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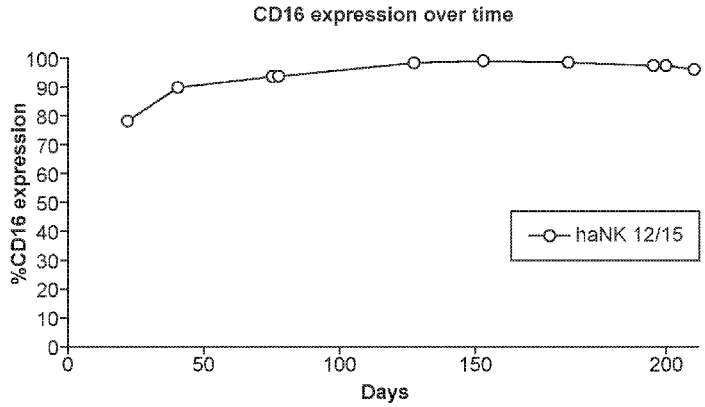

FIG. 3

(57) Abstract: This invention is directed to treatment of a subject having or suspected of having a cancer comprising administering to the subject a monoclonal antibody and NK-92 expressing Fc receptor.

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GENETICALLY MODIFIED NK-92 CELLS AND MONOCLONAL ANTIBODIES FOR THE TREATMENT OF CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority benefit of U.S. provisional application no. 62/139,258, filed March 27, 2015 where is incorporated by reference herein.

BACKGROUND OF THE INVENTION

[0002] Anticancer treatment with monoclonal antibodies (mAbs) has significantly improved the clinical outcome in patients with cancer, especially when combined with chemotherapy. However, often the patients ultimately relapse. Natural killer cells could also be used as cytotoxic effector cells for cell-based immunotherapy.

[0003] NK-92 is a cytolytic cancer cell line which was discovered in the blood of a subject suffering from a non-Hodgkins lymphoma and then immortalized *ex vivo*. NK-92 cells are derived from NK cells, but lack the major inhibitory receptors that are displayed by normal NK cells, while retaining the majority of the activating receptors. NK-92 cells do not, however, attack normal cells nor do they elicit an unacceptable immune rejection response in humans. Characterization of the NK-92 cell line is disclosed in WO 1998/49268 and U.S. Patent Application Publication No. 2002-0068044. NK-92 cells have also been evaluated as a potential therapeutic agent in the treatment of certain cancers.

[0004] Although NK-92 cells retain almost all of the activating receptors and cytolytic pathways associated with NK cells, they do not express CD16 on their cell surfaces. CD16 is an Fc receptor which recognizes and binds to the Fc portion of an antibody to activate NK cells for antibody-dependent cellular cytotoxicity (ADCC). Due to the absence of CD16 receptors, NK-92 cells are unable to lyse target cells via the ADCC mechanism.

[0005] The present invention provides a solution to the aforementioned problems, by augmenting the cytotoxic effect of some molecular antibodies by simultaneously or consequently administering to a subject in need of anticancer treatment NK-92 cells that express Fc receptors.

BRIEF SUMMARY OF ASPECTS OF THE INVENTION

[0006] In one aspect, the inventioncomprises co-administering to a subject in need of anticancer treatment a monoclonal antibody having cytotoxic effects on the target cancer cells and NK-92 cells engineered express an Fc receptor. This combination synergizes the anti-cancer effects of NK cells with the anticancer effects of therapeutic antibodies.

[0007] Thus, in one embodiment, the invention provides a method for treating cancer in a subject in need thereof comprising administering to the subject a monoclonal antibody having a cytotoxic effect on the target cancer cell and FcR-expressing NK-92 cells. In some embodiments, the FcR is CD16. In one aspect of the invention, the NK-92 cells are genetically modified to express an Fc receptor encoding a polypeptide having at least 90% sequence identity with SEQ ID NO:1 (FCγRIII-A or CD16 having a phenylalanine at position 158 (F-158); or at least 90% identity to SEQ ID NO:2 (CD16 having a valine at position 158 (F158V), higher affinity form). In typical embodiments, the CD16 polypeptide has a valine at position 158.

[0008] In further embodiments, the NK-92 cells are additionally modified to express a cytokine, such as IL-2. In some embodiments, the cytokine is targeted to the endoplasmic reticulum. In specific embodiments, the cytokine is interleukin-2 or a variant thereof, that is targeted to the endoplasmic reticulum. In some embodiments, the NK-92 cells are modified to express a polypeptide having a sequence of SEQ ID NO:7.

[0009] In other embodiments, the NK-92 cells are further modified to express a suicide gene. In one aspect, the suicide gene is iCas9.

[0010] The compositions of the invention are useful for the treatment of cancer, including, but not limited to, cancers such as multiple myeloma, leukemias, lymphomas, metastatic breast cancer or gastric carcinoma.

[0011] The monoclonal antibody that is administered to the patient can be a naked monoclonal antibody, a conjugated monoclonal antibody, or a bispecific monoclonal antibody. In some embodiments, the monoclonal antibody is alemtuzumab, rituxumab, trastuzumab, ibritumomab, gemtuzumab, brentuximab, adotranstuzumab, blinatunomab, daratumumab or elotuzumab.

[0012] In some embodiments, the monoclonal antibody and the FcR-expressing NK-92 cells are administered simultaneously to the subject. In other embodiments, the subject is

administered the monoclonal antibody and subsequently administered the FcR-expressing NK-92 cells, *e.g.*, within 24 hours; or within 24 to 72 hours, after administration of the monoclonal antibody.

[0013] In some aspects, the invention relates to use of an NK-92 cells genetically modified to express an FcR, such as CD16, with a cytotoxic monoclonal antibody of the treatment of cancer. Thus, in some embodiments the invention provides use of NK-92 cells that are genetically modified to express CD16 with a cytotoxic monoclonal antibody for a patient that has cancer. In some embodiments, the Fc receptor is a CD16 having a valine at position 158 of the mature form of CD16. In some embodiments, the Fc receptor comprises a polynucleotide sequence encoding a polypeptide having at least 90% sequence identity with SEQ ID NO:1 or SEQ ID NO:2, or the polynucleotide encodes SEQ ID NO:1 or SEQ ID NO:2. In some embodiments, the FcR-expressing NK-92 cells are genetically modified to express a cytokine such as interleukin-2 or a variant thereof. In some embodiments, the interleukin-2 is targeted to the endoplasmic reticulum. In some embodiments, the FcR-expressing NK-92 cells are modified to express an interleukin-2 sequence as set forth in SEQ ID NO:7. In some embodiments, the Fc receptor and at least one cytokine are encoded by different vectors. Alternatively, the Fc receptor and at least one cytokine are encoded by the same vector. In some embodiments, the Fc receptor comprises a CD16 polypeptide having a V at position 158 and the NK-92 cells are further genetically modified to express human interleukin-2, wherein the interleukin 2 is targeted to the endoplasmic reticulum. The FcR-expressing NK-92 cells may also be further modified to express a suicide gene, such as iCas9. In some embodiments, the cancer is leukemia, non-Hodgkin's lymphoma, metastatic breast cancer or gastric carcinoma. The monoclonal antibody may be a naked monoclonal antibody, a conjugated monoclonal antibody, or a bispecific monoclonal antibody. In some embodiments, the monoclonal antibody is alemtuzumab, rituxumab, trastuzumab, ibritumomab, brentuximab, gemtuzumab, adotranstuzumab, blinatunomab, avelumamab, daratumumab or elotuzumab. In some embodiments, the monoclonal antibody and the FcR-expressing NK-92 cells are administered simultaneously to the subject. In some embodiments, the subject is administered the monoclonal antibody and subsequently treated with the genetically modified FcR-expressing NK-92 cells. In some embodiments, the monoclonal antibody is injected intravenously into the subject. In other embodiments, the genetically modified FcR-expressing NK-92 cells are injected into the bone marrow.

[0014] The foregoing general description and the following detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed. Other objects, advantages and novel features will be readily apparent to those skilled in the art from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] The objects, features and advantages of the present invention will be more readily appreciated upon reference to the following disclosure when considered in conjunction with the accompanying drawings.

[0016] Figure 1 shows a schematic representation of a plasmid expressing a modified form of IL-2 with ERRS (endoplasmic reticulum retention signal) and CD16.

[0017] Figure 2a and 2b provide illustrative data showing expression of CD16 is NK-92 cells modified to express CD16 using a plasmid vector depicted in Figure 1 at about 2 weeks (Figure 2a) and about 4 weeks (Figure 2b).

[0018] Figure 3 provides illustrative data showing CD16 expression in modified NK-92 cells that were frozen for storing and then thawed for culture.

[0019] Figure 4 provides illustrative data showing ADCC activity of CD16-expressing NK-92 cells used in combination with monoclonal antibodies.

DETAILED DESCRIPTION OF THE INVENTION

[0020] In one aspect, the disclosure relates to the use of NK-92 cells modified to express FcR and monoclonal antibodies for the treatment of cancer in a subject in need thereof. Malignant cells are able to develop mechanisms to escape the immunological protection that innate immune cells, such as dendritic cells and natural killer cells, and adaptive immune cells, such as T cells and B cells, provide. There is therefore an urgent need for reducing incidence of tumor relapse in subjects having cancer or suspected of having cancer.

[0021] NK-92 cells present the attractive feature that they can easily be propagated and expanded *in vitro*. However, they do not express the IgG Fc receptor FcγRIII, and thus these cells are unable to act via antibody-dependent cell-mediated cytotoxicity (ADCC). The present invention is based on the predicament that genetic transformation of the NK-92 cells to express the IgG Fc receptor FcγRIII would enhance NK-tumor cell interaction and allow the NK cells to work in unison with monoclonal antibodies that kill target cells through

ADCC. Thus, the separate cytotoxic effect of NK-92 cells and monoclonal antibodies may be augmented when the monoclonal antibodies and the NK-92 cells are administered simultaneously or in close temporal relation to a subject that has cancer or is otherwise in need of cancer treatment.

[0022] Accordingly, the present invention provides for the use of NK-92 cells that are genetically modified to express the high affinity form of the transmembrane immunoglobulin γ Fc region receptor III-A (FcγRIII-A or CD16 in which a valine is present at position 158 of the mature form of the polypeptide).

