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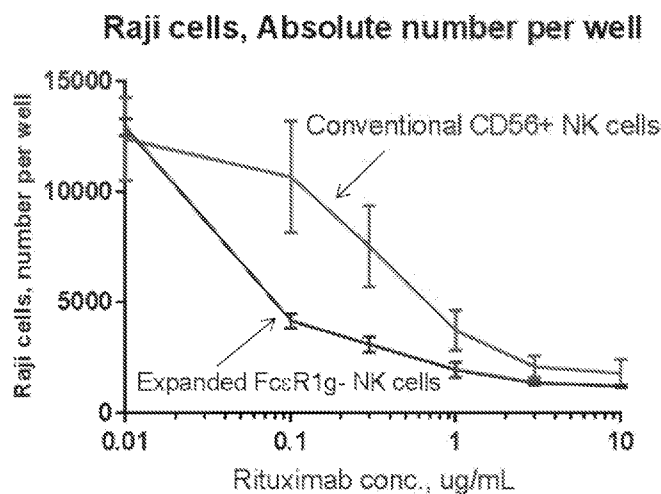
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(54) Title: ENGINEERED NATURAL KILLER (NK) CELLS AND COMPOSITIONS AND METHODS THEREOF

FIG. 1



(57) Abstract: The present invention provides engineered Natural Killer (NK) cells and methods of producing engineered NK cells. The engineered NK cells and compositions containing the engineered NK cells are useful for treating diseases such as cancer.

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ENGINEERED NATURAL KILLER (NK) CELLS AND COMPOSITIONS AND METHODS THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. provisional application No. 62/457,098 filed February 9, 2017, entitled "ENGINEERED NATURAL KILLER (NK) CELLS AND COMPOSITIONS AND METHODS THEREOF," and U.S. provisional application No. 62/484,350 filed April 11, 2017, entitled "ENGINEERED NATURAL KILLER (NK) CELLS AND COMPOSITIONS AND METHODS THEREOF," the contents of which are incorporated by reference in their entirety.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled 776032000140SeqList.txt, created February 6, 2018, which is 12,126 bytes in size. The information in the electronic format of the Sequence Listing is incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present invention provides engineered Natural Killer (NK) cells and methods of producing engineered NK cells. The engineered NK cells and compositions containing the engineered NK cells are useful for treating diseases such as cancer.

BACKGROUND OF THE INVENTION

[0004] NK cells were discovered 40 years ago by their ability to recognize and kill tumor cells without the requirement of prior antigen exposure. Since then, NK cells have been seen as promising agents for cell based cancer therapy. Most cancers lack identifiable, tumor-specific antigens in the HLA context, and thus cannot succumb to antigen specific cytotoxic T lymphocytes. Since a wide range of cancer cells are sensitive to NK cytotoxicity, one major advantage of using NK cells is that transplantation of natural killer (NK) cell can be employed against cancer cells in an allogeneic setting, without risk of graft-versus-host disease. The therapeutic promise of NK cells has been limited by the difficulty in obtaining a theoretically

effective amount of NK cells, particularly NK cells that exhibit cytotoxic effector functions for killing malignant tumor cells or infected cells. Accordingly, there is a need for engineered NK cells for therapeutic use. Provided herein are embodiments that meet such needs.

BRIEF SUMMARY OF THE INVENTION

[0005] Provided herein are engineered natural killer (NK) cells that have reduced expression, activity and/or signaling of the FcR γ chain. In some embodiments, the provided engineered cells exhibit enhanced immune responses, including for the treatment of cancer, microbial infections and/or viral infections in a subject. Also provided are methods of treatment of a subject by administering a therapeutically effective amount of the provided engineered NK cells. In some embodiments, the engineered NK cells are useful for enhancing therapeutic responses to an administered therapeutic antibody, such as to an anti-cancer, anti-viral or anti-microbial monoclonal antibody.

[0006] In some embodiments, provided herein is an engineered NK cell, wherein the NK cell is genetically engineered to reduce FcR γ chain expression, activity and/or signaling in the cell. In some of these embodiments, the engineered NK cell comprises a genetic disruption of a gene resulting in reduced FcR γ chain expression, activity and/or signaling in the cell. In some cases, the genetic disruption can result in a deletion or mutation of the gene. In some embodiments, the engineered NK cell comprises an inhibitory nucleic acid that reduces expression of a gene that results in reduced FcR γ chain expression, activity and/or signaling in the cell.

[0007] In some embodiments, the engineered NK cell comprises a genetic disruption of a gene encoding FcR γ chain and/or a genetic disruption resulting in reduced expression of FcR γ chain in the engineered NK cell. In some embodiments, the engineered NK cell comprises a genetic disruption of a gene encoding a protein regulating expression or activity of FcR γ chain and/or a genetic disruption resulting in reduced expression of a protein regulating expression or activity of FcR γ chain. In some embodiments, the engineered NK cell comprises a genetic disruption of a gene encoding a protein involved in FcR γ chain-dependent signaling and/or a genetic disruption resulting in reduced expression of a protein involved in FcR γ chain-dependent signaling. In some embodiments, the engineered cell can contain one or more of the

above genetic disruptions. In some embodiments, the genetic disruption comprises a deletion, mutation and/or insertion resulting in a premature stop codon in the gene or a frameshift of the open reading frame of the gene. In some embodiments, both alleles of the gene encoding FcR γ chain, the gene encoding a protein regulating expression or activity of FcR γ chain and/or the gene encoding a protein involved in FcR γ chain-dependent signaling are disrupted in the engineered NK cells.

[0008] In some embodiments, the engineered NK cell comprises an inhibitory nucleic acid molecule targeting a gene in the NK cell resulting in reduced expression of FcR γ chain, reduced expression of a protein regulating expression or activity of FcR γ chain and/or reduced expression of a protein involved in FcR γ chain-dependent signaling. In some embodiments, the inhibitory nucleic acid interferes with or reduces expression of a gene encoding FcR γ chain, a gene encoding a protein regulating expression or activity of FcR γ chain and/or a gene encoding a protein involved in FcR γ chain-dependent signaling. In some embodiments, the inhibitory nucleic acid comprises an RNA interfering agent. In some embodiments, the inhibitory nucleic acid comprises siRNA, shRNA, or miRNA.

[0009] In some of any such embodiments, the expression of FcR γ chain, a protein regulating expression or activity of FcR γ chain and/or a protein involved in FcR γ chain-dependent signaling is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % in the engineered NK cell as compared to the expression of the protein in the NK cell that is not genetically engineered.

[0010] In some of any such embodiments, expression of a protein regulating expression or activity of FcR γ chain is reduced in the engineered NK cell. In some of these embodiments, the protein regulating expression or activity of FcR γ chain is a transcription factor. In some embodiments, the transcription factor is PLZF (ZBTB16) or HELIOS (IKZF2).

[0011] In some of any such embodiments, the expression of a protein involved in FcR γ chain-dependent signaling is reduced in the engineered NK cell. In some embodiments, the protein involved in FcR γ chain-dependent signaling is a downstream signaling molecule of FcR γ . In some of these embodiments, the downstream signaling molecule is SYK, DAB2 or EAT-2.

[0012] In some of any such embodiments, expression of FcR γ chain is reduced in the engineered NK cell. In some embodiments, the engineered NK cell comprises a genetic disruption in a gene encoding FcR γ chain. In some of these embodiments, the genetic disruption comprises a deletion, mutation and/or insertion resulting in a premature stop codon in the gene or a frameshift of the open reading frame of the gene. In some embodiments, both alleles of the gene encoding FcR γ chain are disrupted in the genome of the engineered NK cell. In some embodiments, the engineered NK cell comprises an inhibitory nucleic acid molecule that targets a gene encoding FcR γ chain, thereby reducing expression of FcR γ chain in the cell. In some embodiments, the inhibitory nucleic acid molecule comprises a sequence complementary to the gene encoding FcR γ chain. In some embodiments, the inhibitory nucleic acid comprises an RNA interfering agent. In some embodiments, the inhibitory nucleic acid comprises siRNA, shRNA, or miRNA. In some embodiments, the expression of FcR γ chain is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % in the engineered NK cell as compared to the expression in the NK cell that is not genetically engineered.

[0013] In some of any of the provided embodiments, the reduced FcR γ chain expression, activity and/or signaling in the engineered NK cell is permanent, transient or inducible. In some embodiments, the expression, activity and/or signaling of FcR γ chain is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression, activity and/or signaling in the NK cell that is not genetically engineered. In some embodiments, the expression of FcR γ chain expressed in the cell is undetectable in an immunoblot assay.

[0014] In some of any of the embodiments, the engineered NK cell is derived from a primary cell obtained from a subject. In some embodiments, the subject is human.

[0015] In some of any of the embodiments, the engineered NK cell is derived from a clonal cell line. In some embodiments, the clonal cell line is NK-92, NK-YS, KHYG-1, NKL, NKG, SNK-6, or IMC-1.

[0016] In some of any of the embodiments, CD16 is expressed on the surface of the engineered NK cell. In some of any of the embodiments, the engineered NK cell expresses CD3-zeta (CD3 ζ) chain. In some embodiments, the expressed CD16 and/or CD3 ζ chain is endogenous to the NK cell. In some embodiments, the engineered NK cell is additionally engineered to express a recombinant or heterologous CD16 and/or CD3 ζ chain in the NK cell. In some embodiments, the engineered NK cell comprises a recombinant or heterologous CD16 gene and/or a recombinant or heterologous CD3-zeta (CD3 ζ) chain. In some embodiments, the recombinant or heterologous CD16 comprises a CD16-activating mutation. In some embodiments, the CD16 activating mutation is a mutation that results in higher affinity to IgG1. In some embodiments, the CD16 comprises the mutation 158V. In some embodiments, the CD16 comprises the mutation 158F.

[0017] Also provided are engineered NK cells that have reduced surface expression of an NK inhibitory receptor. In some embodiments, any of the provided engineered NK cells that have reduced expression, activity and/or signaling of FcR γ chain can additionally have reduced surface expression on an NK inhibitory receptor. In some embodiments, the engineered NK cells comprises a genetic disruption of a gene encoding a NK inhibitory receptor and/or a genetic disruption resulting in reduced expression of an NK inhibitory receptor. In some embodiments, the engineered NK cell comprises an inhibitory nucleic acid that targets a gene encoding a gene encoding an NK inhibitory receptor and/or results in reduced expression of an NK inhibitory receptor in the cell. In some embodiments, the inhibitory receptor is NKG2A and/or KIR2DL1. In some embodiments, expression of the inhibitory receptor, such as NKG2A and/or KIR2DL1, is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression, activity and/or signaling in the NK cell that is not genetically engineered.

[0018] In some embodiments, the engineered NK cell exhibits increased activity when stimulated through CD16 compared to the NK cell that is not genetically engineered. In some embodiments, the increased activity is observed following CD16 engagement by CD16 crosslinking, such as can occur in the presence of an antibody by binding of the Fc portion of the antibody to CD16.

[0019] In some embodiments, the engineered NK cell has reduced surface expression of NKp46, NKp30, and/or NKp44 compared to the NK cell without the modification. In some embodiments, expression of NKp46, NKp30, and/or NKp44 is reduced in the cell by greater than or greater than about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95 % or more compared to the expression in the NK cell that is not genetically engineered.

[0020] Also provided herein is a method of producing an engineered NK cell, comprising genetically engineering the NK cell to reduce FcR γ chain expression, activity, and/or signaling in the cell. In some of these embodiments, the methods involve disrupting a gene or repressing expression a gene to effect reduced FcR γ chain expression, activity and/or signaling in the cell. In some cases, the genetic disruption can result in a deletion or mutation of the gene. In some embodiments, the methods involves introducing into the cell an inhibitory nucleic acid that reduces expression of a gene that results in reduced FcR γ chain expression, activity and/or signaling in the cell.

[0021] In some embodiments, the method comprises disrupting a gene encoding FcR γ chain and/or disrupting a gene that results in reduced expression of FcR γ chain in the engineered NK cell. In some embodiments, the method comprises disrupting a gene encoding a protein regulating expression or activity of FcR γ chain and/or a disrupting a gene that results in reduced expression of a protein regulating expression or activity of FcR γ chain. In some embodiments, the method comprises disrupting a gene encoding a protein involved in FcR γ chain-dependent signaling and/or a disrupting a gene that results in reduced expression of a protein involved in FcR γ chain-dependent signaling. In some embodiments, the method of disrupting the gene results in introducing a deletion, mutation, or insertion into the gene.

[0022] In some embodiments, the method of disrupting the gene comprises introducing into an NK cell an endonuclease that has been engineered to target to the gene. In some embodiments, the endonuclease is a TAL nuclease, meganuclease, zinc-finger nuclease, CRISPR- associated protein 9 (Cas9), or Argonaute. In some embodiments, the endonuclease is a Cas9 that is a fusion with or is complexed with at least one guide RNA that is complementary with a target domain or region of the gene. In some embodiments, the Cas9 is an *S. aureus* Cas9 molecule. In some embodiments, the Cas9 molecule is an *S. pyogenes*

Cas9. In some embodiments, the method involves introducing into the cell an endonuclease, such as Cas9, that targets to the gene encoding FcR γ chain in the NK cell.

[0023] In some embodiments, the method for repressing expression of a gene comprises introducing an inhibitory nucleic acid into the cell that targets a gene to result in reduced FcR γ chain expression, activity and/or signaling in the cell. In some embodiments, one or more inhibitory nucleic acid molecule is introduced into the cell that targets one or more genes resulting in reduced expression of a gene encoding FcR γ chain, a gene encoding a protein regulating expression or activity of FcR γ chain and/or a gene encoding a protein involved in FcR γ chain-dependent signaling. In some embodiments, the inhibitory nucleic acid comprises a sequence complementary to a gene encoding FcR γ chain. In some embodiments, the inhibitory nucleic acid comprises a sequence complementary to a gene encoding a protein regulating expression or activity of FcR γ chain. In some embodiments, the inhibitory nucleic acid comprises a sequence complementary to a gene encoding a protein involved in FcR γ chain-dependent signaling. In some embodiments, the inhibitory nucleic acid comprises an RNA interfering agent. In some embodiments, the nucleic acid is siRNA, shRNA, or miRNA.

[0024] In some of any such embodiments, the method is carried out to reduce expression of a protein regulating expression or activity of FcR γ chain in the NK cell. In some of these embodiments, the protein regulating expression or activity of FcR γ chain is a transcription factor. In some embodiments, the transcription factor is PLZF (ZBTB16) or HELIOS (IKZF2).

[0025] In some of any such embodiments, the method is carried out to reduce expression of a protein involved in FcR γ chain-dependent signaling in the NK cell. In some embodiments, the protein involved in FcR γ chain-dependent signaling is a downstream signaling molecule of FcR γ . In some of these embodiments, the downstream signaling molecule is SYK, DAB2 or EAT-2.

[0026] In some of any such embodiments, the method is carried out to reduce expression of FcR γ chain in the NK cell.

[0027] In some of any of the embodiments, the method is carried out *in vitro*. In some of any of the embodiments, the method is carried out *ex vivo*, such as from cells isolated from a subject, such as from a human patient. In some of any of the embodiments, the method is carried so that the reduced expression is permanent, transient, or inducible.

[0028] In some embodiments, the methods for producing an engineered NK cell involves carrying out the genetic engineering, such as the disruption or repression of expression of a gene as described, in primary NK cells obtained from a subject. In some embodiments, the subject is mammalian, such as is human.

[0029] In some embodiments, prior to disrupting or repressing expression of the gene, the method comprises (i) isolating a NK cell from a sample from a mammalian subject. In some embodiments, the mammalian subject is human. In some of these embodiments, the sample comprises peripheral blood mononuclear cells (PBMC). In some embodiments, isolating the NK cell comprises selecting NK cells based on surface expression of an NK cell marker. In some embodiments, the NK cell marker is one or more of CD56, CD161, KIR, NKG2A, NKG2D, NKp30, NKp44, NKp46, 2B4, NTB-A, CRACC, DNAM-1, CD69, and/or CD25. In some embodiments, the method comprises selecting NK cells from other lymphocytes by selecting cells that do not express surface CD3, a T-cell antigen receptor (TCR) and/or surface immunoglobulins (Ig) B cell receptor. In some embodiments, the NK cells are selected or are additionally selected to have surface expression of CD16. In some embodiments, the NK cells express CD3 ζ .

[0030] In some embodiments, the methods for producing an engineered NK cell involves carrying out the genetic engineering on an NK cell line. In some embodiments, the cell line is NK-92, NK-YS, KHYG-1, NKL, NKG, SNK-6, or IMC-1.

[0031] In some embodiments, the method comprises engineering the NK cell line to express a recombinant or heterologous CD16 and/or CD3 ζ . In some embodiments, the method comprises introducing into the NK cell a nucleic acid encoding the CD16 and/or CD3 ζ . In some embodiments, the recombinant or heterologous CD16 contains an activating mutation. In some embodiments, the activating mutation increases affinity of CD16 for IgG. In some

embodiments, the CD16 comprises a 158V mutation. In some embodiments, the CD16 comprises a 158F mutation.

[0032] In some embodiments, the method comprises virally transducing the NK cell with nucleic acid encoding the CD16 and/or CD3 ζ gene. In some embodiments, the method comprises transfecting the NK cell with nucleic acid encoding the CD16 and/or CD3 ζ gene. In some embodiments, the method comprises transiently, inducibly, or permanently expressing CD16 or CD3 ζ in the NK cell.

[0033] In some embodiments, the method is carried out so that FcR γ chain expression, signaling and/or activity is reduced in the NK cell by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression in the NK cell that is not genetically engineered by the present method. In some embodiments, the FcR γ adaptor protein expression level is not detectable by an immunoblot assay.

