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(54) Title: NATURAL KILLER CELLS WITH NOVEL IL-15 KNOCK-IN AND METHODS OF USE THEREOF

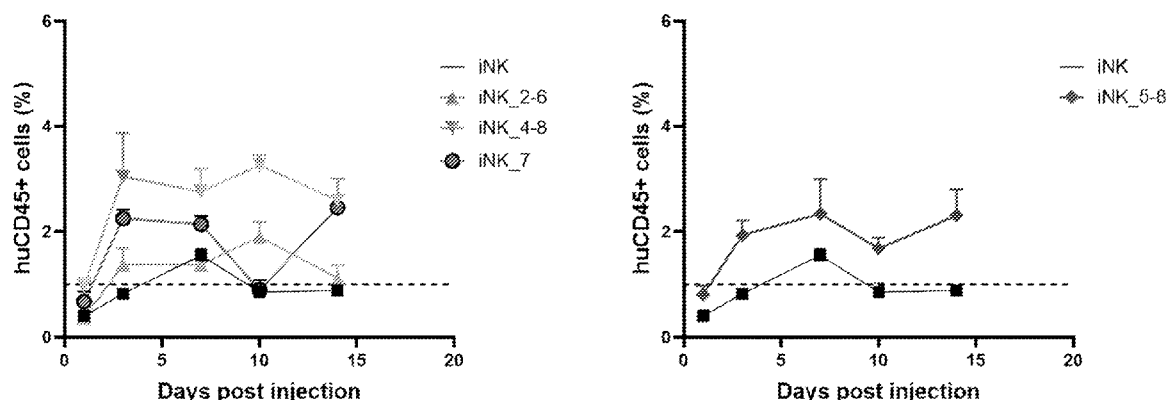


Figure 18

(57) Abstract: The present disclosure provides a cell population comprising induced pluripotent stem cells (iPSCs) or NK cells wherein the iPSCs or NK cells comprise an IL-15 construct comprising (i) sequences encoding IL-15 or a functional fragment thereof, wherein the IL-15 or a functional fragment thereof is trapped in the endoplasmic reticulum, or (ii) sequences encoding IL-15 or a functional fragment thereof and IL-2Rb or a functional fragment thereof. In one embodiment, the NK cells are derived from the iPSCs (iNK cells), and the iNK cells can be used to treat cancer in a subject.

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NATURAL KILLER CELLS WITH NOVEL IL-15 KNOCK-IN AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority to U.S. provisional patent application serial no. 63/506,123, filed June 4, 2023, the entire contents of which is incorporated herein by reference.

SEQUENCE LISTING

[0002] A Sequence Listing conforming to the rules of WIPO Standard ST.26 is hereby incorporated by reference. Said Sequence Listing has been filed as an electronic document via PatentCenter in XML file format. The electronic document, created on June 3, 2024, is entitled "P-625313-PC_ST26.xml", and is 266,736 bytes in size.

FIELD OF THE INVENTION

[0003] The present disclosure relates generally to populations of Natural Killer (NK) cells with novel IL-15 knock-in and methods of use thereof.

BACKGROUND OF THE INVENTION

[0004] Lymphocytes such as natural killer (NK) cells are potent anti-tumor effectors that play an important role in innate and adaptive immunity. There are several activating receptors found on NK cells, including Nkp30, Nkp44, and Nkp46, which are collectively known as Natural Cytotoxicity Receptors (NCRs), as well as NKG2D, CD16 and TRAIL. Nkp46 is an established marker for the identification of NK cells. Nkp46 is an NK cell specific triggering molecule found on both resting and activated NK cells. It is an important mediator in NK cell activation against numerous targets, including tumors and virally infected cells. NK cells are a subpopulation of lymphocytes that have spontaneous cytotoxicity against a variety of tumor cells, virus-infected cells, and some normal cells in the bone marrow and thymus. NK cells are critical effectors of the early innate immune response toward transformed and virus-infected cells. NK cells constitute about 10% of the lymphocytes in human peripheral blood. NK cells are effector cells known as large granular lymphocytes because of their larger size and the presence of characteristic azurophilic granules in their cytoplasm. NK cells differentiate and mature in the bone marrow,

lymph nodes, spleen, tonsils, and thymus. NK cells can be detected by specific surface markers, such as CD56 and CD45 in humans. NK cells do not express T cell antigen receptors, the pan T marker CD3, or surface immunoglobulin B cell receptors.

[0005] Stimulation of NK cells may be achieved through a cross-talk of signals derived from cell surface activating and inhibitory receptors. The activation status of NK cells is regulated by a balance of intracellular signals received from an array of germ-line-encoded activating and inhibitory receptors (MacFarlane and Campbell, *Curr Top Microbiol Immunol.* 2006; 298:23-57). When NK cells encounter an abnormal cell (e.g., tumor or virus-infected cell) and activating signals predominate, the NK cells can rapidly induce apoptosis of the target cell through directed secretion of cytolytic granules containing perforin and granzymes or engagement of death domain-containing receptors. Activated NK cells can also secrete type I cytokines, such as interferon- γ , tumor necrosis factor- α and granulocyte-macrophage colony-stimulating factor (GM-CSF), which activate both innate and adaptive immune cells as well as other cytokines and chemokines (Wu and Lanier, *Adv Cancer Res.* 2003; 90:127-56). Production of these soluble factors by NK cells in early innate immune responses significantly influences the recruitment and function of other hematopoietic cells. Also, through physical contacts and production of cytokines, NK cells are central players in a regulatory crosstalk network with dendritic cells and neutrophils to promote or restrain immune responses.

[0006] NK cells have several characteristics which make them advantageous to use in therapeutic settings. For example, they do not require antigen priming and they are HLA agnostic, which decreases the risk of developing of Graft vs. Host Disease in allogeneic use. Nonetheless, the use of immune cells for adoptive cell therapies remains challenging and there are unmet needs for improvement. For example, NK cells are usually short-lived, and the clinical efficacy of NK cells is often compromised by their limited persistence. Therefore, there is a need to screen for optimal version(s) of IL-15 to be knocked into NK cells to help their physical and functional persistence. There are significant opportunities that remain to harness the full potential of NK cells in adoptive immunotherapy.

SUMMARY OF THE DISCLOSURE

[0007] In one embodiment, provided herein is a cell population comprising induced pluripotent stem cells (iPSCs) or NK cells wherein the iPSCs or NK cells comprise an IL-15 construct comprising sequences encoding IL-15 or a functional fragment thereof, wherein the IL-15 or a

functional fragment thereof is trapped in the endoplasmic reticulum. In one embodiment, the IL-15 construct further comprises sequences encoding IL-2R β or a functional fragment thereof.

[0008] In one embodiment, provided herein is a cell population comprising induced pluripotent stem cells (iPSCs) or NK cells wherein the iPSCs or NK cells comprise an IL-15 construct comprising sequences encoding (i) IL-15 or a functional fragment thereof, and (ii) IL-2R β or a functional fragment thereof. In one embodiment, the IL-15 construct encodes a cell membrane-bound form of IL-15 (mbIL-15) or a functional fragment thereof. In another embodiment, the IL-15 construct encodes a soluble form of IL-15 or a functional fragment thereof. In another embodiment, the IL-15 construct encodes an IL-15 trapped in the endoplasmic reticulum (ER) or a functional fragment thereof.

[0009] In one embodiment, the IL-15 construct encodes a fusion protein comprising IL-15 or a functional fragment thereof and IL-2R β or a functional fragment thereof. In another embodiment, the IL-15 or a functional fragment thereof and IL-2R β or a functional fragment thereof are expressed as individual proteins. The IL-15 can be a membrane-bound IL-15 or a functional fragment thereof, soluble IL-15 or a functional fragment thereof, or an ER-trapped IL-15 or a functional fragment thereof.

[0010] In one embodiment, the above IL-15 constructs further comprise sequences encoding IL-15 Receptor alpha (IL-15R α) or a functional fragment thereof. Accordingly, in one embodiment, the IL-15 construct encodes a fusion protein comprising (i) IL-15 or a functional fragment thereof, (ii) IL-2R β or a functional fragment thereof, and (iii) IL-15R α or a functional fragment thereof. In another embodiment, the IL-15 or a functional fragment thereof, IL-2R β or a functional fragment thereof, and IL-15R α or a functional fragment thereof are expressed as individual proteins. The IL-15 can be a membrane-bound IL-15 or a functional fragment thereof, soluble IL-15 or a functional fragment thereof, or an ER-trapped IL-15 or a functional fragment thereof.

[0011] In one embodiment, the NK cells (iNK cells) are derived from iPSCs. In one embodiment, the iPSCs or iNK cells comprise a knock-in of the IL-15 construct into a B2M gene or a TGF β R2 gene of the iNK cells.

[0012] In one embodiment, the IL-15 construct is operably linked to a promoter which is silent or minimally active (e.g., <10% of its full activity) in iPSC. Examples of promoters that are silent or minimally active in iPSC include, but are not limited to, a NK cell-specific promoter. In one embodiment, the NK cell-specific promoter can be a NKp46 promoter.

[0013] In one embodiment, the iNK cells further comprise a knockout of a cytokine inducible SH2 containing protein (CISH). In another embodiment, the iNK cells further comprise one or more knockouts of one or more NK inhibitory receptors. For example, the NK inhibitory receptor can be T-cell immunoglobulin and ITIM domain (TIGIT) receptor.

[0014] In yet another embodiment, the iNK cells further comprise both a knockout of a cytokine inducible SH2 containing protein (CISH) and one or more knockouts of one or more NK inhibitory receptors. For example, the NK inhibitory receptor can be T-cell immunoglobulin and ITIM domain (TIGIT) receptor.

[0015] In another embodiment, there is provided a method of producing a cell population comprising NK cells (iNK cells) differentiated from induced pluripotent stem cells (iPSCs), the method comprising (i) genetically editing a population of iPSCs with an IL-15 construct comprising sequences encoding IL-15 or a functional fragment thereof, wherein the IL-15 or a functional fragment thereof is trapped in the endoplasmic reticulum; (ii) generating a monoclonal population of edited iPSCs; and (iii) differentiating said edited iPSCs into a population of iNK cells.

[0016] In another embodiment, there is provided a method of producing a cell population comprising NK cells (iNK cells) differentiated from induced pluripotent stem cells (iPSCs), the method comprising (i) genetically editing a population of iPSCs with an IL-15 construct comprising sequences encoding IL-15 or a functional fragment thereof and IL-2R β or a functional fragment thereof; (ii) generating a monoclonal population of edited iPSCs; and (iii) differentiating said edited iPSCs into a population of iNK cells. In one embodiment, the IL-15 construct encodes a cell membrane-bound form of IL-15 (mbIL-15) or a functional fragment thereof. In another embodiment, the IL-15 construct encodes a soluble form of IL-15 or a functional fragment thereof. In another embodiment, the IL-15 construct encodes an IL-15 trapped in the endoplasmic reticulum or a functional fragment thereof.

[0017] In one embodiment, the IL-15 construct encodes a fusion protein comprising IL-15 or a functional fragment thereof and IL-2R β or a functional fragment thereof. In another embodiment, the IL-15 or a functional fragment thereof and IL-2R β or a functional fragment thereof are expressed as individual proteins.

[0018] In another embodiment, the above IL-15 constructs further comprise sequences encoding IL-15 Receptor alpha (IL-15R α) or a functional fragment thereof. In one embodiment, the IL-15

construct encodes a fusion protein comprising (i) IL-15 or a functional fragment thereof, (ii) IL-2R β or a functional fragment thereof, and (iii) IL-15R α or a functional fragment thereof. In another embodiment, the IL-15 or a functional fragment thereof, IL-2R β or a functional fragment thereof, and IL-15R α or a functional fragment thereof are expressed as individual proteins.

[0019] In one embodiment, the IL-15 construct in the above method is operably linked to a promoter which is silent or minimally active (e.g., <10% of its full activity) in iPSC. Examples of promoters that are silent or minimally active in iPSC include, but are not limited to, a NK cell-specific promoter. In one embodiment, the NK cell-specific promoter can be a NKp46 promoter.

[0020] In one embodiment, the genetic editing of the above method comprises a knock-in of the IL-15 construct into a B2M gene or a TGF β 2 gene of the iPSCs. In another embodiment, the genetic editing further comprises a knockout of a cytokine inducible SH2 containing protein (CISH). In another embodiment, the genetic editing further comprises one or more knockouts of one or more NK inhibitory receptors. For example, the NK inhibitory receptor can be T-cell immunoglobulin and ITIM domain (TIGIT) receptor. In yet another embodiment, the genetic editing further comprises both a knockout of a cytokine inducible SH2 containing protein (CISH) and one or more knockouts of one or more NK inhibitory receptors. In one embodiment, the genetic editing comprises using a TALEN construct or a Cas9 or Cas12 enzyme.

[0021] In one embodiment, the present disclosure includes cell populations disclosed herein. In another embodiment, the present disclosure comprises cell populations produced by the methods disclosed herein.

[0022] In another embodiment, there is provided a pharmaceutical composition comprising the cell populations disclosed herein, or the cell populations produced by the methods disclosed herein.

[0023] In another embodiment, there is provided a method of treating cancer or autoimmune diseases in a subject in need thereof, comprising administering to the subject an effective amount of the pharmaceutical composition disclosed herein. In one embodiment, the cancer is a solid tumor or a hematological cancer. In another embodiment, the subject is further administered with one or more immune checkpoint inhibitors. In one embodiment, the immune checkpoint inhibitors can be inhibitors of one or more NK cell inhibitory receptors, for example, antibodies against the NK cell inhibitory receptors. In another embodiment, the immune checkpoint inhibitor can be an anti-PD-1 antibody, an anti-PDL-1 antibody, an anti-CTLA-4 antibody, or an anti-TIGIT antibody.