[0023] In some embodiments the FcR-expressing NK-92 cells may be further modified to express IL-2. In such cells, the expression of IL-2 in the cells is typically directed to the endoplasmic reticulum. This feature prevents undesirable effects of systemic administration of IL-2, such as toxicity affecting the cardiovascular, gastrointestinal, respiratory and nervous systems. In some embodiments, when the FcR-expressing NK-92 cells are further modified to express IL-2, a suicide gene may also be inserted into these cells to prevent unregulated endogenous expression of IL-2, that could lead to the potential development of mutants with autonomous growth. In some embodiments, the suicide gene is iCas9.

[0024] The FcR-expressing NK-92 cells produced according to the invention are administered in conjunction with a monoclonal antibody targeting cancerous cells to a subject having or suspected of having cancer for effective treatment of cancerous diseases.

[0025] Administration of the FcR-expressing NK-92 cells may be carried out simultaneously with the administration of the monoclonal antibody, or in a sequential manner. In some embodiments, the FcR-expressing NK-92 cells are administered to the subject within 24 hours after the subject has been treated with the monoclonal antibody.

Terminology

[0026] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0027] In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings:

[0028] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0029] All numerical designations, e.g., pH, temperature, time, concentration, amounts, an molecular weight, including ranges, are approximations which are varied (+) or (-) by increments of 0.1 or 1.0, where appropriate. It is to be understood, although not always explicitly stated, that all numerical designations may be preceded by the term "about." It is also to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are known in the art.

[0030] "Optional" or "optionally" means that the subsequently described event or circumstance can or cannot occur, and that the description includes instances where the event or circumstance occurs and instances where it does not.

[0031] The term "comprising" is intended to mean that the compositions and methods include the recited elements, but do not exclude others. "Consisting essentially of" when used to define compositions and methods, refers to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s) of the claimed invention. "Consisting of" shall mean excluding more than trace amounts of other ingredients and substantial method steps recited. Embodiments defined by each of these transition terms are within the scope of this invention.

[0032] As used to describe the present invention, "immunotherapy" refers to the use of NK-92 cells, modified or unmodified, in combination with antibody, naturally occurring or modified NK cell or T-cell, whether alone or in combination, and which are capable of inducing cytotoxicity when contacting a target cell.

[0033] As used to describe the present invention, "natural killer (NK) cells" are cells of the immune system that kill target cells in the absence of a specific antigenic stimulus, and without restriction according to MHC class. Target cells may be tumor cells or cells harboring viruses. NK cells are characterized by the presence of CD56 and the absence of CD3 surface markers.

[0034] The term "endogenous NK cells" is used to refer to NK cells derived from a donor (or the patient), as distinguished from the NK-92 cell line. Endogenous NK cells are

generally heterogeneous populations of cells within which NK cells have been enriched. Endogenous NK cells may be intended for autologous or allogeneic treatment of a patient.

[0035] "NK-92 cells" refer to the immortal NK cell line, NK-92, which was originally obtained from a patient having non-Hodgkin's lymphoma. For purposes of this invention and unless indicated otherwise, the term "NK-92" is intended to refer to the original NK-92 cell lines as well as NK-92 cell lines that have been modified (*e.g.*, by introduction of exogenous genes). NK-92 cells and exemplary and non-limiting modifications thereof are described in U.S. Patent Nos. 7,618,817; 8,034,332; and 8,313,943, all of which are incorporated herein by reference in their entireties.

[0036] "Modified NK-92 cell" refers to an NK-92 cell that further comprises a vector that encodes for transgenes, including CD16. In some embodiments, the modified FcR-expressing NK-92 cells may be further modified to express a cytokine such as IL-2, and/or suicide genes.

[0037] As used herein, "non-irradiated NK-92 cells" are NK-92 cells that have not been irradiated. Irradiation renders the cells incapable of growth and proliferation. In some embodiments, it is envisioned that the NK-92 cells for administration will be irradiated at a treatment facility or some other point prior to treatment of a patient, since the time between irradiation and infusion should be no longer than four hours in order to preserve optimal activity. Alternatively, NK-92 cells may be inactivated by another mechanism.

[0038] As used to describe the present invention, "inactivation" of the NK-92 cells renders them incapable of growth. Inactivation may also relate to the death of the NK-92 cells. It is envisioned that the NK-92 cells may be inactivated after they have effectively purged an *ex vivo* sample of cells related to a pathology in a therapeutic application, or after they have resided within the body of a mammal a sufficient period of time to effectively kill many or all target cells residing within the body. Inactivation may be induced, by way of non-limiting example, by administering an inactivating agent to which the NK-92 cells are sensitive.

[0039] As used to describe the present invention, the terms "cytotoxic" and "cytolytic", when used to describe the activity of effector cells such as NK cells, are intended to be synonymous. In general, cytotoxic activity relates to killing of target cells by any of a variety of biological, biochemical, or biophysical mechanisms. Cytolysis refers more specifically to activity in which the effector lyses the plasma membrane of the target cell, thereby destroying

its physical integrity. This results in the killing of the target cell. Without wishing to be bound by theory, it is believed that the cytotoxic effect of NK cells is due to cytolysis.

[0040] The term "kill" with respect to a cell/cell population is directed to include any type of manipulation that will lead to the death of that cell/cell population.

[0041] The term "Fc receptor" refers to a protein found on the surface of certain cells (e.g., natural killer cells) that contribute to the protective functions of the immune cells by binding to part of an antibody known as the Fc region. Binding of the Fc region of an antibody to the Fc receptor (FcR) of a cell stimulates phagocytic or cytotoxic activity of a cell via antibody-mediated phagocytosis or antibody-dependent cell-mediated cytotoxicity (ADCC). FcRs are classified based on the type of antibody they recognize. For example, Fc-gamma receptors (FCγR) bind to the IgG class of antibodies. FCγRIII-A (also called CD16) is a low affinity Fc receptor bind to IgG antibodies and activate ADCC. FCγRIII-A are typically found on NK cells. A representative polynucleotide sequence encoding a native form of CD16 is shown in SEQ ID NO:5.

[0042] The terms "polynucleotide", "nucleic acid" and "oligonucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three dimensional structure and may perform any function, known or unknown. The following are non limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polynucleotide. The sequence of nucleotides can be interrupted by non nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component. The term also refers to both double and single stranded molecules. Unless otherwise specified or required, any embodiment of this invention that is a polynucleotide encompasses both the double stranded form and each of two complementary single stranded forms known or predicted to make up the double stranded form.

[0043] A polynucleotide is composed of a specific sequence of four nucleotide bases: adenine (A); cytosine (C); guanine (G); thymine (T); and uracil (U) for thymine when the polynucleotide is RNA. Thus, the term "polynucleotide sequence" is the alphabetical representation of a polynucleotide molecule.

[0044] As used herein, "percent identity" refers to sequence identity between two peptides or between two nucleic acid molecules. Percent identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are identical at that position. As used herein, the phrase "homologous" or "variant" nucleotide sequence," or "homologous" or "variant" amino acid sequence refers to sequences characterized by identity, at the nucleotide level or amino acid level, of at least a specified percentage. Homologous nucleotide sequences include those sequences coding for naturally occurring allelic variants and mutations of the nucleotide sequences set forth herein. Homologous nucleotide sequences include nucleotide sequences encoding for a protein of a mammalian species other than humans. Homologous amino acid sequences include those amino acid sequences which contain conservative amino acid substitutions and which polypeptides have the same binding and/or activity. In some embodiments, a homologous nucleotide or amino acid sequence has at least 60% or greater, for example at least 70%, or at least 80%, at least 85% or greater, with a comparator sequence. In some embodiments, a homologous nucleotide or amino aicd sequence has at leaset 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% dentity with a comparator sequence. In some embodiments, a homologous amino acid sequence has no more than 15, nor more than 10, nor more than 5 or no more than 3 conservative amino acid substitutions. Percent identity can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for UNIX, Genetics Computer Group, University Research Park, Madison Wis.), using default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482-489).

[0045] The term "express" refers to the production of a gene product. The term "transient" when referred to expression means a polynucleotide is not incorporated into the genome of the cell.

[0046] The term "cytokine" or "cytokines" refers to the general class of biological molecules which effect cells of the immune system. Exemplary cytokines for use in

practicing the invention include but are not limited to interferons and interleukins (IL), in particular IL-2, IL-12, IL-15, IL-18 and IL-21. In preferred embodiments, the cytokine is IL-2.

[0047] As used herein, the term "vector" refers to a non-chromosomal nucleic acid comprising an intact replicon such that the vector may be replicated when placed within a permissive cell, for example by a process of transformation. A vector may replicate in one cell type, such as bacteria, but have limited ability to replicate in another cell, such as mammalian cells. Vectors may be viral or non-viral. Exemplary non-viral vectors for delivering nucleic acid include naked DNA; DNA complexed with cationic lipids, alone or in combination with cationic polymers; anionic and cationic liposomes; DNA-protein complexes and particles comprising DNA condensed with cationic polymers such as heterogeneous polylysine, defined-length oligopeptides, and polyethylene imine, in some cases contained in liposomes; and the use of ternary complexes comprising a virus and polylysine-DNA.

[0048] As used herein, the term "targeted" is intended to include, but is not limited to, directing proteins or polypeptides to appropriate destinations in the cell or outside of it. The targeting is typically achieved through signal peptides or targeting peptides, which are a stretch of amino acid residues in a polypeptide chain. These signal peptides can be located anywhere within a polypeptide sequence, but are often located on the N-terminus. Polypeptides can also be engineered to have a signal peptide on the C-terminus. Signal peptides can direct a polypeptide for extracellular section, location to plasma membrane, golgi, endosomes, endoplasmic reticulum, and other cellular compartments. For example, polypeptides with a particular amino acid sequence on their C-terminus (e.g., KDEL) are retained in the ER lumen or transported back the ER lumen.

[0049] The term "suicide gene" is one that allows for the negative selection of the cells. A suicide gene is used as a safety system, allowing the cells expressing the gene to be killed by introduction of a selective agent. This is desirable in case the recombinant gene causes a mutation leading to uncontrolled cell growth. A number of suicide gene systems have been identified, including the herpes simplex virus thymidine kinase (TK) gene, the cytosine deaminase gene, the varicella-zoster virus thymidine kinase gene, the nitroreductase gene, the *Escherichia coli* gpt gene, and the *E. coli* Deo gene (also see, for example, Yazawa K, Fisher W E, Brunicardi F C: Current progress in suicide gene therapy for cancer. World J. Surg.