[0034] Also provided is a method of producing an engineered NK cell that has reduced surface expression of an NK inhibitory receptor. In some embodiments, any of the provided methods for producing an engineered NK cell that have reduced expression, activity and/or signaling of FcR γ chain can additionally involve reducing surface expression on an NK inhibitory receptor. In some embodiments, such engineered NK cells are produced by a method that includes disrupting a gene encoding a NK inhibitory receptor. In some embodiments, such engineered NK cells are producing by introducing into the NK cell an inhibitory nucleic acid that targets a gene encoding an NK inhibitory receptor. In some embodiments, the inhibitory receptor is NKG2A and/or KIR2DL1. In some embodiments, such methods are carried out so that expression of an NK inhibitory receptor, such as NKG2A and/or KIR2DL1, is reduced in the NK cell by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression of the inhibitory receptor in the NK cell that is not genetically engineered by the present method.

[0035] In some embodiments, the provided methods for producing genetically engineered NK cell can further include a step of expanding the engineered NK cells. In some embodiments, expanding the engineered NK cells involves culturing or incubating the engineered NK cells in the presence of feeder cells or cytokines. In some embodiments, expanding the engineered

NK cells in carried out in vitro. In some embodiments, expanding the engineered NK cells is carried out in vivo.

[0036] Also provided are engineered NK cells produced by any of the above methods.

[0037] Also provided herein are compositions comprising any of the provided engineered NK cells. In one embodiment, the composition comprises a therapeutically effective amount of the engineered NK cells provided herein and a pharmaceutically acceptable carrier. In some embodiments, the carrier is a saline solution, a dextrose solution, or 5% human serum albumin. In some embodiments, the composition comprises between 1×10^5 and 1×10^8 cells/mL.

[0038] In some embodiments, the composition is a cryopreserved composition and/or comprises the engineered NK cells and a cryoprotectant.

[0039] Also provided here are kits comprising an engineered NK cell and an additional agent. In some embodiments, the kits further comprise instructions for use, for example, instructions for administering the engineered NK cell and additional agent for treatment of a disease. In some embodiments, the additional agent is an antibody or an Fc-fusion protein. In some embodiments, the antibody recognizes a tumor associated antigen, a viral antigen, a microbial antigen. In some embodiments, the antibody is selected from the group consisting of an anti-CD20 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-EGFR antibody and an anti-CD38 antibody. In some embodiments, the antibody comprises an Fc domain.

[0040] Also provided herein is a method of treating a condition comprising administering any of the provided engineered NK cells to an individual in need thereof. In some embodiments, the individual is a mammalian subject, such as a human subject. In some embodiments, the subject is one that has a cancer or an infection, such as a viral infection or microbial infection. In some embodiments, the method comprises administering 1×10^8 to 1×10^{10} cells/m² to the individual.

[0041] In some embodiments, the provided methods further include administering an additional agent. In some embodiments, the additional agent is an antibody or an Fc-fusion protein. In some embodiments, the antibody recognizes a tumor associated antigen, a viral

antigen, a microbial antigen. In some embodiments, the antibody is an anti-CD20 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-EGFR antibody or an anti-CD38 antibody. In some embodiments, the antibody comprises an Fc domain.

[0042] In some embodiments, the additional agent and the engineered NK cells are administered sequentially. In some embodiments, the additional agent is administered prior to administration of the engineered NK cells. In some embodiments, the additional agent and the engineered NK cells are administered simultaneously.

[0043] In some embodiments, the method comprises administering NK cells to treat an inflammatory condition, an infection, and/or cancer. In some embodiments, the infection is a viral infection or a bacterial infection. In some embodiments, the cancer is leukemia or lymphoma. In some embodiments, the individual expresses a low affinity Fc γ RIIA. In some embodiments, the individual is a human.

[0044] In some embodiments, the engineered NK cell is allogenic to the subject. In some embodiments, the engineered NK cell is autologous to the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0045] FIG. 1 depicts activity of g-NK cells and conventional NK cells in an antibody dependent cell cytotoxicity (ADCC) assay in the absence or presence of anti-CD20 antibody Rituximab.

DETAILED DESCRIPTION OF THE INVENTION

[0046] Provided herein are engineered Natural Killer (NK) cells that are genetically engineered to have reduced expression, activity and/or signaling of the FcR γ (also known as Fc ϵ RI γ). In some aspects, the provided engineered NK cells are genetically engineered to knockout (e.g. by genetic disruption) or knockdown (e.g. by gene silencing or repression) a gene encoding FcR γ , a gene encoding a protein controlling expression of the FcR γ , such as a transcription factor, or a gene encoding a protein involved in FcR γ -dependent signaling, such as a downstream signaling molecule of FcR γ .

[0047] Natural killer (NK) cells are innate lymphocytes important for mediating anti-viral and anti-cancer immunity through cytokine and chemokine secretion, and through the release of cytotoxic granules (Vivier et al. *Science* 331(6013):44-49 (2011); Caligiuri, *Blood* 112(3):461-469 (2008); Roda et al., *Cancer Res.* 66(1):517-526 (2006)). NK cells are effector cells that comprise the third largest population of lymphocytes and are important for host immuno-surveillance against tumor and pathogen-infected cells.

[0048] However, unlike T and B lymphocytes, NK cells are thought to have only a limited capacity for target recognition using germline-encoded activation receptors (Bottino et al., *Curr Top Microbiol Immunol.* 298:175-182 (2006); Stewart et al., *Curr Top Microbiol Immunol.* 298:1-21 (2006)). Instead, NK cells express the activating Fc receptor CD16, which recognizes IgG-coated target cells, thereby broadening target recognition (Ravetch & Bolland, *Annu Rev Immunol.* 19:275-290 (2001); Lanier *Nat. Immunol.* 9(5):495-502 (2008); Bryceson & Long, *Curr Opin Immunol.* 20(3):344-352 (2008)). In some cases, antibody-dependent cellular cytotoxicity (ADCC) is triggered when receptors on the NK cell surface (such as CD16) recognize IgG1 or IgG3 antibodies bound to the surface of a cell. This triggers release of cytoplasmic granules containing perforin and granzymes, leading to target cell death. ADCC and antibody-dependent cytokine/chemokine production are primarily mediated by NK cells.

[0049] In some cases, ADCC is a mechanism of action of therapeutic antibodies, including anti-cancer antibodies. While significant advances have been made in cancer treatment by use of antibodies directed against cancer antigens, the responsiveness of patients to such antibodies varies. Investigation of such variable responses has typically focused on the direct inhibitory effects of these antibodies on the tumor cells (e.g. inhibition of growth factor receptors and the subsequent induction of apoptosis) and the *in vivo* effects of these antibodies may be more complex and may involve the host immune system, including through ADCC. In some aspects, cell therapy by administering NK cells can be used in concert with antibodies for therapeutic and related purposes.

[0050] Upon activation, NK cells produce cytokines and chemokines abundantly and at the same time exhibit potent cytolytic activity. Activation of NK cells can occur through the direct binding of NK cell receptors to ligands on the target cell, as seen with direct tumor cell

killing, or through the crosslinking of the Fc receptor (CD 16; Fc γ RIII) by binding to the Fc portion of antibodies bound to an antigen-bearing cell. The expression and signal transduction activity of several NK cell activation receptors requires physically associated adaptors, which transduce signals through immunoreceptor tyrosine-based activation motifs (ITAMs). Among these adaptors, FcR γ and CD3 ζ chains can associate with CD16 and natural cytotoxicity receptors (NCRs) as either disulfide-linked homo-dimers or hetero-dimers, and these chains have been thought to be expressed by all mature NK cells.

[0051] In some aspects, CD16 engagement (CD16 crosslinking) initiates NK cell responses via intracellular signals that are generated through one, or both, of the CD16-associated adaptor chains, FcR γ or CD3 ζ . Triggering of CD16 leads to phosphorylation of the γ or ζ chain, which in turn recruits tyrosine kinases, syk and ZAP-70, initiating a cascade of signal transduction leading to rapid and potent effector functions. The most well-known effector function is the release of cytoplasmic granules carrying toxic proteins to kill nearby target cells through the process of antibody-dependent cellular cytotoxicity. CD16 crosslinking also results in the production of cytokines and chemokines that, in turn, activate and orchestrate a series of immune responses.

[0052] This release of cytokines and chemokines can play a role in the anti-cancer activity of NK cells *in vivo*. NK cells also have small granules in their cytoplasm containing perforin and proteases (granzymes). Upon release from the NK cell, perforin forms pores in the cell membrane of targeted cells through which the granzymes and associated molecules can enter, inducing apoptosis. The fact that NK cells induce apoptosis rather than necrosis of target cells is significant—necrosis of a virus-infected cell would release the virions, whereas apoptosis leads to destruction of the virus inside the cells.

[0053] The provided engineered NK cells include cells that are engineered to have lower activity or expression of FcR γ signaling adaptor. In some cases, the engineered cells express the signaling adaptor CD3 ζ -chain abundantly, but are deficient in the expression of the signaling adaptor FcR γ -chain. In some embodiments, compared to NK cells that express the signaling adaptor FcR γ -chain, these engineered NK cells exhibit dramatically enhanced activity when activated by engagement of CD16, such as can occur in the presence of antibodies. For example, the engineered cells can be activated by antibody-mediated

crosslinking of CD16 or by antibody-coated tumor cells. Also provided are methods of producing the engineered cells. The provided engineered NK cells and methods address problems related to selecting or identifying NK cells of a particular phenotype, which may only be present as a small percentage of total NK cells in a subject and/or which may not normally exist in all subjects in a population. In some aspects, the provided embodiments offer an improved NK cell therapy in which NK cells can be engineered with enhanced activities and yet can be more easily obtained in sufficient amounts for therapeutic use, including for administration in concert with antibodies, compared to NK cells of a similar phenotype isolated directly from a subject.

[0054] All references cited herein, including patent applications, patent publications, and scientific literature and databases, are herein incorporated by reference in their entirety for all purposes to the same extent as if each individual reference were specifically and individually indicated to be incorporated by reference.

[0055] For clarity of disclosure, and not by way of limitation, the detailed description is divided into the subsections that follow. The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

I. Definitions

[0056] Unless defined otherwise, all terms of art, notations and other technical and scientific terms or terminology used herein are intended to have the same meaning as is commonly understood by one of ordinary skill in the art to which the claimed subject matter pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art.

[0057] As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a molecule" optionally includes a combination of two or more such molecules, and the like.

[0058] The term “about” as used herein refers to the usual error range for the respective value readily known to the skilled person in this technical field. Reference to “about” a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se.

[0059] It is understood that aspects and embodiments of the invention described herein include “comprising,” “consisting,” and “consisting essentially of” aspects and embodiments.

[0060] As used herein, “optional” or “optionally” means that the subsequently described event or circumstance does or does not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not. For example, an optionally substituted group means that the group is unsubstituted or is substituted.

[0061] As used herein, “antibody” refers to immunoglobulins and immunoglobulin fragments, whether natural or partially or wholly synthetically, such as recombinantly, produced, including any fragment thereof containing at least a portion of the variable heavy chain and/or light chain region of the immunoglobulin molecule that is sufficient to form an antigen binding site and, when assembled, to specifically bind antigen. Hence, an antibody includes any protein having a binding domain that is homologous or substantially homologous to an immunoglobulin antigen-binding domain (antibody combining site). Typically, antibodies minimally include all or at least a portion of the variable heavy (V_H) chain and/or the variable light (V_L) chain. In general, the pairing of a V_H and V_L together form the antigen-binding site, although, in some cases, a single V_H or V_L domain is sufficient for antigen-binding. The antibody also can include all or a portion of the constant region. Reference to an antibody herein includes full-length antibody and antigen-binding fragments. The term “immunoglobulin” (Ig) is used interchangeably with “antibody” herein.

[0062] The terms “full-length antibody,” “intact antibody” or “whole antibody” are used interchangeably to refer to an antibody in its substantially intact form, as opposed to an antibody fragment. A full-length antibody is an antibody typically having two full-length heavy chains (*e.g.*, $VH-CH1-CH2-CH3$ or $VH-CH1-CH2-CH3-CH4$) and two full-length light chains ($VL-CL$) and hinge regions, such as antibodies produced from mammalian species (*e.g.* human, mouse, rat, rabbit, non-human primate, etc.) by antibody secreting B cells and

antibodies with the same domains that are produced synthetically. Specifically whole antibodies include those with heavy and light chains including an Fc region. The constant domains may be native sequence constant domains (e.g., human native sequence constant domains) or amino acid sequence variants thereof. In some cases, the intact antibody may have one or more effector functions.

[0063] An “antibody fragment” comprises a portion of an intact antibody, the antigen binding and/or the variable region of the intact antibody. Antibody fragments, include, but are not limited to, Fab fragments, Fab' fragments, F(ab')₂ fragments, Fv fragments, disulfide-linked Fvs (dsFv), Fd fragments, Fd' fragments; diabodies; linear antibodies (see U.S. Pat. No. 5,641,870, Example 2; Zapata *et al.*, *Protein Eng.* 8(10): 1057-1062 [1995]); single-chain antibody molecules, including single-chain Fvs (scFv) or single-chain Fabs (scFab); antigen-binding fragments of any of the above and multispecific antibodies from from antibody fragments. For purposes herein, an antibody fragment typically includes one that is sufficient to engage or crosslink CD16 on the surface of an NK cell.

[0064] The term “autologous” refers to cells or tissues originating within or taken from an individual’s own tissues. For example, in an autologous transfer or transplantation of NK cells, the donor and recipient are the same person.

[0065] The term “allogeneic” refers to cells or tissues that belong to or are obtained from the same species but that are genetically different, and which, in some cases, are therefore immunologically incompatible. Typically, the term “allogeneic” is used to define cells that are transplanted from a donor to a recipient of the same species.

[0066] The term “expression” refers to the process by which a polynucleotide is transcribed from a DNA template (such as into an mRNA or other RNA transcript) and/or the process by which a transcribed mRNA is subsequently translated into peptide, polypeptides or proteins. Transcripts and encoded polypeptides may be collectively referred to as “gene product.” If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

[0067] The term "heterologous" with reference to a protein or nucleic acid refers to a protein or nucleic acid originating from a different genetic source. For example, a protein or nucleic acid that is heterologous to a cell originates from an organism or individual other than the cell in which it is expressed.

[0068] As used herein, the term "introducing" encompasses a variety of methods of introducing DNA into a cell, either in vitro or in vivo, such methods including transformation, transduction, transfection (e.g. electroporation), and infection. Vectors are useful for introducing DNA encoding molecules into cells. Possible vectors include plasmid vectors and viral vectors. Viral vectors include retroviral vectors, lentiviral vectors, or other vectors such as adenoviral vectors or adeno-associated vectors.

[0069] The term "composition" refers to any mixture of two or more products, substances, or compounds, including cells or antibodies. It may be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous or any combination thereof. The preparation is generally in such form as to permit the biological activity of the active ingredient (e.g. antibody) to be effective.

[0070] A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable carrier includes, but is not limited to, a buffer, excipient, stabilizer, or preservative.

[0071] As used herein, combination refers to any association between or among two or more items. The combination can be two or more separate items, such as two compositions or two collections, can be a mixture thereof, such as a single mixture of the two or more items, or any variation thereof. The elements of a combination are generally functionally associated or related.

[0072] As used herein, a kit is a packaged combination that optionally includes other elements, such as additional agents and instructions for use of the combination or elements thereof, for a purpose including, but not limited to, therapeutic uses.

[0073] As used herein, the term “treatment” or “treating” refers to clinical intervention designed to alter the natural course of the individual or cell being treated during the course of clinical pathology. Desirable effects of treatment include decreasing the rate of disease progression, ameliorating or palliating the disease state, and remission or improved prognosis. An individual is successfully “treated”, for example, if one or more symptoms associated with a disorder (*e.g.*, an eosinophil-mediated disease) are mitigated or eliminated. For example, an individual is successfully “treated” if treatment results in increasing the quality of life of those suffering from a disease, decreasing the dose of other medications required for treating the disease, reducing the frequency of recurrence of the disease, lessening severity of the disease, delaying the development or progression of the disease, and/or prolonging survival of individuals.

[0074] An “effective amount” refers to at least an amount effective, at dosages and for periods of time necessary, to achieve the desired or indicated effect, including a therapeutic or prophylactic result. An effective amount can be provided in one or more administrations. A “therapeutically effective amount” is at least the minimum dose of cells required to effect a measurable improvement of a particular disorder. A therapeutically effective amount herein may vary according to factors such as the disease state, age, sex, and weight of the patient. A therapeutically effective amount may also be one in which any toxic or detrimental effects of the antibody are outweighed by the therapeutically beneficial effects. A “prophylactically effective amount” refers to an amount effective, at the dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at the earlier stage of disease, the prophylactically effective amount can be less than the therapeutically effective amount.

[0075] As used herein, an “individual” or a “subject” is a mammal. A “mammal” for purposes of treatment includes humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, rabbits, cattle, pigs, hamsters, gerbils, mice, ferrets, rats, cats, *etc.* In some embodiments, the individual or subject is human.

II. Engineered NK Cells

[0076] Provided herein is an engineered NK cell that is genetically engineered to reduce FcR γ chain expression, activity and/or signaling in the cell. Also provided are methods for engineering the cells. In some embodiments, the methods include introducing a genetic disruption or inhibitory nucleic acid that disrupts a gene or represses expression of a gene resulting in reduced expression, activity and/or signaling of FcR γ chain in the NK cell.

