

STIMULATORY CELL LINES FOR EX VIVO EXPANSION AND ACTIVATION OF NATURAL KILLER CELLS

RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/477,311, filed March 27, 2017, the entirety of which is incorporated by reference herein.

BACKGROUND

[0002] The emergence and persistence of many diseases are characterized by an insufficient immune response to aberrant cells, including malignant and virally infected cells. Immunotherapy is the use and manipulation of the patient's immune system for treatment of various diseases.

SUMMARY

[0003] Immunotherapy presents a new technological advancement in the treatment of disease, wherein immune cells are engineered to express certain targeting and/or effector molecules that specifically identify and react to diseased or damaged cells. This represents a promising advance due, at least in part, to the potential for specifically targeting diseased or damaged cells, as opposed to more traditional approaches, such as chemotherapy, where all cells are impacted, and the desired outcome is that sufficient healthy cells survive to allow the patient to live. Immunotherapy approaches employing the adoptive transfer of Natural Killer (NK) cells to patients are presently in development. However, such treatments require large numbers of ex vivo pure NK cells suitable for genetic manipulation and clinical applications. Methods and compositions (and uses thereof) are disclosed herein for the ex vivo expansion and activation of NK cells from a mixed cell culture.

[0004] A variety of engineered cell types, DNA constructs, and methods for expanding and activating NK cells are provided for herein. For example, in several embodiments, there is provided a genetically engineered cell population that does not express major histocompatibility complex (MHC) I molecules, wherein co-culture of said engineered cell population with a population of immune cells results in the activation and expansion of at least one subpopulation of immune cells. In several embodiments, the engineered cell

population is derived from a cell line that is immortal. For example, a cell line that exhibits one or more characteristics of an immortal cell while in culture. In several embodiments, the engineered cell population is derived from a cell line that is naturally immortal (e.g. stem cell lines). In several embodiments, the engineered cell population is derived from an immortalized (e.g., cancerous cell) line. In some embodiments, the engineered cell population is derived from cells that have been immortalized through engineering (e.g. genetically engineered to alter telomerase expression). In some embodiments, the engineered cell population is derived from a cancerous cell. In several embodiments, the engineered cell population is modified to express one or more membrane-bound factors that facilitate and/or enhance either activation and/or expansion of NK cells. For example, in several embodiments, the engineered cells express membrane-bound interleukin-15 (mbIL15). In still additional embodiments, the engineered cell population is modified to express membrane-bound 4-1BB ligand (4-1BBL), either in addition to, or in place of mbIL15. In further embodiments, the engineered cell population is modified to express at least one additional membrane bound interleukin that stimulates immune cell activation, in addition to, or in place of mbIL15, 4-1BBL, and/or other activating/expansion promoting factors.

[0005] In some embodiments, the engineered cell population comprises at least a first plurality of cells that expresses mbIL15 and a second plurality of cells that expresses 4-1BBL, such that the population *as a whole* expresses both mbIL15 and 4-1BBL. In other embodiments, the engineered cell population comprises a plurality of cells that expresses both mbIL15 and 4-1BBL. In yet other embodiments, the engineered cell population comprises some cells that express mbIL15, some cells that express 4-1BBL, and some cells that express both. In some embodiments, other ligands and/or activation factors may be additionally expressed in addition to or in lieu of mbIL15 and/or 4-1BBL.

[0006] In several embodiments, the mbIL15 expressed by the engineered cell population is encoded by a nucleic acid sequence that comprises SEQ ID NO. 1. In several embodiments, the 4-1BBL expressed by the engineered cell population is encoded by a nucleic acid sequence that comprises SEQ ID NO. 13.

[0007] Depending on the embodiment, each of the membrane bound molecules is coupled to a transmembrane domain. In several embodiments, the transmembrane domain of human CD8 α is used to link the molecules to the membrane. In several embodiments, the transmembrane domain of human CD8 α comprises the sequence of SEQ ID NO. 18. Other transmembrane domains are also used, depending on the embodiment, for example, other receptor or signaling domains (optionally truncated) known to reside in or across a cellular membrane may be used.

[0008] In several embodiments, the engineered cell population is derived from a cell line selected from the group consisting of K562 cells, Wilms tumor cell line HFWT, endometrial tumor cell line HHUA, melanoma cell line HMTV-II, hepatoblastoma cell line HuH-6, lung small cell carcinoma cell lines Lu-130 or Lu-134-A, neuroblastoma cell lines NB19 or NB69, embryonal carcinoma testis cell line NEC14, cervical carcinoma cell line TCO-2, and neuroblastoma cell line TNB1. In several embodiments, the overall population is derived from two or more of the cell lines above (or others) and combined to yield a cell population that enables unexpectedly enhanced activation/expansion of immune cells, such as NK cells.

[0009] In several embodiments, the engineered cell population lacks expression of MHC II molecules. In several embodiments, the engineered cell population is derived from K562 cells.

[0010] Depending on the embodiment, an engineered cell population as disclosed herein expresses one or more interleukin molecules. In several embodiments, the interleukin comprises IL12A, or a fragment thereof. In several embodiments, the IL12A comprises the sequence of SEQ ID NO. 4 (or fragment thereof). In one embodiment, the interleukin comprises IL12B, or a fragment thereof. In one embodiment, the IL12B comprises the sequence of SEQ ID NO. 6 (or fragment thereof). In several embodiments, the interleukin comprises IL12A and IL12B, or fragments thereof. In such embodiments, the IL12A and IL12B can be oriented in the polynucleotide in an A-B, B-A, A-B-A-B, A-B-B-A, B-A-A-B orientation in either duplicate, triplicate, or larger repeats.

[0011] In several embodiments, the engineered cell population further expresses membrane bound IL18 (mbIL18), or fragment thereof. In several embodiments, the IL18

comprises the sequence of SEQ ID NO. 8. In still further embodiments, the engineered cell population further expresses membrane bound IL21 (mbIL21), or fragment thereof. In one embodiment, the IL21 comprises the sequence of SEQ ID NO. 10, or a fragment thereof.

[0012] In several embodiments, the cells express membrane bound IL22 (mbIL22), or fragment thereof. In one embodiment, the IL22 comprises the sequence of SEQ ID NO. 12, or a fragment thereof. As discussed above, combinations of various interleukins can be expressed, in a variety of combinations, repeats, triplets, etc. In several embodiments, such repeated patterns of expression in the polynucleotides yield unexpectedly enhanced activation and/or expansion of NK cells.

[0013] In several embodiments, the engineered cells may further comprise a membrane bound anti-CD3 antibody (mb α -CD3), an antibody fragment thereof, or scFv. In one embodiment, the mb α -CD3 is a monoclonal antibody. In several such embodiments, the mb α -CD3 targets an epitope within the nucleic acid sequence of CD3 epsilon of SEQ ID NO. 15. In additional embodiments, the mb α -CD3 is a scFv. In one embodiment, the scFv comprises the sequence of SEQ ID NO. 17.

[0014] Also provided for herein, as discussed in more detail below, are methods for expanding immune cells, the methods comprising co-culturing a blood sample comprising immune cells with any of the engineered cell populations disclosed herein. In several embodiments, the immune cells are Natural Killer (NK) cells.

[0015] According to several embodiments, there is provided a genetically engineered cell population derived from a cancerous cell, wherein the engineered cell population is modified to express one, two, or more of: membrane-bound interleukin-15 (mbIL15), membrane-bound 4-1BB ligand (4-1BBL), and at least one additional membrane bound interleukin that stimulates immune cell activation, and wherein co-culture of the engineered cell population with a population of immune cells results in the activation and expansion of at least one subpopulation of immune cells. In several embodiments, the genetically engineered cell population does not express major histocompatibility complex (MHC) I molecules.

[0016] Also provided for in several embodiments is a genetically engineered cell population that does not express major histocompatibility complex (MHC) I molecules, the

engineered cell population being derived from a cancerous cell, engineered cell population being modified to express mbIL15, 4-1BBL, a membrane-bound anti-CD3 antibody (mb α -CD3) that stimulates immune cell activation, and wherein co-culture of the engineered cell population with a population of immune cells results in the activation and expansion of at least one subpopulation of immune cells.

[0017] In several embodiments, the engineered cells are modified to express an additional membrane-bound interleukin that comprises IL12A, or a fragment thereof. In one embodiment, the membrane bound IL12A comprises the sequence of SEQ ID NO. 4. In one embodiment, the membrane bound IL12A has the sequence of SEQ ID NO. 4. In several embodiments, the additional membrane-bound interleukin comprises IL12B, or a fragment thereof. In one embodiment, the membrane bound IL12B comprises the sequence of SEQ ID NO. 6. In one embodiment, the membrane bound IL12B has the sequence of SEQ ID NO. 6. In several embodiments, the additional membrane bound interleukin comprises membrane bound IL12A and membrane bound IL12B or fragments thereof of either. Additionally, any of such embodiments of engineered cells can further comprise expression of membrane bound IL18 (mbIL18), or a fragment thereof. In one embodiment, the membrane bound IL18 comprises the sequence of SEQ ID NO. 8. In one embodiment, the membrane bound IL18 has the sequence of SEQ ID NO. 8.

[0018] In still additional embodiments, the engineered cells express membrane bound IL21 (mbIL21), or a fragment thereof. In several embodiments, the membrane bound IL21 has the sequence of SEQ ID NO. 10, or a fragment thereof. Either in combination with IL21, or alone, several embodiments provide for engineered cells that express membrane bound IL22 (mbIL22), or a fragment thereof. In several embodiments, the membrane bound IL22 has the sequence of SEQ ID NO. 12, or a fragment thereof.

[0019] Additionally, several embodiments provide for engineered cells that express a membrane bound anti-CD3 antibody (mb α -CD3), an antibody fragment or a single chain fragment variable (scFv) construct thereof. In one embodiment, the mb α -CD3 is a monoclonal antibody. In several embodiments, the mb α -CD3 targets an epitope within the nucleic acid sequence of CD3 portion of a T cell receptor. For example, in one embodiment one or more of the delta, epsilon or gamma subunit of the CD3 receptor is targeted by the

membrane bound anti-CD3 antibody. In one embodiment, the CD3 receptor epsilon subunit of SEQ ID NO. 15 is targeted by the membrane bound antibody expressed on the engineered cells. In several embodiments the mb α -CD3 is a scFv. In one embodiment, the scFv comprises, consists essentially of or consists of the sequence of SEQ ID NO. 17. In one embodiment, the scFv has the sequence of SEQ ID NO. 17.

[0020] Additionally, in several embodiments, there is provided a stimulatory cell that is engineered to express the alpha subunit of the IL15 receptor with a high affinity for IL15, allowing it to engulf and present soluble IL15 on the surface of the cell. Combinations of any of the additional interleukins or antibodies can also be used, depending on the embodiment, to essentially allow for modular engineering of a stimulatory cell that provides for unexpectedly superior expansion and activation of NK cells.

[0021] In several embodiments, the engineered cell population is derived from a cell line including, but not limited to, the following: K562 cells, Wilms tumor cell line HFWT), endometrial tumor cell line HHUA, melanoma cell line HMV-II, hepatoblastoma cell line HuH-6, lung small cell carcinoma cell lines Lu-130 or Lu-134-A, neuroblastoma cell lines NB19 or NB69, embryonal carcinoma testis cell line NEC14, cervical carcinoma cell line TCO-2, and neuroblastoma cell line TNB1. In several embodiments, the engineered cells lack expression of MHC II molecules. In several embodiments, the engineered cells are derived from K562 cells.