2002 July; 26(7):783-9). In one embodiment, the suicide gene is inducible caspase 9 (iCas9) (Di Stasi, (2011) "Inducible apoptosis as a safety switch for adoptive cell therapy." N Engl J Med 365: 1673–1683. See also Morgan, "Live and Let Die: A New Suicide Gene Therapy Moves to the Clinic" *Molecular Therapy* (2012); 20: 11–13). The TK gene may be a wild-type or mutant TK gene (e.g., tk30, tk75, sr39tk). Cells expressing the TK protein can be killed using ganciclovir.

[0050] The term "monoclonal antibody" as used herein, refers to a pure, target-specific antibody produced from a single clone of cells grown in culture and that is capable of proliferating indefinitely. Monoclonal antibodies that may be used according to the invention include naked antibodies, that attach to and block antigens on cancerous cells. In one embodiment, the naked monoclonal antibody is alemtuzumab, which binds to the CD52 antigen in lymphocytes. Also included in the monoclonal antibodies that may be used according to the invention are conjugated monoclonal antibodies, such as tagged, labeled or loaded antibodies. Specifically, the antibodies may be tagged or loaded with a drug or a toxin, or radioactively labeled. Examples of such antibodies include, but are not limited to, ibritumomab, which targets the CD20 antigen; brentuximab, which targets the CD30 antigen, and trastuzumab, which targets the HER2 protein. Other monoclonal antibodies that may be used according to the invention are bispecific monoclonal antibodies, such as blinatunomab, which targets CD19 in lymphoma cells, and CD3 in T cells.

[0051] The terms "patient," "subject," "individual," and the like are used interchangeably herein, and refer to any animal, or cells thereof whether in vitro or in situ, amenable to the methods described herein. In certain non-limiting embodiments, the patient, subject or individual is a human.

[0052] The term "treating" or "treatment" covers the treatment of a disease or disorder described herein, in a subject, such as a human, and includes: (i) inhibiting a disease or disorder, i.e., arresting its development; (ii) relieving a disease or disorder, i.e., causing regression of the disorder; (iii) slowing progression of the disorder; and/or (iv) inhibiting, relieving, or slowing progression of one or more symptoms of the disease or disorder. The term "administering" or "administration" of a monoclonal antibody or a natural killer cell to a subject includes any route of introducing or delivering the antibody or cells to perform the intended function. Administration can be carried out by any route suitable for the delivery of the cells or monoclonal antibody. Thus, delivery routes can include intravenous,

intramuscular, intraperitoneal, or subcutaneous deliver. In some embodiments a monoclonal antibody and/or NK-92 cells are administered directly to the tumor, *e.g.*, by injection into the tumor. Administration includes self-administration and the administration by another.

NK-92 Cells

[0053] The NK-92 cell line is a unique cell line that was discovered to proliferate in the presence of interleukin 2 (IL-2). Gong et al., *Leukemia* 8:652-658 (1994). These cells have high cytolytic activity against a variety of cancers. The NK-92 cell line is a homogeneous cancerous NK cell population having broad anti-tumor cytotoxicity with predictable yield after expansion. Phase I clinical trials have confirmed its safety profile.

[0054] The NK-92 cell line is found to exhibit the $CD56^{bright}$, CD2, CD7, CD11a, CD28, CD45, and CD54 surface markers. It furthermore does not display the CD1, CD3, CD4, CD5, CD8, CD10, CD14, CD16, CD19, CD20, CD23, and CD34 markers. Growth of NK-92 cells in culture is dependent upon the presence of recombinant interleukin 2 (rIL-2), with a dose as low as 1 IU/mL being sufficient to maintain proliferation. IL-7 and IL-12 do not support long-term growth, nor do other cytokines tested, including IL-1α, IL-6, tumor necrosis factor α, interferon α, and interferon γ. NK-92 has high cytotoxicity even at a low effector:target (E:T) ratio of 1:1. Gong, et al., *supra*. NK-92 cells are deposited with the American Type Culture Collection (ATCC), designation CRL-2407.

[0055] Heretofore, studies on endogenous NK cells have indicated that IL-2 (1000 IU/mL) is critical for NK cell activation during shipment, but that the cells need not be maintained at 37 °C and 5% carbon dioxide. Koepsell, et al., *Transfusion* 53:398-403 (2013).

Suicide gene

[0056] The term "suicide gene" is one that allows for the negative selection of the cells. A suicide gene is used as a safety system, allowing the cells expressing the gene to be killed by introduction of a selective agent. This is desirable in case the recombinant gene causes a mutation leading to uncontrolled cell growth. A number of suicide gene systems have been identified, including the herpes simplex virus thymidine kinase (TK) gene, the cytosine deaminase gene, the varicella-zoster virus thymidine kinase gene, the nitroreductase gene, the *Escherichia coli* gpt gene, and the *E. coli* Deo gene (also see, for example, Yazawa K, Fisher W E, Brunicardi F C: Current progress in suicide gene therapy for cancer. World J. Surg. 2002 July; 26(7):783-9). As used herein, the suicide gene is active in NK-92 cells.

Typically, the suicide gene encodes for a protein that has no ill-effect on the cell but, in the presence of a specific compound, will kill the cell. Thus, the suicide gene is typically part of a system.

[0057] In one embodiment, the suicide gene is the thymidine kinase (TK) gene. The TK gene may be a wild-type or mutant TK gene (*e.g.*, tk30, tk75, sr39tk). Cells expressing the TK protein can be killed using ganciclovir.

[0058] In another embodiment, the suicide gene is Cytosine deaminase which is toxic to cells in the presence of 5-fluorocytosine. Garcia-Sanchez et al. "Cytosine deaminase adenoviral vector and 5-fluorocytosine selectively reduce breast cancer cells 1 million-fold when they contaminate hematopoietic cells: a potential purging method for autologous transplantation." *Blood* 1998 Jul 15;92(2):672-82.

[0059] In another embodiment, the suicide gene is cytochrome P450 which is toxic in the presence of ifosfamide, or cyclophosphamide. See e.g. Touati et al. "A suicide gene therapy combining the improvement of cyclophosphamide tumor cytotoxicity and the development of an anti-tumor immune response." *Curr Gene Ther.* 2014;14(3):236-46.

[0060] In another embodiment, the suicide gene is iCas9. Di Stasi, (2011) "Inducible apoptosis as a safety switch for adoptive cell therapy." N Engl J Med 365: 1673–1683. See also Morgan, "Live and Let Die: A New Suicide Gene Therapy Moves to the Clinic" *Molecular Therapy* (2012); 20: 11–13. The iCas9 protein induces apoptosis in the presence of a small molecule AP1903. AP1903 is biologically inert small molecule, that has been shown in clinical studies to be well tolerated, and has been used in the context of adoptive cell therapy.

Fc receptors

[0061] Fc receptors bind to the Fc portion of antibodies. Several Fc receptors are known, and differ according to their preferred ligand, affinity, expression, and effect following binding to the antibody.

Table 1. Illustrative Fc receptors

Receptor name	Principal antibody ligand	Affinity for ligand	Cell distribution	Effect following binding to antibody

FcγRI (CD64)	IgG1 and IgG3	High (Kd ~ 10^{-9} M)	Macrophages Neutrophils Eosinophils Dendritic cells	Phagocytosis Cell activation Activation of respiratory burst Induction of microbe killing
FcγRIIA (CD32)	IgG	Low (Kd > 10^{-7} M)	Macrophages Neutrophils Eosinophils Platelets Langerhans cells	Phagocytosis Degranulation (eosinophils)
FcγRIIB1 (CD32)	IgG	Low (Kd > 10^{-7} M)	B Cells Mast cells	No phagocytosis Inhibition of cell activity
FcγRIIB2 (CD32)	IgG	Low (Kd > 10^{-7} M)	Macrophages Neutrophils Eosinophils	Phagocytosis Inhibition of cell activity
FcγRIIIA (CD16a)	IgG	Low (Kd > 10^{-6} M)	NK cells Macrophages (certain tissues)	Induction of antibody-dependent cell-mediated cytotoxicity (ADCC) Induction of cytokine release by macrophages
FcγRIIIB (CD16b)	IgG	Low (Kd > 10^{-6} M)	Eosinophils Macrophages Neutrophils Mast cells Follicular dendritic cells	Induction of microbe killing
FcεRI	IgE	High (Kd ~ 10^{-10} M)	Mast cells Eosinophils Basophils Langerhans cells Monocytes	Degranulation Phagocytosis
FcεRII (CD23)	IgE	Low (Kd > 10^{-7} M)	B cells Eosinophils Langerhans cells	Possible adhesion molecule IgE transport across human intestinal epithelium Positive-feedback mechanism to enhance allergic sensitization (B

				cells)
FcαRI (CD89)	IgA	Low (Kd > 10^{-6} M)	Monocytes Macrophages Neutrophils Eosinophils	Phagocytosis Induction of microbe killing
Fcα/μR	IgA and IgM	High for IgM, Mid for IgA	B cells Mesangial cells Macrophages	Endocytosis Induction of microbe killing
FcRn	IgG		Monocytes Macrophages Dendritic cells Epithelial cells Endothelial cells Hepatocytes	Transfers IgG from a mother to fetus through the placenta Transfers IgG from a mother to infant in milk Protects IgG from degradation

[0062] In some embodiments NK-92 cells are modified to express an Fc receptor protein on the cell surface.

[0063] In some embodiments, the Fc receptor is CD16. For purposes of this disclosure, specific amino acid residues of CD16 are designated with reference to SEQ ID NO:2, or to SEQ ID NO:1, which differs at one position relative to SEQ ID NO:2. Thus, an amino acid residue "at position 158" of a CD16 polypeptide in accordance with the invention is the amino acid residue that corresponds to position 158 of SEQ ID NO:2 (or SEQ ID NO:1), when the CD16 polypeptide and SEQ ID NO:2 are maximally aligned. In some embodiments, NK-92 cells are modified to express a human CD16 that has a phenylalanine at position 158 of the mature form of the protein, *e.g.*, SEQ ID NO:1. In typical embodiments, NK-92 cells are modified to express a high affinity form of human CD16 having a valine at position 158 of the mature form of the protein, *e.g.*, SEQ ID NO:2. Position 158 of the mature protein corresponds to position 176 of the CD16 sequence that includes the native signal peptide. In some embodiments, a CD16 polypeptide is encoded by a polynucleotide that encodes the precursor (i.e., has a native signal peptide) polypeptide sequence of SEQ ID NO:3 or of SEQ ID NO:4.