[0077] In some embodiments, the engineered NK cell is genetically engineered to directly reduce or eliminate expression or activity of FcR γ chain. In some embodiments, the engineered NK cell is genetically engineered to indirectly reduce or eliminate expression or activity of FcR γ chain, such as by reducing or eliminating expression of a protein that regulates expression or activity of FcR γ , for example a transcription factor that controls expression of FcR γ . In some embodiments, the engineered NK cell is genetically engineered to reduce or eliminate expression or activity of a molecule involved in the downstream signaling of FcR γ . In some aspects, the engineered cell retains or is additionally engineered to transduce signals through CD3 ζ , such as upon engagement or crosslinking of CD16.

Targets for Engineering

[0078] In some embodiments the NK cell is genetically engineered to reduce or eliminate expression or activity of human FcR γ chain protein. In some embodiments, the engineered NK cell comprises a genetic disruption of a gene encoding FcR γ chain or of a gene encoding a protein that regulates expression or activity of FcR γ chain protein. In some embodiments, the genetic disruption results in an insertion, deletion or mutation in the gene, such as a frameshift mutations and/or premature stop codons within the open reading frame. In some embodiments, one allele is disrupted. In some embodiments, both alleles are disrupted. In some embodiments the engineered NK cell comprises an inhibitory nucleic acid, such as an siRNA or other inhibitory nucleic acid molecule, that reduces expression of a gene encoding FcR γ chain or of a gene encoding a protein that regulates expression or activity of FcR γ chain protein.

[0079] An amino acid sequence for FcR γ chain (*Homo sapiens*, also called the High affinity immunoglobulin gamma Fc receptor D) is available in the NCBI database as accession number NP_004097.1 (GI:4758344), and is reproduced below as SEQ ID NO:1.

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1 MIPAVVLLLL LLVEQAAALG EPQLCYILDA ILFLYGIVLT
41 LLYCRLKIQV RKAAITSYEK SDGVYTGLST RNQETYETLK
81 HEKPPQ
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[0080] The NCBI database genomic reference sequence for FcR γ chain is NG_029043.1 RefSeqGene. The mRNA reference sequence is U004106.1.

[0081] The engineered NK cell may contain a mutation, disruption, or deletion in the gene encoding FcR γ signaling adaptor, for example an inactivating mutation in an exon of the FcR γ chain gene. The mutation, disruption, or deletion can also be in a regulatory element of the FcR γ chain gene, for example, in the promoter.

[0082] Various inactivating mutations or deletions can also be used to decrease expression of FcR γ chain. For example, a mutation in the immunoreceptor tyrosine-based activation motif (ITAM) may be used. In some embodiments, the engineered NK cells may comprise an inactivating mutation in the ITAM motif. In other embodiments, the transmembrane region of FcR γ chain may be mutated to inactivate the protein.

[0083] In some embodiments, it is also possible to decrease FcR γ chain expression or activity, indirectly by increasing or decreasing expression of a protein that regulates FcR γ chain. In some embodiments, the engineered NK cell is genetically engineered to reduce or eliminate expression of a protein that regulates expression or activity of FcR γ chain, such as a transcription factor, e.g. PLZF or HELIOS. In some embodiments, the engineered NK cell comprises a genetic disruption of a gene encoding a protein that regulates expression or activity of FcR γ chain protein. In some embodiments, the genetic disruption results in an insertion, deletion or mutation in the gene, such as a frameshift mutations and/or premature stop codons within the open reading frame. In some embodiments, one allele is disrupted. In some embodiments, both alleles are disrupted. In some embodiments the engineered NK cell

comprises an inhibitory nucleic acid that reduces expression of a gene encoding a protein that regulates expression or activity of FcR γ chain protein, such as a transcription factor, e.g. PLZF or HELIOS.

[0084] In some embodiments, the engineered NK cell may contain a genetic disruption, deletion, or mutation in a gene that promotes transcription of FcR γ signaling adaptor. In some embodiments, the engineered NK cell may have a genetic disruption, mutation, or deletion in a gene encoding a transcription factor that promotes transcription of the FcR γ chain. For example, the engineered NK cell may have a genetic disruption, in a gene encoding the PLZF or HELIOS transcription factor. In some embodiments, one allele of PLZF and/or HELIOS is disrupted. In some embodiments, both alleles of PLZF or both alleles of HELIOS are disrupted. In some embodiments, the engineered NK cell contains an inhibitory nucleic acid molecule, such as an siRNA or other inhibitory nucleic acid molecule, that reduces expression of a gene that promotes transcription of FcR γ signaling adaptor. In some embodiments, the engineered NK cell may have an inhibitory nucleic acid molecule that targets a transcription factor that promotes transcription of the FcR γ chain, such as an inhibitory nucleic acid molecule that targets a gene encoding the PLZF or HELIOS transcription factor.

[0085] The amino acid sequence of PLZF is available in the NCBI database as accession number NP_001018011.1, and is reproduced below as SEQ ID NO:3. The NCBI database genomic reference sequence is NG_012140.1. The mRNA reference sequence is NM_001018011.1.

MDLTKMGMIQLQNPSHPTGLLCKANQMRLAGTLCDDVIMVDSQEFHA
 HRTVLACTSKMFEILFHRNSQHYTELDFLSPKTFQQILEYAYTATLQAKA
 EDLDDLLYAAEILEIEYLEEQCLKMLETIQASDDNDTEATMADGGAAE
 EEDRKARYLKNIFISKHSSEESGYASVAGQSLPGPMVDQSPSVSTSFGLS
 AMSPTKAAVDSLMTIGQSLLQGTLLQPPAGPEEPTLAGGGRHPGVAEVK
 TEMMQVDEVPSQDSPGAAESSISGGMGDKVEERGKEGPGTPTRSSVITS
 ARELHYGREESAQVPPPAEAGQAPTGRPEHPAPPPEKHLGIYSVLPNH
 KADAVLSMPSSVTSGLVHQPALAVSMDFSTYGGLLPQGGFIQRELFKSL
 GELAVGMKSESRTIGEQCVCVVELPDNEAVEQHRKLLHSGMKTYGCE

LCGKRFLDSLRLRMHLLAHSAGAKAFVCDQCGAQFSKEDALETHRQT
 HTGTDMAVFCLLCGKRFQAQSALQQHMEVHAGVRSYICSECNRTFPS
 HTALKRHLRSHTGDHPYECEFCGSCFRDESTLKSHKRIHTGEKPYECNG
 CGKKFSLKHQLETHYRVHTGEKPFECKLCHQRSRDYSAMIKHLRTHN
 GASPYQCTICTEYCPSLSSMQKHMKGHKPEEIPPDWRIEKTYLYLCYV

[0086] The amino acid sequence of HELIOS (IKZF2) is available in the NCBI database as accession number NP_057344.2 (isoform 1) and NP_001072994.1 (isoform 2) The NCBI gene identifier is NC_000002.12. The mRNA reference sequence is NM_016260.2 (isoform 1) NM_001079526.1 (isoform 2). The gene ID is 22807.

[0087] In some embodiments, the engineered NK cell is genetically engineered to reduce or eliminate a protein involved in FcR γ chain –dependent signaling. In some embodiments, the engineered NK cell comprises a reduction in FcR γ chain –dependent signaling. For example, the engineered NK cell may have reduced expression of downstream molecules that is involved in FcR γ chain –dependent signaling. For example, Lee and Schlums describe members of the FcR γ signaling pathway that can be suitable targets for modification and/or deletion (Lee et al., Immunity 42:431-42 (2015); Schlums et al. Immunity, 42:443-56 (2015)). In some of these embodiments, the downstream molecules comprise SYK, DAB2 or EAT2. The gene ID for SYK is 6850. The NCBI gene ID for DAB2 is 1601. The genomic reference sequence for DAB2 is NG_030312.1. The mRNA and protein sequences for isoform 2 of DAB2 are 001244871.1 and NP_001231800.1. The mRNA and protein sequences for isoform 1 of DAB2 are 2.NM_001343.3 and NP_001334.2. The NCBI gene ID for EAT2 is 175072.

[0088] In some embodiments, the engineered NK cell may contain a have a genetic disruption, mutation, or deletion in a gene encoding a protein involved in FcR γ chain –dependent signaling. For example, the engineered NK cell may have a genetic disruption, in a gene encoding the SYK, DAB2 and/or EAT2. In some embodiments, one allele of SYK, DAB2 and/or EAT2 is disrupted. In some embodiments, both alleles of SYK, both alleles of DAB2 and/or both alleles of EAT2 are disrupted. In some embodiments, the engineered NK cell contains an inhibitory nucleic acid molecule, such as an siRNA or other inhibitory nucleic acid molecule, that reduces expression of a gene that is involved in FcR γ chain –dependent

signaling. In some embodiments, the engineered NK cell may have an inhibitory nucleic acid molecule that targets a signaling molecule, such as an inhibitory nucleic acid molecule that targets a gene encoding SYK, DAB2 and/or EAT2.

[0089] Also provided are engineered NK cells that are genetically engineered to reduce or eliminate expression of an NK inhibitory receptor. In some aspects, any of the provided engineered that are engineered to have decreased expression, signaling, and/or activity of Fc γ chain as described above additionally can be engineered to reduce or eliminate expression of an NK inhibitory receptor. In some cases, the engineered cell contains a disruption of a gene encoding the NK inhibitory receptor. In some embodiments, the engineered cell contains an inhibitory nucleic acid molecule, such as an siRNA or other inhibitory nucleic acid molecule, that reduces expression of a gene encoding the NK inhibitory receptor. Inhibitory NK receptors are known in the art and include NKG2A (also known as KLRC1; NCBI gene ID 3821) and KIR2DL1 (NCBI gene ID 3802). The NK cell may be engineered to decrease expression of the inhibitory NK receptor using any of the methods provided herein, for example using an interfering RNA or genetic disruption, including a gene insertion, mutation, or deletion that produces a stop codon or frameshift.

[0090] In the above embodiments, such engineered NK cells include those that comprise genetic disruptions produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce non-conservative amino acid substitutions, deletions or additions. In some embodiments, the genetic disruption comprises an insertion, deletion, or mutation that results in a premature stop codon in the gene or a frameshift of the open reading frame of the gene.

[0091] In some embodiments, the expression of a particular gene product, such as any described above, in the engineered cell is reduced by greater than about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95 %, 98% or 99% as compared to the expression of the gene product in the NK cell that is not genetically engineered. In some embodiments, the expression of a particular gene product in the engineered cell is reduced to a level that is undetectable. In some embodiments, the expression of a particular gene product in the engineered cell is completely eliminated. In some of these embodiments, the gene is

FcR γ chain. In other embodiments, the gene may encode a protein that regulates FcR γ chain, such as a transcription factor that promotes expression of FcR γ chain, such as PLZF or HELIOS. In other embodiments, the gene may encode a protein that is involved in FcR γ chain-mediated signaling, such as a such as downstream signaling molecules SYK, DAB2 or EAT2.

[0092] In some embodiments, the expression, activity and/or signaling of FcR γ chain in the provided engineered NK cells is reduced by greater than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95 %, 98% or 99% as compared to the expression of the gene in the NK cell that is not genetically engineered. In some of these embodiments, the level of FcR γ chain expression is reduced to an undetectable level using an immunoblot assay.

[0093] In some embodiments, the engineered NK cell may express CD16 (also referred to as CD16A or Fc γ RIIIa). Thus, reference to CD16 herein is not meant to refer to the glycosylphosphatidylinositol-anchored form (Fc γ RIIIB or CD16B). In some of these embodiments, the CD16 is human CD16. In humans or as a glycosylphosphatidylinositol-anchored form (Fc γ RIIIB or CD16B). Typically, the provided engineered cells express a polypeptide-anchored CD16 form that is able to associated with the ζ chain of the TCR-CD3 complex. CD16 binds to antibody Fc regions and initiates ADCC. In some embodiments, the expression of other Fc receptor proteins is maintained in the engineered NK cell.

[0094] The genomic sequence for CD16A is available in the NCBI database at NG_009066.1. The gene ID for CD16A is 2214. Sequence information for CD16, including gene polymorphisms, is available at UniProt Acc. No. P08637. Nucleic acid and protein sequences for CD16a are publicly available. For example, GenBank Accession Nos. NM_000569 (SEQ ID NO: 1), NM_001127596, NM_001127595, NM_001127593, and NM_001127592 disclose exemplary human CD16a nucleic acid sequences, and GenBank Accession Nos. NP_000560 (SEQ ID NO: 2), NP_001121068, NP_001121067, NP_00112065, and NP_001121064 disclose exemplary human CD16a protein sequences. One of ordinary skill in the art can identify additional CD16a nucleic acid and amino acid sequences that vary from those provided herein, but that retain at least one activity of CD16a, such as Fc binding activity.

[0095] CD16 is most commonly found in a form that has a relatively low binding affinity for the Fc portion of IgG molecules. An alternative form that exhibits a higher binding affinity is found in some individuals. The low and high affinity forms of CD16 differ only by the substitution of valine (high affinity) for phenylalanine (low affinity) at position 158 in the mature (processed) form of the polypeptide chain. The sequence of CD16 (158F) is set forth in SEQ ID NO:4 (residue 158F is bold and underlined). In some embodiments, CD16 (158F) further comprises a signal peptide set forth as MWQLLLPTALLLLVSA (SEQ ID NO:5).

GMRTEDLPKAVVFLEPQWYRVLEKDSVTLKQCQAYSPEDNSTQWFHNESLIS
SQASSYFIDAATVDDSGEYRCQTNLSTLSDPVQLEVHIGWLLLQAPRWVFKEE
DPIHLRCHSWKNTALHKVTYLQNGKGRKYFHHNSDFYIPKATLKDSGSYFCR
GL**F**GSKNVSSETVNITITQGLAVSTISSFFPPGYQVSFCLVMVLLFAVDTGLYFS
VKTNIRSSTRDWKDHKFKWRKDPQDK (SEQ ID NO:4)

[0096] The sequence of CD16 158V (polymorphism resulting in F158V) is known as VAR_003960 and has the sequence set forth in SEQ ID NO:6 (158V polymorphism is in bold and underline). In some embodiments, CD16 (158V) further comprises a signal peptide set forth as MWQLLLPTALLLLVSA (SEQ ID NO:5).

GMRTEDLPKAVVFLEPQWYRVLEKDSVTLKQCQAYSPEDNSTQWFHNESLISSQ
ASSYFIDAATVDDSGEYRCQTNLSTLSDPVQLEVHIGWLLLQAPRWVFKEEDPIHL
RCHSWKNTALHKVTYLQNGKGRKYFHHNSDFYIPKATLKDSGSYFCRGL**V**GSKN
VSSETVNITITQGLAVSTISSFFPPGYQVSFCLVMVLLFAVDTGLYFSVKTNIRSSTR
DWKDHKFKWRKDPQDK (SEQ ID NO:6)

[0097] In some embodiments, the engineered NK cell may comprise a CD16 gene that comprises an activating mutation. In some of embodiments, the mutation results in higher affinity to an IgG region. In some of these embodiments, the CD16 mutation results in higher affinity of CD16 for IgG1, IgG2, or IgG4. In some embodiments, the CD16 mutation results in higher affinity of CD16 for IgG1. In some embodiments, the CD16 contains the 158V mutation. In some embodiments, the CD16 has the sequence of amino acids set forth in SEQ

ID NO:6 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:6 and that contains the 158V polymorphism. In some embodiments, the mutation in CD16 is a 158F mutation. In some embodiments, the CD16 has the sequence of amino acids set forth in SEQ ID NO:4 or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:4 and that contains the 158F polymorphism.

[0098] In some aspects, the engineered NK cells can be engineered to express a recombinant or heterologous CD16. In some embodiments, even if the NK cells may express CD16, the NK cells may be engineered to further express a higher level of CD16 and/or express a modified form of CD16 than is expressed by the NK in the absence of the genetic engineering. In such examples, the cells can be engineered to contain nucleic acid comprising a strong or constitutive promotor followed by a CD16 gene.

[0099] The engineered NK cell may also express other signaling adaptor molecules. For example, signaling molecules with an ITAM domain other than FcR γ . In some embodiments, the engineered NK cell expresses CD3 ζ adaptor chain. In some of these embodiments, CD3 ζ is human CD3 ζ .

[0100] An amino acid sequence for the human CD3 ζ (*Homo sapiens*) is available in the NCBI database as accession number ABQ28690.1 (GI:146399947), and is reproduced below as SEQ ID NO:2.

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1  AILQAQLPIT  EAQSFGLLDP  KLCYLLDGIL  FIYGVILTAL
41 FLRVKFSRSA  DAPAYQQGQN  QLYNELNLGR  REEYDVLDKR
81 RG

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[0101] The NCBI gene ID for CD3 ζ (CD247) is 919.

[0102] In some aspects, the engineered NK cells can be engineered to express a recombinant or heterologous CD3 ζ . In some embodiments, even if the NK cells may express CD3 ζ , the NK cells may be engineered to further express a higher level of CD3 ζ and/or express a modified

form of CD3 ζ than is expressed by the NK in the absence of the genetic engineering. In such examples, the cells can be engineered to contain nucleic acid comprising a strong or constitutive promoter followed by a CD3 ζ gene. In some embodiments, the engineered cells may comprise a CD3 ζ gene with an activating mutation. In other embodiments, the engineered NK cells may express CD3 ζ at a higher level after engineering.

[0103] In some embodiments, the engineered NK cell may have reduced expression of NK cell surface receptors, including natural cytotoxicity receptors. The engineered NK cell may have reduced expression of NKp46, NKp30, and/or NKp44 compared to the NK cell without the modification. In some of these embodiments, the expression of the cell surface receptor is reduced by greater than 1.2 fold, 1.5 fold, 2 fold, 3 fold, 4 fold, 10 fold, 100 fold or 1000 fold. In some of these embodiments the NK cell surface receptor may not be detectable in the engineered NK cell. In other embodiments, the NK cell may be negative for NK cell surface receptors, such as NKp46, NKp30, and/or NKp44.

