencing read obtained was equivalent to a single labelled DSB-end from a cell. This generated a DNA break readout, enabling the direct detection and quantification of genomic DSBs by sequencing without the need for error-correction and enabled mapping a clear list of off-target mutations.

Table 1 below summarizes the results of the editing strategy in two representative HLA edited clones relative to wild-type cells (gHSCs).

TABLE 1: Clonal HSC HLA knockouts.

Sample ID	Locus	Allele 1	Allele 2	Comments
WT (gHSC)	A	A*01:01:01	A*01:01:01	Not affected
	B	B*08:01:01	B*08:01:01	Not affected
	C	C*07:01:01	C*07:01:01	Not affected
	DPA1	DPA1*01:03:01	DPA1*02:01:02	Not affected

	DPB1	DPB1*01:01:01	DPB1*04:01:01	Not affected
	DQA1	DQA1*05:01:01	DQA1*05:01:01	Not affected
	DQB1	DQB1*02:01:01	DQB1*02:01:01	Not affected
	DRB1	DRB1*03:01:01	DRB1*03:01:01	Not affected
	DRB345	DRB3*01:01:02	DRB3*01:01:02	Not affected
Clone #1	A	xxx	xxx	Deletion in Exon 2
	B	B*08:01:01	B*08:01:01	Not affected
	C	C*07:01:01	C*07:01:01	Not affected
	DPA1	DPA1*01:03:01	DPA1*02:01:02	Not affected
	DPB1	xxx	xxx	Deletion in Exon 2
	DQA1	DQA1*05:01:01	DQA1*05:01:01	Not affected
	DQB1	xxx	xxx	Deletion in Exon 2
	DRB1	DRB1*03:01:01	DRB1*03:01:01	Not affected
Clone #2	DRB345	DRB3*01:01:02	DRB3*01:01:02	Not affected
	A	xxx	xxx	Deletion in Exon 2
	B	B*08:01:01	B*08:01:01	Not affected
	C	C*07:01:01	C*07:01:01	Not affected
	DPA1	DPA1*01:03:01	DPA1*02:01:02	Not affected
	DPB1	xxx	xxx	Deletion in Exon 2

	DQA1	DQA1*05:01:01	DQA1*05:01:01	Not affected
	DQB1	xxx	xxx	Deletion in Exon 2
	DRB1	DRB1*03:01:01	DRB1*03:01:01	Not affected
	DRB345	DRB3*01:01:02	DRB3*01:01:02	Not affected

Table 2 provides a non-limiting example of gRNAs used in the experiments which can be used to knock out expression of indicated HLA genes.

TABLE 2: Exemplary gRNA sequences

gRNA ID	Spacer sequence
HLA-A	CGTAGCCACGCGATGAAG (SEQ ID NO: 1)
	ATTTCTTCACATCCGTGTCC (SEQ ID NO: 2)
	GTCTCCTGGTCCCAATACTC (SEQ ID NO: 3)
	GTAGCCACGCGATGAAGC (SEQ ID NO: 4)
	TAGCCACGCGATGAAGCG (SEQ ID NO: 5)
	GAGGGTTCGGGGCGCCATGA (SEQ ID NO: 6)
	CCGGAACACACGGAATGTGA (SEQ ID NO: 7)
	ACAGCGACGCCGCGAGCCAG (SEQ ID NO: 8)
	CCACTCACAGATTGACCGAG (SEQ ID NO: 9)
	CGACGCCGCGAGCCAGAGGA (SEQ ID NO: 10)
	CAGATTGACCGAGTGGACCT (SEQ ID NO: 11)