[0064] In some embodiments, a polynucleotide encoding a CD16 polypeptide has at least about 70% polynucleotide sequence identity with a polynucleotide sequence encoding a full-

length, including signal peptide, naturally occuring CD16 that has a phenylalanine at position 176 of the full-length CD16 (which corresponds to position 158 of the mature CD16 protein). In some embodiments, a polynucleotide encoding a CD16 polypeptide has at least about 70% polynucleotide sequence identity with a polynucleotide sequence encoding a full-length, including the signal peptide, naturally occuring CD16 that has a valine at position 176 (which corresponds to position 158 of the mature protein). In some embodiments, a polynucleotide encoding CD16 has at least 70% identity to SEQ ID NO:5 and comprises a codon encoding valine at the position of the polynucleotide that encodes position 176 of the full-length, including the signal peptide, CD16 polypeptide. In some embodiments, a polynucleotide encoding CD16 has at least 90% identity to SEQ ID NO:5 and comprises a codon encoding valine at position 176 of the full-length CD16. In some embodiments, a polynucleotide encoding CD16 comprises SEQ ID NO:5, but with a codon encoding valine at position 176 of the full-length CD16.

[0065] In some embodiments, the CD16 polynucleotide encodes a polypeptide having at least 70%, 80%, 90%, or 95% identity to SEQ ID NO:1 or SEQ ID NO:2. In some embodiments, the polynucleotide encodes a polypeptide having at least 70% identity, or at least 80% identity, to SEQ ID NO:2 and comprises a valine at position 158 as determined with reference to SEQ ID NO:2. In some embodiments, the polynucleotide encodes a polypeptide having at least 90% identity to SEQ ID NO:2 and comprises a valine at position 158 as determined with reference to SEQ ID NO:2. In some embodiments, the polynucleotide encodes a polypeptide having at least 95% identity to SEQ ID NO:2 and comprises a valine at position 2 as determined with reference to SEQ ID NO:2. In some emboidments the polynucletide encodes SEQ ID NO:2. In some embodiments, a CD16 polynucleotide encodes an extracellular domain of CD16 with or without the signal sequence, or any other fragment of a full length CD16, or a chimeric receptor encompassing at least partial sequence of CD16 fused to an amino acid sequence of another protein. In other embodiments, an epitope tag peptide, such as FLAG, myc, polyhistidine, or V5 can be added to the amino terminal domain of the mature polypeptide to assist in cell surface detection by using anti-epitope tag peptide monoclonal or polyclonal antibodies.

[0066] In some embodiments, homologous CD16 polynucleotides may be about 150 to about 700, about 750, or about 800 polynucleotides in length, although CD16 variants having more than 700 to 800 polynucleotides are within the scope of the disclosure.

[0067] Homologous polynucleotide sequences include those that encode polypeptide sequences coding for variants of CD16. Homologous polynucleotide sequences also include naturally occurring allelic variations related to SEQ ID NO:5. Transfection of an NK-92 cell with any polynucleotide encoding a polypeptide having the amino acid sequence shown in either SEQ ID. NO: 1 or SEQ ID NO: 2, a naturally occurring variant thereof, or a sequence that is at least 70 % identical, or at least 80%, 90%, or 95% identical to SEQ ID. NO: 1 or SEQ ID NO: 2 is within the scope of the disclosure. In some embodiments, homologous polynucleotide sequences encode conservative amino acid substitutions in SEQ ID. NO: 1 or SEQ ID NO: 2. In some embodiments, NK-92 cells are transfected using a degenerate homologous CD16 polynucleotide sequence that differs from a native polynucleotide sequence, but encodes the same polypeptide.

[0068] In other examples, cDNA sequences having polymorphisms that change the CD16 amino acid sequences are used to modify the NK-92 cells, such as, for example, the allelic variations among individuals that exhibit genetic polymorphisms in CD16 genes. In other examples, CD16 genes from other species that have a polynucleotide sequence that differs from the sequence of SEQ ID NO:5 are used to modify NK-92 cells.

[0069] In examples, variant polypeptides are made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site direct mutagenesis (Carter, 1986; Zoller and Smith, 1987), cassette mutagenesis, restriction selection mutagenesis (Wells et al., 1985) or other known techniques can be performed on the cloned DNA to produce CD16 variants (Ausubel, 2002; Sambrook and Russell, 2001).

[0070] In some embodiments, a polynucleotide encoding a CD16 is mutated to alter the amino acid sequence encoding for CD16 without altering the function of CD16. For example, polynucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues can be made in SEQ ID NO:1 or SEQ ID NO:2.

[0071] Conservative substitutions in SEQ ID. NO:1 or SEQ ID NO:2, whereby an amino acid of one class is replaced with another amino acid of the same class, fall within the scope of the disclosed CD16 variants as long as the substitution does not materially alter the activity of the polypeptide. Conservative substitutions are well known to one of skill in the art. Non-conservative substitutions that affect(1) the structure of the polypeptide backbone, such as a β-sheet or α-helical conformation, (2) the charge, (3) the hydrophobicity, or (4) the bulk of

the side chain of the target site can modify CD16 polypeptide function or immunological identity. Non-conservative substitutions entail exchanging a member of one of these classes for another class. Substitutions may be introduced into conservative substitution sites or more preferably into non-conserved sites.

[0072] In some embodiments, CD16 polypeptide variants are at least 200 amino acids in length and have at least 70 % amino acid sequence identity, or at least 80%, or at least 90% identity to SEQ ID NO:1 or SEQ ID NO:2. In some embodiments, CD16 polypeptide variants are at least 225 amino acid in length and have at least 70 % amino acid sequence identity, or at least 80%, or at least 90% identity to SEQ ID NO:1 or SEQ ID NO:2. In some embodiments, CD16 polypeptide variants have a valine at position 158 as determined with reference to SEQ ID NO:2.

[0073] In some embodiments a nucleic acid encoding a CD16 polypeptide may encode a CD16 fusion protein. A CD16 fusion polypeptide includes any portion of CD16 or an entire CD16 fused with a non-CD16 polypeptide. Fusion polypeptides are conveniently created using recombinant methods. For example, a polynucleotide encoding a CD16 polypeptide such as SEQ ID NO:1 or SEQ ID NO:2 is fused in-frame with a non-CD16 encoding polynucleotide (such as a polynucleotide sequence encoding a signal peptide of a heterologous protein). In some embodiment, a fusion polypeptide may be created in which a heterologous polypeptide sequence is fused to the C-terminus of CD16 or is positioned internally in the CD16. Typically, up to about 30 % of the CD16 cytoplasmic domain may be replaced. Such modification can enhance expression or enhance cytotoxicity (e.g., ADCC responsiveness). In other examples, chimeric proteins, such as domains from other lymphocyte activating receptors, including but not limited to Ig-a, Ig-B, CD3-e, CD3-d, DAP-12 and DAP-10, replace a portion of the CD16 cytoplasmic domain.

[0074] Fusion genes can be synthesized by conventional techniques, including automated DNA synthesizers and PCR amplification using anchor primers that give rise to complementary overhangs between two consecutive gene fragments that can subsequently be annealed and reamplified to generate a chimeric gene sequence (Ausubel, 2002). Many vectors are commercially available that facilitate sub-cloning CD16 in-frame to a fusion moiety.

Cytokines

[0075] The cytotoxicity of NK-92 cells is dependent on the presence of cytokines (e.g., interleukin-2 (IL-2)). The cost of using exogenously added IL-2 needed to maintain and expand NK-92 cells in commercial scale culture is significant. The administration of IL-2 to human subjects in sufficient quantity to continue activation of NK92 cells would cause adverse side effects.

[0076] In some embodiments, FcR-expressing NK-92 cells are further modified to express at least one cytokine and a suicide gene. In specifici embodiments, the at least one cytokine is IL-2, IL-12, IL-15, IL-18, IL-21 or a variant thereof. In preferred embodiments, the cytokine is IL-2. In certain embodiments the IL-2 is a variant that is targeted to the endoplasmic reticulum, and the suicide gene is iCas9.

[0077] In one embodiment, the IL-2 is expressed with a signal sequence that directs the IL-2 to the endoplasmic reticulum. In some embodiments, a polynucleotide that encodes IL-2 encodes a polypeptide having a sequence of SEQ ID NO:7. Not to be bound by theory, but directing the IL-2 to the endoplasmic reticulum permits expression of IL-2 at levels sufficient for autocrine activation, but without releasing IL-2 extracellularly. See Konstantinidis et al "Targeting IL-2 to the endoplasmic reticulum confines autocrine growth stimulation to NK-92 cells" *Exp Hematol.* 2005 Feb;33(2):159-64. Continuous activation of the FcR-expressing NK-92 cells can be prevented, *e.g.*, by the presence of the suicide gene.

Immunotherapy

[0078] Antibodies may be used to target cells that are infected or express cancer-associated markers. A number of antibodies have been approved for the treatment of cancer, alone.

Table 2. Illustrative therapeutic monoclonal antibodies

Examples of FDA-approved therapeutic monoclonal antibodies				
Antibody	**Brand name**	**Company**	**Target**	**Indication (Targeted disease)**
Alemtuzumab	Campath®	Genzyme	CD52	Chronic lymphocytic leukemia
Brentuximab vedotin	Adcetris®		CD30	Anaplastic large cell lymphoma (ALCL) and Hodgkin

Examples of FDA-approved therapeutic monoclonal antibodies

Antibody	Brand name	Company	Target	Indication (Targeted disease)
				lymphoma
Cetuximab	Erbitux®	Bristol-Myers Squibb/Eli Lilly/Merck KGaA	epidermal growth factor receptor	Colorectal cancer, Head and neck cancer
Gemtuzumab	Mylotarg®	Wyeth	CD33	Acute myelogenous leukemia (with calicheamicin)
Ibritumomab tiuxetan	Zevalin®	Spectrum Pharmaceuticals, Inc.	CD20	Non-Hodgkin lymphoma (with yttrium-90 or indium-111)
Ipilimumab (MDX-101)	Yervoy®		blocks CTLA-4	Melanoma
Ofatumumab	Arzerra®		CD20	Chronic lymphocytic leukemia
Palivizumab	Synagis®	MedImmune	an epitope of the RSV F protein	Respiratory Syncytial Virus
Panitumumab	Vectibix®	Amgen	epidermal growth factor receptor	Colorectal cancer
Rituximab	Rituxan®, Mabthera®	Biogen Idec/Genentech	CD20	Non-Hodgkin lymphoma
Tositumomab	Bexxar®	GlaxoSmithKline	CD20	Non-Hodgkin lymphoma
Trastuzumab	Herceptin®	Genentech	ErbB2	Breast cancer
Blinatunomab			bispecific CD19-directed CD3 T-cell engager	Philadelphia chromosome-negative relapsed or refractory B cell precursor acute lymphoblastic leukemia (ALL)
Avelumamab			anti-PD-L1	Non-small cell lung cancer, metastatic Merkel cell carcinoma; gastic cancer, breast cancer, ovarian cancer,

Examples of FDA-approved therapeutic monoclonal antibodies

Antibody	Brand name	Company	Target	Indication (Targeted disease)
				bladder cancer, melanoma, meothelioma, including metastatic or locally advanced solid tumors
Daratumumab			CD38	Multiple myeloma
Elotuzumab			a SLAMF7-directed (also known as CD 319) immunostimulatory antibody	Multiple myeloma

[0079] Antibodies may treat cancer through a number of mechanisms. Antibody-dependent cellular cytotoxicity (ADCC) occurs when immune cells, such as NK cells, bind to antibodies that are bound to target cells through Fc receptors, such as CD16.