Cell Types

[0104] One of skill in the art will appreciate that both primary cells obtained from human tissue and existing cell lines are suitable for engineering. For example, in some embodiments, the engineered NK cell may be derived from a primary cell obtained from a subject, such as a human.

Primary Cells

[0105] Primary cells can be engineered using any of the methods provided herein. According to some embodiments the population of cells comprising said NK cells is obtained from a sample from a mammalian subject, such as a human subject. The sample or source can be cord blood, bone marrow or peripheral blood.

[0106] In some aspects, Natural killer cells expressing one or more natural killer cell-specific markers are isolated from the cell population. Methods of isolating and identifying NK cells are well known in the art, and include those discussed in Dahlberg et al, *Frontiers in Immunology*, vol. 6 article 605 pp. 1-18 (2015).

[0107] Techniques for the in vitro isolation and large-scale expansion of NK cells are known. An exemplary procedure is described in US Pat. App. Publ. No. 2014/0086890, incorporated herein by reference in its entirety. One of ordinary skill in the art can identify additional methods for expanding NK cells, for example as described in Childs et al., Hematol. The Education Program 2013:234-246, 2013, incorporated herein by reference in its entirety.

[0108] In some embodiments, mononuclear cells are collected from a subject (such as a donor subject or a subject with a tumor or hyperproliferative disease). In some examples, mononuclear cells are collected by an apheresis procedure. The mononuclear cells are enriched for NK cells, for example by negative depletion using an immuno-magnetic bead strategy. In some examples, NK cells are enriched by depleting the mononuclear cell sample of T cells, B cells, monocytes, dendritic cells, platelets, macrophages, and erythrocytes utilizing a mixture of biotinylated monoclonal antibodies. The non- NK cells in the sample are removed with magnetic beads coupled to streptavidin, resulting in an enriched preparation of NK cells. An exemplary commercially available kit for this method is Dynabeads® Untouched™ Human NK Cells kit (Thermo Fisher Scientific, Waltham, MA). In another example, NK cells are enriched by positive selection of CD56⁺ NK cells, for example utilizing magnetic beads conjugated to an anti-CD56 antibody (such as CD56 MicroBeads, Miltenyi Biotec, Inc., Auburn, CA). In other examples, a two-step method including negative depletion (such as T cell depletion) followed by positive selection of CD56⁺ NK cells is used for enriching NK cells.

[0109] In some embodiments, Natural killer cells may be identified as those expressing typical human natural killer cell markers such as KIR, NKG2A, NKG2D, NKp30, NKp44, NKp46, CD56, and CD161. For studies involving mice, natural killer cells can be identified and/or isolated using typical mouse markers such as NK1.1, CD 122, LY49 Family (Ly49A, Ly49C, Ly49D, Ly49E, Ly49F, Ly49G, Ly49H, and Ly49I), or NKG2A/C/E. In some embodiments, cell staining, or FACS may be used to identify cells that express a certain marker.

[0110] In some embodiments, NK cells can be selectively enriched using either positive or negative selection. For example, NK cells that do not express CD3 can be selected by exposing a mixture of cells to an immobilized anti-CD3 antibody and removing the unbound cells. According to some embodiments of the present invention the NK cells comprise CD56⁺CD3⁻

cells. According to some embodiments of the present invention the NK cells comprise CD56+CD16+CD3⁻ cells.

[0111] In one embodiment, cytokines can be administered to a subject prior to isolating primary NK cells. For example, IL-12, IL-15, IL-18, IL-2, and/or CCL5 can be administered to a subject prior to isolating the primary NK cells.

[0112] It may also be beneficial to enrich the isolated NK primary cells for those that express CD16 and CD3 ζ . One of skill in the art will appreciate that many methods exist for selectively enriching cells expressing certain markers from population exist, such as fluorescent cell sorting, or selective depletion of cells expressing certain markers using antibodies bound to a solid phase.

[0113] In some embodiments, the methods can be carried out under or adapted for Current Good Manufacturing Practice (cGMP). One of ordinary skill in the art can identify other methods that can be used to prepare an enriched population of NK cells.

[0114] In some embodiments, enriched NK cells (typically >99% CD3 negative and >85% CD56+) are expanded in vitro prior to or after genetically engineering the cells.

[0115] In one non-limiting example, the enriched NK cells are cultured with an irradiated EBV-LCL feeder cell line (SMI-LCL) in X-VIVO™ 20 medium (Lonza, Basel, Switzerland) with 10% human AB serum and 500 IU/ml of interleukin-2 (IL-2), for up to 21 days. Utilizing this technique, expansions of NK cells in the range of 200- to 1000-fold may be achieved (expanded NK cells are typically >99% CD3 negative and >90% CD56+). In some examples, the starting population of enriched NK cells is about 0.8-1.6 x 10⁸ total NK cells, which over a 2-4 week period expand up to 1000-fold or greater in vitro. Similar numbers of NK cells have been expanded in scaled up experiments using GMP conditions. In some examples, NK cells are expanded in G-Rex® containers (Wilson Wolf, New Brighton, MN). The G-Rex®100 container support NK expansions to doses of 2.5 x 10⁸ NK cells/kg or higher. NK cells cultured in G-Rex®100 containers could be cultured at concentrations up to 4 x 10⁶ NK cells/ml.

[0116] In some embodiments, bulk NK cells or NK cells subsets isolated by additional enriching procedures, such as through the use of immune-magnetic beads or flow sorting, may be grown in

cell culture medium, e.g., Cellgro SCGM serum-free media (CellGenix, Gaithersburg, MD) containing 10% human AB serum, 50 U /mL penicillin, 50 µg/mL streptomycin, and 500 IU/mL IL-2 or in X-VIVO™ 20 media containing 10% heat inactivated human AB serum or 10% autologous serum.

[0117] Non-expanded and expanded NK cells can be analyzed by flow cytometry for the expression of markers such as CD56, CD16, TRAIL, FasL, NKG2D, LFA-1, perforin, and granzymes A and B. In some examples, expression of one or more of the markers is measured at baseline and >10 days following in vitro expansion. Chromium release assays can be used to assess fresh vs. expanded NK cell cytotoxicity against cancer cell targets. One of ordinary skill in the art can identify other methods to assess the NK cell population (for example, purity), viability, and/or activity.

[0118] In some embodiments, expand primary cells derived from a subject may be expanded and/or cultured before genetic engineering. In some embodiments the engineered primary cells are cultured and/or expanded following engineering and prior to administration to a patient.

NK Cell Line

[0119] In some embodiments, NK cell lines can be engineered as described herein. In some embodiments, the engineering NK cells include engineered NK cell lines. In some aspects, engineered cell lines allow production of higher amounts of cells without having to expand small numbers of NK cells that are derived from a subject. Engineered cell lines also have the advantage of being well characterized.

[0120] In some embodiments, the cell line is a clonal cell line. In some embodiments, the cell line is derived from a patient with NK-cell leukemia or lymphoma. In some embodiments, the NK cell line comprises NK-92, NK-YS, KHYG-1, NKL, NKG, SNK-6, or IMC-1.

[0121] The NK-92 is a NK-like cell line that was initially isolated from the blood of a subject suffering from a large granular lymphoma and subsequently propagated in cell culture. The NK-92 cell line has been described (Gong et al., 1994; Klingemann, 2002). NK-92 cells have a CD3⁻/CD56⁺ phenotype that is characteristic of NK cells. They express all of the known NK cell-activating receptors except CD16, but lack all of the known NK cell inhibitory receptors

except NKG2A/CD94 and ILT2/LIR1, which are expressed at low levels. Furthermore, NK-92 is a clonal cell line that, unlike the polyclonal NK cells isolated from blood, expresses these receptors in a consistent manner with respect to both type and cell surface concentration. Similarly, NK-92 cells are not immunogenic and do not elicit an immune rejection response when administered therapeutically to a human subject. Indeed NK-92 cells are well tolerated in humans with no known detrimental effects on normal tissues.

[0122] Some existing cell lines, such as NK-92 do not naturally express CD16. In some embodiments, the method provided herein includes engineering a NK cell line, such as NK-92, to express CD16. Klingemann et al., also discuss the advantages of the NK92 cell lines. Klingemann et al, *Frontiers in Immunology*, vol 7, article 91 pp 1-7 (2016) In some embodiments, the methods provided herein include engineering a cell line to express CD3 ζ . In some embodiments CD16 and/or CD3 ζ can be expressed inducibly or transiently. In such embodiments, the cell line is not engineered to heterologously or recombinantly express FcR γ chain or is engineered to reduce or eliminate expression of FcR γ chain in the cell.

Methods of Producing Engineered NK Cells

[0123] Provided herein are methods of producing an engineered NK cell that is genetically engineered to reduce FcR γ chain expression, activity and/or signaling in the cell as described. For example, the method may comprise introducing a genetic disruption of a gene encoding FcR γ chain, a gene encoding a protein that regulates expression or activity of FcR γ signaling adaptor (e.g. a transcription factor, such as PLZF or HELIOS) and/or a gene encoding a protein that is involved in FcR γ -mediated signaling (e.g. a downstream signaling molecule, such as SYK, DAP2 or EAT2) as described. In some embodiments, the method may comprise introducing an inhibitory nucleic acid molecule that targets a gene encoding FcR γ chain, a gene encoding a protein that regulates expression or activity of FcR γ signaling adaptor (e.g. a transcription factor, such as PLZF or HELIOS) and/or a gene encoding a protein that is involved in FcR γ -mediated signaling (e.g. a downstream signaling molecule, such as SYK, DAP2 or EAT2) as described.

[0124] In some embodiments, the method provided herein comprises isolating an NK cell from a subject, such as by the methods as described above or known to a skilled artisan, and reducing the expression of FcR γ chain expression, activity and/or signaling in the cell in accord with the provided methods. In some embodiments, the method provided herein comprises obtaining an NK cell line, such as any described herein, and engineering the cell to reduce expression of FcR γ expression, activity and/or signaling in the cell in accord with the provided methods.

[0125] One of ordinary skill in the art will appreciate that there are many ways of decreasing the expression or activity of FcR γ . For example, the level of transcription can be decreased. One method of decreasing gene expression, such as FcR γ chain expression, involves modifying an endogenous gene to decrease transcription. For example, the FcR γ chain gene may be deleted, disrupted, or mutated. In addition to targeting the FcR γ RNA, mutating, or modifying the FcR γ gene, FcR γ protein level can be decreased by effecting a molecule that increases FcR γ gene expression or activity, such as a transcription factor that regulates transcription of FcR γ . In some embodiments a gene that regulates transcription or translation of the FcR γ chain gene may be deleted, disrupted, or mutated. In some of these embodiments, the gene is a transcription factor that regulates expression of the FcR γ chain gene. Specifically, inhibition of a transcription factor that positively regulates FcR γ expression will result in decreased FcR γ expression. Transcription factors that regulate FcR γ transcription include HELIOS and PLZF.

[0126] One of ordinary skill in the art will understand that there are many suitable methods for disrupting FcR γ chain gene or other gene, such as those described herein. For example, the entire gene locus, such as FcR γ locus, may be deleted. In some cases, it is also suitable to delete a portion of the gene, for example an exon, or a domain. Specifically, the ITAM signaling domain of FcR γ may be deleted. Alternatively, the provided methods also include introducing one or more amino acid substitutions into the gene locus, such as FcR γ locus, such as an inactivating mutation. In some embodiments, a stop codon can be introduced into the mRNA, such as FcR γ mRNA, to produce a truncated and/or inactivated form of the expressed gene, such as FcR γ signaling adaptor. In some embodiments, regulatory elements of the gene, such as FcR γ gene, can also be mutated or deleted in order to reduce expression, activity and/or signaling of FcR γ signaling adaptor.

[0127] In some embodiments, gene disruption can be carried out in mammalian cells using site-specific endonucleases. Endonucleases that allow for site-specific deletion of a gene are well known in the art and may include TAL nucleases, meganucleases, zinc-finger nucleases, Cas9, and Argonaute. Methods for producing engineered, site-specific endonucleases are known in the art. The site-specific endonuclease can be engineered to recognize and delete or modify a specific gene, such as the FcR γ chain gene.

[0128] In one embodiment, zinc-finger nucleases (ZFNs) can be engineered to recognize and cut predetermined sites in a genome. ZFNs are chimeric proteins comprising a zinc finger DNA-binding domain fused to the nuclease domain of the FokI restriction enzyme. The zinc finger domain can be redesigned through rational or experimental means to produce a protein which binds to a pre-determined DNA sequence, about or approximately 18 basepairs in length. By fusing this engineered protein domain to the FokI nuclease, it is possible to target DNA breaks with genome-level specificity. ZFNs have been used extensively to target gene addition, removal, and substitution in a wide range of eukaryotic organisms (reviewed in S. Durai et al., *Nucleic Acids Res* 33, 5978 (2005)).

[0129] In other embodiments, TAL-effector nucleases (TALENs) can be generated to cleave specific sites in genomic DNA. Like a ZFN, a TALEN comprises an engineered, site-specific DNA-binding domain fused to the FokI nuclease domain (reviewed in Mak, et al. (2013) *Curr Opin Struct Biol.* 23:93-9). In this case, however, the DNA binding domain comprises a tandem array of TAL-effector domains, each of which specifically recognizes a single DNA basepair. Because ZFNs and TALENs are heterodimeric so that the production of a single functional nuclease in a cell requires co-expression of two protein monomers, compact TALENs provide an alternative endonuclease architecture that avoids the need for dimerization (Beurdeley, et al. (2013) *Nat Commun.* 4: 1762). A Compact TALEN comprises an engineered, site-specific TAL-effector DNA-binding domain fused to the nuclease domain from the I-TevI homing endonuclease. Unlike FokI, I-TevI does not need to dimerize to produce a double-strand DNA break so a Compact TALEN is functional as a monomer.

[0130] In some embodiments, engineered endonucleases based on the CRISPR/Cas9 system are also known in the art and can be employed in the provided methods to engineer the cells (Ran, et al. (2013) *Nat Protoc.* 8:2281-2308; Mali et al. (2013) *Nat Methods.* 10:957- 63). A CRISPR

endonuclease comprises two components: (1) a caspase effector nuclease, typically microbial Cas9; and (2) a short "guide RNA" that directs the nuclease to a location of interest in the genome. In some embodiments, the guide RNA comprises an approximately 20 nucleotide targeting sequence. By expressing multiple guide RNAs in the same cell, each having a different targeting sequence, it is possible to target DNA breaks simultaneously to multiple sites in the genome. Methods of using CRISPR-Cas9 are well known in the art.

[0131] In some aspects, the guide sequence is any polynucleotide sequence comprising at least a sequence portion that has sufficient complementarity with a target polynucleotide sequence, such as a gene encoding FcR γ , PLZF, HELIOS, SYK, DAB2 or EAT2, to hybridize with the target sequence and direct sequence-specific binding of the CRISPR complex to the target sequence. Typically, in the context of formation of a CRISPR complex, "target sequence" generally refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between the target sequence and a guide sequence promotes the formation of a CRISPR complex. Full complementarity is not necessarily required, provided there is sufficient complementarity to cause hybridization and promote formation of a CRISPR complex. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. In some embodiments, a guide sequence is selected to reduce the degree of secondary structure within the guide sequence. Secondary structure may be determined by any suitable polynucleotide folding algorithm.

[0132] In some embodiments, a CRISPR enzyme (e.g. Cas9 nuclease) in combination with (and optionally complexed with) a guide sequence is delivered to the cell. In some embodiments, one or more elements of a CRISPR system is derived from a type I, type II, or type III CRISPR system. In some embodiments, one or more elements of a CRISPR system are derived from a particular organism comprising an endogenous CRISPR system, such as *Streptococcus pyogenes* or *Staphylococcus aureus*.

[0133] In the one embodiment of the invention, the DNA break-inducing agent is an engineered homing endonuclease (also called a "meganuclease"). Homing endonucleases are a group of naturally-occurring nucleases which recognize 15-40 base-pair cleavage sites commonly found

in the genomes of plants and fungi. They are frequently associated with parasitic DNA elements, such as group 1 self-splicing introns and inteins. They naturally promote homologous recombination or gene insertion at specific locations in the host genome by producing a double-stranded break in the chromosome, which recruits the cellular DNA- repair machinery (Stoddard (2006), *Q. Rev. Biophys.* 38: 49-95). Homing endonucleases are commonly grouped into four families: the LAGLIDADG family, the GIY-YIG family, the His-Cys box family and the HNH family. These families are characterized by structural motifs, which affect catalytic activity and recognition sequence. For instance, members of the LAGLIDADG family are characterized by having either one or two copies of the conserved LAGLIDADG motif (see Chevalier et al. (2001), *Nucleic Acids Res.* 29(18): 3757- 3774). The LAGLIDADG homing endonucleases with a single copy of the LAGLIDADG motif form homodimers, whereas members with two copies of the LAGLIDADG motif are found as monomers.

[0134] Another method of decreasing FcR γ chain expression, activity and/or signaling involves introducing an inhibitory nucleic acid, such as an inhibitory RNA, into the cell that targets, e.g. is complementary to, a target gene transcript, such as an FcR γ , PLZF, HELIOS, SYK, DAB2 or EAT2 gene transcript, thereby reducing expression of the gene product. For example the nucleic acid may target FcR γ chain mRNA. In other embodiments, the inhibitory nucleic acid may target the mRNA of a gene that regulates transcription or translation of the FcR γ chain gene, such as a transcription factor, for example PLZF or HELIOS mRNA. In some embodiments the nucleic acid targets the mRNA of gene encoding a protein involved in FcR γ - mediated signaling, such as SYK, DAB2 or EAT-2 mRNA.