	<p>CAGACTGACCGAGTGGACCT (SEQ ID NO: 12)</p> <p>ACAGACTGACCGAGTGGACC (SEQ ID NO: 13)</p> <p>GCCGGGCCGGGACACGGATG (SEQ ID NO: 14)</p> <p>CCAGGAGACACGGAATGTGA (SEQ ID NO: 15)</p> <p>CCAGTCACAGACTGACCGAG (SEQ ID NO: 16)</p> <p>TCGACAGCGACGCCGCGAGC (SEQ ID NO: 17)</p>
HLA-DPB1	<p>GAGATACATCTACAACCGGG (SEQ ID NO: 18)</p> <p>TGGAGAGATACATCTACAAC (SEQ ID NO: 19)</p> <p>GGTTGTAGATGTATCTCTCC (SEQ ID NO: 20)</p> <p>GGAGAGATACATCTACAACC (SEQ ID NO: 21)</p> <p>AGGAATGCTACGCGTTTAAT (SEQ ID NO: 22)</p>
HLA-DQB1	<p>CAGATACATCTATAACCGAG (SEQ ID NO: 23)</p> <p>CGAGTACTGGAACAGCCAGA (SEQ ID NO: 24)</p> <p>CCCGTTGGTGAAGTAGCACA (SEQ ID NO: 25)</p> <p>GTGCTACTTCACCAACGGGA (SEQ ID NO: 26)</p> <p>AGGTCGTGCGGAGCTCCAAC (SEQ ID NO: 27)</p> <p>AGGTGACAGTTGCTACCCGA (SEQ ID NO: 28)</p> <p>TCCCGTTGGTGAAGTAGCAC (SEQ ID NO: 29)</p> <p>AACTACGAGGTGGCGTACCG (SEQ ID NO: 30)</p>

	CTGTTCCAGTACTCGGCAAC (SEQ ID NO: 31)
	ACTACGAGGTGGCGTACCGC (SEQ ID NO: 32)
	CGAAGCGCACGTACTCCTCT (SEQ ID NO: 33)

The results show that the editing strategy was successful in selectively targeting the HLA-A, DPB1, and DQB1 genes without affecting the other HLA genes or introducing major deletion elsewhere.

5 These results were confirmed by a phenotypic analysis of the HLA edited clones by FACS and immunofluorescence. As shown in **FIGS. 9A** and **B**, HLA edited cells tested positive for overall expression of HLA class-I molecules, comparable to the overall expression of HLA class-I molecules of wild-type cells. Specific expression of HLA-A via immunofluorescence confirmed that HLA-A was not expressed in the HLA edited cells,
10 corroborating the finding that the gene editing strategy was successful in deleting only the HLA-A gene. Specifically, **FIG. 9A**, shows that the HLA edited cells were all positive for class-I like HLA to the same extent as the wild type (WT) (i.e., non-HLA-edited) cells. This result indicates that despite the deletion of HLA-A, other class-I molecules like HLA-B and C were expressed and not affected by the gene editing strategy.

15 To confirm that the HLA-A gene was deleted, specific expression of the HLA-A was analyzed with immunofluorescence. As can be seen in **FIG. 9B**, HLA-A was not expressed in the HLA edited clone indicating that the gene editing strategy was efficient in specifically deleting the HLA-A gene only. Such preservation of overall class-I expression with deletion of HLA-A will facilitate patient matching while avoiding NK-cell mediated rejection.

20 *Example 4 – Evaluating Pluripotency and Immunocompatibility of HLA edited HSCs*

The ability of HLA edited cells to preserve pluripotency was evaluated. As shown in **FIG. 11**, immunofluorescence evaluation of the HLA edited iPSC clones indicated that they maintained trilineage differentiation, with ectoderm differentiation indicated by NESTIN-

488 and PAX6-594 staining, mesoderm differentiation indicated by GATA-488 staining, and endoderm differentiation indicated by CXCR4-488 and FOX2A-594 staining.

HLA class I molecules are expressed on the surface of all nucleated cells and if the HLA class I molecules are mismatched between donor and recipient, then the cells could be recognized and killed by CD8⁺ T cells. Additionally, HLA mismatching could lead to cytokine release syndrome (CRS) and graft-versus-host disease (GVHD). Conversely, the complete deletion of HLA-I molecules, via B2M KO, would make the cell a target of NK cell-mediated cytotoxicity. The preservation of overall class-I expression with deletion of HLA-A can facilitate patient matching while preventing the NK-cell mediated rejection. Thus, the immunocompatibility of the HLA edited HSCs was tested by co-culture with peripheral blood mononuclear cells (PBMCs) to evaluate if the immune cells would reject a graft of the HLA edited and wild type HSCs (gHSCs).