[0022] Depending on the embodiment, the membrane bound molecule is imparted with the ability to be bound to the cell surface by being coupled to a transmembrane domain. The term “transmembrane” shall be given its ordinary meaning and shall refer to at least a portion of a polypeptide (e.g., domain) that is embedded in a cell membrane. In additional embodiments, at least one of the membrane bound molecules can be coupled to a single transmembrane domain. Additionally, in several embodiments, multiple types or multiple copies of the membrane bound molecules can be coupled to a transmembrane domain. Additionally, in several embodiments, multiple types or multiple copies of transmembrane domains can be coupled to the membrane bound molecules. In several embodiments, the membrane bound molecule is coupled to a transmembrane domain of human CD8 α . In several embodiments, the transmembrane domain of human CD8 α comprises the sequence of

SEQ ID NO. 18. In several embodiments, the transmembrane domain of human CD8 α has the sequence of SEQ ID NO. 18. In several embodiments, the mbIL15 is encoded by the nucleic acid of SEQ ID NO. 1. In several embodiments, the mb4-1BBL is encoded by the nucleic acid of SEQ ID NO. 13. In several embodiments, the mbIL15 is encoded by a nucleic acid sequence that comprises, consists essentially of or consists of the sequence of SEQ ID NO. 1. In several embodiments, the mb4-1BBL is encoded by a nucleic acid sequence that comprises, consists essentially of or consists the sequence of SEQ ID NO. 13.

[0023] In several embodiments, the engineered cell populations provided for herein are suitable for the expansion and/or activation of immune cells. “Expansion” of cells, as used herein, is given its ordinary meaning, and refers to increase in the number of a characteristic cell type, or cell types, from an initial population of cells, which may or may not be identical. The initial cells used for expansion need not be the same as the cells generated from expansion. For instance, the expanded cells may be produced by growth and differentiation of the initial engineered cell population. “Activation” of immune cells, as used herein, refers to the ability of immune cells to respond and exhibit, on a measurable level, an immune function of the corresponding cell known to a person of skill in the art. Methods to measure the activity of immune cells are also known to a person of skill in the art. “Immune cells” as used herein, is given its ordinary meaning and includes any cells of the immune system that may be assayed, including, but not limited to, B lymphocytes, also called B cells, T lymphocytes, also called T cells, natural killer (NK) cells, lymphokine-activated killer (LAK) cells, monocytes, macrophages, neutrophils, granulocytes, mast cells, platelets, Langerhans cells, stem cells, dendritic cells, peripheral blood mononuclear cells, tumor-infiltrating (TIL) cells, gene modified immune cells including hybridomas, drug modified immune cells, and derivatives, precursors or progenitors of the above cell types. In several embodiments, the expanded and/or activated immune cells are NK cells. As used herein, the term “Natural Killer Cells” (“NK cells”) is given its ordinary meaning and refers to a type of cytotoxic lymphocyte of the immune system that provides rapid responses to virally infected cells and responds to transformed cells. Typically, immune cells detect peptides from pathogens presented by Major Histocompatibility Complex (MHC) molecules on the surface of infected cells, triggering cytokine release, causing lysis or apoptosis. NK

cells are unique, however, as they have the ability to recognize stressed cells regardless of whether peptides from pathogens are present on MHC molecules. In some aspects, the NK cell is a mammalian NK cell. Examples of “mammalian” or “mammals” include primates (e.g., human), canines, felines, rodents, porcine, ruminants, and the like. Specific examples include humans, dogs, cats, horses, cows, sheep, goats, rabbits, guinea pigs, rats and mice. In a particular aspect, the mammalian NK cell is a human NK cell.

[0024] In several embodiments, the expansion and/or activation comprises co-culturing a blood sample, such as a peripheral blood sample, comprising NK cells with one of the engineered cell populations provided for herein.

[0025] In several embodiments, the engineered cell populations provided for herein can be prepared by a method comprising transducing the cells with a first construct encoding mbIL15, thereby generating a first transduced population of cells, expanding the first transduced population of cells, transducing the first transduced population of cells with a second construct encoding 4-1BBL, thereby generating a second transduced population of cells, transducing the second transduced population of cells with a third construct encoding at least one additional molecule capable of stimulating immune cells, thereby generating a third transduced population of cells, and expanding the third transduced population of cells. In several embodiments, the engineered cell populations provided for herein can be prepared by simultaneously transducing a population of cells with a first construct encoding mbIL15, a second construct encoding 4-1BBL, and a third construct encoding at least one additional molecule capable of stimulating immune cells. In still additional embodiments, the engineered cell populations provided for herein can be prepared by transducing a population of cells with a single construct encoding mbIL15, 4-1BBL, and at least one additional molecule capable of stimulating immune cells.

[0026] Additionally, in several embodiments, engineered cell populations can be prepared by a method comprising transducing the cells with a construct encoding mbIL15, 4-1BB, and one or more of mbIL12A, mbIL12B, mbIL18, mbIL21, mbIL22, and mb α -CD3 or fragments thereof.

[0027] Also provided for is a plurality of nucleic acids, for use in generating the engineered cell populations disclosed herein, comprising at least 3 from the group of nucleic

acids comprising a nucleic acid encoding mbIL15, a nucleic acid encoding 4-1BBL, a nucleic acid encoding mbIL12A, a nucleic acid encoding mbIL12B, a nucleic acid encoding mbIL18, a nucleic acid encoding mbIL21, a nucleic acid encoding mbIL22, and a nucleic acid encoding mb α -CD3. In several embodiments, the plurality of nucleic acids is optionally configured as a single construct (e.g., encoded or operationally linked). In several embodiments, the plurality of nucleic acids is configured as part of more than one construct. In several embodiments, the mbIL15 is encoded by SEQ ID NO. 1. In several embodiments, the 4-1BBL is encoded by SEQ ID NO. 13. In several embodiments, the mbIL12A is encoded by SEQ ID NO. 3. In several embodiments, the mbIL12B is encoded by SEQ ID NO. 5. In several embodiments, the mbIL18 is encoded by SEQ ID NO. 7. In several embodiments, the mbIL21 is encoded by SEQ ID NO. 9 or a fragment thereof. In several embodiments, the mbIL22 is encoded by SEQ ID NO. 11 or a fragment thereof. In several embodiments, the mb α -CD3 is encoded by SEQ ID NO. 16. Depending on the embodiment, one or more of the plurality of nucleic acids optionally comprises a tag, such as a FLAG tag, a HIS tag, GFP, or other tags and /or markers known to a person of skill in the art.

[0028] Also provided for herein, in several embodiments, is a method for expanding NK cells, comprising obtaining a peripheral blood sample comprising a mixed population of immune cells comprising NK cells and T cells, contacting the mixed population of cells with an engineered cell population that exhibits reduced expression of MHC I and has been modified to express membrane-bound interleukin-15 (mbIL15), 4-1BB ligand (4-1BBL), and at least one additional molecule that stimulates immune cell activation, and co-culturing the mixed population of cells with the engineered cells for a period of time sufficient to expand the NK cells of the mixed population. In several embodiments, the method optionally comprises adding IL2 to the media used in the co-culture. In several embodiments, the method optionally further comprises removing T cells from the mixed population either prior to or after co-culturing. Methods of removing and/or separating T cells from a mixed population of immune cells comprising NK cells and T cells are well known in the relevant art.

[0029] In one embodiment, there is provided a modified cell line comprising K562 myeloid leukemia cells that lack major histocompatibility complex I molecules that are

genetically modified to express membrane-bound interleukin-15, 4-1BB ligand, and membrane-bound anti-CD3. The terms “genetically modified” and “genetically engineered” shall be given their ordinary meaning, shall be used interchangeably, and shall refer to use of use of biotechnology to manipulate one or more aspect of at least a portion of an organism’s genome.

[0030] In one embodiment, there is provided a modified cell line comprising K562 myeloid leukemia cells that lack major histocompatibility complex I molecules that are genetically modified to express membrane-bound interleukin-15, membrane-bound 4-1BB ligand, and at least one additional membrane-bound interleukin. In several embodiments, the at least one additional membrane-bound interleukin is one or more of interleukin-12, interleukin-18, and a combination of interleukin-12 and interleukin-18. In several embodiments, the modified cells further comprise a membrane-bound anti-CD3 antibody. Combinations of these additional membrane bound signaling molecules are used in several embodiments. As used herein, the term “signaling molecules” shall be given its ordinary meaning and shall include, but not be limited to interleukins, CD3, 4-1BB, etc.

[0031] In one embodiment, there is provided a modified cell line comprising K562 myeloid leukemia cells that lack major histocompatibility complex I molecules that are genetically modified to express membrane-bound interleukin-15, 4-1BB ligand, membrane-bound anti-CD3 antibody, and at least one additional membrane-bound interleukin.

[0032] Also provided for herein is a population of NK cells expanded and/or activated by culturing a mixed cell culture comprising NK cells and T lymphocytes with any of the modified cell lines disclosed herein. Such a population of NK cells can be used for the treatment of cancer or infectious disease, and/or in the preparation of a medicament for such treatment. The engineered cell populations disclosed herein are suitable for use in the activation of NK cells, such activated NK cells for use in the treatment of cancer or infectious disease.

[0033] Methods for treating diseases using expanded and/or activated NK cells are also provided for herein. For example, in several embodiments, there is provided a method of treating cancer or an infectious disease comprising administering to a subject having cancer (e.g., a tumor, whether solid or suspension) or an infectious disease a

composition comprising an expanded population of immune cells, the immune cells having been expanded by co-culturing the immune cells with an engineered cell population that has been modified to express membrane-bound interleukin-15 (mbIL15) and 4-1BB ligand (4-1BBL), and has been modified to express at least one additional membrane bound interleukin that stimulates immune cell activation. In several embodiments, the co-culturing results in the activation and expansion of at least one subpopulation of immune cells, and wherein the at least one subpopulation of immune cells is administered to the subject. In several embodiments, the engineered cell population is derived from a cancerous cell, e.g., an immortalized cell line. In several embodiments, the administered subpopulation of immune cells comprises NK cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034] The descriptions of the figures below are related to experiments and results that represent non-limiting embodiments of the inventions disclosed herein.

[0035] Figures 1A-1G represent non-limiting examples of engineered cells for use in expanding immune cells in accordance with several embodiments disclosed herein. Constructs are provided wherein a K562 cell (as an example) expresses a ligand for 4-1BB (4-1BBL) and membrane bound IL15 (mbIL15) in conjunction with other cytokines, such as membrane bound IL12A/12B (mbIL12A/12B; Figure 1A), membrane bound IL18 (mbIL18; Figure 1B), or combinations thereof (Figure 1C). Also provided are constructs wherein cells (using K562 as an example) co-express 4-1BBL and mbIL15 in conjunction with combinations of cytokines (such as mbIL12A/12B and/or mbIL18, Figures 1D-1F) and antibodies (for example membrane bound anti-CD3 (mbantiCD3, Figure 1G)).

[0036] Figures 2A-2F depict flow cytometry measurements of expression of various genes by K562, according to several embodiments disclosed herein. Figure 2A depicts expression of mbIL15, Figure 2B depicts expression of 4-1BBL, Figure 2C depicts expression of mbIL18, Figure 2D depicts expression of mbIL12A, Figure 2E depicts expression of mbIL12B, and Figure 2F depicts expression of mb-anti-CD3.