[0135] The presently disclosed subject matter takes advantage of RNAi technology (for example shRNA, siRNA and miRNA molecules and ribozymes) to cause the down regulation of cellular genes, a process referred to as RNA interference (RNAi). As used herein, "RNA interference" (RNAi) refers to a process of sequence-specific post-transcriptional gene silencing mediated by a small interfering RNA (siRNA) or short hairpin RNA (shRNA) molecules, miRNA molecules or synthetic hammerhead ribozymes. See generally Fire et al., *Nature* 391:806-811, 1998, and U.S. Pat. No. 6,506,559. The process of RNA interference (RNAi) mediated post-transcriptional gene silencing is thought to be an evolutionarily conserved cellular defense mechanism that has evolved to prevent the expression of foreign genes (Fire, *Trends Genet* 15:358-363, 1999).

[0136] In some embodiments, a recombinant virus comprising nucleic acid encoding the RNA can be produced. Engineering retroviral vectors is known to those having ordinary skill in the art. Such a skilled artisan would readily appreciate the multiple factors involved in selecting the appropriate virus and vector components needed to optimize recombinant virus production for use with the presently disclosed subject matter without the necessity of further detailed discussion herein. As one non-limiting example, a retrovirus can be engineered comprising DNA encoding an shRNA comprising an siRNA.

[0137] The gene expression may be reduced permanently, transiently, or inducibly. Suitable inducible systems are well known and include eukaryotic promoters responsive to heavy metals, Lac/VP16, and the tetracycline repressor system.

[0138] On the other hand, it may be beneficial to permanently reduce expression of the gene, for example by producing a cell line with a deletion, substitution, or insertion that causes inactivation of the gene.

[0139] Retroviral systems can be used to introduce cDNAs into NK cells. Methods of eukaryotic cell transfection and prokaryotic cell transformation are well known in the art. The choice of host cell dictates the preferred technique for introducing the polynucleotide of interest. Introduction of polynucleotides into an organism may also be done with *ex vivo* techniques that use an *in vitro* method of transfection, as well as established genetic techniques, if any, for that particular organism.

[0140] Other vectors and packaging cell lines have been used in the preparation of genetically modified variants of NK cells and can be used equivalently herein. Retroviral transduction systems have also been successfully used to transduce a variety of genes into NK cells. By way of example, these alternative methods include, but are not limited to the p-JET vector in conjunction with FLYA13 packaging cells (Gerstmayer et al., 1999), the plasmid-based kat retroviral transduction system, and DFG-hIL-2-neo/CRIP (Nagashima et al., 1998). Electroporation and “gene gun” introduction of the vector into the packaging cells is also practiced. Use of the pBMN-IRES-EGFP vector in combination with the Phoenix-Amphotropic packaging cell line is convenient for the purpose of this and the following Examples in that it provides high efficiencies of Phoenix-Amphotropic cell transfection; the use of Moloney LTR

promoters results in a high level of CD16 expression; the virus is produced at high titers; the efficiency of NK transduction is improved over other vectors that have been used to transduce NK cells; and the vector provides adequate space to accommodate the CD16 cDNA or alternative inserts. The pBMN-IRES-EGFP vector further incorporates genes for enhanced green fluorescent protein (EGFP), which can be used as an endogenous surrogate marker for gene expression. The Phoenix cell line stably expresses this vector in episomal form along with producing other viral components, thus allowing the cells to stably produce virus for an extended period of time.

[0141] Methods of introducing and expressing genes into a cell are known in the art. In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

[0142] Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al. (2001, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York). A preferred method for the introduction of a polynucleotide into a host cell is calcium phosphate transfection.

[0143] Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

[0144] Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle).

[0145] In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (in vitro, ex vivo or in vivo). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

[0146] Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma, St. Louis, Mo.; dicityl phosphate ("DCP") can be obtained from K & K Laboratories (Plainview, N.Y.); cholesterol ("Choi") can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, Ala.). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20 deg. C. Chloroform is used as the only solvent since it is more readily evaporated than methanol. "Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh et al., 1991

Glycobiology 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules.

[0147] Regardless of the method used to introduce exogenous nucleic acids into a host cell or otherwise engineer the cell in accord with the provided methods, in order to confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR or "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots).

Features of Engineered Cells

[0148] The expression of gene products associated with the engineering of genes as described can be assessed following or in connection with the engineering and/or culturing of the NK cells. Any available procedure known to a skilled artisan can be employed to detect the gene products so long as the procedure does not injure the cells. For example, the NK cells can be detected, identified and/or isolated by flow cytometry for detection of a cell surface marker that correlates with expression of the gene product, such as that correlates with FcR γ expression. The targets for modulating expression by genetic engineering as described, such as FcR γ proteins, are intracellular proteins that are not easily detected unless the cells are treated to allow intracellular proteins to be detected, for example, by fixation and permeabilization.

[0149] In some embodiments, the methods provided herein result in expression of a particular gene product in the engineered cell that is reduced by greater than about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95 %, 98% or 99% as compared to the expression of the gene product in the NK cell that is not genetically engineered. In some embodiments, the methods provided herein reduce FcR γ chain expression by greater than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95 %, 98% or 99% as compared to the expression of FcR γ chain in the NK cell that is not genetically engineered. In some of these embodiments, the level of FcR γ chain expression is reduced to an undetectable level using an immunoblot assay.

[0150] Expression of gene products as described, such as FcR γ chain expression, can be measured in a variety of ways. For example, RNA expression can be measured by northern blot, qPCR, and FISH. Protein expression can also be measured, for example using flow cytometry, western blot, immunohistochemistry, ELISA, and the like.

[0151] In some aspects, the provided engineered NK cells exhibit enhanced activity when activated by antibody, such as can occur by antibody-mediated crosslinking of CD16 or by antibody-coated cells, e.g. antibody-coated tumor cells. In some embodiments, the provided engineered NK cells are particularly responsive in the presence of an antibody or other Fc-containing protein and can be used in methods in combination with an administered monoclonal antibody or other Fc-containing protein specific to a tumor, virus or microbial cell. In some cases, the provided engineered NK cells exhibit properties or features that are the same as or similar to g⁻NK cells, which are a specific subset of NK cells deficient in FcR γ present in small numbers in individuals, but only in about one-third of the population (see e.g. published patent appl. No. US2013/0295044; see also Hwang et al. (2012) *Int. Immunol.*, 24:793-802 and Lee et al. (2015) *Cell Immunity*, 42:431-442)).

[0152] In some embodiments, the increased activity is observed following CD16 engagement by CD16 crosslinking, such as can occur in the presence of an antibody by binding of the Fc portion of the antibody to CD16 and initiation of ADCC. In some embodiments, the increased activity can be determined by monitoring phosphorylation of the CD3 ζ chain, signaling molecules, CA²⁺ flux, expression or secretion of cytokines by the cell (e.g. IFN- γ or TNF- α), expression or secretion of chemokines by the engineered cell (MIP-1 α , MIP-1 β or RANTES), degranulation response, expression of Granzyme B and/or cytotoxic killing response. Any of a number of well-known assays can be used to assess the properties or activities of the engineered NK cells (see e.g. Hwang et al. (2012) *Int. Immunology*, 24:793-802; published patent appl. No. US2013/0295044). In some embodiments, the activity of the engineered NK cells following CD16 crosslinking or engagement is increased by greater than or greater than about 1.2-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold or more compared to the activity in the engineered NK cell in the same assay but in the absence of CD16 crosslinking or engagement.

[0153] In vitro assays are commonly employed for assessing antibody-dependent cellular cytotoxicity (ADCC). In one example, target cells (e.g. cells that express an antigen that is appropriate to the antibody being evaluated) are loaded with an indicator material (such as ⁵¹Cr), and the indicator-loaded target cells are treated with the antibody to be evaluated. The resulting cells are exposed to NK effector cells as described herein. Lysis of the target cells is indicated by the release of the indicator material into the assay supernatant where its concentration can be measured by a suitable method such as scintillation counting (⁵¹Cr) or fluorescence intensity or lifetime determination. Efficacy can likewise be assessed by the measurement of surrogate indicators such as cytokine release by the NK cells; the up-regulation of NK cell activation markers, such as CD25, CD69 and/or CD95L; activation of NK cell transcription factors, such as NF-AT or NF-κB; or the activation of caspases or other markers of apoptosis in the target cells. Parental NK cells (such as non-genetically engineered NK cells) serve as a control because they permit differentiating between ADCC-mediated cytotoxicity and other cytolytic effects that NK cells exert on the target cells.

[0154] In some embodiments, the engineered NK cells are able to persist in an individual for an extended period and therefore the number of times that the cells need to be administered to have a therapeutic effect can be decreased. In some embodiments, the engineered cells provided herein persist for at least one month, at least two months, at least three months, at least four months, at least five months, at least six months, or at least months after administration.

III. Compositions and Kits Comprising Engineered NK Cells

[0155] Provided herein are compositions comprising the provided engineered NK cells. Among the compositions are pharmaceutical compositions and formulations for administration, such as for adoptive cell therapy. In some embodiments, the engineered cells are formulated with a pharmaceutically acceptable carrier.

[0156] A pharmaceutically acceptable carrier can include all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration (Gennaro, 2000, Remington: The science

and practice of pharmacy, Lippincott, Williams & Wilkins, Philadelphia, PA). Examples of such carriers or diluents include, but are not limited to, water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. Supplementary active compounds can also be incorporated into the compositions. The pharmaceutical carrier should be one that is suitable for NK cells, such as a saline solution, a dextrose solution or a solution comprising human serum albumin.

[0157] In certain embodiments, the number of cells in the composition provides the engineered NK cells at a therapeutically effective amount. In some embodiments, the amount is an amount that reduces the severity, the duration and/or the symptoms associated with cancer, viral infection, microbial infection, or septic shock in an animal. In certain other embodiments, a therapeutically effective amount is a dose of cells that results in a reduction of the growth or spread of cancer by at least 2.5%, at least 5%, at least 10%, at least 15%, at least 25%, at least 35%, at least 45%, at least 50%, at least 75%, at least 85%, by at least 90%, at least 95%, or at least 99% in a patient or an animal administered a composition described herein relative to the growth or spread of cancer in a patient (or an animal) or a group of patients (or animals) not administered the composition. In some embodiments, an effective amount for cytotoxicity is defined as amount of engineered NK cells that is able to inhibit or reduce the growth of cancer, viral and microbial cells. In some embodiments, the composition comprises a dose of engineered NK cells that is from or from about 10^5 to about 10^{12} cells, or about 10^5 to about 10^8 cells, or about 10^6 to about 10^{12} cells, or about 10^8 to about 10^{11} cells, or about 10^9 to about 10^{10} cells. In some embodiments, the composition comprises greater than or greater than about 10^5 , about 10^6 , about 10^7 , about 10^8 , about 10^9 , about 10^{10} , about 10^{11} , or about 10^{12} cells.

[0158] In some embodiments, the volume of the composition is at least or at least about 10 mL, 50 mL, 100 mL, 200 mL, 300 mL, 400 mL or 500 mL, such as is from or from about 10 mL to 500 mL, 10 mL to 200 mL, 10 mL to 100 mL, 10 mL to 50 mL, 50 mL to 500 mL, 50 mL to 200 mL, 50 mL to 100 mL, 100 mL to 500 mL, 100 mL to 200 mL or 200 mL to 500 mL, each inclusive. In some embodiments, the composition has a cell density of at least or at least about 1×10^5 cells/mL, 5×10^5 cells/mL, 1×10^6 cells/mL, 5×10^6 cells/mL, 1×10^7 cells/mL, 5×10^7 cells/mL or 1×10^8 cells/mL. In some embodiment, the cell density of the composition is between or between about 1×10^5 cells/mL to 1×10^8 cells/mL, 1×10^5 cells/mL to 1×10^7

cells/mL, 1×10^5 cells/mL to 1×10^6 cells/mL, 1×10^6 cells/mL to 1×10^7 cells/mL, 1×10^6 cells/mL to 1×10^8 cells/mL, 1×10^6 cells/mL to 1×10^7 cells/mL or 1×10^7 cells/mL to 1×10^8 cells/mL, each inclusive.

[0159] Depending upon the method of engineering the NK cell, it may be necessary or desirable to culture the NK cells to expand them prior to formulating them as a composition for administration. In some of the embodiments, methods of producing a composition comprising engineered NK cells comprises culturing or incubating the engineered NK cells, such as to expand the cells to a therapeutically effective amount prior to administering the NK cells to an individual in need thereof.

[0160] Suitable methods for culturing and expanding NK cells are known. For example, the NK cells may be cultured using feeder cells, or in the presence of cytokines to enhance their growth and/or activation. As used herein “culturing” includes providing the chemical and physical conditions (e.g., temperature, gas) which are required for NK cell maintenance, and growth factors. In one embodiment, culturing the NK cells includes providing the NK cells with conditions for proliferation. Examples of chemical conditions which may support NK cell proliferation include but are not limited to buffers, nutrients, serum, vitamins and antibiotics as well as cytokines and other growth factors which are typically provided in the growth (i.e., culture) medium. In one embodiment, the NK culture medium includes MEM α comprising 10% FCS or CellGro SCGM (Cell Genix) comprising 5% Human Serum/LiforCell® FBS Replacement (Lifeblood Products). Other media suitable for use with the invention include, but are not limited to Glasgow's medium (Gibco Carlsbad Calif.), RPMI medium (Sigma-Aldrich, St Louis Mo.) or DMEM (Sigma-Aldrich, St Louis Mo.). It will be noted that many of the culture media contain nicotinamide as a vitamin supplement for example, MEM α (8.19 μ M nicotinamide), RPMI (8.19 μ M nicotinamide), DMEM (32.78 μ M nicotinamide) and Glasgow's medium (16.39 μ M nicotinamide).

[0161] In some embodiments, such as for applications in which cells are introduced (or reintroduced) into a human subject, culturing is carried out using serum-free formulations, such as AIM V™ serum free medium for lymphocyte culture or MARROWMAX™ bone marrow medium. Such medium formulations and supplements are available from commercial sources such as Invitrogen (GIBCO) (Carlsbad, Calif.). The cultures can be supplemented with amino

acids, antibiotics, and/or with cytokines to promote optimal viability, proliferation, functionality and/or survival.

[0162] In some embodiments, culturing the population of cells comprising the engineered NK cells is effected without a feeder layer or feeder cells. In some of these embodiments, the engineered NK cells can be cultured with a growth factor. According to some embodiments, the at least one growth factor comprises a growth factor selected from the group consisting of SCF, FLT3, IL-2, IL-7, IL-15, IL-12 and IL-21. According to some embodiments, the at least one growth factor is IL-2 or IL-2 and IL-15. According to some embodiments, the at least one growth factor is solely IL-2.

[0163] In some embodiments, the provided compositions include those in which the genetically engineered NK cells, such as engineered NK cells that are reduced for FcR γ chain expression, activity and/or signaling in the cell, make up at least or at least about 60%, 70%, 80%, 85%, 90%, 95% or more of the cells in the composition or of the NK cells in the composition.

[0164] Also provided herein are compositions that are suitable for cryopreserving engineered NK cells. In some embodiments, the composition comprises an engineered NK cell and a cryoprotectant. In some embodiments, the cryoprotectant is or comprises DMSO and/or s glycerol. In some embodiments, compositions formulated for cryopreservation can be stored at low temperatures, such as ultra low temperatures, for example, storage with temperature ranges from -40 °C to -150 °C, such as or about 80 °C \pm 6.0 ° C.

[0165] In some embodiments, the engineered NK cells can be preserved at ultra low temperature before the administration to a patient. The engineered NK cells can also be preserved at ultra low temperature after isolation from a mammalian subject and prior to the genetic engineering. For example, lymphocytes or another source of engineered NK cells can be isolated, stored at ultra low temperature and then processed to yield engineered NK cells. Alternatively, the lymphocytes or another source of engineered NK cells can be isolated, processed to yield engineered NK cells and then stored at ultra-low temperature.

[0166] A typical method for the preservation at ultra low temperature in small scale is described, for example, in U.S. Pat. No. 6,0168,991. For small-scale, cells can be preserved at ultra low temperature by low density suspension (e.g., at a concentration of about 200 \times 10⁶/ml) in 5%

human albumin serum (HAS) which is previously cooled. An equivalent amount of 20% DMSO can be added into the HAS solution. Aliquots of the mixture can be placed into vials and frozen overnight inside an ultra low temperature chamber at about -80° C.

[0167] In some embodiments, the cryopreserved NK cells are prepared for administration by thawing. In some cases, the NK cells can be administered to a subject immediately after thawing. In such an embodiment, the composition is ready-to-use without any further processing. In other cases, the NK cells are further processed after thawing, such as by resuspension with a pharmaceutically acceptable carrier, incubation with an activating or stimulating agent, or are activated washed and resuspended in a pharmaceutically acceptable buffer prior to administration to a subject.

[0168] Kits comprising engineered cells are also provided herein. For example, in some embodiment provided herein is a kit comprising an engineered cell and an additional agent. In some embodiments, the additional agent comprises an Fc domain. In some embodiment the additional agent is an Fc fusion protein or an antibody. In some embodiments, the additional agent is a human, humanized, or chimeric antibody. In some of these embodiments, the additional agent is a full length antibody. Exemplary antibodies are described below.

IV. Methods of Treatment

[0169] In some embodiments, provided herein is a method of treating a condition in an individual, comprising administering engineered NK cells to an individual in need thereof.