Wild-type (gHSCs) and HLA edited HSCs were co-cultured with PBMCs matching the HLA-B and HLA-C markers, but with mismatched HLA-A. B2M KO HSCs lacking expression of HLA class-I molecules and CIITA KO HSCs lacking expression of class-II molecules were used as controls to compare the degree of PBMC-mediated cytotoxicity for HLA-null and mismatched HLA, respectively. **FIG. 12** shows the results of the PBMC-mediated cytotoxicity assay in the co-cultures as measured by an annexin V staining. The results show that deletion of HLA-A in the HLA edited HSCs protects the cells from PBMC-mediated cytotoxicity, while WT, B2M KO, and CIITA KO were susceptible to PBMC-mediated cytotoxicity. Co-cultured HSCs with sorted CD8⁺ T cells from the same PBMC donor protected HLA edited and B2M KO HSCs from CD8⁺ T cell cytotoxicity. Conversely, co-cultured HSCs with sorted NK cells only protected the WT and HLA edited cells from the NK cell-mediated cytotoxicity.

In summary, the immune compatibility results show that the CD8⁺ T cells present in the PBMC samples were responsible for killing the cells with mismatched HLA molecules (WT and CIITA KO), while the NK cells present in the PBMCs were responsible for killing the HLA-null cells (B2M KO). However, HLA edited HSCs are protected from CD8⁺ T cell-mediated cytotoxicity (because the mismatched HLA-A had been knocked out), and

protected from NK cell-mediated cytotoxicity (because HLA class I molecule expression was largely preserved).

Example 5 – Evaluating the in vivo engraftment potential of HLA edited HSCs

To evaluate the engrafting potential of HLA edited HSCs, the cells' ability to engraft
5 in vivo was evaluated by a competitive transplant against WT HSCs. Equal proportions of
mCherry HLA edited HSCs and wild-type HSCs were admixed and transplanted into mice,
where bone marrow (BM) and peripheral blood samples were recovered and evaluated by
FACS to compare the relative amounts of each cell type present in the samples. As shown
10 in **FIG. 12**, both the HLA edited HSCs and the WT HSCs contributed to approximately
equal engraftment in the BM and peripheral blood samples. These results confirm that HLA
edited HSCs (prepared according to this disclosure) are comparable to WT HSCs in their
engraftment and reconstitution potential. Hence, it is expected that properties of the WT
(unedited, parent) HSCs are consistent with that of the HLA edited HSCs of the present
disclosure, for generating T cell lineages.

15 *Example 6 – Evaluating the degranulation and cytotoxicity of immunocompatible HSC-
derived NK cells*

Next, HSCs were evaluated for their ability to differentiate into NK cells which
maintained their degranulation and cytotoxicity capabilities. As shown in **FIG. 13**, HSCs
effectively differentiated into NK cells as determined by fluorescence-activated cell sorting
20 (FACS) experiments, gated based on expression of the known NK cell surface marker,
CD56. The HSCs demonstrated the ability to differentiate at least as well as CD34+ BM and
iPSC-EB CD34+ cell populations. To measure the ability of the HSC-derived NK cells to
effectively kill tumor cells, an experimental protocol as shown in **FIG. 14A** was performed.
The HSC-derived NK cells were co-cultured for 3.5 hours with K562 HLA-null cells, which
25 are a human erythromyeloblastoid leukemia cell line derived from pleural effusion of a
chronic myeloid leukemia patient. These cells expressed ligands for aNKR, and their lack of
HLA cell-surface expression also contributes to NK cell activation by abrogating negative
signaling through iNKRs. Therefore, these HLA-null cell lines have the potential to induce
different functional profiles in NK cells and their subsets. After co-culturing, the degree of

NK cell degranulation is measured using FACS and AnnexinV staining, as well as a cytotoxicity assay. As shown in **FIG. 14B**, AnnexinV staining demonstrated that HSC-derived NK cells exhibited a greater degree of activation from the HLA-null K562 cells than did the CD34+ BM and iPSC-EB CD34+ cells. This was confirmed by the cytotoxicity assay results as shown in **FIG. 14C**.