[0037] Figures 3A-3B depict data related to the expansion of NK cells by various K562 constructs according to several embodiments disclosed herein. Figure 3A depicts data

related to the percentage of NK cells recovered after 7 days of culture (with IL-2) with various K562 cell lines, relative to the number of peripheral blood mononucleated cells initially seeded. Figure 3B depicts data related to the percentage of NK cells recovered after 7 days of culture with various K562 cell lines, relative to the number of PBMCs initially seeded (P value calculated by paired t test).

[0038] Figures 4A-4F depict data related to the long-term expansion and function of NK cells stimulated with various genetically-modified K562 cells. Figure 4A depicts data related to the expansion of NK cells over time when co-cultured with the indicated K562 variant. PBMCs were co-cultured with irradiated K562 cells expressing mbIL15 and 4-1BBL (K562-mb15-41BBL) (Figure 4A), or with K562-mb15-41BBL cells also expressing mbIL12 (+mb12) (Figure 4B), mbIL18 (+mb18) (Figure 4C), or both mbIL12 and mbIL18 (+mb12+mb18) (Figure 4D). Figure 4E depicts data related to the cytotoxicity of expanded NK cells against K562 cells at the indicated effector:target (E:T) ratios. Figure 4F relates to the cytotoxicity of expanded NK cells against K562 cells at the indicated E:T ratios. Shown are means ($\pm$ SD) of triplicate experiments. P value was calculated by paired t test.

DETAILED DESCRIPTION

[0039] The emergence and persistence of aberrant cells (including virally infected and malignant cells) underlying many diseases are enabled by an insufficient immune response to said aberrant cells. A goal of immunotherapy is to initiate or augment the response of the patient's immune system, for example, to boost the ability of immune cells, such as Natural Killer (NK) cells to damage, kill, or otherwise inhibit damaged or diseased cells. Adoptive transfer of immune cells engineered to express certain targeting and/or effector molecules that specifically identify and react to diseased or damaged cells is a particularly promising immunotherapy approach. One variation of this approach involves administering T cells engineered to express chimeric receptors to patients to elicit targeted recognition and destruction of the aberrant cells of interest. However, a drawback of this approach is that it may favor the use of autologous cells (or MHC-compatible donor cells) to prevent the induction of graft-versus-host-disease in the patient. Further, retrieval and use of autologous T cells from cancer patients poses several potentially adverse issues. NK cells,

however, are advantageous in that either autologous or donor-derived allogeneic cells can be employed, according to several embodiments disclosed herein. One challenge associated with NK cell based immunotherapy is obtaining adequately large and sufficiently pure (e.g., free of other cell types) quantities of NK cells for genetic manipulation and infusion, as NK cells represent a small fraction of the total cells in an immune cell population.

[0040] Thus, there remains a need for greater expansion of NK cells for use in NK cell-based immunotherapy. As such, in several embodiments, there are provided populations of expanded and activated NK cells derived from co-culturing the modified cell lines disclosed herein with a starting population of immune cells. In several embodiments, the starting population of immune cells comprises NK cells and T cells. In several embodiments, there is also provided a method for preferentially expanding NK cells in a mixed cell culture comprising NK cells and T cells, which comprises co-culturing said mixed cell culture with the modified cell lines disclosed herein. Depending on the embodiment, preferential expansion includes, but is not limited to, two-fold, three-fold, 5-fold, 10-fold, or greater, expansion of NK cells as compared to other immune cells. In additional embodiments, preferential expansion refers to NK cell expansion that is at least about 10%, about 20%, about 30%, about 50% or more than expansion of another immune cell type. There is also provided, in several embodiments, methods of using any of the modified cell lines disclosed herein for expanding NK cells in a mixed cell culture comprising NK cells and T cells.

Cells for Use in Immune Cell Expansion

[0041] In several embodiments, cell lines are used in a co-culture with a population of immune cells that are to be expanded. Such cell lines are referred to herein as “stimulatory cells,” which can also be referred to as “feeder cells”. In several embodiments, the entire population of immune cells is to be expanded, while in several embodiments, a selected immune cell subpopulation is preferentially expanded. For example, in several embodiments, NK cells are preferentially expanded relative to other immune cell subpopulations. While in some embodiments, stimulatory cells are wild type cells, in several embodiments, the stimulatory cells are genetically modified to render them particularly suitable for expanding and/or activating immune cells. As discussed in more detail below,

various cell lines are amenable to genetic modification that can result in surface expression of certain molecules that stimulate NK activation. Certain cell lines are particularly amenable to expanding NK cells, for example, those that do not express MHC I molecules, which have an inhibitory effect on NK cells. In some embodiments, the cells need not entirely lack MHC I expression, however they may express MHC I molecules at a lower level than a wild type cell. For example, in several embodiments, if a wild type cell expresses an MHC at a level of X, the cell lines used may express MHC at a level less than 95% of X, less than 90% of X, less than 85% of X, less than 80% of X, less than 70% of X, less than 50% of X, less than 25% of X, and any expression level between (and including) those listed. In several embodiments, the stimulatory cells are immortalized, e.g., a cancer cell line. However, in several embodiments, the stimulatory cells are primary cells.

[0042] Cell types that lack, or have reduced, MHC I expression include, but are not limited to, K562 cells, certain Wilm's Tumor cell lines (for example Wilms tumor cell line HFWT), endometrial tumor cells (for example, HHUA), melanoma cells (e.g., HMV-II), hepatoblastoma cells (e.g., HuH-6), lung small cell carcinoma cells (e.g., Lu-130 and Lu-134-A), neuroblastoma cells (e.g., NB19 and NB69), embryonal carcinoma testis cells (e.g., NEC14), cervical carcinoma cells (TCO-2), neuroblastoma cells (e.g., TNB1), 721.221 EBV transformed B cell line, among others. In several embodiments, the stimulatory cells also have reduced (or lack) MHC II expression, as well as having reduced (or lacking) MHC I expression. In some embodiments, other cell lines that may initially express MHC class I molecules can be used, in conjunction with genetic modification of those cells to reduce or knock out MHC I expression. Genetic modification can be accomplished through the use of gene editing techniques (e.g. the crispr/cas-9 system), inhibitory RNA (e.g., siRNA), or other molecular methods to disrupt and/or reduce the expression of MHC I molecules on the surface of the cells. Additionally, or alternatively, other approaches to block binding or other interactions with the MHC I molecules can be used (e.g., blocking antibodies, interfering ligands, etc.).

[0043] In several embodiments, certain ratios of stimulatory cells to cells to be expanded/stimulated are used. For example, in several embodiments a stimulatory cell : "target" cell ratio of about 5:1 is used. In several embodiments, 1:1 ratios are used, while in

additional embodiments, can range from about: 1:10, 1:20, 1:50, 1:100, 1:1,000, 1:10,000, 1:50,000, 1:100,000, 100,000:1, 50,000:1, 10,000:1, 1,000:1, 100:1, 50:1, 20:1, 10:1, and any ratio in between those listed, including endpoints. In some embodiments, combinations of cell types are used (e.g., K562 with one or more additional cell types), with the resultant activation and/or expansion of NK cells being greater than could be achieved with the use of any single cell type alone (e.g., as a result of synergy between the cell types). In some such embodiments, MHC I expression need not necessarily be reduced and/or absent in each of the cell lines used in combination. In some embodiments the relative frequency of one cell type versus the others in combination can be varied in order to maximize the expansion and activation of the desired immune cell population. For example, if two cell populations are used, the relative frequency can range from a ratio of 1:10, 1:20, 1:50, 1:100, 1:1,000, 1:10,000, 1:50,000, 1:100,000, 100,000:1, 50,000:1, 10,000:1, 1,000:1, 100:1, 50:1, 20:1, 10:1, and any ratio in between those listed, including endpoints.

[0044] As discussed in more detail below, certain stimulatory molecules (e.g. interleukins, CD3, 4-1BBL, etc.) can be expressed on or by the cells that promote immune cell expansion and activation (e.g., the stimulatory cells). However, in several embodiments, either in conjunction with, or in place of cells to promote immune cell expansion, a solid support is used. For example, a solid support is a surface that is capable of having a molecule attached to the surface, including, but not limited to, metal, glass, plastic, polymeric materials, particles (e.g., beads or microspheres), and/or lipids (either natural or synthetic). In some embodiments, compositions are used that can elute a stimulatory molecule, such as those disclosed herein, into the culture medium in order to facilitate the expansion of a desired immune cell population.

Stimulatory Molecules

[0045] As discussed briefly above, certain molecules promote the expansion of immune cells. Depending on the embodiment, the stimulatory molecule, or molecules, can be expressed on the surface of the stimulatory cells used to expand the immune population, while in some embodiments the stimulatory cells can be engineered to express and secrete one or more stimulatory molecules into the culture medium. In still additional embodiments,

one or more stimulatory molecules are used to supplement the cell culture media. In some embodiments, the immune cell population is expanded relatively uniformly (e.g., no particular subpopulation is preferentially expanded). In some such embodiments, following expansion of all immune cell populations, desired subpopulations are selectively separated (e.g., NK cells are separated from T cells, or vice versa) for further use. In several embodiments, certain specific immune cell subpopulations, such as NK cells, are preferentially expanded.

[0046] In several embodiments, the general construct for engineering a stimulatory cell line to express a membrane bound molecule employs a signal peptide that ultimately drives expression of the membrane bound molecule, the nucleic acid sequence that encodes the membrane bound molecule, an optional linker, and a transmembrane domain. This general construct can vary with the embodiment based on, at least in part, the complexity, size or ability to express a given membrane bound molecule.

[0047] In some embodiments interleukin 15 (IL15) is used to facilitate expansion of NK cells. In some embodiments, the IL15 is membrane bound on the stimulatory cells (referred to herein as “mbIL15”). In some embodiments, IL15 is membrane bound by virtue of being coupled or conjugated to a transmembrane molecule or integral membrane protein. In several embodiments, a transmembrane domain of CD8 α is used (SEQ ID NO. 18). In several embodiments, wild type (e.g., a full-length) IL15 is expressed on, or by, the stimulatory cells. In some embodiments, the IL15 is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% homologous with full-length IL15. In some embodiments, truncated forms of IL15 are used. In several embodiments, mbIL15 is encoded by the nucleic acid sequence of SEQ ID NO: 1. In several embodiments, mbIL15 is encoded by the amino acid sequence of SEQ ID NO: 2. In several embodiments, the stimulatory molecule may have one or more additional mutations from SEQ ID NO. 1 or 2, but retains, or in some embodiments, has enhanced stimulatory activity.

[0048] In several embodiments, the stimulatory cells are engineered to express all, or a portion of the IL15 receptor. In several embodiments, the portion of the IL15 receptor is a functional portion of the IL15 receptor. For example, in some embodiments, the stimulatory cells are engineered to express the IL15 receptor alpha subunit. In several

embodiments, the cells produce, or are engineered to produce (e.g., secrete) soluble IL15. The soluble IL15 can thereby bind its receptor expressed by the stimulatory cells and subsequently be internalized (e.g., endocytosed) and presented to another cell. In essence, in some embodiments, rather than engineering the stimulatory cells to express an engineered mbIL15, the stimulatory cells could be engineered to express the IL15 receptor alpha subunit, which can bind IL15 (even in the absence of the remaining IL15 receptor CD122 and CD132 subunits), and present it on the cell surface, thus resulting in IL15 expression in an alternative way to mbIL15.