[0170] In some embodiments, the method comprises administering an effective amount of engineered cells to an individual. In some embodiments, from or from about 10^5 to about 10^{12} cells, or about 10^5 to about 10^8 cells, or about 10^6 to about 10^{12} cells, or about 10^8 to about 10^{11} cells, or about 10^9 to about 10^{10} cells. In some embodiments, the composition comprises, about 10^5 , about 10^6 , about 10^7 , about 10^8 , about 10^9 , about 10^{10} , about 10^{11} , or about 10^{12} cells are administered to the individual. In some embodiments, from or from about 10^6 to 10^{10} engineered NK cells/kg are administered to the subject.

[0171] In some embodiments, the engineered NK cells are administered to an individual soon after isolation and the engineering of the NK cells. In some embodiments, the engineered NK cells are administered to an individual within 1, 2, 3, 4, 5, 6, 7, 14, 21, or 28 days of isolation and the engineering.

[0172] In other embodiments, the engineered NK cells are stored or expanded by growth in culture prior to administration and/or engineering, such as by methods described above. For example, the NK cells can be stored for greater than 6, 12, 18, or 24 months prior to engineering and/or administration to the individual.

[0173] In some cases, clonal cell lines of NK cells are derived from cancerous cells and thus may divide out of control upon administration to a patient. In some embodiments, the engineered NK cells, such as those from clonal cell lines, may be irradiated prior to administration to a subject to prevent them from dividing out of control.

[0174] The engineered NK cells can be administered to a subject by any convenient route including parenteral routes such as subcutaneous, intramuscular, intravenous, and/or epidural routes of administration.

[0175] The provided engineered NK cells and compositions can be used in methods of treating an individual with a tumor or hyperproliferative disorders or microbial infection such as a viral infection, yeast infection, fungal infection, protozoan infection and/or bacterial infection. The disclosed methods of treating a subject with the engineered cells can be in combination with a therapeutic monoclonal antibody, such as an anti-tumor antigen or anti-cancer antibody, anti-viral antibody or anti-bacterial antibody. The engineered NK cells can be administered for treatment of animals, such as mammalian animals, for example human subjects.

[0176] In some examples, the methods include treating a hyperproliferative disorder, such as a hematological malignancy or a solid tumor. Examples of types of cancer and proliferative disorders that can be treated with the compositions described herein include, but are not limited to, leukemia (e.g., myeloblastic, promyelocytic, myelomonocytic, monocytic, erythroleukemia, chronic myelocytic (granulocytic) leukemia, and chronic lymphocytic leukemia), lymphoma (e.g., Hodgkin's disease and non-Hodgkin's disease), fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, angiosarcoma, endotheliosarcoma, Ewing's tumor, colon

carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, renal cell carcinoma, hepatoma, Wilm's tumor, cervical cancer, uterine cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, oligodendroglioma, melanoma, neuroblastoma, retinoblastoma, dysplasia and hyperplasia. The treatment and/or prevention of cancer includes, but is not limited to, alleviating one or more symptoms associated with cancer, the inhibition or reduction of the progression of cancer, the promotion of the regression of cancer, and/or the promotion of the immune response.

[0177] In some examples, the methods include treating a viral infection, such as an infection caused by the presence of a virus in the body. Viral infections include chronic or persistent viral infections, which are viral infections that are able to infect a host and reproduce within the cells of a host over a prolonged period of time-usually weeks, months or years, before proving fatal. Viruses giving rise to chronic infections that which may be treated in accordance with the present invention include, for example, the human papilloma viruses (HPV), Herpes simplex, and other herpes viruses, the viruses of hepatitis B and C as well as other hepatitis viruses, human immunodeficiency virus, and the measles virus, all of which can produce important clinical diseases. Prolonged infection may ultimately lead to the induction of disease which may be, e.g., in the case of hepatitis C virus liver cancer, fatal to the patient. Other chronic viral infections which may be treated in accordance with the present invention include Epstein Barr virus (EBV), as well as other viruses such as those which may be associated with tumors.

[0178] Examples of viral infections which can be treated or prevented with the compositions and methods described herein include, but are limited to, viral infections caused by retroviruses (e.g., human T-cell lymphotropic virus (HTLV) types I and II and human immunodeficiency virus (HIV)), herpes viruses (e.g., herpes simplex virus (HSV) types I and II, Epstein-Ban virus and cytomegalovirus), arenaviruses (e.g., lassa fever virus), paramyxoviruses (e.g., morbillivirus virus, human respiratory syncytial virus, and pneumovirus), adenoviruses, bunyaviruses (e.g., hantavirus), coronaviruses, filoviruses (e.g., Ebola virus), flaviviruses (e.g., hepatitis C virus (HCV), yellow fever virus, and Japanese encephalitis virus), hepadnaviruses (e.g., hepatitis B viruses (HBV)), orthomyoviruses (e.g., Sendai virus and influenza viruses A, B and C), papovaviruses (e.g., papillomaviruses), picornaviruses (e.g., rhinoviruses, enteroviruses and

hepatitis A viruses), poxviruses, reoviruses (e.g., rotaviruses), togaviruses (e.g., rubella virus), and rhabdoviruses (e.g., rabies virus). The treatment and/or prevention of a viral infection includes, but is not limited to, alleviating one or more symptoms associated with said infection, the inhibition, reduction or suppression of viral replication, and/or the enhancement of the immune response.

[0179] In some embodiments, the compositions are used in a method of treating a yeast or bacterial infection. For example, the compositions and methods described herein can treat infections relating to *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Neisseria gonorrhoea*, *Neisseria meningitidis*, *Corynebacterium diphtheriae*, *Clostridium botulinum*, *Clostridium perfringens*, *Clostridium tetani*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Klebsiella rhinoscleromatis*, *Staphylococcus aureus*, *Vibrio cholera*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Campylobacter (Vibrio) fetus*, *Campylobacter jejuni*, *Aeromonas hydrophila*, *Bacillus cereus*, *Edwardsiella tarda*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Salmonella typhimurium*, *Treponema pallidum*, *Treponema caratense*, *Borrelia vincentii*, *Borrelia burgdorferi*, *Leptospira icterohemorrhagiae*, *Mycobacterium tuberculosis*, *Toxoplasma gondii*, *Pneumocystis carinii*, *Francisella tularensis*, *Brucella abortus*, *Brucella suis*, *Brucella melitensis*, *Mycoplasma spp.*, *Rickettsia prowazekii*, *Rickettsia tsutsugumushi*, *Chlamydia spp.*, *Helicobacter pylori* or combinations thereof.

V. Combination Therapy

[0180] In some embodiments, the presently engineered NK cells exhibit enhanced activity when activated by antibodies or Fc-containing proteins. For example, the engineered cells can be activated by antibody-mediated crosslinking of CD16 or by antibody-coated tumor cells.

[0181] In some embodiments, provided herein is a method of treating a condition in an individual comprising administering an engineered NK cell and an antibody. One of ordinary skill in the art can select an appropriate therapeutic (e.g., anti-cancer) monoclonal antibody to administer to the subject with the engineered NK cells described herein, such as depending on the particular disease or condition of the individual. Suitable antibodies may include polyclonal,

monoclonal, fragments (such as Fab fragments), single chain antibodies and other forms of specific binding molecules.

[0182] Abs may further comprise humanized or human Abs. Humanized forms of non-human Abs are chimeric Igs, Ig chains or fragments (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of Abs) that contain minimal sequence derived from non-human Ig. In some embodiments, the antibody comprises an Fc domain.

[0183] Generally, a humanized antibody has one or more amino acid residues introduced from a non-human source. These non-human amino acid residues are often referred to as “import” residues, which are typically taken from an “import” variable domain. Humanization is accomplished by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody (Jones et al., 1986; Riechmann et al., 1988; Verhoeyen et al., 1988). Such “humanized” Abs are chimeric Abs (1989), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized Abs are typically human Abs in which some CDR residues and possibly some Fc residues are substituted by residues from analogous sites in rodent Abs. Humanized Abs include human Igs (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit, having the desired specificity, affinity and capacity. In some instances, corresponding non-human residues replace Fv framework residues of the human Ig. Humanized Abs may comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody comprises substantially all of at least one, and typically two, variable domains, in which most if not all of the CDR regions correspond to those of a non-human Ig and most if not all of the FR regions are those of a human Ig consensus sequence. The humanized antibody optimally also comprises at least a portion of an Ig constant region (Fc), typically that of a human Ig (Jones et al., 1986; Presta, 1992; Riechmann et al., 1988).

[0184] Human Abs can also be produced using various techniques, including phage display libraries (Hoogenboom et al., 1991; Marks et al., 1991) and the preparation of human mAbs

(Boerner et al., 1991; Reisfeld and Sell, 1985). Similarly, introducing human Ig genes into transgenic animals in which the endogenous Ig genes have been partially or completely inactivated can be exploited to synthesize human Abs. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire (1997a; 1997b; 1997c; 1997d; 1997; 1997; Fishwild et al., 1996; 1997; 1997; 2001; 1996; 1997; 1997; 1997; Lonberg and Huszar, 1995; Lonberg et al., 1994; Marks et al., 1992; 1997; 1997; 1997).

[0185] Specifically, the cells of the present invention can be targeted to tumors by administration with an antibody that recognizes a tumor associated antigen. One of ordinary skill in the art will appreciate that the present engineered NK cells are suitable for use with a wide variety of antibodies that recognize tumor associated antigens. Non-limiting examples of a tumor associated antigen includes CD19, CD20, CD22, CD30, CD33, CD37, CD38, CD40, CD52, CD56, CD70, CD74, CD140, EpCAM, CEA, gpA33, mesothelin, α -fetoprotein, Mucin, PDGFR-alpha, TAG-72, CAIX, PSMA, folate-binding protein, scatter factor receptor kinase, a ganglioside, cytokerain, frizzled receptor, VEGF, VEGFR, Integrin α V β 3, integrin α 5 β 1, EGFR, EGFL7, ERBB2 (HER2), ERBB3, fibronectin, HGF, HER3, LOXL2, MET, IGF1R, IGLF2, EPHA3, FR-alpha, phosphatidylserine, Syndecan 1, SLAMF7 (CD319), TRAILR1, TRAILR2, RANKL, FAP, vimentin or tenascin. In some cases, the antibody is an anti-CD20 antibody, an anti-HER2 antibody, an anti-CD52 antibody, an anti-EGFR antibody and an anti-CD38 antibody. Exemplary antibodies include rituximab, trastuzumab, aletuzumab, certuximab, daratumumab, veltuzumab, ofatumumab, ublituximab, ocaratuzumab. Antibodies specific for a selected cancer type can be chosen, and include any antibody approved for treatment of cancer. Examples include trastuzumab (Herceptin) for breast cancer, rituximab (Rituxan) for lymphoma, and cetuximab (Erbix) for head and neck squamous cell carcinoma. A skilled artisan is familiar with FDA-approved monoclonal antibodies able to bind particular tumor or disease antigens, any of which can be used in accord with the provided methods for treating the tumor or disease.

[0186] The engineered NK cells and the additional agent can be administered sequentially or simultaneously. In some embodiments, the additional agent can be administered before administration of the engineered NK cells. In some embodiments, the additional agent can be

administered after administration of the NK cells. For example, the engineered NK cells can be administered simultaneously with antibodies specific for a selected cancer type. Alternatively, the engineered NK cells can be administered at selected times that are distinct from the times when antibodies specific for a selected cancer type are administered.

[0187] In particular examples, the subject is administered an effective dose of an antibody before, after, or substantially simultaneously with the population of engineered NK cells. In some examples, the subject is administered about 0.1 mg/kg to about 100 mg/kg of the antibody (such as about 0.5- 10 mg/kg, about 1-20 mg/kg, about 10-50 mg/kg, about 20-100 mg/kg, for example, about 0.5 mg/kg, about 1 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 8 mg/kg, about 10 mg/kg, about 16 mg/kg, about 20 mg/kg, about 24 mg/kg, about 36 mg/kg, about 48 mg/kg, about 60 mg/kg, about 75 mg/kg, or about 100 mg/kg). An effective amount of the antibody can be selected by a skilled clinician, taking into consideration the particular antibody, the particular disease or conditions (e.g. tumor or other disorder), the general condition of the subject, any additional treatments the subject is receiving or has previously received, and other relevant factors. The subject is also administered a population of modified NK cells described herein. Both the antibody and the population of modified NK cells are typically administered parenterally, for example intravenously; however, injection or infusion to a tumor or close to a tumor (local administration) or administration to the peritoneal cavity can also be used. One of skill in the art can determine appropriate routes of administration.

[0188] The engineered NK cells can also be administered simultaneously or sequentially with anti-microbial, anti-viral and other therapeutic agents. In some embodiments of the present invention, the engineered cells can be administered to an individual in combination with cytokines and/or growth factors. According to some embodiments of the present invention the at least one growth factor comprises a growth factor selected from the group consisting of SCF, FLT3, IL-2, IL-7, IL-15, IL-12 and IL-21. In some embodiments, the engineered NK cells and the cytokines or growth factors are administered sequentially. For example, the engineered NK cells may be administered first, followed by administration of the cytokines and/or growth factors. In some embodiments, the engineered NK cells are administered simultaneously with

the cytokines or growth factors.

[0189] In some embodiments, the subject is administered one or more cytokines (such as IL-2, IL- 15, IL-21, and/or IL-12) to support survival and/or growth of NK cells. The cytokine(s) can be administered before, after, or substantially simultaneously with the NK cells. In some examples, the cytokine(s) can be administered after the NK cells. In one specific example, the cytokine(s) is administered to the subject within about 1-8 hours (such as within about 1-4 hours, about 2-6 hours, about 4-6 hours, or about 5-8 hours) of the administration of the NK cells.

[0190] In some embodiments, the provided methods also can include administering engineered NK cells to an individual in combination with a chemotherapeutic agent. In some embodiments, the chemotherapeutic agent may comprise cyclophosphamide, fludarabine, methyl prednasone. In some embodiments, the chemotherapeutic agent is selected from the group consisting of: thalidomide, cisplatin (cis-DDP), oxaliplatin, carboplatin, anthracenediones, mitoxantrone; hydroxyurea, methylhydrazine derivatives, procarbazine (N-methylhydrazine, MM), adrenocortical suppressants, mitotane (.omicron.,.rho.'-DDD), aminoglutethimide, RXR agonists, bexarotene, tyrosine kinase inhibitors, imatinib, mechlorethamine, cyclophosphamide, ifosfamide, melphalan (L- sarcolysin), chlorambucil, ethylenimines, methylmelamines, hexamethylmelamine, thiotepa, busulfan, carmustine (BCNU), semustine (methyl-CCNTJ), lomustine (CCNU), streptozocin (streptozotocin), DNA synthesis antagonists, estramustine phosphate, triazines, dacarbazine (OTIC, dimethyl-triazenoimidazolecarboxamide), temozolomide, folic acid analogs, methotrexate (amethopterin), pyrimidine analogs, fluorouracil (5-fluorouracil, 5-FU, 5FTJ), floxuridine (fluorodeox>'uridine, FUdR), cytarabine (cytosine arabinoside), gemcitabine, purine analogs, mercaptopurine (6-mercaptopurine, 6- MP), thioguanine (6-thioguanine, TG), pentostatin (2'-deoxycoformycin, deoxycoformycin), cladribine and fludarabine, topoisomerase inhibitors, amsacrine, vinca alkaloids, vinblastine (VLB), vincristine, taxanes, paclitaxel, protein bound paclitaxel (Abraxane(R)), docetaxel (Taxotere(R)); epipodophyllotoxins, etoposide, teniposide, camptothecins, topotecan, irinotecan, dactinomycin (actinomycin D), daunorubicin (daunomycin, rubidomycin), doxorubicin, bleomycin, mitomycin (mitomycin C), idarubicin, epirubicin, buserelin, adrenocorticosteroids, prednisone, progestins , hydroxyprogesterone caproate, medroxyprogesterone acetate, megestrol acetate, diethylstilbestrol, ethinyl estradiol, tamoxifen, anastrozole; testosterone propionate,

fluoymesterone, flutamide, bicalutamide, and leuprolide.

[0191] In some embodiments, the cancer drug is thalidomide or its derivatives. In some embodiments, the cancer drug is selected from the group consisting of cisplatin, carboplatin, and oxaliplatin. In certain embodiments, the cancer drug is selected from the group consisting of paclitaxel, Abraxane(R), and Taxotere(R). In one embodiment, the chemotherapeutic agent is selected from the group consisting of asparaginase, bevacizumab, bleomycin, doxorubicin, epirubicin, etoposide, 5-fluorouracil, hydroxyurea, streptozocin, and 6- mercaptopurine, cyclophosphamide, paclitaxel, and gemcitabine.

VI. Exemplary Embodiments

[0192] Among the provided embodiments herein are:

1. An engineered natural killer (NK) cell, wherein the NK cell is genetically engineered to reduce FcR γ chain expression, activity and/or signaling in the cell.
2. The engineered NK cell of embodiment 1, wherein the engineered NK cell comprises:
 - a genetic disruption of a gene encoding FcR γ chain and/or a genetic disruption resulting in reduced expression of FcR γ chain in the engineered NK cell;
 - a genetic disruption of a gene encoding a protein regulating expression or activity of FcR γ chain and/or a genetic disruption resulting in reduced expression of a protein regulating expression or activity of FcR γ chain; and/or
 - a genetic disruption of a gene encoding a protein involved in FcR γ chain-dependent signaling and/or a genetic disruption resulting in reduced expression of a protein involved in FcR γ chain-dependent signaling.
3. The engineered NK cell of embodiment 2, wherein the genetic disruption comprises a deletion, mutation and/or insertion resulting in a premature stop codon in the gene or a frameshift of the open reading frame of the gene.
4. The engineered NK cell of embodiment 2 or embodiment 3, wherein both alleles of the gene encoding FcR γ chain, the gene encoding a protein regulating expression or activity

of FcR γ chain and/or the gene encoding a protein involved in FcR γ chain-dependent signaling are disrupted in the engineered NK cells.