[0024] These and other aspects of the invention will be appreciated from the ensuing descriptions

of the figures and detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

[0026] **Figure 1** shows a map of donor 107 plasmid.

[0027] **Figure 2** shows a map of donor 107ER plasmid.

[0028] **Figure 3** shows a map of donor 108 plasmid.

[0029] **Figure 4** shows a map of donor 108B2M plasmid.

[0030] **Figure 5** shows a map of donor 115 plasmid.

[0031] **Figure 6** shows a map of a vector 1 encoding a membrane-bound IL-15.

[0032] **Figure 7** shows a map of a vector 2 encoding a membrane-bound IL-15 and IL-15Ra.

[0033] **Figure 8** shows a map of a vector 3 encoding a membrane-bound IL-15, IL-15Ra, IL-15Rb, and a full cytoplasmic portion of the common cytokine receptor γ chain.

[0034] **Figure 9** shows a map of a vector 4 encoding a membrane-bound IL-15, IL-15Ra, IL-15Rb with a portion of the cytoplasmic region deleted, and a full cytoplasmic portion of the common cytokine receptor γ chain.

[0035] **Figure 10** shows a map of a vector 5 encoding an ER-trapped IL-15.

[0036] **Figure 11** shows a map of a vector 6 encoding an IL-15 and IL-15Ra that are both trapped in the ER.

[0037] **Figure 12** shows a map of a vector 7 encoding IL-15Ra, IL-15Rb, and a soluble IL-15.

[0038] **Figure 13** shows a map of a vector 8 encoding IL-15Ra and a soluble IL-15.

[0039] **Figure 14** shows a map of a vector 9 encoding an ER-trapped fusion protein comprising IL-15, IL-15Ra, IL-15Rb, and a full cytoplasmic portion of the common cytokine receptor γ chain.

[0040] **Figure 15** shows the IL-15-engineered iNK cells showed proliferative advantage compared to control iNK cells during *ex vivo* expansion. iNK cells were transduced at the beginning of the stage 3 week 2, and expanded with K562 feeders in 1:5 ratio for a total of 20 days. At day 3, day 9, day 16, and day 20 the transduced iNK cells were harvested and measured for the viable cell

number (tdT: tdTomato-transduced; control: no transduction). 2-6 are iNK cells incorporating vector 2; 3-2 are iNK cells incorporating vector 3; 4-8 are iNK cells incorporating vector 4; 5-8 are iNK cells incorporating vector 5; 7 are iNK cells incorporating vector 7.

[0041] Figure 16 shows the IL-15-engineered iNK cells showed advantage in survivability in cytokine-starved condition. Following 20 days of *ex vivo* expansion, the IL-15-engineered iNK cells were grown in cytokine-free medium for 6 days and the viable cell number was monitored at day 0, day 2, and day 6.

[0042] Figure 17 shows the IL-15-engineered, cytokine-starved iNK cells maintain pSTAT5 activation in response to IL2 and Ki67 proliferation marker. Following 26 days of *ex vivo* expansion, the IL-15-engineered iNK cells were grown in cytokine-free medium for 6 days, or in cytokine-free medium for 6 days and then pulse-treated with 100 IU/mL of IL2 for 30 minutes. The % positive population of phospho-STAT5 and proliferation marker Ki-67 were analyzed at day 0 and day 6.

[0043] Figure 18 shows the IL-15-engineered iNK cells showed persistence in peripheral blood *in vivo*. The IL-15-engineered iNK cells were injected into mice. The mice looked healthy post injection, and peripheral blood was withdrawn from the mice at hour 3, day 3, day 7, day 10, day 14, and day 17. The persistence of the IL-15-engineered iNK cells was examined by flow cytometry on huCD45+ cell population. Compared to the control iNK cells, the IL-15-engineered iNK cells exhibited better survivability *in vivo* during the time course.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0044] Unless otherwise defined, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures utilized in connection with, and techniques of, cell and tissue culture, molecular biology, and protein and oligo- or polynucleotide chemistry and hybridization described herein are those well-known and commonly used in the art. Standard techniques are used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (e.g., electroporation, lipofection). Enzymatic reactions and purification techniques are performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures are generally performed according to conventional methods well-known in the art and as described in

various general and more specific references that are cited and discussed throughout the present specification. See e.g., Sambrook et al. *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)). The nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients. The materials, methods, and examples disclosed herein are for illustrative purpose only and are not intended to be necessarily limiting. Each literature reference or other citation referred to herein is incorporated herein by reference in its entirety.

[0045] As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0046] As used in the specification and claims, the singular form “a”, “an” and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a cell” includes a plurality of cells, including mixtures thereof.

NK CELLS

[0047] In one aspect, provided herein is a cell population comprising differentiated cells derived from induced pluripotent stem cells (iPSCs), said differentiated cells having a Natural Killer cell phenotype. These NK cells derived from iPSCs are also referred to interchangeably as “iPSC-NK cells” or “iNK cells.”

METHODS OF MAKING IPSC-NK CELLS

[0048] Any suitable source of iPSC may be used to generate the iPSC-NK cells provided herein. Thus, in one embodiment, provided herein is a method of producing a cell population comprising iPSC-NK cells, wherein the iPSC-NK cells express novel IL-15 constructs as disclosed herein, the method comprising (i) genetically editing a population of induced pluripotent stem cells (iPSCs); (ii) differentiating said population of iPSCs into a population of Natural Killer (NK) cells; and (iii) expanding the population of NK cells.

[0049] In one embodiment, iPSC may be produced by reprogramming somatic cells to induce pluripotency. The reprogramming involves the activation of pluripotency genes and repression of somatic genes. In its simplest form, this process includes the expression of certain transcription factors in the somatic cells, in particular Octamer 3/4 (Oct3/4), SRY-box containing gene 2 (Sox2),

Krüppel-like factor 4 (Klf4), and the protooncogene cytoplasmic Myc protein (c-Myc) (see Takahashi and Yamanaka, *Cell* 126:663–676). The delivery of these transcription factors into the somatic cells can be accomplished by any suitable method known in the art, for example, using viral vectors, mRNA transfection, or delivery of recombinant proteins (see, e.g. Chang et al., 2019, *J Korean Neurosurg Soc.*62(5):493-501). Alternatively or additionally, small molecules targeting cell signaling pathways, metabolic pathways, and epigenetic modifications may be used to induce pluripotency and reprogram somatic cells into iPSCs. In particular, repression of DNA methylation, activation of Wnt signaling, activation of MAPK/ERK signaling, and induction of glycolytic metabolism have been described as mechanisms to aid in reprogramming. Such small molecules include, for example, Gsk3 β inhibitors, transforming growth factor β (TGF β) inhibitors, TGFR inhibitors, MEK inhibitors, AMPK inhibitors, mTOR inhibitors, VEGF inhibitors, Wnt activators, cAMP activators, retinoic acid receptor (RAR) α agonists, RAR γ agonists, pyruvate dehydrogenase kinase, isozyme 1 (PDK-1) activators, HMT inhibitors, DNMT inhibitors, KDM inhibitors, HDAC inhibitors, and others. See, e.g., Liu et al., 2019, *Cell Chem Biol*; 23:893-916, which is incorporated herein in its entirety for examples of small molecules that may be used in the methods of generating iPSC.

[0050] Commercial iPSC lines are also available and may be used to generate the iPSC-NK cells described herein.

[0051] In some embodiments, the iPSCs used to generate the iPSC-NK cells provided herein are generated, maintained and differentiated under Good Manufacturing Protocol (GMP) conditions.

[0052] iPSCs may be differentiated into NK cells using any suitable method known in the art or described herein. A description of such methods is described in, for example, Zhu, H., Kaufman, D.S. (2019). An Improved Method to Produce Clinical-Scale Natural Killer Cells from Human Pluripotent Stem Cells. In: Kaneko, S. (eds) *In Vitro Differentiation of T-Cells. Methods in Molecular Biology*, vol 2048. Humana, New York, NY. For example, the iNK cells may be activated for three days at high concentrations of IL-2 (100 unit/ml to 500 unit/ml), and with additional cytokines IL-15 and IL-21A.

[0053] The iPSC-NK cells provided herein may be cultured under any suitable conditions described herein or known in the art. In some embodiments, the NK cells are cultured on a feeder layer, i.e., in co-culture with another cell line. Such co-cultures can be effective in inducing proliferation in cell types that otherwise proliferate very slowly or not at all. In some embodiments,

provided herein is a feeder layer that is capable of inducing proliferation of iPSC-NK cells. In some embodiments, provided herein is a feeder layer that is capable of activating iPSC-NK cells. [0054] Examples of feeder layers that may be used for the culture of NK cells provided herein include, without limitation, K562 cells and 221 cells. In some embodiments, the feeder layer cells may be genetically modified, e.g., the feeder layer cells may be transduced with IL-15, IL21 and/or 4-1-BB. Prior to being used in the co-culture, the feeder layer cells may be irradiated with doses sufficient to induce cell cycle arrest, such that the feeder layer cells do not proliferate in the co-culture.

[0055] In some embodiments, about 40% to about 45%, about 45% to about 50%, about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90 to about 95%, or about 95% to 100% of cells in a population of iPSC-NK cells provided herein express one or more NK cell marker such as CD56 and/or CD45.

MODIFICATION OF NK CELLS

[0056] In some embodiments, the iPSC-NK cells provided herein cells are genetically modified by introducing (“integrating” or “knocking in”) or deleting (“knocking out”) one or more genes. Without wishing to be bound by theory, knocking out or integrating genes of interest involved in NK cell exhaustion, activation, tolerance, and/or memory are thought to improve the clinical utility of the iPSC-NKs provided herein.

[0057] The genetic modification of the iPSC-NK cells provided herein may be achieved by any suitable method known in the art or described herein. For example, the genome of the iPSC-NK cells provided herein may be modified by introducing DNA double strand breaks, which are then repaired by the cell’s endogenous repair mechanisms, such as homologous recombination. DNA double strand breaks may be introduced using targeted endonucleases, such as Zinc-finger nucleases, transcription activation-like effector-nucleases (TALENs), meganucleases, or the CRISPR/Cas system, which relies on the Cas9 endonuclease for inducing the DNA breaks and a guide RNA (gRNA) for site-specificity.

[0058] Alternatively, a Dualase™ platform may be used to edit the iPSC cells described herein. The Dualase™ is a gene editing technology which cuts DNA twice and leaves non-compatible DNA ends, which is hypothesized to lead to higher fidelity repair than non-compatible ends.

[0059] TALENs employ a bacterial DNA cleavage domain and specifically bind DNA via highly

conserved 33–35 amino acid TALE repeats which resemble the DNA-binding domains of transcription factors. The TALE repeats each bind a single base pair of DNA. The specificity of TALEN DNA binding is dictated by two hypervariable residues. The repeats form a two-helix structure connected by a loop which presents the hypervariable residue into the major groove of the DNA. Multiple modular TALE repeats can be linked together into a longer array with custom DNA-binding specificities. See e.g., Maeder and Gersbach, 2016, *Mol Ther.*24(3):430–446; Carrol, 2017, *Yale J Biol Med* 90:653-659. Methods for designing TALEN sequences targeting a desired locus are well known in the art and described in, e.g., Cermak et al., *Nucleic Acids Res.*2011 Jul;39(12):e82.

[0060] Cas-based DNA editing systems are well known in the art. Any suitable Cas enzyme can be used to edit the iPSC-NK cells described herein, including, without limitation, Cas9 and Cas12.

[0061] In the cases of knock-in, the polynucleotide encoding the knocked-in gene is introduced in such a way that the polynucleotide is operatively linked to a promoter. The term “operably linked” as used herein refers to positions of components so described are in a relationship permitting them to function in their intended manner. A control sequence “operably linked” to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

[0062] The term “control sequence” as used herein refers to polynucleotide sequences which are necessary to affect the expression and processing of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism. In prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence. In eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term “control sequences” is intended to include, at a minimum, all components whose presence is essential for expression and processing, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. The term “polynucleotide” as referred to herein means a polymeric chain of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

[0063] In some embodiments, the iPSC-NK cells are modified by knocking in an IL-15 construct as disclosed herein. In one embodiment, the IL-15 construct encodes a cell membrane-bound form

of IL-15 (mbIL-15) or a functional fragment thereof. In another embodiment, the IL-15 construct encodes a soluble form of IL-15 or a functional fragment thereof. In another embodiment, the IL-15 construct encodes an IL-15 trapped in the endoplasmic reticulum (ER) or a functional fragment thereof.

[0064] In some embodiments, the IL-15 construct comprises (i) IL-2R β or a functional fragment thereof, and (ii) a form of IL-15 as disclosed herein, or a functional fragment thereof. In some embodiments, the IL-15 construct comprises (i) IL-2R β or a functional fragment thereof, (ii) IL-15Ra or a functional fragment thereof, and (iii) a form of IL-15 as disclosed herein, or a functional fragment thereof. By “functional fragment” is meant a fragment of a protein (e.g., IL-15 or IL-2R β) which retains one or more desired activities of the parental protein.

[0065] Several isoforms of IL-15 are known in the art and may be used in the iPSC-NK cells provided herein. Exemplary sequences of IL-15 isoforms are known in the art, e.g. SEQ ID NOs:1-2. In some embodiments, an iPSC-NK cells provided herein expresses a polypeptide comprising the sequence of human IL-15 isoform S48AA (SEQ ID NO:1). In some embodiments, an iPSC-NK cells provided herein expresses a polypeptide comprising a sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO:1. In some embodiments, an iPSC-NK cells provided herein expresses a polypeptide comprising the sequence of human IL-15 isoform S21AA (SEQ ID NO:2). In some embodiments, an iPSC-NK cells provided herein expresses a polypeptide comprising a sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98% identical to the sequence of SEQ ID NO:2.