[0080] Accordingly, in some embodiments, NK-92 cells that express CD16 are administered to a patient along with antibodies directed against a specific cancer-associated protein.

[0081] Administration of the FcR-expressing NK-92 cells may be carried out simultaneously with the administration of the monoclonal antibody, or in a sequential manner. Genetic modification of the NK-92 cells to express the FcR enables the cells to recognize Ab-coated target cells and trigger NK cell-mediated ADCC, thus resulting in rapid NK-cell activation. In some embodiments, the FcR-expressing NK-92 cells are administered to the subject after the subject has been treated with the monoclonal antibody. In some embodiments, the FcR-expressing NK-92 cells are administered within 24 hours, or within 18 hours, or within 12 hours, or within 8 hours or within 6, 5, 4, 3, 2, or 1 hours of administering the monoclonal antibody. In some emodiments, the FcR-expressing NK-92 cells are administered from 24 to 72 hours after administration of the antibody. In some embodiments, the FcR-expressing NK-92 cells are administered within 1, 2, 3, or 4 days, or greater, of administering the antibody.

[0082] In some embodiments, the FcR-expressing NK-92 cells and monoclonal antibody are administered intravenously. In some embodiments the FcR-expressing NK-92 cells are infused directly into the bone marrow.

[0083] In one aspect of the invention, the FcR-expressing NK-92 cells are administered to a subject suffering from leukemia combination with a therapeutic monoclonal antibody, *e.g.*, alemtuzumab. In some embodiments, the FcR-expressing NK-92 cells are administered simultaneously with alemtuzumab. In some embodiments, the FcR-expressing NK-92 cells are administered after the subject has been treated with alemtuzumab. In some embodiments, the FcR-expressing NK-92 cells are administered within 24 hours, or within 18 hours, or within 12 hours, or within 8 hours or within 6, 5, 4, 3, 2, or 1 hour of administration of alemtuzumab. In some embodiments, the FcR-expressing NK-92 cells are administered 24 to 72 hours, or longer, following administration of alemtuzumab.

[0084] In a further aspect, the FcR-expressing NK-92 cells are administered in combination with trastuzumab to a subject suffering from a cancer such as breast cancer or stomach cancer. In some embodiments, the FcR-expressing NK-92 cells are administered simultaneously with trastuzumab. In some embodiments, the FcR-expressing NK-92 cells are administered after trastuzumab. In some embodiments, the FcR-expressoin NK-92 cells are administeredwithin 24 hours, or within 18 hours, or within 12 hours, or within 8 hours or within 6, 5, 4, 3, 2, or 1 hour of administration of trastuzumab. In some embodiments, the FcR-expressing NK-92 cells are administered 24 to 72 hours, or longer, following administration of trastuzumab.

[0085] In an additional aspect, the FcR-expressing NK-92 cells are administered to a subject suffering from Hodgkin lymphoma in combination with brentuximab. In some embodiments, the FcR-expressing NK-92 cells are administered simultaneously with brentuximab. In some embodiments, the FcR-expressing NK-92 cells are administered after brentuximab. In some embodiments, the FcR-expressing NK-92 cells are administeredwithin 24 hours, or within 18 hours, or within 12 hours, or within 8 hours or within 6, 5, 4, 3, 2, or 1 hour of administration of brentuximab. In some embodiments, the FcR-expressing NK-92 cells are administered 24 to 72 hours, or longer, following administration of brentuximab.

[0086] In an additional aspect, the FcR-expressing NK-92 cells are administered to a subject suffering from multiple myeloma in combination with daratumumab. In some embodiments, the FcR-expressing NK-92 cells are administered simultaneously with

daratumumab. In some embodiments, the FcR-expressing NK-92 cells are administered after daratumumab. In some emboidments, the FcR-expressin g NK-92 cells are administered within 24 hours, or within 18 hours, or within 12 hours, or within 8 hours or within 6, 5, 4, 3, 2, or 1 hour of administration of daratumumab. In some embodiments, the FcR-expressing NK-92 cells are administered 24 to 72 hours, or longer, following administration of daratumumab.

Transgene expression

[0087] Transgenes (e.g. CD16 and IL-2) can be engineered into an expression plasmid by any mechanism known to those of skill in the art. Transgenes may be engineered into the same expression plasmid or different. In preferred embodiments, the transgenes are expressed on the same plasmid.

[0088] Transgenes can be introduced into the NK-92 cells using any transient transfection method known in the art, including, for example, electroporation, lipofection, nucleofection, or "gene-gun."

[0089] Any number of vectors can be used to express CD16 and IL-2. In some emboidments, the vector is a retroviral vector. In some emboidments, the vector is a plasmid vector. Other viral vectors that can be used include adenoviral vectors, adeno-assoicated viral vectors, herpes simplex viral vectors, pox viral vectors, and others.

[0090] NK-92 cells can be administered to such an individual by absolute numbers of cells, e.g., said individual can be administered from about 1000 cells/injection to up to about 10 billion cells/injection, such as at about, at least about, or at most about, 1×10^8, 1×10^7, 5×10^7, 1×10^6, 5×10^6, 1×10^5, 5×10^5, 1×10^4, 5×10^4, 1×10^3, 5×10^3 (and so forth) NK-92 cells per injection, or any ranges between any two of the numbers, end points inclusive. In other embodiments, NK-92 cells can be administered to such an individual by relative numbers of cells, e.g., said individual can be administered about 1000 cells to up to about 10 billion cells per kilogram of the individual, such as at about, at least about, or at most about, 1×10^8, 1×10^7, 5×10^7, 1×10^6, 5×10^6, 1×10^5, 5×10^5, 1×10^4, 5×10^4, 1×10^3, 5×10^3 (and so forth) NK-92 cells per kilogram of the individual, or any ranges between any two of the numbers, end points inclusive. In some embodiments, between about 1 billion and about 3 billion NK-92 cells are administered to a patient. In other embodiments, the total dose may be calculated

based on m^2 of body surface area, including 11×10^{11}, 1×10^{10}, 1×10^{9}, 1×10^{8}, 1×10^{7}, per m^2. The average person is 1.6-1.8 m^2.

[0091] The NK-92 cells, monoclonal antibody and/or other anti-cancer agents as described below, can be administered once to a patient with cancer or infected with a virus or can be administered multiple times, *e.g.*, once every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23 hours, or once every 1, 2, 3, 4, 5, 6 or 7 days, or once every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more weeks during therapy, or any ranges between any two of the numbers, end points inclusive.

EXAMPLES

[0092] The following examples are for illustrative purposes only and should not be interpreted as limitations of the claimed invention. There are a variety of alternative techniques and procedures available to those of skill in the art which would similarly permit one to successfully perform the intended invention.

Example 1: CD16 Recombinant Retrovirus Preparation

[0093] CD16 cDNA X52645.1 encoding the low affinity mature form (SEQ ID NO:1) of the transmembrane immunoglobulin γ Fc region receptor III-A (FcγRIII-A or CD16) [Phenylalanine-158 (F158), complete sequence: SwissProt P08637 (SEQ ID NO:3)] or a polymorphic variant encoding a higher affinity mature form of the CD16 receptor [Valine-158 (F158V) (SEQ ID NO:2), complete sequence: SwissProt VAR_008801 (SEQ ID NO:4)] was sub-cloned into the bi-cistronic retroviral expression vector, pBMN-IRES-EGFP (obtained from G. Nolan, Stanford University, Stanford, Calif.) using the BamHI and NotI restriction sites in accordance with standard methods.

[0094] The recombinant vector was mixed with 10 µL of PLUS™ Reagent (Invitrogen; Carlsbad, Calif.); diluted to 100 µL with pre-warmed, serum-free Opti-MEM® (Invitrogen; MEM, minimum essential media); further diluted by the addition of 8 µL LipofectamineTM (Invitrogen) in 100 µL pre-warmed serum-free Opti-MEM®; and incubated at room temperature for 15 minutes. This mixture was then brought to a total volume of 1 mL by the addition of pre-warmed serum-free Opti-MEM®. Phoenix-Amphotropic packaging cells (obtained from G. Nolan, Stanford University, Stanford, Calif.; (Kinsella and Nolan, 1996)) were grown to 70-80% confluence in a 6-well plate and washed with 6 mL of pre-warmed serum-free Opti-MEM® medium (Invitrogen). After removal of the medium, 1 mL of the

solution of recombinant vector in Lipofectamine™ PLUS™ Reagent was added to each well, and the cells were incubated for at least three hours at 37° C under a 7% CO_2/balance air atmosphere. Four mL of pre-warmed RPMI medium containing 10% fetal bovine serum (FBS) was added to each well, and the cells incubated overnight at 37° C., under a 7% CO_2/balance air atmosphere. The medium was then removed; the cells washed with 6 mL pre-warmed serum-free Opti-MEM®; 2 mL serum-free Opti-MEM® added; and the cells incubated at 37° C., under a 7% CO_2/balance air atmosphere for an additional 48 hours.

[0095] The virus-containing supernate was collected into a 15 mL plastic centrifuge tube; centrifuged at 1300 rpm for 5 minutes to remove cells and cell fragments; and the supernate transferred to another 15 mL plastic centrifuge tube. Immediately before use, 20 μL of PLUS™ Reagent was added to the virus suspension; the mixture incubated at room temperature for 15 minutes; 8 μL Lipofectamine™ added to the mixture; and the mixture incubated for an additional 15 minutes at room temperature.