5. The engineered NK cell of embodiment 1, wherein the engineered NK cell comprises an inhibitory nucleic acid molecule targeting a gene in the NK cell resulting in reduced expression of FcR γ chain, reduced expression of a protein regulating expression or activity of FcR γ chain and/or reduced expression of a protein involved in FcR γ chain-dependent signaling.

6. The engineered NK cell of any of embodiments 2-5, wherein the expression of FcR γ chain, a protein regulating expression or activity of FcR γ chain and/or a protein involved in FcR γ chain-dependent signaling is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression of the protein in the NK cell that is not genetically engineered.

7. The engineered NK cell of any of embodiments 2-6, wherein expression of a protein regulating expression or activity of FcR γ chain is reduced in the engineered NK cell and the protein is a transcription factor.

8. The engineered NK cell of embodiment 7, wherein the transcription factor is PLZF (ZBTB16) or HELIOS (IKZF2).

9. The engineered NK cell of any of embodiments 2-6, wherein the expression of a protein involved in FcR γ chain-dependent signaling is reduced in the engineered NK cell and the protein is a downstream signaling molecule.

10. The engineered NK cells of embodiment 9, wherein the downstream signaling molecule is SYK, DAB2 or EAT-2.

11. The engineered NK cell of any of embodiments 2-6, wherein expression of FcR γ chain is reduced in the engineered cell.

12. An engineered NK cell comprising a genetic disruption in a gene encoding FcR γ chain, wherein expression of FcR γ is reduced in the cell.

13. The engineered NK cell of embodiment 12, wherein the genetic disruption comprises a deletion, mutation and/or insertion resulting in a premature stop codon in the gene or a frameshift of the open reading frame of the gene.

14. The engineered NK cell of embodiment 12 or embodiment 13, wherein both alleles of the gene encoding FcR γ chain are disrupted in the genome of the engineered NK cell.

15. The engineered NK cell of any of embodiments 1, 5 or 6, wherein the engineered NK cell comprises an inhibitory nucleic acid molecule that targets a gene encoding FcR γ chain.
16. The engineered NK cell of embodiment 15, wherein the inhibitory nucleic acid molecule comprises a sequence complementary to the gene encoding FcR γ chain.
17. The engineered NK cell of any of embodiments 11-16, wherein the expression of FcR γ chain is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression in the NK cell that is not genetically engineered.
18. The engineered NK cell of any of embodiments 5-6 and 15-17, wherein the inhibitory nucleic acid comprises an RNA interfering agent.
19. The engineered NK cell of any of embodiments 5-6 and 15-18, wherein the inhibitory nucleic acid comprises siRNA, shRNA, or miRNA.
20. The engineered NK cell of any of embodiments 1-19, wherein the reduced expression, activity and/or signaling of FcR γ is permanent, transient or inducible.
21. The engineered NK cell of any of embodiments 1-20, wherein the expression, activity and/or signaling of FcR γ chain is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression, activity and/or signaling in the NK cell that is not genetically engineered.
22. The engineered NK cell of any one of embodiments 1-21, wherein the expression of FcR γ chain expressed in the cell is undetectable in an immunoblot assay.
23. The engineered NK cell of any of embodiments 1-22, wherein CD16 is expressed on the surface of the engineered NK cell.
24. The engineered NK cell of any one of embodiments 1-23, wherein the engineered NK cell expresses CD3-zeta (CD3 ζ) chain.
25. The engineered NK cell of any one of embodiments 1-24 that is derived from a primary cell obtained from a subject.
26. The engineered NK cell of embodiment 25, wherein the subject is human.
27. The engineered NK cell of any one of embodiments 1-24 that is derived from a clonal cell line.
28. The engineered NK cell of embodiment 27, wherein the clonal cell line is NK-92, NK-YS, KHYG-1, NKL, NKG, SNK-6, or IMC-1.

29. The engineered NK cell of any of embodiments 1-28, wherein the engineered NK cell comprises a recombinant or heterologous CD16 gene and/or a recombinant or heterologous CD3-zeta (CD3 ζ) chain.

30. The engineered NK cell of embodiment 29, wherein the CD16 comprises a CD16-activating mutation, a mutation that results in higher affinity to IgG1, a 158V mutation and/or a 158F mutation.

31. The engineered cell of any of embodiments 1-30, wherein the engineered NK cell exhibits increased activity when stimulated through CD16 compared to the NK cell without the modification.

32. The engineered NK cell of any one of embodiments 1-31, wherein the engineered NK cell has reduced surface expression of NKp46, NKp30, and/or NKp44 compared to the NK cell without the modification.

33. A method of producing an engineered NK cell, comprising genetically engineering an NK cell to reduce FcR γ chain expression, activity, and/or signaling in the cell.

34. The method of embodiment 33, wherein reducing expression comprises:
disrupting or repressing a gene encoding FcR γ chain and/or disrupting or repressing a gene that results in reduced expression of FcR γ chain in the NK cell;

disrupting or repressing a gene encoding a protein regulating expression or activity of FcR γ chain and/or a disrupting or repressing a gene that results in reduced expression of a protein regulating expression or activity of FcR γ chain in the NK cell; and/or

disrupting or repressing a gene encoding a protein involved in FcR γ chain-dependent signaling and/or a disrupting or repressing a gene that results in reduced expression of a protein involved in FcR γ chain-dependent signaling in the NK cell.

35. The method of embodiment 33 or embodiment 34, comprising introducing a deletion, mutation, or insertion into the gene.

36. The method of any one of embodiments 33-35, wherein the gene encodes FcR γ chain.

37. The method of any one of embodiments 33-35 wherein the gene encodes a protein regulating expression or activity of FcR γ chain that is a transcription factor.

38. The method of embodiment 37, wherein the transcription factor is PLZF or HELIOS.

39. The method of any one of embodiments 33-35, wherein the gene encodes a protein involved in FcR γ chain-dependent signaling that is a downstream signaling molecule.
40. The method of embodiment 38, wherein the downstream signaling molecule is SYK, DAB2 or EAT2.
41. The method of any of embodiments 33-40, wherein disruption or repression of the gene is effected by introducing an endonuclease that target to the gene in the NK cell under conditions that allow disruption or repression of the gene.
42. The method of embodiment 41, wherein the endonuclease is selected from the group consisting of TAL nucleases, meganucleases, zinc-finger nucleases, Cas9, and Argonaute.
43. The method of any of embodiments 33-40, wherein disruption or repression is effected by introducing an inhibitory nucleic acid that targets the gene into the NK cell under conditions that results in repression of the gene.
44. The method of embodiment 43, wherein the inhibitory nucleic acid molecule comprises a sequence complementary to the gene encoding FcR γ chain.
45. The method of embodiment 43 or embodiment 44, wherein the inhibitory nucleic acid comprises an RNA interfering agent.
46. The method of any one of embodiments 43-45, wherein the nucleic acid is siRNA, shRNA, or miRNA.
47. The method of any one of embodiments 33-46, wherein the reduced expression is permanent, transient, or inducible.
48. The method of any one of embodiments 33-47, wherein the expression, activity and/or signaling of FcR γ chain is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression in the NK cell in that is not genetically engineered.
49. The method of any one of embodiments 33-48, wherein the FcR γ chain expression level is not detectable by an immunoblot assay.
50. The method of any one of embodiments 33-49, comprising, prior to the genetically engineering, isolating the NK cell from a human subject.
51. The method of embodiment 50, comprising isolating the NK cell from peripheral blood mononuclear cells (PBMC).

52. The method of embodiment 50 or 51, wherein isolating the NK cell comprises selecting NK cells from PBMC using a NK cell marker.
53. The method of embodiment 52, wherein the NK cell marker is CD56, Cd161, KIR, NKG2A, NKG2D, NKp30, NKp44, and/or NKp46.
54. The method of any one of embodiments 50-53, further comprising selecting NK cells that express CD16 and/or CD3 ζ .
55. The method of any one of embodiments 50-54, further comprising selecting NK cells that do not express surface CD3.
56. The method of any one of embodiments 33-49, wherein the NK cell is an NK cell line.
57. The method of embodiment 56, wherein the cell line is NK-92, NK-YS, KHYG-1, NKL, NKG, SNK-6, or IMC-1.
58. The method of embodiment 56 or embodiment 57, further comprising engineering the NK cell line to express a recombinant or heterologous CD16 and/or CD3 ζ .
59. The method of embodiment 58, comprising introducing nucleic acid encoding the CD16 and/or CD3 ζ into the NK cell.
60. The method of embodiment 58 or embodiment 59, wherein the CD16 gene contains an activating mutation and/or a mutation that increases affinity of CD16 for IgG.
61. The method of any of embodiments 58-60, wherein the CD16 comprises a mutation that is 158V.
62. The method of any of embodiments 58-60, wherein the CD16 comprises a mutation that is 158F.
63. The method of any of embodiments 58-62, comprising virally transducing the NK cell with nucleic acid encoding the CD16 and/or CD3 ζ .
64. The method of any of embodiments 58-62, comprising transfecting the NK cell with nucleic acid encoding the CD16 and/or CD3 ζ .
65. The method of any one of embodiments 58-64, comprising transiently, inducibly, or permanently expressing CD16 or CD3 ζ in the NK cell.
66. The method of any one of embodiments 33-65, further comprising culturing or expanding the engineered NK cells.

67. The method of embodiment 66, comprising culturing the engineered NK cells with feeder cells.
68. The method of embodiment 66 or embodiment 67 comprising culturing the engineered NK cells with cytokines.
69. An engineered NK cell produced by the methods of any one of embodiments 33-68.
70. A composition comprising an effective amount of the engineered NK cell of any one of embodiments 1-32, or 69.
71. The composition of embodiment 70, further comprising a pharmaceutically acceptable carrier.
72. The composition of embodiment 71, wherein the carrier is a saline solution, a dextrose solution, or 5% human serum albumin.
73. The composition of any one of embodiments 70-72, wherein the composition comprises between 1×10^5 and 1×10^8 cells/mL.
74. The composition of any of embodiments 70-73 comprising a cryoprotectant.
75. A kit comprising the engineered cell of any one of embodiments 1-32, or 69 or a composition of any one of embodiments 70-74 and an additional agent for treatment of a disease.
76. The kit of embodiment 75, wherein the additional agent is an antibody or an Fc-fusion protein.
77. The kit of embodiment 76, wherein the antibody recognizes or specifically binds a tumor associated antigen.
78. The kit of embodiment 77, wherein the tumor associated antigen is CD19, CD20, CD22, CD30, CD33, CD37, CD38, CD40, CD52, CD56, CD70, CD74, CD140, EpCAM, CEA, gpA33, mesothelin, α -fetoprotein, Mucin, PDGFR-alpha, TAG-72, CAIX, PSMA, folate-binding protein, scatter factor receptor kinase, a ganglioside, cytokerain, frizzled receptor, VEGF, VEGFR, Integrin α V β 3, integrin α 5 β 1, EGFR, EGFL7, ERBB2 (HER2), ERBB3, fibronectin, HGF, HER3, LOXL2, MET, IGF1R, IGLF2, EPHA3, FR-alpha, phosphatidylserine, Syndecan 1, SLAMF7 (CD319), TRAILR1, TRAILR2, RANKL, FAP, vimentin or tenascin.
79. The kit of any one of embodiments 76-78, wherein the antibody is a full length antibody and/or comprises an Fc domain.

80. A method of treating a condition comprising administering engineered NK cells of any one of embodiments 1-32, or 69 or a composition of any one of embodiments 70-74 to an individual in need thereof.

81. The method of embodiment 80, wherein prior to administering the engineered NK cells producing the engineered NK cells by the method of any of embodiments 33-68.

82. The method of embodiment 80 or embodiment 81, comprising administering from or from about 1×10^8 to 1×10^{10} cells/m² to the individual or administering from or from about 1×10^6 to 1×10^{10} NK cells/kg.

83. The method of any one of embodiments 80-82 further comprising administering an additional agent.

84. The method of embodiment 83, wherein the additional agent is an antibody or an Fc-fusion protein.

85. The method of embodiment 84 wherein the antibody recognizes a tumor associated antigen.

86. The method of embodiment 84 or embodiment 85, wherein the tumor associated antigen is CD19, CD20, CD22, CD30, CD33, CD37, CD38, CD40, CD52, CD56, CD70, CD74, CD140, EpCAM, CEA, gpA33, mesothelin, α -fetoprotein, Mucin, PDGFR-alpha, TAG-72, CAIX, PSMA, folate-binding protein, scatter factor receptor kinase, a ganglioside, cytokeratin, frizzled receptor, VEGF, VEGFR, Integrin α V β 3, integrin α 5 β 1, EGFR, EGFL7, ERBB2 (HER2), ERBB3, fibronectin, HGF, HER3, LOXL2, MET, IGF1R, IGLF2, EPHA3, FR-alpha, phosphatidylserine, Syndecan 1, SLAMF7 (CD319), TRAILR1, TRAILR2, RANKL, FAP, vimentin or tenascin.

87. The method of any one of embodiments 84-86, wherein the antibody comprises an Fc domain and/or is a full-length antibody.

88. The method of any one of embodiments 83-87, wherein the additional agent and the engineered NK cells are administered sequentially.

89. The method of embodiment 88, wherein the additional agent is administered prior to administration of the engineered NK cells.

90. The method of any one of embodiments 83-87, where the additional agent and the engineered NK cells are administered simultaneously

91. The method of any one of embodiments 80-90, wherein the condition is selected from the group consisting of an inflammatory condition, an infection, and cancer.
92. The method of embodiment 91, wherein the infection is a viral infection or a bacterial infection.
93. The method of embodiment 92, wherein the cancer is leukemia or lymphoma.
94. The method of embodiment 92, wherein the cancer comprises a solid tumor.
95. The method of any one of embodiments 80-94, wherein the individual is a human.
96. The method of any one of embodiments 80-95, wherein the engineered NK cell is allogenic to the individual.
97. The method of any one of embodiments 80-95, wherein the engineered NK cell is autologous to the subject.
98. The engineered NK cell of any one of embodiments 1-32, wherein the cell is genetically engineered to reduce expression of a NK inhibitory receptor.
99. The engineered NK cell of embodiment 98, wherein:
the cell comprises a genetic disruption of a gene encoding a NK inhibitory receptor and/or a genetic disruption resulting in reduced expression of an NK inhibitory receptor; or
the cell comprises an inhibitory nucleic acid that targets a gene encoding a NK inhibitory receptor and/or that reduces expression of an NK inhibitory receptor.
100. The engineered NK cell of embodiment 98 or embodiment 99, wherein the inhibitory receptor is NKG2A or KIR2DL1.
101. The method of any one of embodiments 32-68, further comprising genetically engineering the NK cell to reduce expression or activity of an inhibitory receptor.
102. The method of embodiment 101, wherein reducing expression or activity comprises disrupting or repressing expression of a gene encoding the NK inhibitory receptor or a gene that results in reduced expression of the NK inhibitory receptor.
103. The method of embodiment 102, wherein the inhibitory receptor is NKG2A or KIR2DL1.

VII. Examples

[0193] The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1: Assessment of Cell Killing of Expanded G-NK Cells and Conventional NK Cells

[0194] 25×10^6 peripheral blood mononuclear cells (PBMCs) from a healthy human donor were obtained, and fluorescence-activated cell sorting was used to enrich $\sim 2 \times 10^5$ FcR γ -deficient (g⁻ NK) cells based on certain cell surface phenotypes. G-NK cells were expanded 250-fold in 14 days by mixing 0.5 mL (1×10^6) of feeder cells (irradiated autologous PBMC stimulated with 10 ng/mL OKT3) with 0.5 mL (2×10^5) of sorted g-NK cells. The expansion was spiked with 10 ng/mL IL-2 every 2 days and on day 7, expanding g-NK cells were re-fed the cells with irradiated feeders (pre-activated with 10 ng/mL OKT3) at a 5:1 feeder:g-NK cell ratio. Conventional NK cells also were obtained and expanded.

[0195] To assess antibody-directed activity of expanded g-NK cells, serial dilutions of the enriched g-NK cells (E/T ratio ranging from 50:1 to 1:1) were incubated in a 96-well plate with ⁵¹Cr-labeled Raji lymphoma cells in the presence or absence of anti-CD20 antibody rituximab (5 μ g/mL). After incubation for 4 hours, antibody dependent cell cytotoxicity (ADCC) was assessed by determining ⁵¹Cr activity per well. As shown in **FIG. 1**, the results showed that g-NK cells exhibited greater ability to mediate ADCC when co-cultured with rituximab and Raji lymphoma cells than conventional NK cells.

[0196] The present invention is not intended to be limited in scope to the particular disclosed embodiments, which are provided, for example, to illustrate various aspects of the invention. Various modifications to the engineered cells, compositions and methods described will become apparent from the description and teachings herein. Such variations may be practiced without departing from the true scope and spirit of the disclosure and are intended to fall within the scope of the present disclosure.