[0066] In some embodiments, an iPSC-NK cells provided herein comprises a polynucleotide encoding the amino acid sequences of human IL-15 isoform S48AA (SEQ ID NO:1). In some embodiments, an iPSC-NK cells provided herein comprises a polynucleotide encoding a sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98% identical to the amino acid sequences of SEQ ID NO:1. In some embodiments, an iPSC-NK cells provided herein comprises a polynucleotide encoding the amino acid sequences of human IL-15 isoform S21AA (SEQ ID NO:2). In some embodiments, an iPSC-NK cells provided herein comprises a polynucleotide encoding a sequence that is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98% identical to the amino acid sequences of SEQ ID NO:2.

[0067] In some embodiments, the IL-15 or functional fragment thereof may be a membrane bound form of IL-15 (mbIL-15) or a functional fragment thereof. Expression of a membrane-bound form of IL-15 may be achieved by expressing a nucleic acid encoding a soluble form of IL-15 (e.g., SEQ ID NO:41) as a fusion protein with the hinge and transmembrane domains of CD8. An illustrative nucleic acid sequence encoding the hinge and transmembrane domains of CD8 is set forth in SEQ ID NO:46.

[0068] In one embodiment, the IL-15 or functional fragment thereof may be a form of IL-15 that is trapped in the endoplasmic reticulum (ER). This may be achieved by, for example, expressing a polynucleotide encoding IL-15 or a functional fragment thereof (e.g., SEQ ID NO:41) as a fusion protein with an ER retention signal. An illustrative ER retention signal sequence is set forth as SEQ ID NO:54.

[0069] In one embodiment, the IL-15 or functional fragment thereof may be a form of IL-15 (e.g., a membrane-bound form of IL-15, a soluble form of IL-15, or an ER-trapped form of IL-15) or functional fragment thereof fused to the IL-15 receptor alpha (IL-15R α). This may be accomplished by expressing the IL-15 or functional fragment thereof as a fusion protein with IL-15R α . An illustrative nucleic acid sequence encoding IL-15R α is set forth in SEQ ID NO:43.

[0070] In some embodiments, the iPSC-NK cells provided herein are modified by knocking in an IL-15 construct encoding IL-2R β or a functional fragment thereof, and interleukin 15 (IL-15) or a functional fragment thereof. The IL-15 can be in a soluble form, a membrane-bound form, or an ER-trapped form. Tab

[0071] In some embodiments, the iPSC-NK cells provided herein are modified by knocking in an IL-15 construct encoding IL-2R β or a functional fragment thereof, interleukin 15 (IL-15) or a functional fragment thereof, and IL-15R α or a functional fragment thereof. The IL-15 can be in a soluble form, a membrane-bound form, or an ER-trapped form.

[0072] In another aspect, provided herein are polypeptides and vectors comprising the gRNAs and/or the donor DNA sequences provided herein. The gRNAs provided herein may be used in combination with any suitable DNA editing enzyme known in the art or described herein, including, for example, Cas9 and Cas12.

[0073] The IL-15 construct disclosed herein may be knocked into the iPSC-NK cell genome at any suitable position. In some embodiment, the IL-15 construct comprises sequences encoding IL-15, IL-15R α and IL-2R β . In certain embodiments the integration locus is the B2M locus. In certain

embodiments, the integration locus is the CD38 locus. In certain embodiments, the integration locus is the TGF β R2 locus. An illustrative gene editing strategy for a non-disruptive knock-in of IL-15 into the B2M locus is shown in FIG. 2A of WO 2023/060136. An illustrative gene editing strategy for a disruptive knock-in of IL-15 into the TGF β R2 locus is shown in FIG. 2B of WO 2023/060136.

[0074] Exemplary gRNAs that may be used for the knock-in of the IL-15 construct at the B2M locus are shown in SEQ ID NOs:3-4.

[0075] In some embodiments, provided herein are nucleic acid donor constructs that may be used to deliver the IL-15 construct disclosed herein to a target site in the genome. Illustrative donor sequences that may be used to insert the IL-15 construct into a cell are set forth in SEQ ID NOs:27-38.

[0076] Generally, a construct that may be used to deliver the IL-15 construct disclosed herein to a target site (e.g., a B2M target site or a TGF β R2 target site) comprises a nucleic acid sequence encoding the desired polypeptide (e.g. IL-15 and IL-2R β , or IL-15, IL-15Ra and IL-2R β), flanked by a left homology arm (LHA) and a right homology arm (RHA). The LHA and RHA of a given donor construct comprise nucleic acid sequences with homology to the target site (e.g., the B2M locus or the TGF β R2 locus).

[0077] The sequence and length of the RHA and LHA sequences may vary based on the targeted site. In some embodiments, the LHA sequence comprises a nucleic acid sequence that is homologous to the 5' upstream sequence of the B2M gene. In some embodiments, the RHA sequence comprises a nucleic acid sequence that is homologous to exon 1 and intron 1 of B2M. An illustrative LHA-RHA sequence pair that may be used for targeted insertion into the B2M locus is the pair of sequences set forth in SEQ ID NO:39 (LHA) and SEQ ID NO:45 (RHA).

[0078] In some embodiments, the LHA sequence comprises a nucleic acid sequence that is homologous to intron 2 and exon 3 of TGF β R2. In some embodiments, the RHA sequence comprises a nucleic acid sequence that is homologous to exon3 and intron 3 of TGF β R2. An illustrative LHA-RHA sequence pair that may be used for targeted insertion into the TGF β R2 locus is the pair of sequences set forth in SEQ ID NO:52 (LHA) and SEQ ID NO:47 (RHA).

[0079] In some embodiments, the donor construct further comprises one or more of the following: spacer domains, one or more insulator domains, a CD8-hinge-transmembrane domain, a promoter, an endoplasmic reticulum (ER) retention signal sequence, a polyA sequence (e.g., a bGHpA

sequence), and/or an IRES element (e.g., an IRES2 element). In some embodiments, the elements of a nucleic acid construct may be separated by spacer elements, insulators, and/or 2A sequences (e.g., a P2A sequence).

[0080] An illustrative donor sequence (donor 107 plasmid) for inserting an IL-15 construct into the TGFβ2 locus of a cell is shown in **Fig. 1**: RHA(TGFβ2)-Promoter(NKp46)-CD8aSP-IL15-IL15Ra-IL2Rβ-ICD-bGHpA-LHA(TGFβ2) (SEQ ID NO:64).

[0081] An illustrative donor sequence (donor 107ER plasmid) for inserting an IL-15 construct (comprising an ER-trapped IL-15) into the TGFβ2 locus of a cell is shown in **Fig. 2**: RHA(TGFβ2)-Promoter(NKp46)-CD8aSP-GSEKDEL-IL15-IL15Ra-IL2Rβ-ICD-bGHpA-LHA(TGFβ2) (SEQ ID NO:65).

[0082] An illustrative donor sequence (donor 108 plasmid) for inserting an IL-15 construct into the TGFβ2 locus of a cell is shown in **Fig. 3**: RHA(TGFβ2)-Promoter(NKp46)-IL15Ra-P2A-IL2Rβ-P2A-IL15-bGHpA-LHA(TGFβ2) (SEQ ID NO:66).

[0083] An illustrative donor sequence (donor 108B2M plasmid) for inserting an IL-15 construct into the B2M locus of a cell is shown in **Fig. 4**: LHA(B2M)-B2M-IRES-IL15Ra-P2A-IL2Rβ-P2A-IL15-bGHpA-RHA(B2M) (SEQ ID NO:67).

[0084] An illustrative donor sequence (donor 115 plasmid) for inserting an IL-15 construct into the B2M locus of a cell is shown in **Fig. 5**: LHA(B2M)-IL15(soluble)-IRES2-IL15Ra-P2A-IL2Rβ-P2A-RHA(B2M) (SEQ ID NO:68).

[0085] In one embodiment, **Figure 6** shows a map of a vector 1 comprising sequences encoding a membrane-bound IL-15. The vector has the polynucleotide sequence of SEQ ID NO:55.

Components of Vector 1 (SEQ ID NO:55)	
LTR 5'	Nucleotides #395-986 of SEQ ID NO:55
CD8a-SP (signal peptide)	Nucleotides #1858-1920 of SEQ ID NO:55
Membrane-bound IL-15 polypeptide	Nucleotides #1921-2262 of SEQ ID NO:55
CD8a hinge TM	Nucleotides #2263-2460 of SEQ ID NO:55
EF1 promoter	Nucleotides #2488-3033 of SEQ ID NO:55
tdTomato	Nucleotides #3047-4474 of SEQ ID NO:55
LTR 3'	Nucleotides #4682-5275 of SEQ ID NO:55

Amino acid sequences

Membrane-bound IL-15	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCCKVTAMKC FLLELQVISLES GDASIHTVENLIILANNSLSSNGNVTESGCK
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ECEELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO:69)

[0086] In one embodiment, **Figure 7** shows a map of a vector 2 comprising sequences encoding a membrane-bound IL-15 and IL-15Ra. The vector has the polynucleotide sequence of SEQ ID NO:56.

Components of Vector 2 (SEQ ID NO:56)	
LTR 5'	Nucleotides #395-986 of SEQ ID NO:56
CD8a-SP (signal peptide)	Nucleotides #1858-1920 of SEQ ID NO:56
Membrane-bound IL-15 polypeptide	Nucleotides #1921-2262 of SEQ ID NO:56
IL-15Ra polypeptide	Nucleotides #2341-3051 of SEQ ID NO:56
EF1 promoter	Nucleotides #3070-3615 of SEQ ID NO:56
tdTomato	Nucleotides #3629-5056 of SEQ ID NO:56
LTR 3'	Nucleotides #5264-5857 of SEQ ID NO:56

Amino acid sequences

Membrane-bound IL-15	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKC FLEELQVISLES GDASIHDTVENLILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO:70)
IL-15Ra polypeptide	ITCPPPMSVEHADIWVKSYSLSRERYICNSGFKRKAGTSSLT ECVLNKATNV AHWTTPSLK CIRD PALVHQRPAPPSTVTTAGV TPQPESLSPSGKEPAASSPSSNNTAATTAIVPGSQLMPKSPS TGTTEISSHESHGTPSQTTAKNWELTASASHQPPGVYPQGHS DTTVAISTSTVLLCGLSAV SLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDL ENC SHHL (SEQ ID NO:71)

[0087] In one embodiment, **Figure 8** shows a map of a vector 3 comprising sequences encoding a membrane-bound IL-15, IL-15Ra, IL-15Rb, and a full cytoplasmic portion of the common cytokine receptor γ chain. The vector has the polynucleotide sequence of SEQ ID NO:57.

Components of Vector 3 (SEQ ID NO:57)	
LTR 5'	Nucleotides #395-986 of SEQ ID NO:57
CD8a-SP (signal peptide)	Nucleotides #1858-1920 of SEQ ID NO:57
Membrane-bound IL-15 polypeptide	Nucleotides #1921-2262 of SEQ ID NO:57
IL-15Ra polypeptide	Nucleotides #2341-3051 of SEQ ID NO:57
Cytoplasmic portion of the IL-15Rb polypeptide	Nucleotides #3052-3909 of SEQ ID NO:57
Cytoplasmic portion of the common	Nucleotides #3910-4167 of SEQ ID NO:57

cytokine receptor γ chain	
EF1 promoter	Nucleotides #4186-4733 of SEQ ID NO:57
tdTomato	Nucleotides #4745-6172 of SEQ ID NO:57
LTR 3'	Nucleotides #6380-6973 of SEQ ID NO:57

Amino acid sequences

Membrane-bound IL-15	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKC FLEELQVISLES GDASIHDTVENLILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO:72)
IL-15Ra polypeptide	ITCPPMSVEHADIWVKSYSLSRERYICNSGFKRKAGTSSLT ECVLNKATNVAHWTTPLSKCIRD PALVHQRPAPPSTVTTAGV TPQPESLSPSGKEPAASSPSSNNTAATTAIVPGSQLMPSKSPS TGTTEISSHESHGTPSQTTAKNWELTASASHQPPGVYPQGHS DTTVAISTSTVLLCGLSAV SLLACYLKS RQTPPLASVEMEAM EALPVTWGTSSRDEDL ENCSHHL (SEQ ID NO:73)
Cytoplasmic portion of the IL-15Rb polypeptide	NCRNTGPWLKKVLKCNTPDPSKFFS QLSSEHGGDVQKWLSS PFPSSSFSPGGLAPEISPLEVLERDKVTQLLLQQDKVPEPASLS SNHSLTSCFTNQGYFFFHLPDALEIEACQVYFTYDPYSEEDPD EGVAGAPTGSSPQPLQPLSGEDDAYCTFP SRDDLLLFSPSLLG GPSPPSTAPGGSGAGEERMPPSLQERVPRDWD PQPLGPPTPG VPDLVDFQPPPELVLREAGEEVPDAGPREGV SFPWSRPPGQG EFRALNARLPLNTDAYLSLQELQGQDP THLV (SEQ ID NO:74)
Cytoplasmic portion of the common cytokine receptor γ chain	ERTMPRIPTLKNLEDLVTEYHGNFSAWSGVSKGLAESLQPDY SERLCLVSEIPP KGGALGEGPGASPCNQHS PYWAPPCYTLKPE T (SEQ ID NO:75)

[0088] In one embodiment, **Figure 9** shows a map of a vector 4 comprising sequences encoding a membrane-bound IL-15, IL-15Ra, IL-15Rb with a portion of the cytoplasmic region deleted, and a full cytoplasmic portion of the common cytokine receptor γ chain. The vector has the polynucleotide sequence of SEQ ID NO:58.