Example 2: Cloning the IL-2 Gene and the TK Suicide Gene into the CD16 Recombinant Retrovirus

[0096] The thymidine kinase (TK) gene and a KDEL-tagged construct generating ER-resident IL-2 (Konstantinidis et al. 2005 *Experimental Hematology* 33: 159-64) are used to prepare recombinant retroviruses incorporating the gene for the expression of IL-2 and ligate the corresponding cDNAs into the CD16 pBMN-IRES-EGFP vector (Miah and Campbell 2010 *Methods Mol. Biol.* 612: 199–208). The pBMN-IRES-EGFP vector is then transfected into the Phoenix-Amphotropic packaging cell line in the presence of Lipofectamine™ Plus as

Example 3: Retroviral Transduction of TK, CD16 and IL-2 into NK-92 Cells

[0097] NK-92 cells cultured in A-MEM (Sigma; St. Louis, Mo.) supplemented with 12.5% FBS, 12.5% fetal horse serum (FHS) and 500 IU rhIL-2/mL (Chiron; Emeryville, Calif.) are collected by centrifugation at 1300 rpm for 5 minutes, and the cell pellet re-suspended in 10 mL serum-free Opti-MEM® medium. An aliquot of cell suspension containing 5 x 10^4 cells is sedimented at 1300 rpm for 5 minutes; the cell pellet re-suspended in 2 mL of the retrovirus suspension described in Example 1, and the cells plated into 12-well culture plates. The plates are centrifuged at 1800 rpm for 30 minutes and incubated at 37° C under an atmosphere of 7% CO_2/balance air for 3 hours. This cycle of centrifugation and incubation is repeated a second time. The cells are diluted with 8 mL of α-MEM, transferred to a T-25

flask, and incubated at 37° C. under a 7% CO_2/balance air until the cells are confluent. The transduced cells are collected, re-suspended in serum-free Opti-MEM® medium, and sorted on the basis of their level of EGFP expression using a fluorescence activated cell sorter (FACS), EGFP being co-expressed with, and a surrogate marker for, CD16. Cell-surface expression of CD16 is confirmed by immuno-staining the transduced cells with an anti-CD16 antibody. Cell-surface expression of IL-2 is determined by immuno-staining with purified rat anti-human IL-2 antibody, and IL-2 intracellular localization was confirmed by immune-staining with rabbit anti-calreticulin ER-Marker. The transduced cells, designated as NK-92-TK-CD16-IL2, are assayed for cell-surface expression of CD16 and IL-2 intracellular expression before use. The cells are assayed for expression of TK by testing for sensitivity to gangcylovir.

Example 4: Growth of NK-92-TK-CD16-IL-2 and Non-Modified NK-92 Cells in the Presence or Absence of Exogenous IL-2

[0098] NK-92-TK-CD16-IL-2 and non-modified NK-92 cells are initially cultured in the presence of exogenous IL-2 (1,200 IU/mL) for 4 to 5 weeks, and then transferred to an IL-2-free medium and cultured in the absence of exogenous IL-2. Proliferation of these cells is then assessed.

[0099] Surface expression of CD16 and IL-2 is measured by flow cytometry. Flow cytometric analysis performed after 24 hours incubation of NK-92-TK-CD16-IL-2 and non-modified NK-92 cells in the absence of exogenous IL-2 shows similar cytotoxic action in NK-92-TK-CD16-IL-2 and non-modified NK-92 cells, with the NK-92-TK-CD16-IL-2 cells presenting increased CD16 surface expression and much lower surface expression of IL-2 as compared to non-modified NK-92 cells.

[0100] These results are confirmed by experiments that determine whether the NK-92-TK-CD16-IL-2 cells support growth of bystander non-modified NK-92 cells, in which the non-modified NK-92 cells are mixed with NK-92-TK-CD16-IL-2 and co-cultured in the absence of exogenous IL-2. These experiments show that the NK-92-TK-CD16-IL-2 do not support the growth of non-modified NK-92 cells because of minimal release of IL-2 into the medium. In fact, the non-modified NK-92 cells stop proliferating after 48 hours incubation in the absence of exogenous IL-2. In contrast, proliferation of NK-92-TK-CD16-IL-2 cells is still visible after 72 hours incubation.

[0101] Overall, these results show that ER-IL-2 stimulates the growth of NK-92-TK-CD16-IL-2 cells when these cells are maintained in an environment not containing exogenous IL-2.

Example 5: Systemic Toxicity and Expansion of NK-92-TK-CD16-IL-2 cells is Effectively Eliminated by the Suicide Gene

[0102] Endogenous expression of IL-2 may lead to the potential development of killer cell mutants with autonomous growth. *In vivo* expansion of NK-92-TK-CD16-IL-2 cells, NK-92-TK-CD16-IL-2 cells and non-modified NK-92 cells is therefore evaluated. SCID mice are sub-lethally irradiated (250 rad) and separated into two groups. Between 15 and 20 days later, when the tumor is palpable (0.5–0.8 cm in diameter), NK-92-TK-CD16-IL-2 cells are injected intravenously into the first group of irradiated mice, and non-modified NK-92 cells are injected intravenously into the second group of mice. No exogenous cytokines are administered to the mice. Detection of EGFP expression with a fluorescence activated cell sorter (FACS) is used to monitor localization and expansion. Both groups of mice show targeted localization and expansion 24 hours after injection. After 24 hours, the non-modified NK-92 cells in the control mice group stop expanding, whereas the NK-92-TK-CD16-IL-2 cells continue to expand significantly. Forty-eight hours after injection apoptosis of non-modified NK cells in the control mice and exponential expansion of NK-92-TK-CD16-IL-2 cells in the test group of mice are visible. In control mice the tumor quickly reaches a size equal to or greater than 1.2 cm in diameter, and the mice are euthanized.

[0103] Mice from the test group with smaller tumors or complete tumor regression are segregated into two groups to evaluate the functionality of the suicide gene. The mice in the first group are treated with two or three doses of ganciclovir (50 μg) intraperitoneally every other day. The mice in the second group are treated with placebo. Administration of ganciclovir to the mice leads to a significant reduction in NK-92-TK-CD16-IL-2 cells within 24 hours to 72 hours, with the cells returning to a pre-expansion level. Expansion of NK-92-TK-CD16-IL-2 cells continues to increase over time in mice treated with placebo.

[0104] These results show that the presence of the TK gene ensures that the NK-92-TK-CD16-IL-2 remain sensitive to ganciclovir and prevents exponential expansion of NK-92-TK-CD16-IL-2 cells. This,the combination of TK and IL-2 on the same retroviral vector, incorporated into the chromosome of NK92 cells, provides enhanced biological safety. Because the cells are dependent on IL-2, there is a strong selection for retaining the TK-CD16-IL-2 sequence. As such, the cells are sensitive to ganciclovir. Those cells that lose the

TK gene, and become resistant to ganciclovir, would also have lost the IL-2 gene that is necessary for their growth.

Example 6: Cytotoxic Activity of NK-92-TK-CD16-IL-2 Against Different Leukemic Cell Lines

[0105] NK-92-TK-CD16-IL-2 effector cells are washed by suspension in α-MEM (without IL-2) and sedimented at 1300 rpm for 5 minutes. The cell pellet is suspended in α-MEM, cells counted, and aliquots prepared at cell concentrations of 1 x 105/mL (effector to target cell ratio (E:T)=1:1), 5 x 10^5/mL (E:T=5:1), 1 x 10^6/mL (E:T=10:1), 2 x 10^6/mL (E:T=20:1) or as appropriate to the determination being performed.

[0106] The cytotoxic activity of NK-92-TK-CD16-IL-2 effector cells against K562, Daudi, TF-1, AML-193, and SR-91 cells is determined (Gong et al. (1994)). K562 (erythroleukemia) and Daudi (Burkitt) lymphoma cell lines are obtained from ATCC. They are maintained in continuous suspension culture in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). TF-1 is a myelomonocytic cell line (Kitamura et al., *J. Cell Physiol.* 140:323-334 (1989)) that requires the presence of medium containing 2 ng/mL of human GM-CSF. AML-193 is a myeloid cell line that is maintained in the presence of 10% 5637-conditioned medium (Lange et al., *Blood* 70:192-199 (1987)). Both TF-1 and AML-193 cells are obtained from Dr. D. Hogge, Terry Fox Laboratory, University of British Columbia, Vancouver, BC. SR-91 is a cell line with features of early progenitor cells established by Gong et al. (1994) from a patient with acute lymphoblastic leukemia (ALL) (Klingemann et al., *Leuk. Lymphoma*, 12, 463-470 (1994). I t is resistant to both NK and activated-NK (A-NK) cell cytotoxicity. SR-91 is also maintained in RPMI 1640/10% FCS. This cell line can be rendered sensitive to killing by NK-92 by treatment with cytokine.

[0107] The cytotoxic activity of the NK-92-TK-CD16-IL-2 effector cells against these target cells is measured in triplicates in a standard 4-hour 51Cr- release assay in triplicate. Briefly, 1 x 106 NK-92-TK-CD16-IL-2 cells are labeled with 100 µL 51Cr (specific activity of 1 mCi/mL) and incubated for one hour at 37° C. Effector cells are counted using trypan blue dye exclusion and mixed with target cells to obtain an effector: target ratio of 10:1, 3:1, 1:1, and 0.3:1. CellGro medium is used as a negative control, and for positive control, cells are incubated with 1% Triton X. After incubation in a V-bottom-shaped 96-well plate for 4 hours at 37° C, 70 µL of supernatant is aspirated from each well and counted using a Packard Cobra Auto- Gamma 5000 Series counting system (Meriden, CT, USA). The percentage of

spontaneous release is calculated from the following formula: % specific 51Cr release = (sample release – spontaneous release)/(maximum release – spontaneous release) x 100.

[0108] The cytotoxic activity of NK-92-TK-CD16-IL-2 cells against K562 and Daudi cells is significantly higher than the cytotoxic activity of non-modified NK cells-92. The cytolytic activity of NK-92-TK-CD16-IL-2 cells against TF-1 cells and AML-193 cells is less potent but still higher than the cytolytic activity of non-modified NK-92 cells. SR-91 cells are resistant to the cytotoxic effect of both the NK-92-TK-CD16-IL-2 cells and the non-modified NK-92 cells. This lack of cytotoxic activity against SR-91 cells is consistent with the lack in SR-91 cells of adhesion molecules necessary to mediate initial binding with NK-92 cells.