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CLAIMS

1. A method for preparing a NK cell population or progenitors thereof, the method comprising:
enriching for CD34+ cells from a differentiated pluripotent stem cell (PSC)
5 population to prepare a CD34+-enriched population;
inducing endothelial-to-hematopoietic transition of the CD34+-enriched cell
population for at least two days, but no more than 12 days, to prepare a population
comprising hematopoietic stems cells (HSCs) and/or hematopoietic stem progenitor cells
(HSPCs); and
10 differentiating the population comprising HSCs and/or HSPCs to a progenitor NK
cell population or a NK cell population.
2. The method of claim 1, wherein the PSC population is a human iPSC population
derived from lymphocytes, cord blood cells, peripheral blood mononuclear cells, CD34+
15 cells, or human primary tissues.
3. The method of claim 2, wherein the iPSC population is derived from CD34+ cells
isolated from peripheral blood.
- 20 4. The method of claim 2 or 3, wherein the iPSCs are homozygous for one or more
HLA Class I and/or Class II genes.
5. The method of claim 4, wherein the iPSCs are homozygous for HLA-DRB1.
- 25 6. The method of claim 4, wherein the iPSCs are homozygous for both HLA-B and
HLA-C.
7. The method of any one of claims 2 to 6, wherein the iPSCs are gene-edited to delete
one or more HLA Class I genes, delete one or more Class II genes, and/or delete one or more
30 genes governing HLA or MHC expression or presentation capacity.

8. The method of claim 7, wherein the iPSCs comprise a deletion of HLA-A.
9. The method of claim 7 or 8, wherein the iPSCs comprise a deletion of HLA-DPB1
5 and/or HLA-DQB1.
10. The method of any one of claims 2 to 9, wherein the iPSCs are HLA-A^{neg}, homozygous for both HLA-B and HLA-C, HLA-DPB1^{neg}, and HLA-DQB1^{neg}, and optionally further homozygous for HLA-DRB1.
- 10 11. The method of claim 7, wherein the one or more genes governing HLA or MHC expression or presentation capacity is β 2-microglobulin and/or CIITA.
12. The method of any one of claims 1 to 11, wherein CD34+-enrichment and endothelial-to-hematopoietic transition is induced at Day 8 to Day 15 of iPSC
15 differentiation.
13. The method of any one of claims 1 to 12, wherein the CD34+-enriched population is cultured in medium containing combinations of Y-27632, TPO, IL-3, SCF, IL-6, IL-11, IGF-1, VEGF, bFGF, BMP4, and FLT3.
20
14. The method of claim 13, wherein the endothelial-to-hematopoietic transition generates an HSC population comprising one or more of long-term hematopoietic stem cells (LT-HSCs), short-term hematopoietic stem cells, and hematopoietic stem progenitor cells.
- 25 15. The method of any one of claims 12 to 14, wherein CD34+ cells are harvested from culture undergoing endothelial-to-hematopoietic transition, including harvesting of CD34+ floater and/or adherent cells.
16. The method of claim 14 or 15, wherein the HSC population comprises long-term
30 hematopoietic stem cells (LT-HSCs)