Components of Vector 4 (SEQ ID NO:58)	
LTR 5'	Nucleotides #395-986 of SEQ ID NO:58
CD8a-SP (signal peptide)	Nucleotides #1858-1920 of SEQ ID NO:58
Membrane-bound IL-15 polypeptide	Nucleotides #1921-2262 of SEQ ID NO:58
IL-15Ra polypeptide	Nucleotides #2341-3051 of SEQ ID NO:58
IL-15Rb with a portion of the cytoplasmic region deleted	Nucleotides #3052-3333 of SEQ ID NO:58

Cytoplasmic portion of the common cytokine receptor γ chain	Nucleotides #3334-3591 of SEQ ID NO:58
EF1 promoter	Nucleotides #3610-4155 of SEQ ID NO:58
tdTomato	Nucleotides #4169-5596 of SEQ ID NO:58
LTR 3'	Nucleotides #5804-6397 of SEQ ID NO:58

Amino acid sequences

Membrane-bound IL-15	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKC FLLELQVISLES GDASIHDTVENLIILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO:76)
IL-15Ra polypeptide	ITCPPMMSVEHADIWVKSYSLSYRERYICNSGFKRKAGTSSLT ECVLNKATNVAHWTTPLSKCIRD PALVHQR PAPPSTVTTAGV TPQPESLSPSGKEPAASSPSSNNTAATTA AIVPGSQLMPSKSPS TGTTEISSHESHGTPSQTTAKNWELTASASHQPPGVYPQGHS DTTVAISTSTVLLCGLSAVSLLACYLKS RQTPPLASVEMEAM EALPVTWGTSSRDEDL ENC SHHL (SEQ ID NO:77)
IL-15Rb with a portion of the cytoplasmic region deleted	NCRNTGPWLKKVLKCNTPDPSKFFS QLSSEHGGDVQKWLSS PFPSSSFSPGGLAPEISPLEVLERDKVTQLLPLNTDAYLSLQEL QGQDPTHLV (SEQ ID NO:78)
Cytoplasmic portion of the common cytokine receptor γ chain	ERTMPRIPTLKNLEDLVTEYHGNFSAWSGVSKGLAESLQPDY SERLCLVSEIPP KGGALGEGPGASPCNQHS PYWAPPCYTLKPE T (SEQ ID NO:79)

[0089] In one embodiment, **Fig. 10** shows a map of a vector 5 comprising sequences encoding an ER-trapped IL-15. The vector has the polynucleotide sequence of SEQ ID NO:59.

Components of Vector 5 (SEQ ID NO:59)	
LTR 5'	Nucleotides #395-986 of SEQ ID NO:59
ER-trapped IL-15 polypeptide	Nucleotides #2002-2343 of SEQ ID NO:59
GSEKDEL signal peptide	Nucleotides #2344-2364 of SEQ ID NO:59
EF1 promoter	Nucleotides #2383-2928 of SEQ ID NO:59
tdTomato	Nucleotides #2942-4369 of SEQ ID NO:59
LTR 3'	Nucleotides #4577-5170 of SEQ ID NO:59

Amino acid sequences

ER-trapped IL-15	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKC FLLELQVISLES GDASIHDTVENLIILANNSLSSNGNVTESGCK
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ECEELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO:80)

[0090] In one embodiment, **Fig. 11** shows a map of a vector 6 comprising sequences encoding an IL-15 and IL-15Ra that are both ER-trapped. The vector has the polynucleotide sequence of SEQ ID NO:60.

Components of Vector 6 (SEQ ID NO:60)	
LTR 5'	Nucleotides #395-986 of SEQ ID NO:60
CD8a-SP (signal peptide)	Nucleotides #1858-1920 of SEQ ID NO:60
GSEKDEL signal peptide	Nucleotides #1921-1941 of SEQ ID NO:60
ER-trapped IL-15 polypeptide	Nucleotides #1942-2283 of SEQ ID NO:60
IL-15Ra polypeptide	Nucleotides #2362-3072 of SEQ ID NO:60
EF1 promoter	Nucleotides #3091-3636 of SEQ ID NO:60
tdTomato	Nucleotides #3650-5077 of SEQ ID NO:60
LTR 3'	Nucleotides #5285-5878 of SEQ ID NO:60

Amino acid sequences

ER-trapped IL-15	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKC FLLELQVISLES GDASIHDTVENLIILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO:81)
IL-15Ra polypeptide	ITCPPMSVEHADIWVKSYSLSRERYICNSGFKRKAGTSSLT ECVLNKATNVAHWTTPLSKCIRD PALVHQR PAPPSTVTTAGV TPQPESLSPSGKEPAASSPSSNNTAATTAIVPGSQLMPSKSPS TGTTEISSHESHGTPSQTTAKNWELTASASHQPPGVYPQGHS DTTVAISTSTVLLCGLSAV SLLACYLKS RQTPPLASVEMEAM EALPVTWGTSSRDEDL ENCSHHL (SEQ ID NO:82)

[0091] In one embodiment, **Fig. 12** shows a map of a vector 7 comprising sequences encoding IL-15Ra, IL-15Rb, and a soluble IL-15. The vector has the polynucleotide sequence of SEQ ID NO:61.

Components of Vector 7 (SEQ ID NO:61)	
LTR 5'	Nucleotides #395-986 of SEQ ID NO:61
IL-15Ra polypeptide (codon optimized)	Nucleotides #1858-2658 of SEQ ID NO:61
P2A sequence	Nucleotides #2668-2724 of SEQ ID NO:61
IL-15Rb polypeptide	Nucleotides #2725-4377 of SEQ ID NO:61
P2A sequence	Nucleotides #4387-4443 of SEQ ID NO:61
Soluble IL-15 polypeptide	Nucleotides #4588-4929 of SEQ ID NO:61

EF1 promoter	Nucleotides #4948-5493 of SEQ ID NO:61
tdTomato	Nucleotides #5507-6934 of SEQ ID NO:61
LTR 3'	Nucleotides #7142-7735 of SEQ ID NO:61

Amino acid sequences

IL-15Ra polypeptide (codon optimized)	MAPRRARGCRTLGLPALLLLLLRPPATRGITCPPPMSVEH ADIWVKSYSLYSRERYICNSGFKRKAGTSSLTECVLNKATN VAHWTTPSLKCIRDPALVHQRPAPPSTVTTAGVTPQPESLSP SGKEPAASSPSSNNTAATTAIVPGSQLMPSKSPSTGTTEISS HESSHGTPSQTTAKNWELTASASHQPPGVYPQGHSDTTVAI STSTVLLCGLSAVSLACYLKSRQTPPLASVEMEAMEALPV TWGTSSRDELENC SHHL (SEQ ID NO:83)
IL-15Rb polypeptide	MAAPALSWRLPLLILLPLATSWASAAVNGTSQFTCFYNSR ANISCVWSQD GALQD TSCQVHAWPDRRRWNQTCELLPVS QASWACNLILGAPDSQKLT TVDIVTLRVLCREGVRWRVMA IQDFKPFENLR LMAPISLQVVHVETHRCNISWEISQASHYFE RHLEFEARTLSPGHTWEEAPLLTLKQKQE WICLETLTPDTQ YEFQVRVKPLQGEFTTWSPWSQPLAFRTKPAALGKDTIPW LGHLLVGLSGAFGFILVYLLINCRNTGPWLKKVLKCNTPD PSKFFSQLSSEHGGDVQKWLSSPFPSSSFSPGGLAPEISPLEV LERDKVTQLLLQQDKVPEPASLSSNHSLTSCFTNQG YFFFH LPDALEIEACQVYFTYDPYSEEDPDEGVAGAPTGSSPQPLQ PLSGEDDAYCTFPSRDDLLLFSPSLLGGPSPSTAPGGSGAG EERMPPSLQERVPRDWD PQLGPPTPGVPDLVDFQPPPELV LREAGEEVPDAGPREGV SFPWSRPPGQGEFRALNARLPLNT DAYLSLQELQGQDPTHLV (SEQ ID NO:84)
Soluble IL-15 polypeptide	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMK CFLLELQVISLESGDASIHDTVENLIILANNLSNNGNVTESG CKECEEELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO:85)

[0092] In one embodiment, **Fig. 13** shows a map of a vector 8 comprising sequences encoding IL-15Ra and a soluble IL-15. The vector has the polynucleotide sequence of SEQ ID NO:62.

Components of Vector 8 (SEQ ID NO:62)	
LTR 5'	Nucleotides #395-986 of SEQ ID NO:62
IL-15Ra polypeptide (codon optimized)	Nucleotides #1858-2658 of SEQ ID NO:62
P2A sequence	Nucleotides #2668-2724 of SEQ ID NO:62
Soluble IL-15 polypeptide	Nucleotides #2869-3210 of SEQ ID NO:62
EF1 promoter	Nucleotides #3229-3774 of SEQ ID NO:62

tdTomato	Nucleotides #3788-5215 of SEQ ID NO:62
LTR 3'	Nucleotides #5423-6016 of SEQ ID NO:62

Amino acid sequences

IL-15Ra polypeptide (codon optimized)	MAPRRARGCRTLGLPALLLLLLLRPPATRGITCPPPMSVEHADIWVK SYSLYSRERYICNSGFKRKAGTSSLTECVLNKATNVAHWTTPSLKCI RDPALVHQRPAAPPSTVTTAGVTPQPESLSPSGKEPAASSPSSNNTAAT TAAIVPGSQLMPSKSPSTGTTEISSHESHGTPSQTTAKNWELTASAS HQPPGVYPQGHSDTTVAISTSTVLLCGLSAVSLACYLKSRQTPPLA SVEMEAMEALPVTWGTSSRDEDLNCSHHL (SEQ ID NO:86)
Soluble IL-15 polypeptide	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLEL QVISLESGDASIHDTVENLILANNSLSSNGNVTESGCKECELEEKNI KEFLQSFVHIVQMFINTS (SEQ ID NO:87)

[0093] In one embodiment, **Fig. 14** shows a map of a vector 9 comprising sequences encoding an ER-trapped fusion protein comprising IL-15, IL-15Ra, IL-15Rb, and a full cytoplasmic portion of the common cytokine receptor γ chain. The vector has the polynucleotide sequence of SEQ ID NO:63.

Components of Vector 9 (SEQ ID NO:63)	
LTR 5'	Nucleotides #395-986 of SEQ ID NO:63
CD8a-SP (signal peptide)	Nucleotides #1858-1920 of SEQ ID NO:63
GSEKDEL signal peptide	Nucleotides #1921-1941 of SEQ ID NO:63
ER-trapped IL-15 polypeptide	Nucleotides #1942-2283 of SEQ ID NO:63
IL-15Ra polypeptide	Nucleotides #2362-3072 of SEQ ID NO:63
Cytoplasmic portion of the IL-15Rb polypeptide	Nucleotides #3073-3930 of SEQ ID NO:63
Cytoplasmic portion of the common cytokine receptor γ chain	Nucleotides #3931-4188 of SEQ ID NO:63
EF1 promoter	Nucleotides #4207-4752 of SEQ ID NO:63
tdTomato	Nucleotides #4766-6193 of SEQ ID NO:63
LTR 3'	Nucleotides #6401-6944 of SEQ ID NO:63

Amino acid sequences

ER-trapped IL-15 polypeptide	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLEL LELQVISLESGDASIHDTVENLILANNSLSSNGNVTESGCKECELEE LEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO:88)
IL-15Ra polypeptide	ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLTEC

	VLNKATNV AHWTTPSLK CIRD PALVHQR PAPPSTVTTAGVTPQP ESLSPSGKEPAASSPSSNNTAATTA AIVPGSQLMPSKSPSTGTTEIS SHESHGTPSQTTAKNWELTASASHQPPGVYPQGHSDTTVAISTS TVLLCGLSAV SLLACYLKSRQTPPLASVEMEAMEALPVTWGTSS RDEDL ENC SHHL (SEQ ID NO:89)
Cytoplasmic portion of the IL-15Rb polypeptide	NCRNTGPWLKKVLKCNTPDPSKFFSQLSSEHGGDVQKWLSSPFP SSSFSPGGLAPEISPLEVLERDKVTQLLLQQDKVPEPASLSSNHSL TSCFTNQGYFFFHLPDALEIEACQVYFTYDPYSEEDPDEGVAGAP TGSSPQPLQPLSGEDDAYCTFPSRDDLLL FSPSLLGGPSPSTAPG GSGAGEERMPPSLQERVPRDWD PQPLGPPTPGVPDLVDFQPPPE LVLREAGEEVPDAGPREGVSFPWSRPPGQGEFRALNARLPLNTD AYLSLQELQGQDP THLV (SEQ ID NO:90)
Cytoplasmic portion of the common cytokine receptor γ chain	ERTMPRIPTLKNLEDLVTEYHGNFSAWSGVSKGLAESLQPDYSE RLCLVSEIPP KGGALGEGPGASPCNQHSPYWAPPCYTLKPET (SEQ ID NO:91)

[0094] In some embodiments, the iPSC-NK cells comprise a knock-in of an IL-15 construct disclosed herein into a gene of the iPSC-NK cells, wherein the IL-15 construct is operably linked to a promoter that is not active in iPSC, but becomes active during or after iPSC differentiation. The promoter can be an exogenous or endogenous promoter. The promoter can be expressed in common progenitor/precursor cells during stem cell differentiation, or expressed in many different cell types after differentiation, or can be cell-type specific. Examples for such promoters include, but are not limited to, NKp46 that is active in NK cells; CD3 γ promoter that is active in T cells; CD19 promoter for B cells; VEGFR promoter and ICAM1 promoter that are active in endothelial cells; B2M promoter that is active in many different cells types; IL2R β promoter that is active in many immune cells; Netin promoter, Sox 2 promoter, Olig 1 promoter, Olig 2 promoter, Olig3 promoter, MBP promoter, OSP promoter, MOG promoter, or Sox10 promoter that is active in neural cells; Sca-1 promoter, CD27 promoter, CD34 promoter, CD38 promoter, CD43 promoter, CD48 promoter, CD117 promoter, or CD 150 promoter that is active in hematopoietic progenitor cells; IL-7R promoter that is active lymphoid precursors; CD45RA promoter, IL-3R α promoter, or thrombopoietin receptor promoter that is active in myeloid progenitors; Is11 promoter, sarcomeric proteins α -myosin heavy chain promoter, β -myosin heavy chain promoter, cTnI promoter, MYH7 promoter, MYH6 promoter, cTNNI promoter, MLC-2A promoter, CACNA1C promoter, CACNA1D promoter, hERG promoter, or HCN-2 promoter for cardiomyocyte

precursors.