CLAIMS**What is claimed is:**

1. An engineered natural killer (NK) cell, wherein the NK cell is genetically engineered to reduce FcR γ chain expression, activity and/or signaling in the cell.
2. The engineered NK cell of claim 1, wherein the engineered NK cell comprises:
 - a genetic disruption of a gene encoding FcR γ chain and/or a genetic disruption resulting in reduced expression of FcR γ chain in the engineered NK cell;
 - a genetic disruption of a gene encoding a protein regulating expression or activity of FcR γ chain and/or a genetic disruption resulting in reduced expression of a protein regulating expression or activity of FcR γ chain; and/or
 - a genetic disruption of a gene encoding a protein involved in FcR γ chain-dependent signaling and/or a genetic disruption resulting in reduced expression of a protein involved in FcR γ chain-dependent signaling.
3. The engineered NK cell of claim 2, wherein the genetic disruption comprises a deletion, mutation and/or insertion resulting in a premature stop codon in the gene or a frameshift of the open reading frame of the gene.
4. The engineered NK cell of claim 2 or claim 3, wherein both alleles of the gene encoding FcR γ chain, the gene encoding a protein regulating expression or activity of FcR γ chain and/or the gene encoding a protein involved in FcR γ chain-dependent signaling are disrupted in the engineered NK cells.
5. The engineered NK cell of claim 1, wherein the engineered NK cell comprises an inhibitory nucleic acid molecule targeting a gene in the NK cell resulting in reduced expression of FcR γ chain, reduced expression of a protein regulating expression or activity of FcR γ chain and/or reduced expression of a protein involved in FcR γ chain-dependent signaling.

6. The engineered NK cell of any of claims 2-5, wherein the expression of FcR γ chain, a protein regulating expression or activity of FcR γ chain and/or a protein involved in FcR γ chain-dependent signaling is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression of the protein in the NK cell that is not genetically engineered.

7. The engineered NK cell of any of claims 2-6, wherein expression of a protein regulating expression or activity of FcR γ chain is reduced in the engineered NK cell and the protein is a transcription factor.

8. The engineered NK cell of claim 7, wherein the transcription factor is PLZF (ZBTB16) or HELIOS (IKZF2).

9. The engineered NK cell of any of claims 2-6, wherein the expression of a protein involved in FcR γ chain-dependent signaling is reduced in the engineered NK cell and the protein is a downstream signaling molecule.

10. The engineered NK cells of claim 9, wherein the downstream signaling molecule is SYK, DAB2 or EAT-2.

11. The engineered NK cell of any of claims 2-6, wherein expression of FcR γ chain is reduced in the engineered cell.

12. An engineered NK cell comprising a genetic disruption in a gene encoding FcR γ chain, wherein expression of FcR γ is reduced in the cell.

13. The engineered NK cell of claim 12, wherein the genetic disruption comprises a deletion, mutation and/or insertion resulting in a premature stop codon in the gene or a frameshift of the open reading frame of the gene.

14. The engineered NK cell of claim 12 or claim 13, wherein both alleles of the gene encoding FcR γ chain are disrupted in the genome of the engineered NK cell.
15. The engineered NK cell of any of claims 1, 5 or 6, wherein the engineered NK cell comprises an inhibitory nucleic acid molecule that targets a gene encoding FcR γ chain.
16. The engineered NK cell of claim 15, wherein the inhibitory nucleic acid molecule comprises a sequence complementary to the gene encoding FcR γ chain.
17. The engineered NK cell of any of claims 11-16, wherein the expression of FcR γ chain is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression in the NK cell that is not genetically engineered.
18. The engineered NK cell of any of claims 5-6 and 15-17, wherein the inhibitory nucleic acid comprises an RNA interfering agent.
19. The engineered NK cell of any of claims 5-6 and 15-18, wherein the inhibitory nucleic acid comprises siRNA, shRNA, or miRNA.
20. The engineered NK cell of any of claims 1-19, wherein the reduced expression, activity and/or signaling of FcR γ is permanent, transient or inducible.
21. The engineered NK cell of any of claims 1-20, wherein the expression, activity and/or signaling of FcR γ chain is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression, activity and/or signaling in the NK cell that is not genetically engineered.
22. The engineered NK cell of any one of claims 1-21, wherein the expression of FcR γ chain expressed in the cell is undetectable in an immunoblot assay.

23. The engineered NK cell of any of claims 1-22, wherein CD16 is expressed on the surface of the engineered NK cell.
24. The engineered NK cell of any one of claims 1-23, wherein the engineered NK cell expresses CD3-zeta (CD3 ζ) chain.
25. The engineered NK cell of any one of claims 1-24 that is derived from a primary cell obtained from a subject.
26. The engineered NK cell of claim 25, wherein the subject is human.
27. The engineered NK cell of any one of claims 1-24 that is derived from a clonal cell line.
28. The engineered NK cell of claim 27, wherein the clonal cell line is NK-92, NK-YS, KHYG-1, NKL, NKG, SNK-6, or IMC-1.
29. The engineered NK cell of any of claims 1-28, wherein the engineered NK cell comprises a recombinant or heterologous CD16 gene and/or a recombinant or heterologous CD3-zeta (CD3 ζ) chain.
30. The engineered NK cell of claim 29, wherein the CD16 comprises a CD16-activating mutation, a mutation that results in higher affinity to IgG1, a 158V mutation and/or a 158F mutation.
31. The engineered cell of any of claims 1-30, wherein the engineered NK cell exhibits increased activity when stimulated through CD16 compared to the NK cell without the modification.

32. The engineered NK cell of any one of claims 1-31, wherein the engineered NK cell has reduced surface expression of NKp46, NKp30, and/or NKp44 compared to the NK cell without the modification.
33. A method of producing an engineered NK cell, comprising genetically engineering an NK cell to reduce FcR γ chain expression, activity, and/or signaling in the cell.
34. The method of claim 33, wherein reducing expression comprises:
disrupting or repressing a gene encoding FcR γ chain and/or disrupting or repressing a gene that results in reduced expression of FcR γ chain in the NK cell;
disrupting or repressing a gene encoding a protein regulating expression or activity of FcR γ chain and/or a disrupting or repressing a gene that results in reduced expression of a protein regulating expression or activity of FcR γ chain in the NK cell; and/or
disrupting or repressing a gene encoding a protein involved in FcR γ chain-dependent signaling and/or a disrupting or repressing a gene that results in reduced expression of a protein involved in FcR γ chain-dependent signaling in the NK cell.
35. The method of claim 33 or claim 34, comprising introducing a deletion, mutation, or insertion into the gene.
36. The method of any one of claims 33-35, wherein the gene encodes FcR γ chain.
37. The method of any one of claims 33-35 wherein the gene encodes a protein regulating expression or activity of FcR γ chain that is a transcription factor.
38. The method of claim 37, wherein the transcription factor is PLZF or HELIOS.
39. The method of any one of claims 33-35, wherein the gene encodes a protein involved in FcR γ chain-dependent signaling that is a downstream signaling molecule.

40. The method of claim 38, wherein the downstream signaling molecule is SYK, DAB2 or EAT2.

41. The method of any of claims 33-40, wherein disruption or repression of the gene is effected by introducing an endonuclease that target to the gene in the NK cell under conditions that allow disruption or repression of the gene.

42. The method of claim 41, wherein the endonuclease is selected from the group consisting of TAL nucleases, meganucleases, zinc-finger nucleases, Cas9, and Argonaute.

43. The method of any of claims 33-40, wherein disruption or repression is effected by introducing an inhibitory nucleic acid that targets the gene into the NK cell under conditions that results in repression of the gene.

44. The method of claim 43, wherein the inhibitory nucleic acid molecule comprises a sequence complementary to the gene encoding FcR γ chain.

45. The method of claim 43 or claim 44, wherein the inhibitory nucleic acid comprises an RNA interfering agent.

46. The method of any one of claims 43-45, wherein the nucleic acid is siRNA, shRNA, or miRNA.

47. The method of any one of claims 33-46, wherein the reduced expression is permanent, transient, or inducible.

48. The method of any one of claims 33-47, wherein the expression, activity and/or signaling of FcR γ chain is reduced by greater than or greater than about 50%, 60%, 70%, 80%, 90%, or 95 % as compared to the expression in the NK cell in that is not genetically engineered.

49. The method of any one of claims 33-48, wherein the FcR γ chain expression level is not detectable by an immunoblot assay.

50. The method of any one of claims 33-49, comprising, prior to the genetically engineering, isolating the NK cell from a human subject.

51. The method of claim 50, comprising isolating the NK cell from peripheral blood mononuclear cells (PBMC).

52. The method of claim 50 or 51, wherein isolating the NK cell comprises selecting NK cells from PBMC using a NK cell marker.

53. The method of claim 52, wherein the NK cell marker is CD56, Cd161, KIR, NKG2A, NKG2D, NKp30, NKp44, and/or NKp46.

54. The method of any one of claims 50-53, further comprising selecting NK cells that express CD16 and/or CD3 ζ .

55. The method of any one of claims 50-54, further comprising selecting NK cells that do not express surface CD3.

56. The method of any one of claims 33-49, wherein the NK cell is an NK cell line.

57. The method of claim 56, wherein the cell line is NK-92, NK-YS, KHYG-1, NKL, NKG, SNK-6, or IMC-1.

58. The method of claim 56 or claim 57, further comprising engineering the NK cell line to express a recombinant or heterologous CD16 and/or CD3 ζ .

59. The method of claim 58, comprising introducing nucleic acid encoding the CD16 and/or CD3 ζ into the NK cell.

60. The method of claim 58 or claim 59, wherein the CD16 gene contains an activating mutation and/or a mutation that increases affinity of CD16 for IgG.
61. The method of any of claims 58-60, wherein the CD16 comprises a mutation that is 158V.
62. The method of any of claims 58-60, wherein CD16 comprises a mutation that is 158F.
63. The method of any of claims 58-62, comprising virally transducing the NK cell with nucleic acid encoding the CD16 and/or CD3 ζ .
64. The method of any of claims 58-62, comprising transfecting the NK cell with nucleic acid encoding the CD16 and/or CD3 ζ .
65. The method of any one of claims 58-64, comprising transiently, inducibly, or permanently expressing CD16 or CD3 ζ in the NK cell.
66. The method of any one of claims 33-65, further comprising culturing or expanding the engineered NK cells.
67. The method of claim 66, comprising culturing the engineered NK cells with feeder cells.
68. The method of claim 66 or claim 67 comprising culturing the engineered NK cells with cytokines.
69. An engineered NK cell produced by the methods of any one of claims 33-68.

70. A composition comprising an effective amount of the engineered NK cell of any one of claims 1-32, or 69.
71. The composition of claim 70, further comprising a pharmaceutically acceptable carrier.
72. The composition of claim 71, wherein the carrier is a saline solution, a dextrose solution, or 5% human serum albumin.
73. The composition of any one of claims 70-72, wherein the composition comprises between 1×10^5 and 1×10^8 cells/mL.
74. The composition of any of claims 70-73 comprising a cryoprotectant.
75. A kit comprising the engineered cell of any one of claims 1-32, or 69 or a composition of any one of claims 70-74 and an additional agent for treatment of a disease.
76. The kit of claim 75, wherein the additional agent is an antibody or an Fc-fusion protein.
77. The kit of claim 76, wherein the antibody recognizes or specifically binds a tumor associated antigen.
78. The kit of claim 77, wherein the tumor associated antigen is CD19, CD20, CD22, CD30, CD33, CD37, CD38, CD40, CD52, CD56, CD70, CD74, CD140, EpCAM, CEA, gpA33, mesothelin, α -fetoprotein, Mucin, PDGFR-alpha, TAG-72, CAIX, PSMA, folate-binding protein, scatter factor receptor kinase, a ganglioside, cytokearin, frizzled receptor, VEGF, VEGFR, Integrin α V β 3, integrin α 5 β 1, EGFR, EGFL7, ERBB2 (HER2), ERBB3, fibronectin, HGF, HER3, LOXL2, MET, IGF1R, IGLF2, EPHA3, FR-alpha, phosphatidylserine, Syndecan 1, SLAMF7 (CD319), TRAILR1, TRAILR2, RANKL, FAP, vimentin or tenascin.

79. The kit of any one of claims 76-78, wherein the antibody is a full length antibody and/or comprises an Fc domain.

80. A method of treating a condition comprising administering engineered NK cells of any one of claims 1-32, or 69 or a composition of any one of claims 70-74 to an individual in need thereof.

81. The method of claim 80, wherein prior to administering the engineered NK cells producing the engineered NK cells by the method of any of claims 33-68.

82. The method of claim 80 or claim 81, comprising administering from or from about 1×10^8 to 1×10^{10} cells/m² to the individual or administering from or from about 1×10^6 to 1×10^{10} NK cells/kg.

83. The method of any one of claims 80-82 further comprising administering an additional agent.

84. The method of claim 83, wherein the additional agent is an antibody or an Fc-fusion protein.

85. The method of claim 84 wherein the antibody recognizes a tumor associated antigen.

86. The method of claim 84 or claim 85, wherein the tumor associated antigen is CD19, CD20, CD22, CD30, CD33, CD37, CD38, CD40, CD52, CD56, CD70, CD74, CD140, EpCAM, CEA, gpA33, mesothelin, α -fetoprotein, Mucin, PDGFR-alpha, TAG-72, CAIX, PSMA, folate-binding protein, scatter factor receptor kinase, a ganglioside, cytokerain, frizzled receptor, VEGF, VEGFR, Integrin α V β 3, integrin α 5 β 1, EGFR, EGFL7, ERBB2 (HER2), ERBB3, fibronectin, HGF, HER3, LOXL2, MET, IGF1R, IGLF2, EPHA3, FR-alpha, phosphatidylserine, Syndecan 1, SLAMF7 (CD319), TRAILR1, TRAILR2, RANKL, FAP, vimentin or tenascin.

87. The method of any one of claims 84-86, wherein the antibody comprises an Fc domain and/or is a full-length antibody.

88. The method of any one of claims 83-87, wherein the additional agent and the engineered NK cells are administered sequentially.

89. The method of claim 88, wherein the additional agent is administered prior to administration of the engineered NK cells.

90. The method of any one of claims 83-87, where the additional agent and the engineered NK cells are administered simultaneously

91. The method of any one of claims 80-90, wherein the condition is selected from the group consisting of an inflammatory condition, an infection, and cancer.

92. The method of claim 91, wherein the infection is a viral infection or a bacterial infection.

93. The method of claim 92, wherein the cancer is leukemia or lymphoma.

94. The method of claim 92, wherein the cancer comprises a solid tumor.

95. The method of any one of claims 80-94, wherein the individual is a human.

96. The method of any one of claims 80-95, wherein the engineered NK cell is allogenic to the individual.

97. The method of any one of claims 80-95, wherein the engineered NK cell is autologous to the subject.

98. The engineered NK cell of any one of claims 1-32, wherein the cell is genetically engineered to reduce expression of a NK inhibitory receptor.

99. The engineered NK cell of claim 98, wherein:
the cell comprises a genetic disruption of a gene encoding a NK inhibitory receptor and/or a genetic disruption resulting in reduced expression of an NK inhibitory receptor; or
the cell comprises an inhibitory nucleic acid that targets a gene encoding a NK inhibitory receptor and/or that reduces expression of an NK inhibitory receptor.

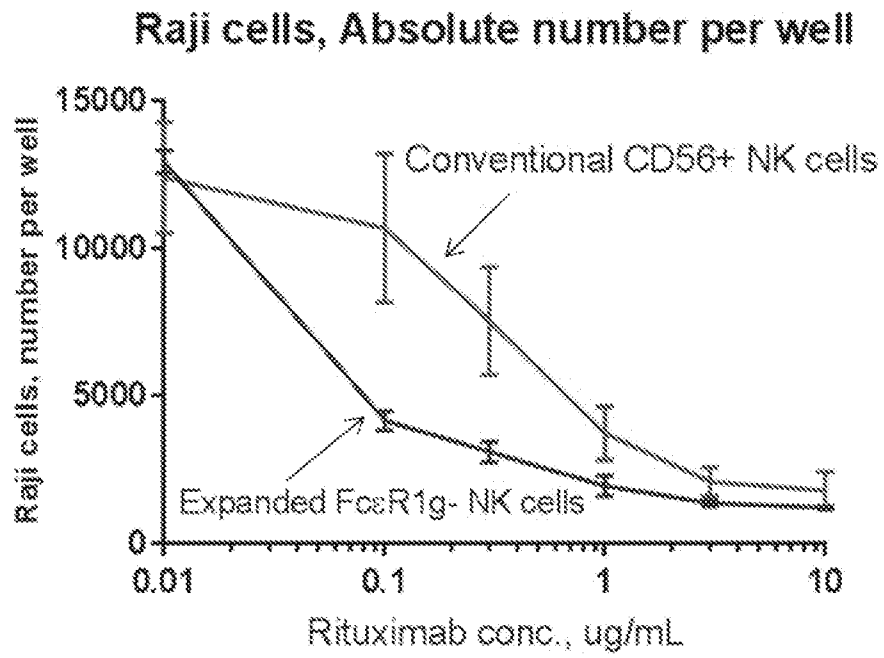
100. The engineered NK cell of claim 98 or claim 99, wherein the inhibitory receptor is NKG2A or KIR2DL1.

101. The method of any one of claims 32-68, further comprising genetically engineering the NK cell to reduce expression or activity of an inhibitory receptor.

102. The method of claim 101, wherein reducing expression or activity comprises disrupting or repressing expression of a gene encoding the NK inhibitory receptor or a gene that results in reduced expression of the NK inhibitory receptor.

103. The method of claim 102, wherein the inhibitory receptor is NKG2A or KIR2DL1.

FIG. 1



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/17493

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/63, C07K 14/705, C12N 15/09 (20108.01)

CPC - A01K 67/0276, A01K 2217/072, C07K 14/70535, C12N 15/8509, C12N 5/0638

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2016/011210 A2 (JUNO THERAPEUTICS, INC.) 21 January 2016 (21.01.2016) para [0003], [0009], [0032], [0175], [0206]	1-5, 12-14, 33-35
Y	US 2013/0295044 A1 (KIM et al.) 7 November 2013 (07.11.2013) para [0032]	1-5, 12-14, 33-35

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

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

13 April 2018

Date of mailing of the international search report

04 MAY 2018

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 18/17493

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

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

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

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

3. Claims Nos.: 6-11, 15-32, 36-103
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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