17. The method of any one of claims 1 to 16, where the induction of endothelial-to-hematopoietic transition comprises increasing the expression or activity of dnmt3b.
18. The method of claim 17, wherein the induction of endothelial-to-hematopoietic transition comprises applying cyclic stretch to the CD34⁺ cells.
19. The method of claim 18, wherein the cyclic stretch is 2D, 3D, or 4D cyclic stretch.
20. The method of any one of claim 1 to 17, wherein the induction of endothelial-to-hematopoietic transition comprises Piezo1 activation.
21. The method of claim 20, wherein the Piezo1 activation is by contacting the CD34⁺ enriched cells or fraction thereof with one or more Piezo1 agonists, which are optionally selected from Yoda1, Jedi1, Jedi2, ssRNA40 or analogues or derivatives thereof.
22. The method of any one of claims 1 to 17, wherein the induction of endothelial-to-hematopoietic transition comprises Trpv4 activation.
23. The method of claim 22, wherein the Trpv4 activation is by contacting the CD34⁺ enriched cells with one or more Trpv4 agonists, which are optionally selected from GSK1016790A, 4alpha-PDD, or analogues or derivatives thereof.
24. The method of any one of claims 1 to 23, wherein the HSC population or fraction thereof is cultured with a partial or full Notch ligand.
25. The method of claim 24, wherein the Notch ligand comprises at least one of DLL1, DLL4, SFIP3, or a functional portion thereof.
26. The method of claim 25, wherein the Notch ligand comprises DLL4 with one or more affinity enhancing mutations.

27. The method of any one of claims 24 to 26, wherein the Notch ligand is immobilized, functionalized, and/or embedded in 2D or 3D culture system.
28. The method of any one of claims 24 to 27, wherein the Notch ligand is incorporated
5 along with a component of extracellular matrix, optionally selected from fibronectin, RetroNectin, and laminin, derivatives or analogues thereof, and/or combinations thereof.
29. The method of claim 28, wherein the Notch ligand and/or component of extracellular
10 matrix are embedded in inert materials providing 3D culture conditions, optionally selected from cellulose, alginate, and combinations thereof.
30. The method of any one of claims 27 to 29, wherein the Notch ligand, a component
of extracellular matrix, or combinations thereof, are in contact with culture conditions
15 providing topographical patterns and/or roughness to cells.
31. The method of any one of claims 24 to 30, wherein the Notch ligand, a component
of extracellular matrix, topographical patterns and/or roughness, or combinations thereof,
are cultured with cytokines and/or growth factors optionally selected from one or more of
TNF-alpha and SHH.
20
32. The method of any one of claims 24 to 31, wherein the HSC population or fraction
thereof is cultured in an artificial thymic organoid optionally comprising a notch ligand,
selected from one or more of BMP2, Delta-Like-1 (DLL1), Delta-Like-4 (DLL4), SFIP3,
and Delta^{Max}.
25
33. The method of any one of claims 24 to 32, HSC population or fraction thereof is
cultured in the presence of one or more growth factors and cytokines selected from TPO,
SCF, Flt3L, IL3, IL7, and SDF-1a.
- 30 34. The method of any one of claims 24 to 33, wherein T cells or progenitor T cells that
arise are further cultured in the presence of IL-3 and/or IL-15.

35. The method of claim 34, wherein IL-15 is added to support NK cell differentiation, and IL-3 is optionally excluded after formation of early NK cells.
- 5 36. The method of any one of claims 1 to 35, wherein the NK cell lineage expresses a chimeric antigen receptor (CAR).
37. The method of any one of claims 1 to 36, wherein the Natural Killer cell is predominately CD56^{DIM}.
- 10 38. The method of any one of claims 1 to 36, wherein the Natural Killer cell is predominately CD56^{BRIGHT}.
39. The method of any one of claims 1 to 36, wherein the Natural Killer cell lineage is an NK cell precursor.
- 15 40. An NK cell population, or pharmaceutically-acceptance composition thereof, produced by the method of any one of claims 1 to 39.
- 20 41. An NK cell population, or pharmaceutically-acceptance composition thereof, wherein the NK cell population is HLA-A^{neg}, homozygous for both HLA-B and HLA-C, HLA-DPB1^{neg}, and HLA-DQB1^{neg}, and optionally further homozygous for HLA-DRB1.
42. A method for cell therapy, comprising administering the NK cell population or pharmaceutically acceptable composition thereof of claim 40 or 41, to a human subject in need thereof.
- 25 43. The method of claim 42, wherein the human subject has a condition comprising one or more of lymphopenia, a cancer, an immune deficiency, an autoimmune disease, viral infection, a skeletal dysplasia, and a bone marrow failure syndrome.
- 30

44. The method of claim 43, wherein the subject has cancer, which is optionally a hematological malignancy or a solid tumor.