[0095] In some embodiments, the NK cells are genetically modified by deleting or inactivating (or “knocking out”) a gene encoding TGF β receptor 2 (TGF β R2). Optionally, such iNK cells comprising a deletion in TGF β R2 further comprise a knock-in of an IL-15 construct as disclosed herein.

[0096] A gene may be inactivated, for example, by introducing a homozygous or heterozygous inactivating mutation into said gene. A homozygous inactivating mutation results in complete loss of protein function and, in some cases, loss of protein expression. In some embodiments, the NK cells provided herein are genetically modified by inactivating the TGF β R2 gene, e.g., by targeting an exon of TGF β R2. In certain embodiments, the TGF β R2 gene may be inactivated by introducing the IL-15 construct as disclosed herein into the TGF β R2 locus. In certain embodiments, the TGF β R2 gene may be inactivated by introducing a dominant negative form of TGF β R2 into the cell. Exemplary sequences of gRNAs that may be used to knockout TGF β R2 in the iPSC NK cells described herein are provided in WO 2023/06013.

[0097] In some embodiments, the iPSC-NK cells provided herein are modified such that they are deficient in TGF β R2 signaling, e.g., by deleting the intracellular signaling domain of TGF β R2. This may be accomplished by, e.g., introducing a stop codon into a suitable position in the TGF β R2 amino acid sequence (for example, introducing a stop codon after the transmembrane domain). Exemplary gRNA sequences and their corresponding donor DNA sequences that may be used to knockout the TGF β R2 signaling domain by introducing a stop codon after the TGF β R2 transmembrane domain are provided in WO 2023/06013.

[0098] In some embodiments, the method described herein result in a population of iPSC-NK cells wherein about 40% to about 45%, about 45% to about 50%, about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90 to about 95%, or about 95% to 100% of cells comprise an inactivating mutation in TGFBR2 and/or express detectable levels of IL-15 or a functional fragment thereof. In some embodiments, the method described herein result in a population of iPSC-NK cells wherein at least 50%, at least 55%, at least 60%, at least 65%, at last 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or more than 98% of cells comprise an inactivating mutation in TGFBR2 and/or express detectable levels of IL-15 or a functional fragment thereof. The expression level of IL-15

or a functional fragment thereof may be determined using any suitable method known in the art or described herein, including, for example, flow cytometry, Western Blotting, Enzyme Linked Immunosorbent Assays (ELISA), quantitative real-time PCR (qPCR) and RNA sequencing. In some embodiments, the iNK cells comprise an inactivating (e.g., a frameshift) mutation in TGF β R2 and express a truncated form of TGF β R2 protein. The inactivating mutation in TGF β R2 may be biallelic or monoallelic.

[0099] In some embodiments, the iNK cells provided herein are resistant to the suppressive effect of TGF β signaling. For example, the expression level of NKG2D, DNAM and/or NKp30 on the surface of an iNK cell may remain comparable after treatment with TGF β 1 to the levels before treatment.

[0100] In some embodiments, the iNK cells provided herein are able to survive without the stimulation of exogenous cytokines. Cell survival may be determined by measuring cell counts or cell viability.

[0101] In some embodiments, the iNK cells provided herein show higher cell killing ability than unmodified NK cells. Cell killing ability may be determined by incubating the iNK (and unmodified control NK) cells with target cells and measuring the disappearance of the target cells using, e.g., a fluorescent marker. In some embodiments, the iNK cells provided herein kill target cells with an efficiency that is about 10% to about 20%, about 20% to about 30%, about 30% to about 40%, about 40% to about 50%, about 50% to about 60%, about 60% to about 70%, about 70% to about 80%, about 80% to about 90%, about 90% to about 100%, about 2-3 times, about 3-4 times, about 4-5 times, about 5-6 times, about 6-7 times, about 7-8 times, about 8-9 times or about 9-10 times higher than that of NK cells not comprising the IL-15 construct disclosed herein.

[0102] In some embodiments, the iNK cells described herein persist longer in the circulation after intravenous administration to a patient. In some embodiments, the iNK cells comprising the IL-15 construct disclosed herein persist in the circulation of a subject about 10% to about 20%, about 20% to about 30%, about 30% to about 40%, about 40% to about 50%, about 50% to about 60%, about 60% to about 70%, about 70% to about 80%, about 80% to about 90%, about 90% to about 100%, about 2-3 times, about 3-4 times, about 4-5 times, about 5-6 times, about 6-7 times, about 7-8 times, about 8-9 times or about 9-10 times longer than NK cells not comprising the IL-15 construct.

[0103] The tumor microenvironment has a suppressive effect on NK cell function and inhibits NK

cell function e.g., via soluble factors (e.g., cytokines), hypoxic conditions and/or low nutrient levels. See, e.g., Melaiu et al., *Front. Immunol.*10:3038. In some embodiments, the iNK cells described herein are resistant to the suppressive effects of the tumor microenvironment.

[0104] In some embodiments, the average expression level of TGF β R2 in a population of iNK cells provided herein is about 10% to about 20%, about 20% to about 30%, about 30% to about 40%, about 40% to about 50%, about 50% to about 60%, about 60% to about 70%, about 70% to about 80%, about 80% to about 90%, about 90% to about 95% or more than 95% lower than the average expression level of TGF β R2 in a population of unedited NK cells. In some embodiments, the expression level of TGF β R2 in the iNK cells is about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or about 95% lower than the expression level of TGFBR2 in unedited NK cells.

[0105] In the case of NK cells differentiated from iPSC, the genetic modifications described herein may be introduced before the differentiation of the iPSCs into iPSC-NK cells (“iNK cells”). Modified iPSC cells may be cryopreserved before differentiation into iNK cells.

[0106] In some embodiments, the iNK cells provided herein comprise polynucleotide sequences encoding IL-15 and IL-2R β , and a knockout of cytokine inducible SH2 containing protein (CISH). In some embodiments, the IL-15 can be a membrane-bound form of IL-15, a soluble form of IL-15, or an ER-trapped form of IL-15. In some embodiments, the IL-15 is further expressed as fusion protein with an IL-15R or a functional fragment thereof, e.g. IL-15Ra or IL-15Rb. Knockout of CISH can be accomplished by genetic editing. In one embodiment, the genetic editing comprises using a TALEN construct or a Cas9 or Cas12 enzyme.

[0107] In some embodiments, the iNK cells provided herein comprise polynucleotide sequences encoding IL-15 and IL-2R β , and a knockout of a NK cell inhibitory receptor, e.g. T-cell immunoglobulin and ITIM domain (TIGIT) receptor. In some embodiments, the IL-15 can be a membrane-bound form of IL-15, a soluble form of IL-15, or an ER-trapped form of IL-15. In some embodiments, the IL-15 is further expressed as fusion protein with an IL-15R or a functional fragment thereof, e.g. IL-15Ra or IL-15Rb. Knockout of TIGIT can be accomplished by genetic editing. In one embodiment, the genetic editing comprises using a TALEN construct or a Cas9 or Cas12 enzyme.

[0108] In some embodiments, the iNK cells provided herein comprise polynucleotide sequences encoding IL-15 and IL-2R β , and a knockout of CISH and a knockout of a NK cell inhibitory

receptor, e.g. T-cell immunoglobulin and ITIM domain (TIGIT) receptor. In some embodiments, the IL-15 can be a membrane-bound form of IL-15, a soluble form of IL-15, or an ER-trapped form of IL-15. In some embodiments, the IL-15 is further expressed as fusion protein with an IL-15R or a functional fragment thereof, e.g. IL-15Ra or IL-15Rb.

PHARMACEUTICAL COMPOSITIONS

[0109] Also provided herein are pharmaceutical compositions and formulations comprising the iPSCs or the iNK cells described herein. In some embodiments, a pharmaceutical composition comprises a dose ranging from about 1×10^5 to about 5×10^5 iNK cells, about 5×10^5 to about 1×10^6 iNK cells, about 1×10^6 to about 5×10^6 iNK cells, about 5×10^6 iNK cells to about 1×10^7 iNK cells, about 1×10^7 to about 5×10^7 iNK cells, about 5×10^7 to 1×10^8 iNK cells, about 1×10^8 to about 5×10^8 iNK cells, about 5×10^8 to about 1×10^9 iNK cells, about 1×10^9 to about 5×10^9 iNK cells, about 5×10^9 to 1×10^{10} iNK cells, about 1×10^{10} to about 5×10^{10} iNK cells, about 5×10^{10} to about 1×10^{11} iNK cell, about 1×10^{11} to about 5×10^{11} iNK cells, about 5×10^{11} to 1×10^{12} iNK cells, about 1×10^{12} to about 5×10^{12} iNK cells, about 5×10^{12} to about 1×10^{13} iNK cells.

[0110] In some embodiments, a pharmaceutical composition is cryopreserved. A composition comprising the iPSCs or the iNK cells provided herein may be cryopreserved for about 1-3 months, about 3-6 months, about 6-9 months, or about 9-12 months. In some embodiments, a composition comprising iPSC-NK cells provided herein may be cryopreserved for more than 3 months, more than 6 months, more than 9 months, more than 12 months, more than 18 months, more than 2 years, or more than 3 years before thawing and use in a method described herein. A formulation comprising the iPSCs or the iPSC-NK cells described herein may further comprise a cryoprotectant. In such embodiments wherein the iPSC NK cells are cryopreserved and thawed before use in a method described herein, the viability of the iPSC-NK cells is at least 30%, at least 50%, or at least 70% as determined by a suitable assay known in the art or described herein.

[0111] The iPSCs or the iNK cells described herein can be incorporated into any pharmaceutical composition suitable for administration. Such compositions typically comprise the iPSCs or the iNK cells and a pharmaceutically acceptable carrier. As used herein, the term “pharmaceutically acceptable carrier” is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent

edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

[0112] A pharmaceutical composition of the present disclosure is formulated to be compatible with its intended route of administration, e.g., intravenous administration. Solutions or suspensions used for intravenous administration can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0113] It is especially advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

[0114] The formulation can also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Alternatively, or in addition, the composition can comprise an agent

that enhances its function, such as, for example, a cytotoxic agent, cytokine, chemotherapeutic agent, or growth-inhibitory agent. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

METHODS OF TREATMENT

[0115] Further provided herein are methods of treating a medical disease or disorder in a subject by administering to the subject a therapeutically effective amount of the iPSCs or the iNK cells provided herein. In one embodiment, therapeutic formulations of the invention, which comprise the iPSC-NK cells of the invention, are used to treat or alleviate a symptom associated with a cancer, including solid cancers and hematological cancers. Examples of cancers that may be treated with a method described herein include, without limitation, leukemias, lymphomas, breast cancer, colon cancer, ovarian cancer, bladder cancer, prostate cancer, glioma, lung & bronchial cancer, colorectal cancer, pancreatic cancer, esophageal cancer, liver cancer, urinary bladder cancer, kidney and renal pelvis cancer, oral cavity & pharynx cancer, uterine corpus cancer, and/or melanoma. Examples of cancers are also disclosed in WO 2023/060136.

[0116] The present invention also provides methods of inhibiting the proliferation of tumor cells in a subject, comprising administering to the subject a population of the iPSCs or the iPSC-NK cells provided herein. A therapeutic regimen is carried out by identifying a subject, e.g., a human patient suffering from (or at risk of developing) a cancer, using standard methods.

[0117] Efficaciousness of treatment is determined in association with any known method for diagnosing or treating the particular immune-related disorder. Alleviation of one or more symptoms of the disease or disorder indicates that the treatment confers a clinical benefit. In some embodiments, a method provided herein results in decreased tumor proliferation in the subject.

[0118] As used herein, the “administration” of an agent, (e.g., a population of iPSC-NK cells), to a subject includes any route of introducing or delivering to a subject a compound to perform its intended function. In a preferred embodiment, the population of iPSC-NK cells is administered intravenously. In some embodiments, the population of iPSC-NK cells provided herein is administered by intravenous infusion, e.g., an intravenous infusion over about 15min, about 30min, about 45min, about 60min, about 90min, about 2 hours, about 3 hours, about 4 hours, or about 5 hours, or an intravenous infusion over about 15min to about 30min, about 30min to about 45min, about 45 min to about 60min, about 60 min to about 90min, about 90min to about 2 hours,

about 2 hours to about 3 hours, about 3 hours to about 4 hours, or about 4 hours to about 5 hours. The rate of infusion may vary with the number of cells being infused to the subject.

[0119] A therapeutically effective amount of the iPSC-NK cells of the invention relates generally to the amount needed to achieve a therapeutic objective. It is also to be appreciated that the various modes of treatment or prevention of medical conditions as described are intended to mean “substantial”, which includes total but also less than total treatment or prevention, and wherein some biologically or medically relevant result is achieved. In some embodiments, about 1×10^5 to about 5×10^5 iNK cells, about 5×10^5 to about 1×10^6 iNK cells, about 1×10^6 to about 5×10^6 iNK cells, about 5×10^6 iNK cells to about 1×10^7 iNK cells, about 1×10^7 to about 5×10^7 iNK cells, about 5×10^7 to 1×10^8 iNK cells, about 1×10^8 to about 5×10^8 iNK cells, about 5×10^8 to about 1×10^9 iNK cells, about 1×10^9 to about 5×10^9 iNK cells, about 5×10^9 to 1×10^{10} iNK cells, about 1×10^{10} to about 5×10^{10} iNK cells, about 5×10^{10} to about 1×10^{11} iNK cell, about 1×10^{11} to about 5×10^{11} iNK cells, about 5×10^{11} to 1×10^{12} iNK cells, about 1×10^{12} to about 5×10^{12} iNK cells, about 5×10^{12} to about 1×10^{13} iNK cells are administered to the subject.