[0049] In some embodiments, interleukin 12A (IL12A) and/or 12B (IL12B) is used to facilitate expansion of NK cells. In some embodiments, the IL12 is membrane bound on the stimulatory cells (referred to herein as “mbIL12”). In some embodiments combinations of IL12A and IL12B are used (referred to herein as “IL12A/12B”, and when membrane bound, “mbIL12A/12B”). In some embodiments, IL15 is membrane bound by virtue of being coupled or conjugated to a transmembrane molecule or integral membrane protein. In several embodiments, a transmembrane domain of CD8 α is used. In several embodiments, wild type (e.g., a full-length) IL12A and/or 12B is expressed on, or by, the stimulatory cells. In some embodiments, IL12A and/or 12B is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% homologous with full-length IL12A or 12B, respectively. In some embodiments, truncated forms of IL12A and/or 12B are used. In several embodiments, mbIL12A is encoded by the nucleic acid sequence of SEQ ID NO: 3. In several embodiments, mbIL12A is encoded by the amino acid sequence of SEQ ID NO: 4. In several embodiments, the stimulatory molecule may have one or more additional mutations from SEQ ID NO. 3 or 4, but retains, or in some embodiments, has enhanced stimulatory activity. In several embodiments, mbIL12B is encoded by the nucleic acid sequence of SEQ ID NO: 5. In several embodiments, mbIL12B is encoded by the amino acid sequence of SEQ ID NO: 6. In several embodiments, the stimulatory molecule may have one or more additional mutations from SEQ ID NO. 5 or 6, but retains, or in some embodiments, has enhanced stimulatory activity. In some embodiments, a mixture of IL12A and IL12B is used. In several embodiments, a particular ratio of expression of IL12A:IL12B is used, for example, 1:10, 1:100, 1:1000, 1:10,000, 10,000:1, 1000:1, 100:1, 10:1 and any ratio there

between, including endpoint. In some embodiments both IL12A and IL12B are expressed, for example, as a fusion protein. In some embodiments, a fragment, or fragments, of IL12A are expressed in conjunction with a fragment, or fragments of IL12B. In several embodiments, expression of IL12 (A and/or B) on the stimulatory cells imparts to the cells the ability to influence the phenotype and function of the expanded cells. In other words, expression of IL12A and/or B (alone or in combination with the other stimulatory molecules disclosed herein, leads to, in several embodiments, selective expansion of an NK cell sub-population. In several embodiments, that particular subpopulation can be advantageous in a specific therapeutic application where a particular phenotype of NK cells is particularly effective.

[0050] In some embodiments interleukin 18 (IL18) is used to facilitate expansion of NK cells. In some embodiments, the IL18 is membrane bound on the stimulatory cells (referred to herein as “mbIL18”). In some embodiments, IL18 is membrane bound by virtue of being coupled or conjugated to a transmembrane molecule or integral membrane protein. In several embodiments, a transmembrane domain of CD8 α is used. In several embodiments, wild type (e.g., a full-length) IL18 is expressed on, or by, the stimulatory cells. In some embodiments, the IL18 is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% homologous with full-length IL18. In some embodiments, truncated forms of IL18 are used. In several embodiments, mbIL18 is encoded by the nucleic acid sequence of SEQ ID NO: 7. In several embodiments, mbIL18 is encoded by the amino acid sequence of SEQ ID NO: 8. In several embodiments, the stimulatory molecule may have one or more additional mutations from SEQ ID NO. 7 or 8, but retains, or in some embodiments, has enhanced stimulatory activity. In several embodiments, expression of IL18 on the stimulatory cells imparts to the cells the ability to influence the phenotype and function of the expanded cells. In other words, expression of IL18 (alone or in combination with the other stimulatory molecules disclosed herein, leads to, in several embodiments, selective expansion of an NK cell sub-population. In several embodiments, that particular subpopulation can be advantageous in a specific therapeutic application where a particular phenotype of NK cells is particularly effective.

[0051] In some embodiments interleukin 21 (IL21) is used to facilitate expansion of NK cells. In some embodiments, the IL21 is membrane bound on the stimulatory cells (referred to herein as “mbIL21”). In some embodiments, IL21 is membrane bound by virtue of being coupled or conjugated to a transmembrane molecule or integral membrane protein. In several embodiments, a transmembrane domain of CD8 α is used. In several embodiments, wild type (e.g., a full-length) IL21 is expressed on, or by, the stimulatory cells. In some embodiments, the IL21 is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% homologous with full-length IL21. In some embodiments, truncated forms of IL21 are used. In several embodiments, the mbIL21 used to stimulate NK cells is derived from the nucleic acid sequence of SEQ ID NO: 9. As discussed herein, in several embodiments, the CD8a transmembrane domain is used to anchor the IL21 of SEQ ID NO: 9 (or fragment thereof) to the membrane of the stimulatory cells. In several embodiments, the mbIL21 used to stimulate NK cells is derived from the amino acid sequence of SEQ ID NO: 10. In several embodiments, the stimulatory molecule may have one or more additional mutations from SEQ ID NO. 9 or 10, but retains, or in some embodiments, has enhanced stimulatory activity.

[0052] In some embodiments interleukin 22 (IL22) is used to facilitate expansion of NK cells. In some embodiments, the IL22 is membrane bound on the stimulatory cells (referred to herein as “mbIL22”). In some embodiments, IL22 is membrane bound by virtue of being coupled or conjugated to a transmembrane molecule or integral membrane protein. In several embodiments, a transmembrane domain of CD8 α is used. In several embodiments, wild type (e.g., a full-length) IL22 is expressed on, or by, the stimulatory cells. In some embodiments, the IL22 is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% homologous with full-length IL22. In some embodiments, truncated forms of IL22 are used. In several embodiments, mbIL22 is encoded by the nucleic acid sequence of SEQ ID NO: 11. In several embodiments, mbIL22 is encoded by the amino acid sequence of SEQ ID NO: 12. In several embodiments, the stimulatory molecule may have one or more additional mutations from SEQ ID NO. 11 or 12, but retains, or in some embodiments, has enhanced stimulatory activity.

[0053] In some embodiments 4-1BB ligand (4-1BBL) is used to facilitate expansion of immune cells. 4-1BBL has an extracellular domain that interacts with its receptor on T cells, 4-1BB, thereby providing the T cells co-stimulatory signals for survival, proliferation, and differentiation. In some embodiments, 4-1BBL is membrane bound by virtue of being coupled or conjugated to a transmembrane molecule or an integral membrane protein. In several embodiments, wild type (e.g., a full-length) 4-1BBL is expressed on, or by, the stimulatory cells. In some embodiments, the 4-1BBL is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% homologous with full-length 4-1BBL. In some embodiments, truncated forms of IL18 are used. In several embodiments, mb4-1BBL is encoded by the nucleic acid sequence of SEQ ID NO: 13. In several embodiments, mb4-1BBL is encoded by the amino acid sequence of SEQ ID NO: 14. In several embodiments, the stimulatory molecule may have one or more additional mutations from SEQ ID NO. 13 or 14, but retains, or in some embodiments, has enhanced stimulatory activity.

[0054] In some embodiments, an anti-CD3 antibody is used to facilitate expansion of immune cells. In some embodiments, the anti-CD3 antibody is membrane bound on the stimulatory cells (referred to herein as “mbantiCD3” or “mb α -CD3”). In several embodiments, a full-length anti-CD3 antibody is expressed on the stimulatory cells. In some embodiments, the anti-CD3 antibody comprises a single chain fragment variable region (scFv) fragment. Depending on the embodiment, the antibody can be monoclonal or polyclonal. In some embodiments, the anti-CD3 antibody comprises a variety of antigenic fragments and/or fusions selected from a Fab', a F(ab')₂, a single domain antibody (e.g., a diabody, a nanobody). In some embodiments, the antibody is selected from the group consisting of muromonab-CD3, oteixizumab, teplizumab and visilizumab. In some embodiments, the antibody is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% homologous with one or more of muromonab-CD3, oteixizumab, teplizumab and visilizumab. In several embodiments, antibodies that bind to one or more subunits of the CD3 portion of the T cell receptor are expressed by the stimulatory cells. In several embodiments, the antibodies expressed are directed against the gamma, epsilon, or delta CD3 subunits. In several embodiments, the antibody expressed by the stimulatory cells are directed against an epitope derived from the CD3 epsilon nucleic acid sequence of SEQ

ID NO: 15. In several embodiments, the anti-CD3 antibody is a single chain fragment variable (scFv). In several embodiments, mbantiCD3 scFv is encoded by the nucleic acid sequence of SEQ ID NO: 16. In some such embodiments, the antibody has the amino acid sequence of SEQ ID NO: 17. In several embodiments, the stimulatory molecule may have one or more additional mutations from SEQ ID NO. 16 or 17, but retains, or in some embodiments, has enhanced stimulatory activity

[0055] In several embodiments, stimulatory cells, such as K562 cells, are genetically modified to express combinations of various stimulatory molecules. Depending on the embodiment, any combination of the stimulatory molecules disclosed herein may be used. For example, in several embodiments, mbIL15, 4-1BBL and mb α -CD3 are co-expressed on the stimulatory cells. In several embodiments, mbIL15, 4-1BBL and mbIL12A/12B are co-expressed on the stimulatory cells. In several embodiments, mbIL15, 4-1BBL and mbIL18 are co-expressed on the stimulatory cells. In several embodiments, mbIL15, 4-1BBL, mbIL18, and mbIL12A/12B are co-expressed on the stimulatory cells. In several embodiments, mbIL15, 4-1BBL, mbIL12A/12B and mbantiCD3 are co-expressed on the stimulatory cells. In several embodiments, mbIL15, 4-1BBL, mbIL18 and mbantiCD3 are co-expressed on the stimulatory cells. In several embodiments, mbIL15, 4-1BBL, mbIL12A/12B, mbIL18 and mbantiCD3 are co-expressed on the stimulatory cells. In some embodiments, mbIL21 and/or mbIL22 can be expressed in addition to, or in place of, any of the stimulatory molecules listed above. In some embodiments, each of these molecules is expressed in the stimulatory cells through transfection with individual plasmids. Alternatively, two or more of the stimulatory molecules can be encoded in a single plasmid.

[0056] Depending on the embodiment, and on the stimulatory molecule in question, the stimulatory molecules may be expressed at particular times during the process of co-culturing with an immune cell population. For example, rather than being constitutively expressed, one or more of the markers may be under the control of an inducible, or otherwise regulatable promoter. As such, a triggering molecule or stimulus can be added to the co-culture at a desired time, resulting in the expression of the desired stimulatory molecule at a particular point during the expansion and activation protocol. As used herein, the terms “inducible promotor” and “regulatable promotor” shall be given their

ordinary meaning and shall also refer to promoters whose transcriptional activity is modulated (e.g., stimulated or inhibited) by the presence of certain biotic or abiotic factors. As used herein, the terms “triggering molecule” or “triggering stimulus” shall be given their ordinary meaning and shall refer to chemical or physical substances or conditions that act on an inducible or regulatable promotor, including but not limited to alcohol, tetracycline, steroids, metal and other compounds, as well as high or low culture temperatures. Additionally, regulatable expression of the stimulatory molecules can also be used to reduce and/or eliminate expression of a particular stimulatory molecule during the culturing process. Such embodiments can facilitate the preferential expansion of certain subpopulations of immune cells, such as NK cells, by for example providing a particular stimulatory signal at a point in time during the activation and expansion process when the NK cells are particularly sensitive to such a signal. In several embodiments, such an approach can lead to an unexpectedly robust activation and expansion of NK cells. In still additional embodiments, the duration of proliferation of the NK cells is extended, ultimately leading to a larger population of activated NK cells for use in, for example, cancer immunotherapy.