Example 7: Cytolysis of Human Primary Leukemic Cells by NK-92-TK-CD16-IL-2 cells

[0109] Samples are obtained, with informed consent, during routine diagnostic blood studies or bone marrow (BM) aspirates from patients with newly diagnosed or relapsed leukemias. Blast-enriched mononuclear cells are isolated by Ficoll Hypaque (Pharmacia, Piscataway, N.J.) density gradient separation and washed in RPMI 1640 medium. NK-92-TK-CD16-IL-2 cells and non-modified NK-92 cells are cultured and maintained in α-MEM medium supplemented with 12.5% FCS and 12.5% horse serum. The cytotoxic activity of NK-92-TK-CD16-IL-2 cells and non-modified NK-92 cells on the leukemic samples is then compared using a standard 4-hour chromium release assay.

[0110] The cytolytic activity of NK-92-TK-CD16-IL-2 cells against leukemic targets is significantly higher than that of non-modified NK-92 cells. The NK-92-TK-CD16-IL-2 cells of the invention are surprisingly and significantly more effective in lysing patient-derived tumor cells, and exert their effect in a shorter time than non-modified NK-92 cells.

Example 8: Antileukemia Effect of NK-92-TK-CD16-IL-2 Cells in Human Leukemia Xenograft SCID Mice Model

[0111] For study of the in vivo tumoricidal capacity of NK-92-TK-CD16-IL-2 cells, leukemic cells derived from a T-lineage-acute lymphoblastic leukemia (ALL) patient, an acute myeloid leukemia (AML) patient, and a pre-B-ALL patient are adoptively grown and expanded in SCID mice by S.C. inoculation. Leukemic cells recovered from the leukemic nodules in the mice (first passage) are used in these experiments. The SCID mice in each group are inoculated I.P. with 5 x 10^6 leukemic cells from the first passage in 0.2 mL PBS,

and 24 hours later 2 x 10^7 NK-92-TK-CD16-IL-2 cells in 0.4 mL PBS are administered by I.P. injection. The animals receive either 1 dose or a series of 5 doses of NK-92-TK-CD16-IL-2 cells which are administered on days 1, 3, 5, 7, and 9, with and without exogenous IL-2.

[0112] All the human leukemias grow aggressively in SCID mice. Leukemic cells derived from the T-ALL patient, the AML patient and the pre-B-ALL patient are highly sensitive *in vitro* to the NK-92-TK-CD16-IL-2 cells, and non-modified NK-92 cells.

[0113] Treatment with NK-92-TK-CD16-IL-2 cells significantly prolong the life and extend survival of the mice compared to treatment with non-modified NK-92 cells. Several animals that received 5 doses of NK-92-TK-CD16-IL-2 cell injections survive without any signs of leukemia development 6 months after inoculation. Mice treated with NK-92 show initial improvement but leukemic cells are observed in a minority of mice at 6 months.

[0114] These results show that *in vivo* treatment of leukemic tumors with NK-92-CD16-IL-2 cells is very effective and results in prolongation of life and health improvement.

Example 9: ADCC Mediated Cell Lysis

[0115] The activity of several antibodies that are highly selective and effective anti-tumor agents depends at least in part on the binding of natural killer cells to the Fc (constant) portion of the antibody, such that lysis of tumor cells occurs via an antibody-dependent cellular cytotoxicity (ADCC) mechanism. Although NK-92 cells retain almost all of the activating receptors and cytolytic pathways associated with NK cells, they do not express the CD16 receptor and, therefore, cannot lyse target cells via the ADCC mechanism. Transgenic insertion of CD16 expression into NK-92 cells allows NK-92 cells to act via the ADCC mechanism if the cells have sufficient binding affinity for an effective antibody.

[0116] The effect of binding to different antibodies is evaluated in FcR-expressing NK-92 cells that are administered to a subject suffering from leukemia 24 to 72 hours after the subject has been treated with Alemtuzumab. FcR-expressing NK-92 cells and the cytotoxic effect of antibody binding on target cancer cells is compared to the cytotoxic effect of non-modified NK-92 cells. The antibodies and corresponding target cancer cells are selected and assayed according to Table 2.

[0117] The selected target cells are labeled with Na [^{51}Cr] chromate. Aliquots of the ^{51}[Cr]-labeled target cells are further incubated with the selected antibody at multiple

concentrations between 0.01 μg and 5 μg/mL for 15 minutes at room temperature, washed with α-MEM, and adjusted to a concentration of 1 x10^5 cells/mL before use. One-hundred μL of the selected type of target cells and 100 μL of effector cells at cell concentrations of 1 x 10^5 cells/mL (E:T=1:1), 5 x 10^5 cells/mL (E:T=5:1), 1 x 10^6 cells/mL (E:T=10:1), 2 x10^6 cells/mL (E:T=20:1) or as appropriate to the determination being performed are added to each well of a 96-well V-bottom plate. Three to six replicate wells are prepared at each E:T ratio to be evaluated. At least 6 wells are allocated to each of a spontaneous lysis control (effector cells replaced with 100 μL of α-MEM) and total release control (effector cells replaced with 100 μL of 2% Triton X-100 detergent in α-MEM). An additional three wells at each E:T ratio are allocated to "non-ADCC" controls in which the target cells were not exposed to the antibody. An additional 6 or more wells are allocated to the use of unmodified NK-92 effector cells that do not express CD16 as a procedural control and internal standard. The plate is then centrifuged at 500 rpm for 3 minutes and incubated for 4 hours at 37° C. in an atmosphere of 7% CO_2/balance air. At the end of the incubation period, the plate is centrifuged at 1500 rpm for 8 minutes and 100 mL of the supernate is collected from each well for counting in a γ counter as a measure of ^{51}[Cr] release due to cytotoxicity. The percentage of specific lysis is then calculated.

[0118] These assays are repeated with FcR-expressing NK-92 cells expressing varying surface levels of CD16.

[0119] FcR-expressing NK-92 cells show high cytotoxic activity against the target cancer cells in the presence of the selected antibody. Non-modified NK-92 cells show lower cytotoxic activity against the target cancer cells. These results demonstrate that the FcR-expressing NK-92 cells have the ability to act via the ADCC mechanism and thus provide enhanced therapeutic effect against tumor cells in the presence of the antibodies.

Example 10: Combined Antileukemia Effect of FcR-expressing NK-92 Cells and Gemtuzumab in Human Leukemia Xenograft SCID Mice Model

[0120] For study of the *in vivo* tumoricidal capacity of FcR-expressing NK-92 cells, leukemic cells derived from an acute myeloid leukemia (AML) patient are adoptively grown and expanded in SCID mice by S.C. inoculation. Leukemic cells recovered from the leukemic nodules in the mice (first passage) are used in these experiments. The SCID mice in each group are inoculated I.P. with 5 x 10^6 leukemic cells from the first passage in 0.2 mL PBS, and 24 hours later 2 x 10^7 FcR-expressing NK-92 cells in 0.4 mL PBS and

Gemtuzumab are administered by I.P. injection. The animals receive either 1 dose or a series of 5 doses of FcR-expressing NK-92 cells which are administered on days 1, 3, 5, 7, and 9, with or without Gemtuzumab. Control animals are treated with non-modified NK-92 cells with or without Gemtuzumab.

[0121] The human leukemias grow aggressively in SCID mice. Mice treated with the FcR-expressing NK-92 cells in combination with Gemtuzumab show tumor regression and the antitumorogenic effect is higher than in mice treated solely with the FcR-expressing NK-92 cells without Gemtuzumab, and more than the mice treated with NK-92.

[0122] These results show that in vivo treatment of leukemic tumors with FcR-expressing NK-92 cells in combination with monoclonal antibodies, such as Gemtuzumab, is very effective and results in prolongation of life and health improvement.

Example 11: Construction of a plasmid expression vector expression CD16 and endoplasmic reticulum-targeted IL-2

[0123] The Gene String program of GeneArt (Life Technologies) was used to design a plasmid backbone de novo. Its minimal structure includes a colE1 bacterial origin of replication, an Ampicillin resistance cassette, and a mammalian expression cassette composed of an EF1α promoter and an SV40 polyadenylation site, flanking a multiple cloning site (MCS).

[0124] The mammalian expression cassette is flanked by BamHI sites that allow not only linearization of the plasmid but also the removal of all non-eukaryotic sequences.

[0125] The expressed transgene is the human CD16 158V sequence followed by an IRES sequence itself followed by the ERIL-2 sequence (IL-2 KDEL) such that the IL-2 is targeted to the endoplasmic reticulum. Both CD16 and ERIL-2 sequences were codon-optimized by GeneArt to maximize expression in a human. The transgene can be excised using EcoRI and NotI. The resulting mRNA is a bicistronic transcript under the control of the EF1α promoter, with the ERIL-2 translated independently from CD16, under the control of the IRES sequence. A schematic of the plasmid is provided in Figure 1. This plasmid was used to transfect NK-92 cells.

Example 12: Transfection of NK-92.W cells using a plasmid expression vector

[0126] NK-92.W cells is the parental line for most clinical trials to date. One vial of the Bioreliance working cell bank (WCB, p15 11/30/00) was thawed into a T25 flask with 12ml X-Vivo10 5%HS + 500IU/ml rhIL-2, and passaged every 2-4 days (dilution x2 to x4 in fresh X-vivo10 5%HS + IL-2, total of 18-20 split/passages).

[0127] NK-92.W cells for transfection were spun at 500g 10min. The supernatant was discarded and the cells pellet was resuspended in 15ml D-PBS 1x and centrifuged at 500g 10min. Pellet was resuspended in buffer R (Neon kit, Invitrogen) at a cell density of 10e7 cells/ml. NK-92.W cells were electroporated with pNEUKv1 CD16(158V)-ERIL2 plasmid, using a Neon electroporator (5ug DNA for 10e6 cells in 100ul buffer R; 1250V/10ms/3 pulses with 3ml buffer E2 in the electroporation tube). Electroporated cells were incubated overnight in medium + IL-2 (in 6-well plate, 4ml medium/well), and transferred to medium without IL-2 on 10/16/14 (one PBS spin/wash). CD16 expression was assayed using an anti-CD16 antibody (clone 3G8, mouse IgG1k) APC-Cy7-conjugated (Bd Pharmingen). At just over two weeks, about 78% of the cells were CD16 positive (right peak, Figure 2a). About 90% of the cells were positive at about four weeks (right peak, Figure 2b).