[0120] In some embodiments, one or more doses of the iPSC-NK cells are administered. If two or more doses of the iPSC-NK cells are administered, the duration between the administrations should be sufficient to allow time for propagation of the cells in the individual. In specific embodiments the duration between doses is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 or more weeks.

[0121] As used herein, “treating” or “treatment” of a disease in a subject refers to (1) inhibiting the disease or arresting its development; or (2) ameliorating or causing regression of the disease or the symptoms of the disease. As understood in the art, “treatment” is an approach for obtaining beneficial or desired results, including clinical results. For the purposes of this disclosure, beneficial or desired results can include one or more, but are not limited to, alleviation or amelioration of one or more symptoms, diminishment of extent of a condition (including a disease), stabilized (i.e., not worsening) state of a condition (including disease), delay or slowing of condition (including disease), progression, amelioration or palliation of the condition (including disease), states and remission (whether partial or total), whether detectable or undetectable.

[0122] In certain embodiments of the present disclosure, iPSC-NK cells are delivered to an individual in need thereof, and the individual has been diagnosed with a cancer. Without wishing to be bound by theory, the cells then enhance the individual's immune system to attack or directly attack the respective cancer or pathogenic cells.

COMBINATION THERAPIES

[0123] The iPSCs or the iPSC-NK cells described herein may be administered in combination with one or more other therapeutic agents. In some embodiments, the additional therapy may be radiation therapy, surgery (e.g., lumpectomy and a mastectomy), chemotherapy, gene therapy, DNA therapy, viral therapy, RNA therapy, immunotherapy, bone marrow transplantation, nanotherapy, monoclonal antibody therapy, or a combination of the foregoing. The additional therapy may be in the form of adjuvant or neoadjuvant therapy.

[0124] In some embodiments, the iPSC-NK cells described herein may be administered in combination with one or more other anti-NK inhibitory receptor agents, such as antibody, RNAi- or small molecule-based agents. The anti-NK inhibitory receptor agents include, but are not limited to, checkpoint inhibitors, such as PD-1/PD-L1 inhibitors, TIGIT inhibitors, TIM-3 inhibitors, and LAG-3 inhibitors. For example, TIGIT/PVR inhibitors include, but are not limited to, Ociperlimab, BAT6005, BMS 986207, PH 804, AGEN 1777, TSRF-786C, liothyronine; TIM-3 inhibitors include but are not limited to Cobolimab BMS-986258, Sabatolimab. The anti-NK inhibitory receptor agents can also be inhibitors of therapeutic targets upstream of NK cell inhibitory receptor, such as Elraglusib, a selective small-molecule inhibitor of glycogen synthase kinase-3 beta that reduces expression of immune checkpoint molecules PD-1, TIGIT and LAG-3.

[0125] In some embodiments, the subject can be administered nonmyeloablative lymphodepleting chemotherapy prior to administering the iNK cells provided herein. The nonmyeloablative lymphodepleting chemotherapy can be any suitable such therapy, which can be administered by any suitable route. The nonmyeloablative lymphodepleting chemotherapy can comprise, for example, the administration of cyclophosphamide and fludarabine. An exemplary route of administering cyclophosphamide and fludarabine is intravenous. Likewise, any suitable dose of cyclophosphamide and fludarabine can be administered. For example, around 60 mg/kg of cyclophosphamide is administered for two days after which around 25 mg/m² fludarabine is administered for five days.

[0126] In some embodiments, the nonmyeloablative lymphodepleting immunotherapy can comprise, for example, the administration of an anti-CD52 agent or anti-CD20 agent. In some embodiments, the lymphodepleting immunotherapy is an anti-CD52 antibody. In some embodiments, the anti-CD52 antibody is alemtuzumab. In some embodiments, the lymphodepleting immunotherapy is an anti-CD20 antibody. Exemplary anti-CD20 antibodies

include, but are not limited to rituximab, ofatumumab, ocrelizumab, obinutuzumab, ibritumomab or iodine 131 tositumomab. An exemplary route of administering anti-CD52 agent or anti-CD20 agent is intravenous. Likewise, any suitable dose of anti-CD52 agent or anti-agent can be administered.

[0127] In some embodiments, immune cell growth factor that promotes the growth and activation of the immune cells is administered to the subject either concomitantly with the iNK cells provided herein or subsequently to the iNK cells provided herein. The immune cell growth factor can be any suitable growth factor that promotes the growth and activation of the immune cells. Examples of suitable immune cell growth factors include, but are not limited to, interleukin (IL)-2, IL-7, IL-15, and IL-12, which can be used alone or in various combinations, such as IL-2 and IL-7, IL-2 and IL-15, IL-7 and IL-15, IL-2, IL-7 and IL-15, IL-12 and IL-7, IL-12 and IL-15, or IL-12 and IL2. In some embodiments, the iPSC-NK cells are not administered in combination with an interleukin.

[0128] In some embodiments, the additional therapy is the administration of small molecule enzymatic inhibitor or anti-metastatic agent. In some embodiments, the additional therapy is the administration of side-effect limiting agents (e.g., agents intended to lessen the occurrence and/or severity of side effects of treatment, such as anti-nausea agents, etc.). In some embodiments, the additional therapy is radiation therapy. In some embodiments, the additional therapy is surgery. In some embodiments, the additional therapy is a combination of radiation therapy and surgery. In some embodiments, the additional therapy is gamma irradiation. In some embodiments, the additional therapy is therapy targeting PBK/AKT/mTOR pathway, HSP90 inhibitor, tubulin inhibitor, apoptosis inhibitor, and/or chemopreventative agent. The additional therapy may be one or more of the chemotherapeutic agents known in the art.

[0129] Combination therapies can include, but are not limited to, one or more anti-microbial agents (for example, antibiotics, anti-viral agents and anti-fungal agents), anti-tumor agents (for example, fluorouracil, methotrexate, paclitaxel, fludarabine, etoposide, doxorubicin, or vincristine), immune-depleting agents (for example, fludarabine, etoposide, doxorubicin, or vincristine), immunosuppressive agents (for example, azathioprine, or glucocorticoids, such as dexamethasone or prednisone), anti-inflammatory agents (for example, glucocorticoids such as hydrocortisone, dexamethasone or prednisone, or non-steroidal anti-inflammatory agents such as acetylsalicylic acid, ibuprofen or naproxen sodium), cytokine antagonists (for example, anti-TNF

and anti-IL-6), cytokines (for example, interleukin-10 or transforming growth factor-beta), hormones (for example, estrogen), or a vaccine. In addition, immunosuppressive or tolerogenic agents including but not limited to calcineurin inhibitors (e.g., cyclosporin and tacrolimus); mTOR inhibitors (e.g., Rapamycin); mycophenolate mofetil, antibodies (e.g., recognizing CD3, CD4, CD40, CD154, CD45, IVIG, or B cells); chemotherapeutic agents (e.g., Methotrexate, Treosulfan, Busulfan); irradiation; or chemokines, interleukins or their inhibitors (e.g., BAFF, IL-2, anti-IL-2R, IL-4, JAK kinase inhibitors) can be administered. Such additional pharmaceutical agents can be administered before, during, or after administration of the iNK cells provided herein, depending on the desired effect. This administration of the iNK cells and the agent can be by the same route or by different routes, and either at the same site or at a different site.

[0130] The iPSC-NK cells provided herein may be administered before, during, or after, an additional therapeutic agent, such as an immune checkpoint inhibitor. The administrations may be in intervals ranging from concurrently to minutes to days to weeks. In embodiments where the iPSC-NK cell therapy is provided to a patient separately from an additional therapeutic agent, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the two compounds would still be able to exert an advantageously combined effect on the patient. In such instances, it is contemplated that one may provide a patient with the iPSC-NK cell therapy and the anti-cancer therapy (e.g., an immune checkpoint inhibitor) within about 12 to 24 or 72 h of each other and, more particularly, within about 6-12 h of each other. In some situations it may be desirable to extend the time period for treatment significantly where several days (2, 3, 4, 5, 6, or 7) to several weeks (1, 2, 3, 4, 5, 6, 7, or 8) lapse between respective administrations.

[0131] The skilled artisan will understand that immunotherapies may be used in combination or in conjunction with methods of the embodiments described herein. In the context of cancer treatment, immunotherapeutics generally rely on the use of immune effector cells and molecules to target and destroy cancer cells. Rituximab (RITUXAN®) is such an example. The immune effector may be, for example, an antibody specific for some marker on the surface of a tumor cell. The antibody alone may serve as an effector of therapy or it may recruit other cells to actually affect cell killing. The antibody also may be conjugated to a drug or toxin (chemotherapeutic, radionuclide, ricin A chain, cholera toxin, pertussis toxin, etc.) and serve as a targeting agent. Alternatively, the effector may be a lymphocyte carrying a surface molecule that interacts, either

directly or indirectly, with a tumor cell target. Examples of immunotherapies are described in WO 2023/060136.

[0132] In some embodiments, the immunotherapy may be an immune checkpoint inhibitor. Immune checkpoints either turn up a signal (e.g., co-stimulatory molecules) or turn down a signal. Inhibitory immune checkpoints that may be targeted by immune checkpoint blockade include adenosine A2A receptor (A2AR), B7-H3 (also known as CD276), B and T lymphocyte attenuator (BTLA), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152), indoleamine 2,3-dioxygenase (IDO), killer-cell immunoglobulin (KIR), lymphocyte activation gene-3 (LAG3), programmed death 1 (PD-1), T-cell immunoglobulin domain and mucin domain 3 (TIM-3) and V-domain Ig suppressor of T cell activation (VISTA). In particular, the immune checkpoint inhibitors target the PD 1 axis and/or CTLA 4. Examples of immune checkpoint inhibitors are described in WO 2023/060136.

[0133] It is contemplated that other agents may be used in combination with certain aspects of the present embodiments to improve the therapeutic efficacy of treatment. These additional agents include agents that affect the upregulation of cell surface receptors and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion, agents that increase the sensitivity of the hyperproliferative cells to apoptotic inducers, or other biological agents. Increases in intercellular signaling by elevating the number of GAP junctions would increase the anti-hyperproliferative effects on the neighboring hyperproliferative cell population. In other embodiments, cytostatic or differentiation agents can be used in combination with certain aspects of the present embodiments to improve the anti-hyperproliferative efficacy of the treatments. Inhibitors of cell adhesion are contemplated to improve the efficacy of the present embodiments. Examples of cell adhesion inhibitors are focal adhesion kinase (FAKs) inhibitors and Lovastatin. It is further contemplated that other agents that increase the sensitivity of a hyperproliferative cell to apoptosis, such as the antibody c225, could be used in combination with certain aspects of the present embodiments to improve the treatment efficacy.

ARTICLES OF MANUFACTURE OR KITS

[0134] An article of manufacture or a kit comprising the iPSCs or the iPSC-NK cells described herein is also provided herein. The article of manufacture or kit can further comprise a package insert comprising instructions for using the iPSC-NK to treat or delay progression of cancer in an individual or to enhance immune function of an individual having cancer. Suitable containers

include, for example, bottles, vials, bags and syringes. The container may be formed from a variety of materials such as glass, plastic (such as polyvinyl chloride or poly olefin), or metal alloy (such as stainless steel or hastelloy). In some embodiments, the container holding the formulation and the label on, or associated with, the container may indicate directions for use. The article of manufacture or kit may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use. In some embodiments, the article of manufacture further includes one or more of another agent (e.g., a chemotherapeutic agent, and anti-neoplastic agent).

[0135] In the description presented herein, each of the steps of the invention and variations thereof are described. This description is not intended to be limiting and changes in the components, sequence of steps, and other variations would be understood to be within the scope of the present invention.

[0136] It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

[0137] While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

EXAMPLE 1

iNK Cells Expressing A Knock-In Construct Encoding IL-15

[0138] The IL-15-engineered iNK cells disclosed herein exhibited proliferative advantage compared to control iNK cells during *ex vivo* expansion (see **Fig. 15**). iNK cells were transduced at the beginning of the stage 3 week 2, and expanded with K562 feeders in 1:5 ratio for a total of 20 days. At day 3, day 9, day 16, and day 20 the transduced iNK cells were harvested and measured for the viable cell number (tdT: tdTomato-transduced; control: no transduction).

[0139] The IL-15-engineered iNK cells disclosed herein showed advantage in survivability in

cytokine-starved condition. Following 20 days of *ex vivo* expansion, the IL-15-engineered iNK cells were grown in cytokine-free medium for 6 days and the viable cell number was monitored at day 0, day 2, and day 6 (**Fig. 16**). The IL-15-engineered iNK cells exhibited advantage in survivability in cytokine-starved condition compared to the tdTomato-transduced iNK cells and control iNK cells (i.e., no transduction).

[0140] The IL-15-engineered, cytokine-starved iNK cells maintain pSTAT5 activation in response to IL2 and Ki67 proliferation marker. Following 26 days of *ex vivo* expansion, the IL-15-engineered iNK cells were grown in cytokine-free medium for 6 days, or in cytokine-free medium for 6 days and then pulse-treated with 100 IU/mL of IL2 for 30 minutes. The % positive population of phospho-STAT5 and proliferation marker Ki-67 were analyzed at day 0 and day 6 (**Fig. 17**). The cytokine-starved IL-15-engineered iNK cells maintained pSTAT5 activation by IL2 and Ki67 proliferation marker, whereas the cytokine-starved control iNK cells failed to be activated and proliferated.

[0141] The IL-15-engineered iNK cells disclosed herein showed persistence in peripheral blood *in vivo*. The IL-15-engineered iNK cells were injected into mice. The mice looked healthy post injection, and peripheral blood was withdrawn from the mice at hour 3, day 3, day 7, day 10, day 14, and day 17. The persistence of the IL-15-engineered iNK cells was examined by flow cytometry on huCD45+ cell population. Compared to the control iNK cells, the IL-15-engineered iNK cells exhibited better survivability *in vivo* during the time course (**Fig. 18**).