[0057] In some embodiments, also provided herein are nucleic acid and amino acid sequences that have homology of at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% (and ranges therein) as compared with the respective nucleic acid or amino acid sequences of the stimulatory molecules disclosed herein, encoded by SEQ ID NOS. 1-17 and that also exhibit one or more of the functions as compared with the respective SEQ ID Nos. 1-17 including but not limited to, (i) activating NK cells, (ii) sensitizing NK cells, (iii) enhanced NK cell proliferation, (iv), enhanced NK cell target affinity, (v) upregulated or otherwise enhanced signal transduction, (vi) enhanced NK cell cytotoxicity, (vii) T cell stimulation (e.g. proliferation, selective expansion of useful subpopulations, etc.), (viii) selective expansion of particular NK cell sub-populations, and (ix) combinations thereof.

Methods of Co-culture and Immune Cell Expansion

[0058] In several embodiments, stimulatory cells can be transduced with multiple constructs (each encoding one or more of the stimulatory molecules to be expressed), or alternatively, single constructs can be used. In several embodiments, the stimulatory cells are

first transduced with a stimulatory molecule coupled to an identifiable marker, such as a fluorescent tag (e.g., green fluorescent protein, GFP, or other fluorescent moiety). In additional embodiments, other tags may be used. For example, in several embodiments a FLAG tag (DYKDDDDK, SEQ ID NO. 19) is used. Also available are other tag sequences, such as a polyhistidine tag (His-tag) (HHHHHH, SEQ ID NO. 20), HA-tag or myc-tag. Combinations of tag types can also be used in certain embodiments. Subsequent to transduction, the stimulatory cells can be queried for presence and degree of expression of the tag, which correlates with expression of the associated stimulatory molecule. Those cells (or individual cells) with high levels of tag expression (and hence high levels of stimulatory molecule expression) can be selected and expanded (clonally if single cells are selected). Subsequently, an additional transduction with one or more additional stimulatory molecules can be performed, followed by an additional query and expansion, until the desired expression of a combination of stimulatory molecules is achieved. In some embodiments, the tag associated with each subsequent transduction is different than those of preceding transductions, so the expression of each stimulatory molecule can be independently verified.

[0059] In several embodiments, stimulatory cells are seeded into culture vessels and allowed to reach near confluence. Immune cells can then be added to the culture at a desired concentration, ranging, in several embodiments from about 0.5×10^6 cells/cm² to about 5×10^6 cells/cm², including any density between those listed, including endpoints. Immune cells can be in a starting sample, such as peripheral blood, an isolated preparation of immune cells, an isolated population of NK cells, etc. depending on the embodiments. In several embodiments, blood samples are pre-processed to segregate certain populations to be expanded, e.g., NK cells. In some embodiments, a peripheral blood sample is co-cultured with the stimulatory cells, and a desired sub-population of expanded immune cells, e.g., NK cells, is optionally isolated from the mixed population of expanded cells. Post expansion, the cells can be maintained in a suitable medium, for example, RPMI-1640, 10% FCS, and 10 IU/mL IL-2.

[0060] As discussed above, there are provided, in several embodiments, engineered cell populations (also referred to herein as stimulatory cells) suitable for activating and/or expanding a population of immune cells. In several embodiments, the

engineered population is derived from a cancerous cell and is modified to express mbIL15, mb 4-1BBL, and at least one additional membrane bound molecule that stimulates immune cell activation, whereby co-culture of the engineered cells with a population of immune cells results in the activation and/or expansion of at least one subpopulation of immune cells, such as NK cells. In several embodiments, the additional molecule comprises one or more interleukins (or fragments thereof), such as IL12A, IL12B, IL18, IL21, and/or IL22. In some embodiments, the additional molecule comprises an antibody. In several embodiments, the antibody comprises a membrane bound anti-CD3 antibody (mb α -CD3), antibody or scFv, or fragments thereof. In several embodiments, the antibody is monoclonal. In several embodiments, the antibody is co-expressed with the at least one interleukin, or fragment thereof. Depending on the embodiment, one or more of the membrane bound molecules is coupled to a transmembrane domain of human CD8 α . Also provided for herein are methods for expanding NK cells, wherein the NK cells are co-expressed with such engineered cells. For example, in several embodiments there is provided a method for expanding NK cells, comprising obtaining a peripheral blood sample comprising a mixed population of immune cells comprising NK cells and T cells, contacting the mixed population of cells with such engineered cell populations and co-culturing the mixed population of cells with the engineered cells for a period of time sufficient to expand the NK cells of the mixed population. In several embodiments, T cells are optionally removed, resulting in a more pure NK cell population.

[0061] In several embodiments, the cell population is derived from one or more of the following cell lines: K562 cells, Wilms tumor cell line HFWT, endometrial tumor cell line HHUA, melanoma cell line HMV-II, hepatoblastoma cell line HuH-6, lung small cell carcinoma cell lines Lu-130 or Lu-134-A, neuroblastoma cell lines NB19 or NB69, embryonal carcinoma testis cell line NEC14, cervical carcinoma cell line TCO-2, and neuroblastoma cell line TNB1. In several embodiments, the cell population lacks expression of MHC I and or/ MHC II molecules.

[0062] In several embodiments, there are also provided kits comprising a plurality of nucleic acids, for use in generating the engineered cell populations to expand immune cells, the kit comprising at least 3 of: a nucleic acid encoding mbIL15, a nucleic acid

encoding 4-1BBL, a nucleic acid encoding mbIL12A, a nucleic acid encoding mbIL12B, a nucleic acid encoding mbIL18, a nucleic acid encoding mbIL21, a nucleic acid encoding mbIL22, and a nucleic acid encoding mb α -CD3. In several embodiments, one or more of the nucleic acids may comprise a tag, such as GFP, FLAG tag, or HIS tag.

[0063] Further provided herein are methods of treating a subject having cancer or an infectious disease comprising administering to the subject a composition comprising genetically engineered cells described herein and/or composition comprising an expanded population of immune cells co-cultured with the genetically engineered cells described herein. As used herein, the terms “treat,” “treating,” and “treatment” in the context of the administration of a therapy to a subject shall be given their ordinary meaning and shall refer to the beneficial effects that a subject derives from a therapy. In certain embodiments, treatment of a subject with the administration of a composition comprising genetically engineered cells described herein and/or composition comprising an expanded population of immune cells co-cultured with the genetically engineered cells described herein achieves one, two, three, four, or more of the following effects, including, for example: (i) reduction or amelioration the severity of disease or symptom associated therewith; (ii) reduction in the duration of a symptom associated with a disease; (iii) protection against the progression of a disease or symptom associated therewith; (iv) regression of a disease or symptom associated therewith; (v) protection against the development or onset of a symptom associated with a disease; (vi) protection against the recurrence of a symptom associated with a disease; (vii) reduction in the hospitalization of a subject; (viii) reduction in the hospitalization length; (ix) an increase in the survival of a subject with a disease; (x) a reduction in the number of symptoms associated with a disease; (xi) an enhancement, improvement, supplementation, complementation, or augmentation of the prophylactic or therapeutic effect(s) of another therapy.

[0064] Administration can be by a variety of routes, including, without limitation, intravenous, intraarterial, subcutaneous, intramuscular, intrahepatic, intraperitoneal and/or local delivery to an affected tissue. Doses of genetically engineered cells and/or the expanded population of immune cells co-cultured with the genetically engineered cells described herein, can be readily determined for a given subject based on their body mass,

disease type and state, and desired aggressiveness of treatment, but range, depending on the embodiments, from about 10^5 cells per kg to about 10^{12} cells per kg (e.g., $10^5 - 10^7$, $10^7 - 10^{10}$, $10^{10} - 10^{12}$ and overlapping ranges therein). In one embodiment, a dose escalation regimen is used. In several embodiments, a range of expanded population of immune cells co-cultured with the genetically engineered cells described herein is administered, for example between about 1×10^6 cells/kg to about 1×10^8 cells/kg. Depending on the embodiment, various types of cancer or infection disease can be treated. Various embodiments provided for herein include treatment or prevention of the following non-limiting examples of cancers including, but not limited to, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), adrenocortical carcinoma, Kaposi sarcoma, lymphoma, gastrointestinal cancer, appendix cancer, central nervous system cancer, basal cell carcinoma, bile duct cancer, bladder cancer, bone cancer, brain tumors (including but not limited to astrocytomas, spinal cord tumors, brain stem glioma, craniopharyngioma, ependymoblastoma, ependymoma, medulloblastoma, medulloepithelioma), breast cancer, bronchial tumors, Burkitt lymphoma, cervical cancer, colon cancer, chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), chronic myeloproliferative disorders, ductal carcinoma, endometrial cancer, esophageal cancer, gastric cancer, Hodgkin lymphoma, non-Hodgkin lymphoma, hairy cell leukemia, renal cell cancer, leukemia, oral cancer, nasopharyngeal cancer, liver cancer, lung cancer (including but not limited to, non-small cell lung cancer, (NSCLC) and small cell lung cancer), pancreatic cancer, bowel cancer, lymphoma, melanoma, ocular cancer, ovarian cancer, pancreatic cancer, prostate cancer, pituitary cancer, uterine cancer, and vaginal cancer.

[0065] Further, various embodiments provided for herein include treatment or prevention of the following non-limiting examples of infectious diseases including, but not limited to, infections of bacterial origin may include, for example, infections with bacteria from one or more of the following genera: *Bordetella*, *Borrelia*, *Brucella*, *Campylobacter*, *Chlamydia* and *Chlamydophila*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Legionella*, *Leptospira*, *Listeria*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Treponema*, *Vibrio*, and *Yersinia*, and mutants or combinations thereof. In

several embodiments, methods are provided to treat a variety to treat viral infections, such as those caused by one or more viruses, such as adenovirus, Cocksackievirus, Epstein-Barr virus, hepatitis a virus, hepatitis b virus, hepatitis c virus, herpes simplex virus, type 1, herpes simplex virus, type 2, cytomegalovirus, ebola virus, human herpesvirus, type 8, HIV, influenza virus, measles virus, mumps virus, human papillomavirus, parainfluenza virus, poliovirus, rabies virus, respiratory syncytial virus, rubella virus, and varicella-zoster virus.

[0066] In several embodiments, the expanded and/or activated cells are administered in a therapeutically effective amount (e.g., an amount that is sufficient to treat a cancer, such as by ameliorating symptoms associated with the cancer, preventing or delaying the onset of the cancer, also lessening the severity or frequency of symptoms of the cancer and/or preventing, delaying or overcoming metastasis of the cancer). The amount that will be therapeutically effective in the treatment of a particular individual will depend on the symptoms and severity of the condition (e.g., cancer), and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the cancer, and should be decided according to the judgment of a practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. In several embodiments, the expanded immune cells are co-administered with one or more stimulatory cells, while in some embodiments, the stimulatory cells (or one or more factors produced, secreted by, or harvested from the stimulatory cells) are administered in order to activate endogenous immune cell populations.