What is claimed is:

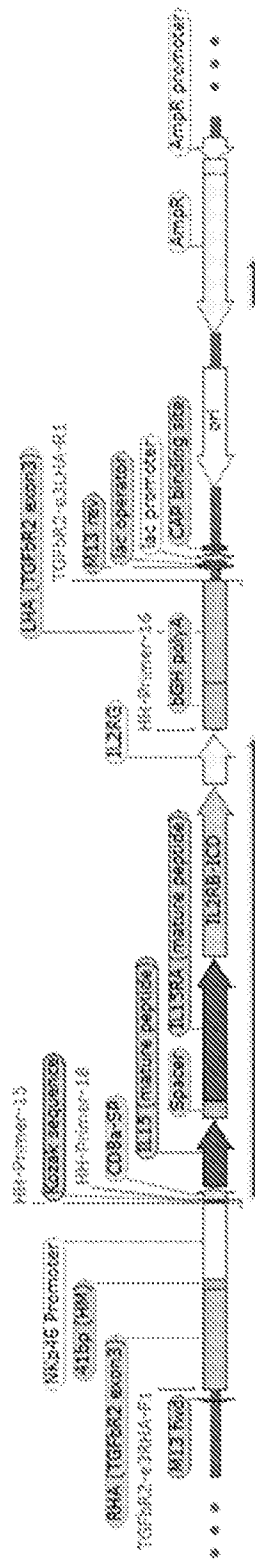
1. A cell population comprising induced pluripotent stem cells (iPSCs) or NK cells wherein the iPSCs or NK cells comprise an IL-15 construct comprising sequences encoding IL-15 or a functional fragment thereof, wherein the IL-15 or a functional fragment thereof is trapped in the endoplasmic reticulum.
2. The cell population of claim 1, wherein the IL-15 construct further comprises sequences encoding IL-2R β or a functional fragment thereof.
3. A cell population comprising induced pluripotent stem cells (iPSCs) or NK cells wherein the iPSCs or NK cells comprise an IL-15 construct comprising sequences encoding (i) IL-15 or a functional fragment thereof, and (ii) IL-2R β or a functional fragment thereof.
4. The cell population of claim 3, wherein the IL-15 construct encodes a cell membrane-bound form of IL-15 (mbIL-15) or a functional fragment thereof, a soluble form of IL-15 or a functional fragment thereof, or an IL-15 trapped in the endoplasmic reticulum or a functional fragment thereof.
5. The cell population of claim 2 or 3, wherein the IL-15 construct encodes a fusion protein comprising (i) IL-15 or a functional fragment thereof and (ii) IL-2R β or a functional fragment thereof, or the IL-15 or a functional fragment thereof and IL-2R β or a functional fragment thereof are expressed as individual proteins.
6. The cell population of any one of claims 1-3, wherein the IL-15 construct further comprises sequences encoding IL-15 Receptor alpha (IL-15R α) or a functional fragment thereof.
7. The cell population of claim 6, wherein the IL-15 construct encodes a fusion protein comprising (i) IL-15 or a functional fragment thereof, (ii) IL-2R β or a functional fragment thereof, and (iii) IL-15R α or a functional fragment thereof, or the IL-15 or a functional fragment thereof, IL-2R β or a functional fragment thereof, and IL-15R α or a functional fragment thereof are expressed as individual proteins.
8. The cell population of any one of claims 1-7, wherein the NK cells are derived from iPSCs.

9. The cell population of any one of claims 1-7, wherein the iPSCs or NK cells comprise a knock-in of the IL-15 construct into a B2M gene or a TGF β R2 gene of the iPSCs or NK cells.
10. The cell population of any one of claims 1-7, wherein the IL-15 construct is operably linked to a promoter which is silent or minimally active in iPSC.
11. The cell population of claim 10, wherein the promoter is a NK cell-specific promoter.
12. The cell population of claim 10, wherein the promoter is a NKp46 promoter or a B2M promoter.
13. The cell population of any one of claims 1-12, wherein the NK cells further comprise one or both of a knockout of a cytokine inducible SH2 containing protein (CISH) and one or more knockouts of one or more NK inhibitory receptors.
14. The cell population of claim 13, wherein the NK inhibitory receptor is T-cell immunoglobulin and ITIM domain (TIGIT) receptor.
15. A method of producing a cell population comprising NK cells (iNK cells) differentiated from induced pluripotent stem cells (iPSCs), the method comprising
 - (i) genetically editing a population of iPSCs with an IL-15 construct comprising sequences encoding IL-15 or a functional fragment thereof, wherein the IL-15 or a functional fragment thereof is trapped in the endoplasmic reticulum;
 - (ii) generating a monoclonal population of edited iPSCs; and
 - (iii) differentiating said edited iPSCs into a population of iNK cells.
16. A method of producing a cell population comprising NK cells (iNK cells) differentiated from induced pluripotent stem cells (iPSCs), the method comprising
 - (i) genetically editing a population of iPSCs with an IL-15 construct comprising sequences encoding (a) IL-15 or a functional fragment thereof, and (b) IL-2R β or a functional fragment thereof;
 - (ii) generating a monoclonal population of edited iPSCs; and
 - (iii) differentiating said edited iPSCs into a population of iNK cells.

17. The method of claim 16, wherein the IL-15 construct encodes a cell membrane-bound form of IL-15 (mbIL-15) or a functional fragment thereof, a soluble form of IL-15 or a functional fragment thereof, or an IL-15 trapped in the endoplasmic reticulum or a functional fragment thereof.
18. The method of claim 15, wherein the IL-15 construct further comprises sequences encoding IL-2R β or a functional fragment thereof.
19. The method of claim 16 or 18, wherein the IL-15 construct encodes a fusion protein comprising (i) IL-15 or a functional fragment thereof and (ii) IL-2R β or a functional fragment thereof, or the IL-15 or a functional fragment thereof and IL-2R β or a functional fragment thereof are expressed as individual proteins.
20. The method of claim 15 or 16 or 18, wherein the IL-15 construct further comprises sequences encoding IL-15 Receptor alpha (IL-15R α) or a functional fragment thereof.
21. The method of claim 20, wherein the IL-15 construct encodes a fusion protein comprising (i) IL-15 or a functional fragment thereof, (ii) IL-2R β or a functional fragment thereof, and (iii) IL-15R α or a functional fragment thereof, or the IL-15 or a functional fragment thereof, IL-2R β or a functional fragment thereof, and IL-15R α or a functional fragment thereof are expressed as individual proteins.
22. The method of any one of claims 15-21, wherein the IL-15 construct is operably linked to a promoter which is silent or minimally active in iPSC.
23. The method of claim 22, wherein the promoter is a NK cell-specific promoter.
24. The method of claim 22, wherein the promoter is a NKp46 promoter or a B2M promoter.
25. The method of claim 15 or 16, wherein said genetic editing comprises a knock-in of the IL-15 construct into a B2M gene or a TGF β R2 gene of the iPSCs .
26. The method of claim 15 or 16, wherein said genetic editing further comprises one or both of a knockout of a cytokine inducible SH2 containing protein (CISH) and one or more knockouts of one or more NK inhibitory receptors.

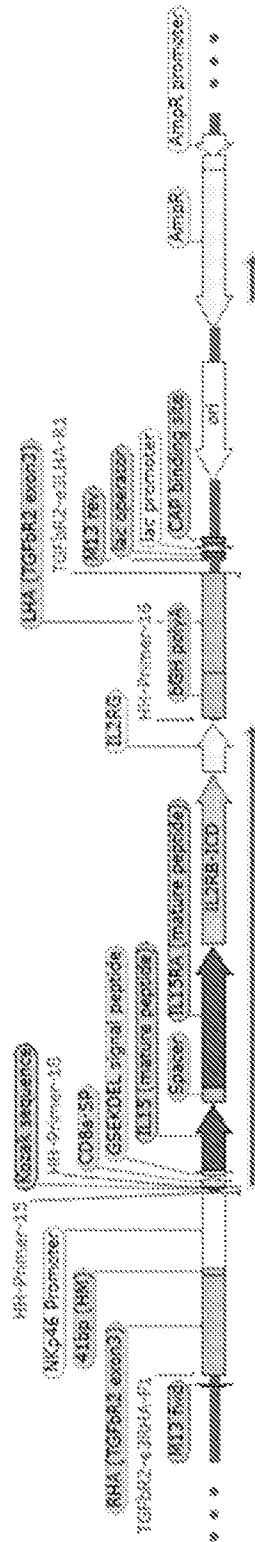
27. The method of claim 26, wherein the NK inhibitory receptor is T-cell immunoglobulin and ITIM domain (TIGIT) receptor.
28. The method of claim 15 or 16, wherein said genetic editing comprises using a TALEN construct or a Cas9 or Cas12 enzyme.
29. A cell population produced by the method of any one of claims 15-28.
30. A pharmaceutical composition comprising the cell population of claim 1, 3 or 29.
31. A method of treating cancer or autoimmune diseases in a subject in need thereof, comprising administering to the subject an effective amount of the pharmaceutical composition of claim 30.
32. The method of claim 31, wherein the cancer is a solid tumor or a hematological cancer.
33. The method of claim 31, wherein the subject is further administered with one or more immune checkpoint inhibitors.
34. The method of claim 33, wherein the immune checkpoint inhibitors are inhibitors of one or more NK cell inhibitory receptors.
35. The method of claim 34, wherein the inhibitor of NK cell inhibitory receptor is an antibody against the NK cell inhibitory receptor.
36. The method of claim 33, wherein the immune checkpoint inhibitor is an anti-PD-1 antibody, an anti-PDL-1 antibody, an anti-CTLA-4 antibody, or an anti-TIGIT antibody.

Figure 1



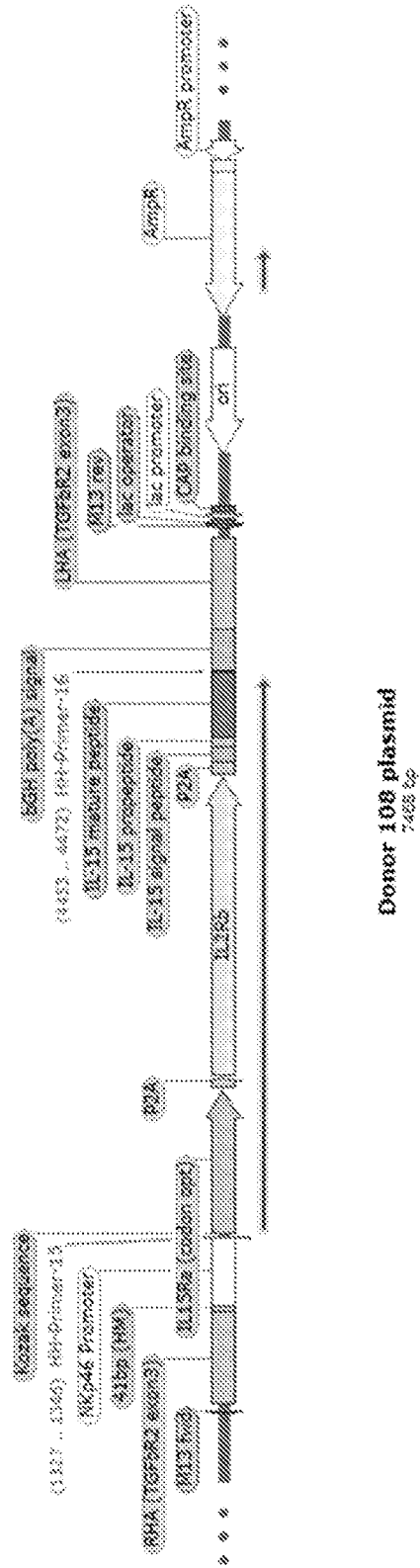
Donor 107 plasmid
6687 bp

Figure 2



Donor 107ER plasmid
8738 bp

Figure 3



Donor 108 plasmid
7428 bp

Figure 4

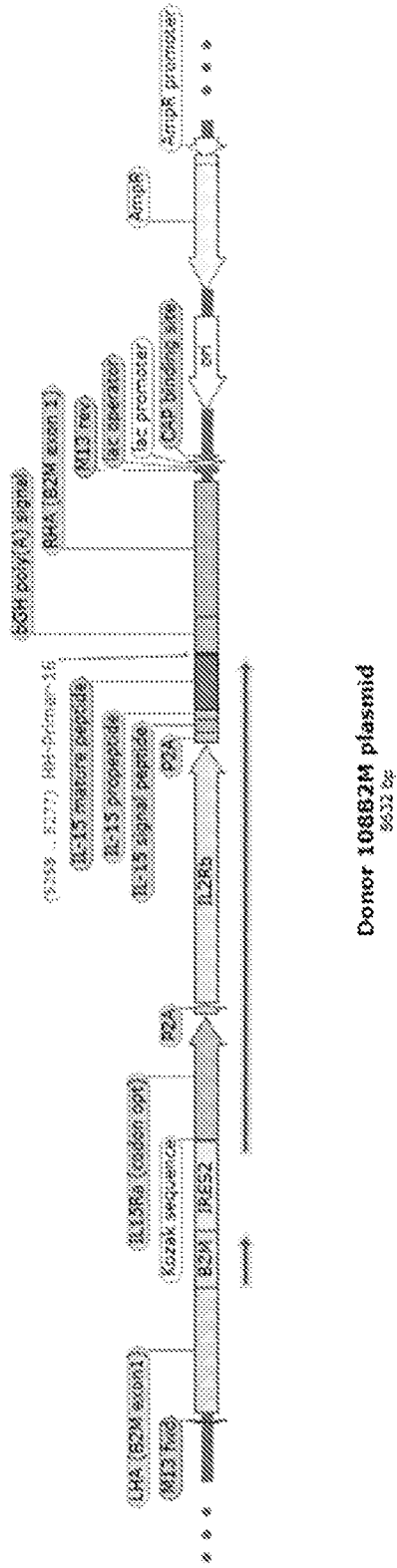


Figure 10

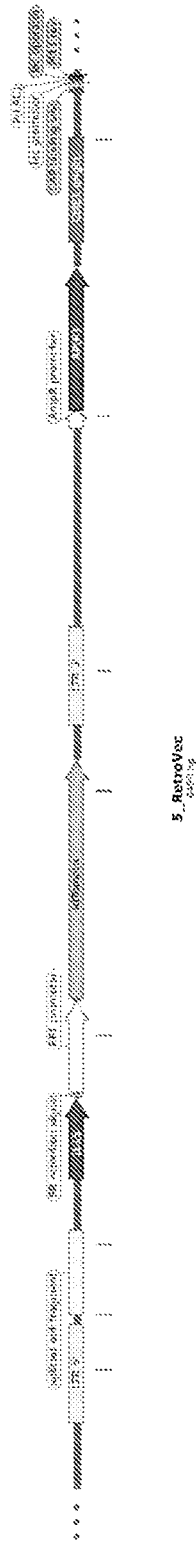


Figure 11



6. RetroVec
2022.20

Figure 13



8. RetroVet
22/2/2024

Figure 14

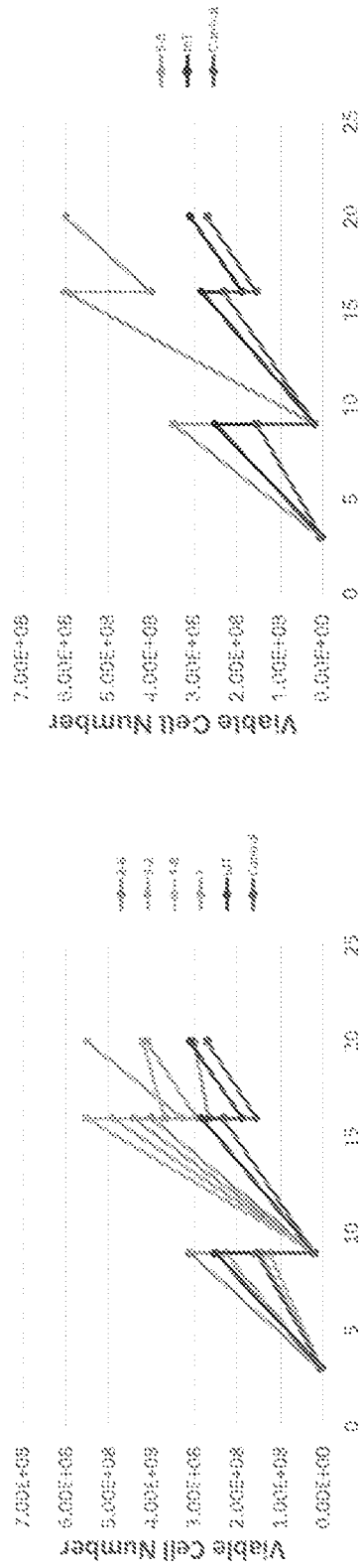


9. RetnoVec
10. 202 30

9. RetnoVec
10. 202 30

15/18

Figure 15

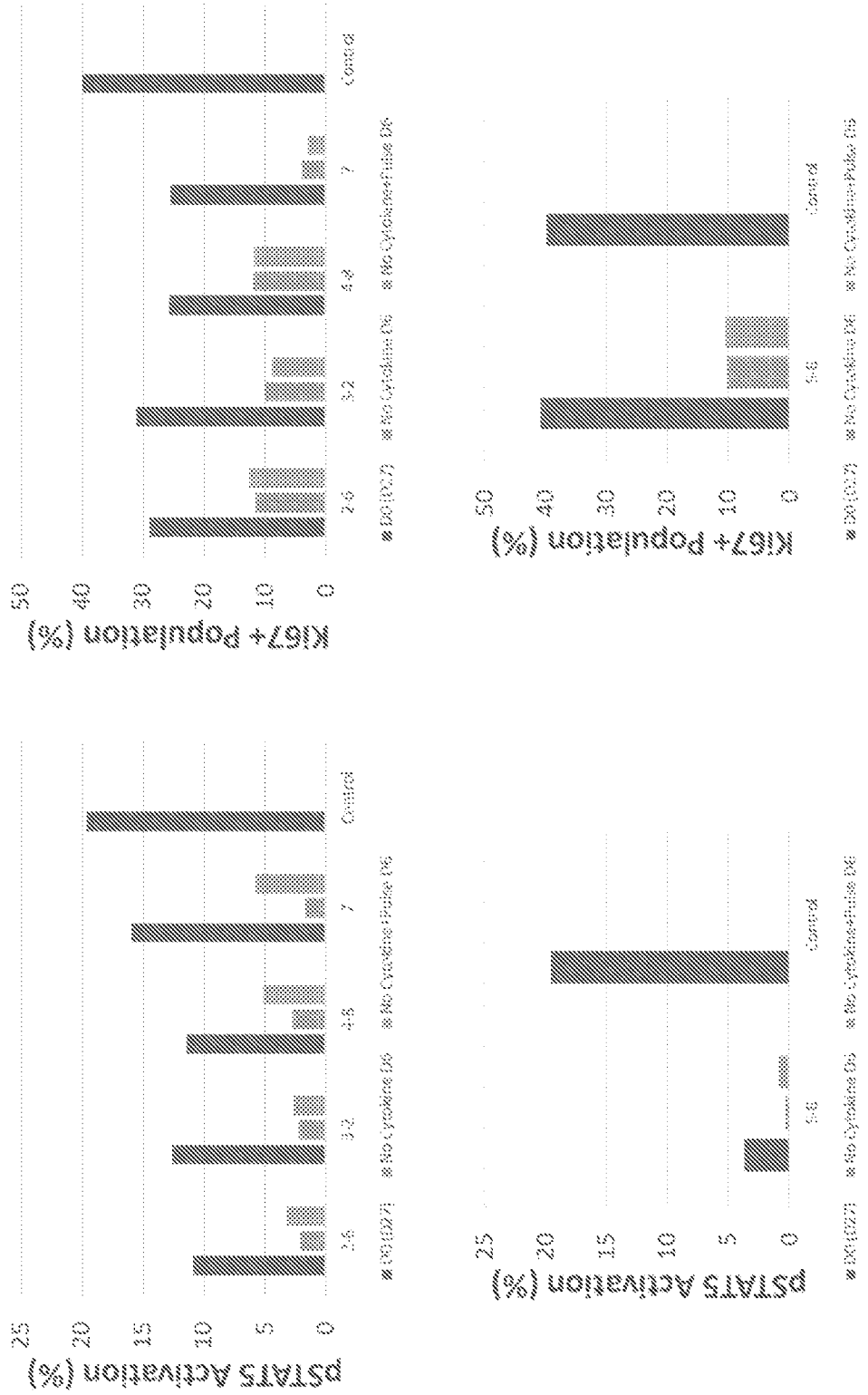


16/18

Figure 16

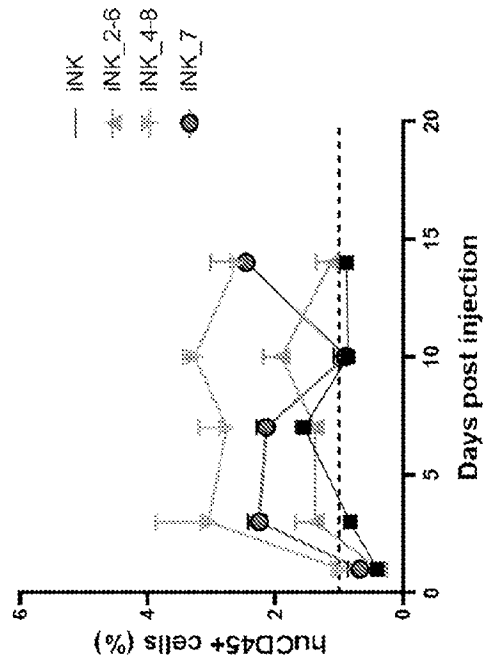
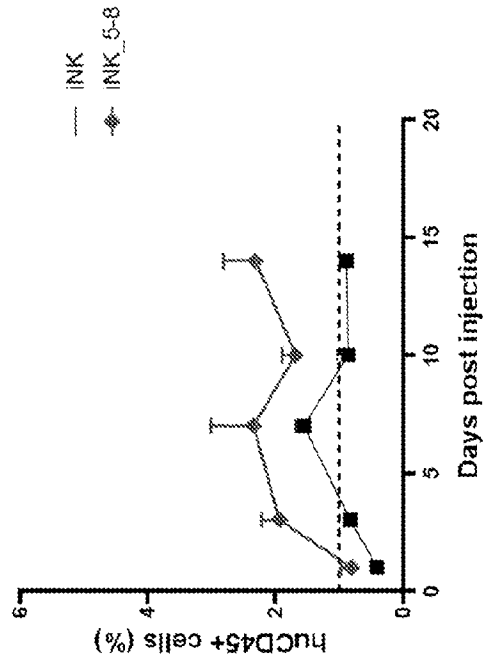


Figure 17



18/18

Figure 18



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/032309

A. CLASSIFICATION OF SUBJECT MATTER

IPC: *A61K 35/17* (2024.01); *A61K 38/20* (2024.01); *A61K 39/395* (2024.01); *A61P 35/00* (2024.01); *A61P 37/00* (2024.01); *C07K 19/00* (2024.01); *C12N 15/79* (2024.01); *C12N 5/074* (2024.01); *C12N 5/0783* (2024.01); *C07K 14/71* (2024.01); *C07K 14/715* (2024.01); *C12N 5/10* (2024.01); *C07K 14/54* (2024.01)

CPC: *A61K 39/4635*; *A61K 38/2086*; *A61K 39/4613*; *A61P 35/00*; *C07K 19/00*; *A61K 39/395*; *A61P 37/00*; *C07K 14/5443*; *C12N 15/79*; *C12N 5/0646*; *C12N 5/0696*; *C07K 2319/32*; *C07K 2319/74*; *A61K 35/17*; *C12N 5/10*; *C07K 14/5446*; *C07K 14/7155*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2023/060136 A1 (CYTOVIA THERAPEUTICS LLC) 13 April 2023 (13.04.2023) entire document	1
Y	entire document	2, 4
X	WO 2022/057851 A1 (BEIGENE LTD.) 24 March 2022 (24.03.2022) entire document	3, 5-7
Y	entire document	2, 4
A	WO 2022/216514 A1 (CENTURY THERAPEUTICS INC.) 13 October 2022 (13.10.2022) entire document	1-7
A	US 2023/0044451 A1 (EXUMA BIOTECH CORP.) 09 February 2023 (09.02.2023) entire document	1-7

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance

“D” document cited by the applicant in the international application

“E” earlier application or patent but published on or after the international filing date

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

30 July 2024 (30.07.2024)

Date of mailing of the international search report

27 September 2024 (27.09.2024)

Name and mailing address of the ISA/US

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Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Authorized officer

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Facsimile No. 571-273-8300

Telephone No. 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/032309

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2022/232796 A1 (THE GENERAL HOSPITAL CORPORATION) 03 November 2022 (03.11.2022) entire document	1-7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/032309

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. forming part of the international application as filed.
 - b. furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
 accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/032309

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: **8-14, 22-24, 29-36**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I: claims 1-7 are drawn to cell populations comprising induced pluripotent stem cells (iPSCs) or NK cells.

Group II: claims 15-21 and 25-28 are drawn to methods of producing a cell population comprising NK cells (iNK cells) differentiated from induced pluripotent stem cells (iPSCs).

The inventions listed in Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The special technical features of Group I, cell populations comprising induced pluripotent stem cells (iPSCs) or NK cells, are not present in Group II; and the special technical features of Group II, methods of producing a cell population comprising NK cells (iNK cells) differentiated from induced pluripotent stem cells (iPSCs), are not present in Group I.

Additionally, even if Groups I and II were considered to share the technical features of cell populations comprising an IL-15 construct comprising sequences encoding IL-15 or a functional fragment thereof, wherein the IL-15 or a functional fragment thereof is trapped in the endoplasmic reticulum, an IL-15 construct comprising sequences encoding (a) IL-15 or a functional fragment thereof, and (b) IL-2Rb or a functional fragment thereof, these shared technical features do not represent a contribution over the prior art.

Specifically, WO 2023/060136 A1 to Cytovia Therapeutics LLC teaches cell populations comprising an IL-15 construct comprising sequences encoding IL-15 or a functional fragment thereof, wherein the IL-15 or a functional fragment thereof is trapped in the endoplasmic reticulum (provided herein is a cell population comprising differentiated cells derived from induced pluripotent stem cells, said differentiated cells having a Natural Killer cell phenotype (iNK cells),... wherein the iNK cells express interleukin 15 (IL 15) or a functional fragment thereof.... wherein the iNK cells express IL-15 trapped in the endoplasmic reticulum (ER), Para. [0008]); and an IL-15 construct comprising sequences encoding (a) IL-15 or a functional fragment thereof (said differentiated cells having a Natural Killer cell phenotype (iNK cells),... wherein the iNK cells express interleukin 15 (IL 15) or a functional fragment thereof, Para. [0008]).

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/032309

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

Further, WO 2022/232796 A1 to The General Hospital Corporation teaches IL-2Rb or a functional fragment thereof (Described herein are immune cells (e.g., natural killer (NK), T cells) expressing a chimeric protein comprising... IL2R β , Pg. 1, Lns. 10-11).

The inventions listed in Groups I and II therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-7

- Remark on Protest**
- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
 - The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
 - No protest accompanied the payment of additional search fees.