[0067] Methods of administration include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, topical, oral and intranasal. Other suitable methods of introduction can also include gene therapy, rechargeable or biodegradable devices, particle acceleration devices (e.g., "gene guns") and slow release polymeric devices. The pharmaceutical compositions of this invention can also be administered as part of a combinatorial therapy with other compounds, in several embodiments.

EXAMPLES

[0068] The following are non-limiting descriptions of experimental methods and materials that were used in examples disclosed below.

Example 1 – Preparation of K562 Derivatives, Expansion of NK Cells

[0069] Peripheral blood samples were obtained from discarded anonymized by-products of platelet donations from healthy adult donors at the National University Hospital Blood Bank, Singapore.

[0070] Mononucleated cells were separated by centrifugation on a Lymphoprep density step (Nycomed, Oslo, Norway) and washed twice in RPMI-1640. To purify primary NK cells from peripheral blood mononucleated cells an NK Cell Isolation Kit from Miltenyi (Bergisch Gladbach, Germany) was used.

[0071] The K562-mb15-41BBL cell line (Figure 1A) was made as previously described (Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. *Blood*. 2005;106:376-383; Fujisaki H, Kakuda H, Shimasaki N, et al. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. *Cancer Res*. 2009;69(9):4010-4017.)

[0072] The other K562 variants were generated by transducing the K562-mb15-41BBL cells with a retroviral vector containing the cDNA sequence encoding membrane-bound interleukin (IL)-12, IL-18, or both, or membrane-bound anti-human CD3 ScFv. The sequences for the cloning constructs are provided in SEQ ID NO: 21 (mbIL15), SEQ ID NO: 23 (mbIL12A), SEQ ID NO: 24 (mbIL12B), SEQ ID NO: 25 (mbIL18), and SEQ ID NO: 26 (mb-anti-CD3 scFv). A RD144-pseudotyped MSCV retrovirus containing the corresponding cDNA was used to transduce the K562-mb15-41BBL cells. Retroviral vector-conditioned medium was added to RetroNectin (Takara, Otsu, Japan)-coated polypropylene tubes; after centrifugation and removal of the supernatant, K562-mb15-41BBL cells were added to the tubes and left at 37°C for 12 hours; fresh viral supernatant was added on two other successive days. Cells were then maintained in RPMI-1640 with FBS and antibiotics.

[0073] Surface expression of IL-12a, IL12b and IL18 was analyzed by flow cytometry using the antibodies anti-IL12a conjugated to allophycocyanin (APC; Miltenyi) or to phycoerythrin (PE; R&D Systems, Minneapolis, MN), anti-IL12b APC (Biolegend, San Diego, CA), anti-IL18 (MBL; Woburn, MA) followed by goat-anti-mouse IgG1 PE (Southern

Biotechnology Associates, Birmingham, AL). Anti-CD3 was detected using a goat-anti-mouse Fab2 antibody conjugated to biotin followed by streptavidin APC (both from Jackson Immunoresearch (West Grove, PA). Subclones expressing high levels of the transgene were enriched by flow cytometry and used to stimulate NK cell expansion.

Human NK cell Expansion

[0074] To expand NK cells, PBMCs and the genetically modified K562 cells were co-cultured. Briefly, peripheral blood mononucleated cells (3×10^6) were cultured in a 6-well tissue culture plate with 2×10^6 irradiated (100 Gy) K562-modified cells in SCGM medium (CellGenix, Freiburg, Germany) containing 10% FBS and 40 IU/mL human interleukin (IL)-2 (Novartis, Basel, Switzerland). Every 2-3 days, fresh tissue culture medium and IL-2 was added. After 7 days of co-culture, residual T cells were removed using Dynabeads CD3 (Thermo Fisher), producing cell populations containing >90% CD56+ CD3- NK cells.

Results

[0075] After generating the constructs and transducing the K562-mb15-41BBL cells with the respective retroviral vector, the K562 cells were evaluated using flow cytometry for expression of the various membrane bound molecules. Figure 2A-2F depict the results of the evaluation. As depicted, each of the six molecules to be expressed showed that nearly 100% of the resulting K562 cell lines expressed the indicated molecule (2A – mbIL15, 2B – 41BBL, 2C – mbIL18, 2D – mbIL12A, 2E – mbIL12B, and 2F – mb-anti-CD3). These data demonstrate that the various constructs generated successfully translate into expression of the desired stimulatory molecule by the K562 cells (or other type of stimulatory cell).

[0076] Having confirmed expression of the desired stimulatory molecule, the ability of the various K562 variants to expand NK cells was evaluated. As discussed above, PBMCs were co-cultured with irradiated K562 cells co-expressing mbIL15 and 4-1BBL (K562-mb15-41BBL). This K562 construct was compared to a construct additionally expressing mbIL12 (+mb12), mbIL18 (+mb18), or both mbIL12 and mbIL18

(+mb12+mb18). The number of NK cells (defined by the expression of CD56 and the lack of CD3) recovered after 7 days of culture relative to those initially seeded was calculated and is depicted in Figure 3A. In all cultures, IL-2 40 IU/mL (Aldeuskin, Novartis) was added. Results of 5 experiments with cells from 4 healthy donors are shown. Horizontal bar corresponds to median value. These data indicate a clear trend towards enhanced NK cell expansion with the addition of mbIL12, mbIL18, and a combination of both. This suggests that supplementing the stimulatory nature of mbIL15-41BBL expressing K562 cells is accomplished using the constructs according to several embodiments herein.

[0077] Figure 3B shows an additional experiment comparing NK cell through co-culture of PBMCs with K562-mb15-41BBL cells or with K562-mb15-41BBL cells expressing mb12 and mb18 for 7 days of culture. This data is from 12 experiments with peripheral blood mononucleated cells from 8 donors. P value was calculated by paired t test. These data demonstrate that a significant increase in the degree of NK cells (as compared to the starting population). Thus, the constructs according to several embodiments disclosed herein result in the significant expansion of NK cell populations. This expansion is particularly advantageous, in several embodiments, because the K562 cells expression multiple stimulatory molecules result in an unexpectedly robust expansion of NK cells, leading to a sizeable population that can be used in therapeutic applications.

Example 2 – Long Term Expansion and Function of NK Cells Stimulated with K562 Variants

Long-term Expansion

[0078] The ability of NK cells to continue to expand was evaluated. PBMCs were co-cultured with irradiated K562 cells expressing mbIL15 and 4-1BBL (K562-mb15-41BBL) (Figure 4A), or with K562-mb15-41BBL cells also expressing mbIL12 (+mb12) (Figure 4B), mbIL18 (+mb18) (Figure 4C), or both mbIL12 and mbIL18 (+mb12+mb18) (Figure 4D). The number of NK cells (defined by the expression of CD56 and the lack of CD3) recovered after different time intervals in each culture relative to those originally seeded was calculated to compute the cell population doublings. To renew the potential expansion of the NK cells, fresh genetically-modified K562 cells were added every 2 weeks, at a K562: NK ratio of 5:1, and IL-2 concentration was maintained at 40 IU/mL during the

first week and at 400 IU/mL subsequently, after T cell depletion. Arrows indicate the time point at which NK cells stopped expanding despite addition of K562 cells, indicating senescence.

[0079] As with the expansion data discussed above, these data indicate that expression of mbIL15-41BBL result in a threshold level of expansion, while the expression of additional stimulatory molecules results in significant enhancements of expansion, in several embodiments. In particular, expression of mbIL12 alone did not appear to alter the ability of NK cells to continue to expand beyond that accomplished using the mbIL15-41BBL expressing cells. However, both the mbIL18 and combination mbIL12-mbIL18 expressing K562 cells resulted in significantly longer durations of NK cell expansions, with each construct stimulating NK cell expansion for nearly 20 weeks (~3 fold greater than the mbIL15-41BBL expressing K562 cells). This demonstrates that, in accordance with several embodiments disclosed herein, the expression of certain stimulatory molecules can unexpectedly enhance NK cell expansion. Additionally, according to several embodiments, co-expressing multiple stimulatory molecules can result in synergistic stimulatory effects.

Cytotoxicity assays

[0080] In addition to the expansion of NK cells, the cytotoxicity of the expanded cells was evaluated to determine whether certain engineered variants of stimulatory cells imparted a greater degree of cytotoxicity to the expanded NK cells.

[0081] Target cells were suspended in RPMI-1640 with 10% FBS, labeled with calcein AM (Sigma), and plated into 96-well flat bottom plates (Costar, Corning, NY). Expanded NK cells, suspended in RPMI-1640 with 10% FBS were then added at various E:T ratios as indicated, and co-cultured with target cells for 4 hours. Cells were then stained with propidium iodide and cytotoxicity was measured by flow cytometry using an Accuri flow cytometer (BD Bioscience), enumerating the number of viable target cells (calcein AM-positive, propidium-iodide negative, and light scattering properties of viable cells).

[0082] Figure 4E depicts data from measurements of NK cell cytotoxicity against K562 cells in 4-hour assays at the effector : target (E:T) ratios shown after 8 and 15 days of culture. At the initial 8-day time point, at both E:T ratios, the NK cells stimulated with

different constructs exhibited differential cytotoxicity. As shown, those NK cells expanded for 8 days with the mbIL15-41BBL+mbIL18 construct and the mbIL15-41BBL showed the greatest cytotoxicity. Interestingly, in those groups cultured for an additional 7 days in culture (15 days in total, with IL-2 at 4400 IU/mL for the second week), the differences in cytotoxicity were reduced, with all groups exhibiting cytotoxic effects at near 100%.

[0083] Figure 4F depicts data related to NK cell cytotoxicity after 64 days in culture (9 weeks in culture), using the E:T ratios shown. These data demonstrate that, even after a substantial amount of time in culture, cytotoxicity is still exhibited. At 1:1 E:T, the NK cells expanded using K562 cells expressing mbIL15-41BBL+mbIL18 demonstrated approximately 30% cytotoxicity, while NK cells expanded using the K562 cells expressing mbIL15-41BBL+mbIL12+mbIL18 demonstrated almost 80% cytotoxicity. When outnumbered by target cells (E:T of 1:2), the respective NK cells still exhibited cytotoxicity, though it was reduced compared to the 1:1 ratio. In contrast, when present in greater quantities than target cells (E:T of 2:1), the NK cells exhibited greater cytotoxicity. These data suggest that, when cultured for long periods of time, there may be a reduction in the potency of the NK cells (albeit with an increase in number). Thus, according to some embodiments, an increased duration of co-culture not only increases the raw number of NK cells, it also leads to an increase in the cytotoxic effects of each NK cell. Accordingly, some embodiments not only result in greater NK cell numbers, but the potency of each member of the expanded NK cell population is enhanced, thereby resulting in overall greater clinical efficacy. According to several embodiments, the duration of culture vs. potency is balanced to strike an optimal balance between cell number and the resultant cytotoxic effects.

[0084] In several embodiments, a nucleic acid encoding 4-1BBL comprises, consists essentially of or consists of the nucleic acid sequence of SEQ ID NO. 22 shown below

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gaattecccc ttccaccatg gaatacgcct ctgacgcttc actggacccc gaagcccccgt
ggcctccccc gccccgcgct cgcgcctgcc gcgtactgcc ttgggccctg gtcgcggggc
tgctgctgct gctgctgctc gctgccgcct gcgccgtctt cctgcctgc ccctgggccg
tgtccggggc tcgcgcctcg cccggetccg cggccagccc gagactccgc gaggggtccc
agctttcgcc cgacgatccc gccggcctct tggacctgcg gcagggcctg ttgcgcgacg
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tgggtggccca aatgttctg ctgategatg ggcccctgag ctggtacagt gaccagggcc
 tggcaggcgt gtccctgacg gggggcctga gctacaaaga ggacacgaag gagctggtgg
 tggccaaggc tggagtctac tatgtcttct ttaactaga gctgcggcgc gtggtggccg
 gcgagggctc aggtccggtt tcaattgcgc tgcacctgca gccactgcgc tctgtgctg
 gggccggcgc cctggcttg accgtggacc tgccacccgc ctctccgag gctcggaact
 cggccttcgg ttccagggc cgcttgctgc acctgagtgc cggccagcgc ctgggcgtcc
 atcttcacac ttaggccagg gcacgccatg cctggcagct taccagggc gccacagct
 tgggactctt ccgggtgacc ccgaaatcc cagccggact ccctcaccg aggtcggaat
 aactcgag (SEQ ID NO. 22).

[0085] It is contemplated that various combinations or subcombinations of the specific features and aspects of the embodiments disclosed above may be made and still fall within one or more of the inventions. Further, the disclosure herein of any particular feature, aspect, method, property, characteristic, quality, attribute, element, or the like in connection with an embodiment can be used in all other embodiments set forth herein. Accordingly, it should be understood that various features and aspects of the disclosed embodiments can be combined with or substituted for one another in order to form varying modes of the disclosed inventions. Thus, it is intended that the scope of the present inventions herein disclosed should not be limited by the particular disclosed embodiments described above. Moreover, while the invention is susceptible to various modifications, and alternative forms, specific examples thereof have been shown in the drawings and are herein described in detail. It should be understood, however, that the invention is not to be limited to the particular forms or methods disclosed, but to the contrary, the invention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the various embodiments described and the appended claims. Any methods disclosed herein need not be performed in the order recited. The methods disclosed herein include certain actions taken by a practitioner; however, they can also include any third-party instruction of those actions, either expressly or by implication. For example, actions such as “administering a population of expanded NK cells” includes “instructing the administration of a population of expanded NK cells.” In addition, where features or aspects of the disclosure are described in terms of

Markush groups, those skilled in the art will recognize that the disclosure is also thereby described in terms of any individual member or subgroup of members of the Markush group.

[0001] The ranges disclosed herein also encompass any and all overlap, sub-ranges, and combinations thereof. Language such as “up to,” “at least,” “greater than,” “less than,” “between,” and the like includes the number recited. Numbers preceded by a term such as “about” or “approximately” include the recited numbers. For example, “90%” includes “90%.” In some embodiments, at least 95% homologous includes 96%, 97%, 98%, 99%, and 100% homologous to the reference sequence. In addition, when a sequence is disclosed as “comprising” a nucleotide or amino acid sequence, such a reference shall also include, unless otherwise indicated, that the sequence “comprises”, “consists of” or “consists essentially of” the recited sequence.

[0086] Articles such as “a”, “an”, “the” and the like, may mean one or more than one unless indicated to the contrary or otherwise evident from the context. The phrase “and/or” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined. Multiple elements listed with “and/or” should be construed in the same fashion, i.e., “one or more” of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause. As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when used in a list of elements, “or” or “and/or” shall be interpreted as being inclusive, i.e., the inclusion of at least one, but optionally more than one, of list of elements, and, optionally, additional unlisted elements. Only terms clearly indicative to the contrary, such as “only one of” or “exactly one of” will refer to the inclusion of exactly one element of a number or list of elements. Thus claims that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present, employed in, or otherwise relevant to a given product or process unless indicated to the contrary. Embodiments are provided in which exactly one member of the group is present, employed in, or otherwise relevant to a given product or process. Embodiments are provided in which more than one, or all of the group members are present, employed in, or otherwise relevant to a given product or process. Any one or more claims may be amended to explicitly exclude

any embodiment, aspect, feature, element, or characteristic, or any combination thereof. Any one or more claims may be amended to exclude any agent, composition, amount, dose, administration route, cell type, target, cellular marker, antigen, targeting moiety, or combination thereof.

WHAT IS CLAIMED IS:

1. A genetically engineered cell population that does not express major histocompatibility complex (MHC) I molecules,
 - wherein said engineered cell population is derived from an immortalized cell,
 - wherein said engineered cell population is modified to express membrane-bound interleukin-15 (mbIL15),
 - wherein said engineered cell population is modified to express membrane-bound 4-1BB ligand (4-1BBL),
 - wherein said engineered cell population is modified to express at least one additional membrane bound interleukin that stimulates immune cell activation,
 - wherein said engineered cell population does not express major histocompatibility complex (MHC) I molecules, and
 - wherein co-culture of said engineered cell population with a population of immune cells results in the activation and expansion of at least one subpopulation of immune cells.
2. The engineered cell population of Claim 1, wherein the mbIL15 is encoded by a nucleic acid sequence that comprises SEQ ID NO. 1.
3. The engineered cell population of Claim 1, wherein the 4-1BBL is encoded by a nucleic acid sequence that comprises SEQ ID NO. 13.
4. The engineered cell population of Claim 1, wherein each of the membrane bound molecules is coupled to a transmembrane domain of human CD8 α .
5. The engineered cell population of Claim 4, wherein the transmembrane domain of human CD8 α comprises the sequence of SEQ ID NO. 18.
6. The engineered cell population of Claim 1, wherein the engineered cell population is derived from a cell line selected from the group consisting of K562 cells, Wilms tumor cell line HFWT, endometrial tumor cell line HHUA, melanoma cell line HMV-II, hepatoblastoma

cell line HuH-6, lung small cell carcinoma cell lines Lu-130 or Lu-134-A, neuroblastoma cell lines NB19 or NB69, embryonal carcinoma testis cell line NEC14, cervical carcinoma cell line TCO-2, and neuroblastoma cell line TNB1.

7. The engineered cell population of Claim 1, wherein the engineered cell population lacks expression of MHC II molecules.

8. The engineered cell population of Claim 1, wherein the engineered cell population is derived from K562 cells.

9. An engineered cell population according to any one of Claims 1-8, wherein the interleukin comprises IL12A, or a fragment thereof.

10. The engineered cell population of Claim 9, wherein the IL12A comprises the sequence of SEQ ID NO. 4.

11. An engineered cell population according to any one of Claims 1-8, wherein the interleukin comprises IL12B, or a fragment thereof.

12. The engineered cell population of Claim 11, wherein the IL12B comprises the sequence of SEQ ID NO. 6.

13. An engineered cell population according to any one of Claims 1-8, wherein the interleukin comprises IL12A and IL12B, or fragments thereof.

14. An engineered cell population according to any one of Claims 1-8, wherein the engineered cell population further expresses membrane bound IL18 (mbIL18), or fragment thereof.

15. The engineered cell population of Claim 14, wherein the IL18 comprises the sequence of SEQ ID NO. 8.

16. An engineered cell population according to any one of Claims 1-8, wherein the engineered cell population further expresses membrane bound IL21 (mbIL21), or fragment thereof.

17. The engineered cell population of Claim 16, wherein the IL21 comprises the sequence of SEQ ID NO. 10, or a fragment thereof.

18. The engineered cell population of Claim 1, wherein the cells express membrane bound IL22 (mbIL22), or fragment thereof.

19. The engineered cell population of Claim 18, wherein the IL22 comprises the sequence of SEQ ID NO. 12, or a fragment thereof.

20. An engineered cell population according to any one of Claims 1-8, wherein the cells further comprise a membrane bound anti-CD3 antibody (mb α -CD3), an antibody fragment thereof, or scFv.

21. The engineered cell population of Claim 20, wherein the mb α -CD3 is a monoclonal antibody.

22. The engineered cell population of Claim 21, wherein the mb α -CD3 targets an epitope within the nucleic acid sequence of CD3 epsilon of SEQ ID NO. 15.

23. The engineered cell population of Claim 20, wherein the mb α -CD3 is a scFv.

24. The engineered cell population of Claim 23, wherein the scFv comprises the sequence of SEQ ID NO. 17.

25. A method for expanding immune cells, comprising co-culturing a blood sample comprising immune cells with the engineered cell population of any one of Claims 1 to 24.

26. The method of Claim 25, wherein the immune cells are Natural Killer (NK) cells.

27. A genetically engineered cell population that does not express major histocompatibility complex (MHC) I molecules,

wherein said engineered cell population is derived from a cancerous cell,

wherein said engineered cell population is modified to express membrane-bound interleukin-15 (mbIL15),

wherein said engineered cell population is modified to express membrane-bound 4-1BB ligand (4-1BBL),

wherein said engineered cell population is modified to express at least one additional membrane bound interleukin that stimulates immune cell activation, and

wherein co-culture of said engineered cell population with a population of immune cells results in the activation and expansion of at least one subpopulation of immune cells.

28. The engineered cell population of Claim 27, wherein the interleukin comprises IL12A, or a fragment thereof.

29. The engineered cell population of Claim 28, wherein the IL12A comprises the sequence of SEQ ID NO. 4.

30. The engineered cell population of Claim 28, wherein the interleukin comprises IL12B, or a fragment thereof.

31. The engineered cell population of Claim 30, wherein the IL12B comprises the sequence of SEQ ID NO. 6.

32. The engineered cell population of Claim 27, wherein the interleukin comprises IL12A and IL12B, or fragments thereof.

33. An engineered cell population according to any one of Claims 27-32, wherein the engineered cell population further expresses membrane bound IL18 (mbIL18), or fragment thereof.

34. The engineered cell population of Claim 33, wherein the IL18 comprises the sequence of SEQ ID NO. 8.

35. An engineered cell population according to any one of Claims 27-34, wherein the engineered cell population expresses membrane bound IL21 (mbIL21), or fragment thereof.

36. The engineered cell population of Claim 35, wherein the IL21 comprises the sequence of SEQ ID NO. 10, or a fragment thereof.

37. An engineered cell population according to any one of Claims 27-36, wherein the engineered cell population expresses membrane bound IL22 (mbIL22), or fragment thereof.

38. The engineered cell population of Claim 37, wherein the IL22 comprises the sequence of SEQ ID NO. 12, or a fragment thereof.

39. An engineered cell population according to any one of Claims 27-38, wherein the engineered cell population further comprises a membrane bound anti-CD3 antibody (mb α -CD3), an antibody fragment thereof, or scFv.

40. The engineered cell population of Claim 39, wherein the mb α -CD3 is a monoclonal antibody.

41. An engineered cell population according to Claim 39 or 40, wherein the mb α -CD3 targets an epitope within the nucleic acid sequence of CD3 epsilon of SEQ ID NO. 15.

42. The engineered cell population of Claim 39, wherein the mb α -CD3 is a scFv.

43. The engineered cell population of Claim 42, wherein the scFv comprises the sequence of SEQ ID NO. 17.

44. An engineered cell population according to any one of Claims 27-43, wherein the engineered cell population is derived from a cell line selected from the group consisting of K562 cells, Wilms tumor cell line HFWT, endometrial tumor cell line HHUA, melanoma cell line HMV-II, hepatoblastoma cell line HuH-6, lung small cell carcinoma cell lines Lu-130 or

Lu-134-A, neuroblastoma cell lines NB19 or NB69, embryonal carcinoma testis cell line NEC14, cervical carcinoma cell line TCO-2, and neuroblastoma cell line TNB1.

45. An engineered cell population according to any one of Claims 27-44, wherein the engineered cells lack expression of MHC II molecules.

46. The engineered cell population of Claim 45, wherein the engineered cells are derived from K562 cells.

47. An engineered cell population according to any one of Claims 27-46, wherein each of the membrane bound molecules is coupled to a transmembrane domain of human CD8 α .

48. The engineered cell population of Claim 47, wherein the transmembrane domain of human CD8 α comprises the sequence of SEQ ID NO. 18.

49. An engineered cell population according to any one of Claims 27-48, wherein the mbIL15 is encoded by a nucleic acid sequence that comprises SEQ ID NO. 1.

50. An engineered cell population according to any one of Claims 27-49, wherein the mb4-1BBL is encoded by a nucleic acid sequence that comprises SEQ ID NO. 13.

51. A genetically engineered cell population that does not express major histocompatibility complex (MHC) I molecules,

wherein said engineered cell population is derived from a cancerous cell,

wherein said engineered cell population is modified to express membrane-bound interleukin-15 (mbIL15),

wherein said engineered cell population is modified to membrane-bound express 4-1BB ligand (4-1BBL),

wherein said engineered cell population is modified to express a membrane-bound anti-CD3 antibody (mb α -CD3) that stimulates immune cell activation, and

wherein co-culture of said engineered cell population with a population of immune cells results in the activation and expansion of at least one subpopulation of immune cells.

52. The engineered cell population of Claim 51, wherein the mb α -CD3 is a monoclonal antibody.

53. An engineered cell population according to Claim 51 or 52, wherein the mb α -CD3 targets an epitope within the nucleic acid sequence of CD3 epsilon of SEQ ID NO. 15.

54. The engineered cell population of Claim 51, wherein the mb α -CD3 is a scFv.

55. The engineered cell population of Claim 54, wherein the scFv comprises the sequence of SEQ ID NO. 17.

56. An engineered cell population according to any one of Claims 51 to 55, wherein the cells further express at least one membrane-bound interleukin, or fragment thereof.

57. The engineered cell population of Claim 56, wherein the interleukin comprises IL12A, or a fragment thereof.

58. The engineered cell population of Claim 57, wherein the IL12A comprises the sequence of SEQ ID NO. 4.

59. An engineered cell population according to any one of Claims 56-58, wherein the at least one membrane-bound interleukin, or fragment thereof comprises IL12B, or a fragment thereof.

60. The engineered cell population of Claim 59, wherein the IL12B at least one membrane-bound interleukin, or fragment thereof the sequence of SEQ ID NO. 6.

61. An engineered cell population according to any one of Claims 56-60, wherein the at least one membrane-bound interleukin, or fragment thereof comprises IL12A and IL12B, or fragments thereof.

62. An engineered cell population according to any one of Claims 56-67, wherein the cells further express membrane bound IL18 (mbIL18), or a fragment thereof.

63. The engineered cell population of Claim 62, wherein the IL18 comprises the sequence of SEQ ID NO. 8.

64. An engineered cell population according to any one of Claims 56-63, wherein the cells express membrane bound IL21 (mbIL21), or a fragment thereof.

65. The engineered cell population of Claim 64, wherein the IL21 comprises the sequence of SEQ ID NO. 10, or a fragment thereof.

66. An engineered cell population according to any one of Claims 56-65, wherein the cells express membrane bound IL22 (mbIL22), or fragment thereof.

67. The engineered cell population of Claim 66, wherein the IL22 comprises the sequence of SEQ ID NO. 12, or a fragment thereof.

68. An engineered cell population according to any one of Claims 25-67, wherein the engineered cells are suitable for the expansion of immune cells.

69. The engineered cell population of Claim 68, wherein the expanded immune cells are NK cells.

70. A method for expanding NK cells, comprising co-culturing a sample comprising NK cells with the engineered cell population of any one of Claims 25 to 69.

71. A method for preparing the engineered cell population of any one of Claims 25 to 69, comprising:

transducing the cells with a first construct encoding mbIL15, thereby
generating a first transduced population of cells,
expanding the first transduced population of cells,

transducing the first transduced population of cells with a second construct encoding 4-1BBL, thereby generating a second transduced population of cells,
transducing the second transduced population of cells with a third construct encoding at least one additional molecule capable of stimulating immune cells, thereby generating a third transduced population of cells, and
expanding the third transduced population of cells.

72. A plurality of nucleic acids, for use in preparing the engineered cell population of any one of Claims 25 to 69, comprising at least 3 of:

- a nucleic acid encoding mbIL15,
- a nucleic acid encoding 4-1BBL,
- a nucleic acid encoding mbIL12A,
- a nucleic acid encoding mbIL12B,
- a nucleic acid encoding mbIL18,
- a nucleic acid encoding mbIL21,
- a nucleic acid encoding mbIL22, and
- a nucleic acid encoding mb α -CD3.

73. The plurality of nucleic acids of Claim 72, wherein the plurality of nucleic acids are configured as a single construct.

74. The plurality of nucleic acids of Claim 72, wherein mbIL15 is encoded by a nucleic acid sequence comprising SEQ ID NO. 1.

75. The plurality of nucleic acids of Claim 72, wherein 4-1BBL is encoded by a nucleic acid sequence comprising SEQ ID NO. 13.

76. The plurality of nucleic acids of Claim 72, wherein mbIL12A is encoded by a nucleic acid sequence comprising SEQ ID NO. 3.

77. The plurality of nucleic acids of Claim 72, wherein mbIL12B is encoded by a nucleic acid sequence comprising SEQ ID NO. 5.

78. The plurality of nucleic acids of Claim 72, wherein mbIL18 is encoded by a nucleic acid sequence comprising SEQ ID NO. 7.

79. The plurality of nucleic acids of Claim 72, wherein mbIL21 is encoded by a nucleic acid sequence comprising SEQ ID NO. 9, or a fragment thereof.

80. The plurality of nucleic acids of Claim 72, wherein mbIL22 is encoded by a nucleic acid sequence comprising SEQ ID NO. 11, or a fragment thereof

81. The plurality of nucleic acids of Claim 72, wherein mb α -CD3 is encoded by a nucleic acid sequence comprising SEQ ID NO. 16.

82. The plurality of nucleic acids of Claim 72, wherein each of the plurality of nucleic further comprises a GFP tag.

83. A method for expanding NK cells, comprising:

- a) obtaining a peripheral blood sample comprising a mixed population of immune cells comprising NK cells and T cells,
- b) contacting mixed population of cells with an engineered cell population that exhibits reduced expression of MHC I and has been modified to express:
 - i) membrane-bound interleukin-15 (mbIL15),
 - ii) 4-1BB ligand (4-1BBL), and
 - iii) at least one additional molecule that stimulates immune cell activation, and
- c) co-culturing the mixed population of cells with the engineered cells for a period of time sufficient to expand the NK cells of the mixed population of immune cells, thereby expanding the NK cells.

84. The method of Claim 83, optionally further comprising removing T cells from the mixed population either prior to or after co-culturing.

85. A modified cell line comprising K562 myeloid leukemia cells that lack major histocompatibility complex I molecules wherein said K562 myeloid leukemia cells are genetically modified to express membrane-bound interleukin-15, 4-1BB ligand, and membrane-bound anti-CD3 antibody.

86. A modified cell line comprising K562 myeloid leukemia cells that lack major histocompatibility complex I molecules wherein said K562 myeloid leukemia cells are genetically modified to express membrane-bound interleukin-15, membrane-bound 4-1BB ligand, and at least one additional membrane-bound interleukin.

87. The modified cell line of Claim 86, wherein the additional membrane-bound interleukin expressed is interleukin-12.

88. The modified cell line of Claim 86, wherein the additional membrane-bound interleukin expressed is interleukin-18.

89. The modified cell line of Claim 86, wherein the additional membrane-bound interleukins expressed are interleukin-12 and interleukin-18.

90. A modified cell line according to any one of Claims 86-89, further comprising a membrane-bound anti-CD3 antibody.

91. A modified cell line according to any one of Claims 86-90, further comprising one or more of mbIL12A, mbIL12B, mbIL18, mbIL21, mbIL22, and mb α -CD3.

92. A modified cell line comprising K562 myeloid leukemia cells that lack major histocompatibility complex I molecules wherein said K562 myeloid leukemia cells are genetically modified to express membrane-bound interleukin-15, membrane-bound 4-1BB ligand, membrane-bound anti-CD3 antibody, and at least one additional membrane-bound interleukin.

93. The modified cell line of Claim 92, wherein the additional membrane-bound interleukin expressed is interleukin-12.

94. The modified cell line of claim 92, wherein the additional membrane-bound interleukin expressed is interleukin-18.

95. The modified cell line of claim 92, wherein the additional membrane-bound interleukins expressed are interleukin-12 and interleukin-18.

96. The modified cell line of Claim 92, wherein the at least one additional interleukin comprises one or more of mbIL12A, mbIL12B, mbIL18, mbIL21, and mbIL22.

97. A population of NK cells expanded by culturing a mixed cell culture comprising NK cells and T lymphocytes with the modified cell line of any one Claims 85-86.

98. Use of the population of NK cells of Claim 97, for the treatment of cancer or infectious disease.

99. Use of the engineered cell population according to any one of Claims 25-69, for use in the expansion of a population of immune cells, wherein the population of immune cells is suitable for use in the treatment of cancer or infectious disease, and wherein the population of immune cells comprises NK cells.

100. A method of treating cancer or an infectious disease comprising:
administering to a subject having cancer or an infectious disease a composition comprising an expanded population of immune cells,
wherein said immune cells have been expanded by co-culturing the immune cells with an engineered cell population,
wherein said engineered cell population is derived from a cancerous cell,
wherein said engineered cell population is modified to express membrane-bound interleukin-15 (mbIL15),

wherein said engineered cell population is modified to express membrane-bound 4-1BB ligand (4-1BBL),

wherein said engineered cell population is modified to express at least one additional membrane bound interleukin that stimulates immune cell activation,

wherein co-culture of said engineered cell population with a population of immune cells results in the activation and expansion of at least one subpopulation of immune cells, and

wherein said at least one subpopulation of immune cells is administered to said subject.

101. The method of Claim 100, wherein the administered subpopulation of cells comprises NK cells.

102. A method of treating cancer or an infectious disease comprising:

administering to a subject having cancer or an infectious disease a composition comprising an expanded population of immune cells,

wherein said immune cells have been expanded by co-culturing the immune cells with an engineered cell population,

wherein said engineered cell population is derived from a cancerous cell,

wherein said engineered cell population is modified to express membrane-bound interleukin-15 (mbIL15),

wherein said engineered cell population is modified to express membrane-bound 4-1BB ligand (4-1BBL),

wherein said engineered cell population is modified to express an anti CD-3 antibody,

wherein co-culture of said engineered cell population with a population of immune cells results in the activation and expansion of at least one subpopulation of immune cells, and

wherein said at least one subpopulation of immune cells is administered to said subject.