[0128] NK-92.W CD16(158V)-ERIL2 cells were frozen (5 vials of ~1x10e6 cells/vial), and on 12/15/14 (5 vials of ~1x10e6 cells/vial). Freezing medium is 10% DMSO, 50% HS, 40%

[0129] Frozen NK-92.W FcR-ERIL2 cells were evaluated. Cells were thawed and cultured in X-Vivo10 5%HS without IL-2 in a T25 flask. Expression of CD16(158V) was followed over time by flow cytometry (Attune) using the anti-CD16 antibody clone 3G8 conjugated to APC-Cy7, using the same settings to allow for comparison of MFI between assays. CD16 expressoin was stable over time (Figure 3).

Example 13: Evaluation of ADCC activity

[0130] ADCC activity was first tested against CD20+ cell line DoHH2 in combination with rituximab. The test was repeated over time (n=9), as well as against the Her2/Neu+ cell line SKOV3 in combination with Herceptin (n=5). The results are shown in Figure 4. The modified NK-92.W cells that expressed CD16 and endoplasmic reticulum-targeted IL-2 (designated HaNK.12/15 in Figure 4) showed enhanced ADCC activity towards SKOV-3 cells when used with Herceptin, and ADCC activity towards DoHH2 cells when used with rituximab. ThehaNK.12/15 cells did not show ADCC activity in the controls (DoHH2 cells,

Herceptin antibody); SKOV-3 cells/rituximab). Unmodified NK-92.2 cells also did not show ADCC activity when administered with antibodies.

[0131] Examples 11-13 thus demonstrate that NK-92 cells that were modified to express CD16 and IL-2 using a plasmid vector exhibited enhanced ADCC activity when used in combination with a monoclonal antibody.

[0132] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, sequence accession numbers, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

Illustrative Sequences

SEQ ID NO: 1 Low Affinity Immunoglobulin Gamma Fc Region Receptor III-A amino acid sequence (mature form). The phenylalanine at position 158 is underlined

Arg Thr Glu Asp Leu Pro Lys Ala Val Val Phe Leu Glu Pro Gln Trp Tyr Arg Val Leu Glu
Lys Asp Ser Val Thr Leu Lys Cys Gln Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp
Phe His Asn Glu Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile Asp Ala Ala Thr Val Asp
Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn Leu Ser Thr Leu Ser Asp Pro Val Gln Leu Glu
Val His Ile Gly Trp Leu Leu Leu Gln Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His
Leu Arg Cys His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn Gly Lys
Gly Arg Lys Tyr Phe His His Asn Ser Asp Phe Tyr Ile Pro Lys Ala Thr Leu Lys Asp Ser Gly
Ser Tyr Phe Cys Arg Gly Leu Phe Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile Thr Ile
Thr Gln Gly Leu Ala Val Ser Thr Ile Ser Ser Phe Phe Pro Pro Gly Tyr Gln Val Ser Phe Cys
Leu Val Met Val Leu Leu Phe Ala Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile
Arg Ser Ser Thr Arg Asp Trp Lys Asp His Lys Phe Lys Trp Arg Lys Asp Pro Gln Asp Lys

SEQ ID NO: 2 High Affinity Variant F158V Immunoglobulin Gamma Fc Region Receptor III-A amino acid sequence (mature form). The valine at position 158 is underlined

Arg Thr Glu Asp Leu Pro Lys Ala Val Val Phe Leu Glu Pro Gln Trp Tyr Arg Val Leu Glu
Lys Asp Ser Val Thr Leu Lys Cys Gln Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp
Phe His Asn Glu Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile Asp Ala Ala Thr Val Asp
Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn Leu Ser Thr Leu Ser Asp Pro Val Gln Leu Glu
Val His Ile Gly Trp Leu Leu Leu Gln Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His
Leu Arg Cys His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn Gly Lys
Gly Arg Lys Tyr Phe His His Asn Ser Asp Phe Tyr Ile Pro Lys Ala Thr Leu Lys Asp Ser Gly
Ser Tyr Phe Cys Arg Gly Leu Val Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile Thr Ile
Thr Gln Gly Leu Ala Val Ser Thr Ile Ser Ser Phe Phe Pro Pro Gly Tyr Gln Val Ser Phe Cys
Leu Val Met Val Leu Leu Phe Ala Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile
Arg Ser Ser Thr Arg Asp Trp Lys Asp His Lys Phe Lys Trp Arg Lys Asp Pro Gln Asp Lys

SEQ ID NO: 3 Low Affinity Immunoglobulin Gamma Fc Region Receptor III-A amino acid sequence (precursor form). Position 176 of the precursor form corresponds to position 158 of the mature form. The Phe at position 176 is underlined.

Met Trp Gln Leu Leu Leu Pro Thr Ala Leu Leu Leu Leu Val Ser Ala Gly Met Arg Thr Glu Asp Leu Pro Lys Ala Val Val Phe Leu Glu Pro Gln Trp Tyr Arg Val Leu Glu Lys Asp Ser Val Thr Leu Lys Cys Gln Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp Phe His Asn Glu Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile Asp Ala Ala Thr Val Asp Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn Leu Ser Thr Leu Ser Asp Pro Val Gln Leu Glu Val His Ile Gly Trp Leu Leu Leu Gln Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His Leu Arg Cys His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn Gly Lys Gly Arg Lys Tyr Phe His His Asn Ser Asp Phe Tyr Ile Pro Lys Ala Thr Leu Lys Asp Ser Gly Ser Tyr Phe Cys Arg Gly Leu <u>Phe</u> Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile Thr Ile Thr Gln Gly Leu Ala Val Ser Thr Ile Ser Ser Phe Phe Pro Pro Gly Tyr Gln Val Ser Phe Cys Leu Val Met Val Leu Leu Phe Ala Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile Arg Ser Ser Thr Arg Asp Trp Lys Asp His Lys Phe Lys Trp Arg Lys Asp Pro Gln Asp Lys

SEQ ID NO: 4 High Affinity Variant Immunoglobulin Gamma Fc Region Receptor III-A amino acid sequence (precursor form). Position 176 of the precursor form corresponds to positions 158 of the mature form. The Val at position 176 is underlined.

Met Trp Gln Leu Leu Leu Pro Thr Ala Leu Leu Leu Leu Val Ser Ala Gly Met Arg Thr Glu Asp Leu Pro Lys Ala Val Val Phe Leu Glu Pro Gln Trp Tyr Arg Val Leu Glu Lys Asp Ser Val Thr Leu Lys Cys Gln Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp Phe His Asn Glu Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile Asp Ala Ala Thr Val Asp Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn Leu Ser Thr Leu Ser Asp Pro Val Gln Leu Glu Val His Ile Gly Trp Leu Leu Leu Gln Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His Leu Arg Cys His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn Gly Lys Gly Arg Lys Tyr Phe His His Asn Ser Asp Phe Tyr Ile Pro Lys Ala Thr Leu Lys Asp Ser Gly Ser Tyr Phe Cys Arg Gly Leu <u>Val</u> Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile Thr Ile Thr Gln Gly Leu Ala Val Ser Thr Ile Ser Ser Phe Phe Pro Pro Gly Tyr Gln Val Ser Phe Cys Leu Val Met Val Leu Leu Phe Ala Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile Arg Ser Ser Thr Arg Asp Trp Lys Asp His Lys Phe Lys Trp Arg Lys Asp Pro Gln Asp Lys

SEQ ID NO: 5 Polynucleotide Encoding the Low Affinity Immunoglobulin Gamma Fc Region Receptor III-A (Precursor) (Encodes phenylalanine at position 158)

atgtggcagc tgctcctccc aactgctctg ctacttctag tttcagctgg catgcggact gaagatctcc caaaggctgt ggtgttcctg gagcctcaat ggtacagggt gctcgagaag gacagtgtga ctctgaagtg ccagggagcc tactccctg aggacaattc cacacagtgg tttcacaatg agagcctcat ctcaagccag gcctcgagct acttcattga cgctgccaca gtcgacgaca gtggagagta caggtgccag acaaacctct ccaccctcag tgacccggtg cagctagaag tccatatcgg ctggctgttg ctccaggccc ctcggtgggt gttcaaggag gaagacccta ttcacctgag gtgtcacagc tggaagaaca ctgctctgca taaggtcaca tatttacaga atggcaaagg caggaagtat tttcatcata attctgactt ctacattcca aaagccacac tcaaagacag cggctcctac ttctgcaggg ggcttttttgg gagtaaaaat gtgtcttcag agactgtgaa catcaccatc actcaaggtt tggcagtgtc aaccatctca tcattctttc cacctgggta ccaagtctct ttctgcttgg tgatggtact ccttttgca gtggacacag gactatattt ctctgtgaag acaaacattc gaagctcaac aagagactgg aaggaccata aatttaaatg gagaaaggac cctcaagaca aatga

SEQ ID NO: 6 Wild-Type IL-2

Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu Val Thr Asn Ser Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr

SEQ ID NO: 7 IL-2-ER

Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu Val Thr Asn Ser Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr Gly Ser Glu Lys Asp Glu Leu

WHAT IS CLAIMED IS:

1. A method for treating cancer in a subject in need there of comprising administering to the subject a monoclonal antibody having a cytotoxic effect and NK-92 cells genetically modified to express a FcR.

2. The method of claim 1, wherein the Fc receptor is a CD16 polypeptide having a valine at position 158 of the mature form of the CD16.

3. The method of claim 1, wherein the Fc receptor comprises a polynucleotide sequence encoding a polypeptide having at least 90% sequence identity to the amino acid sequence of SEQ ID NO:2 and comprises valine at position 158.

4. The method of claim 1, wherein the Fc receptor comprises the amino acid sequence of SEQ ID NO:2.

5. The method of any one of claims 1 to 4, wherein the FcR-expressing NK-92 cells are genetically modified to express a cytokine.

6. The method of claim 5, wherein the cytokine is interleukin-2.

7. The method of claim 8, wherein the interleukin-2 is targeted to the endoplasmic reticulum

8. The method of claim 5, wherein the Fc receptor and at least one cytokine are encoded by different vectors.

9. The method of claim 5, wherein the Fc receptor and at least one cytokine are encoded by the same vector.

10. The method of claim 1, wherein the Fc receptor comprises a CD16 polypeptide comprising the amino acid sequence of SEQ ID NO:2 and the NK-92 cells are further genetically modified to express human interleukin-2, wherein the interleukin 2 is targeted to the endoplasmic reticulum.

11. The method of claim 10, wherein the FcR-expressing NK-92 cells are further modified to express a suicide gene.

12. The method of claim 11, wherein the suicide gene is iCas9.

13. The method of any one of claims 1 to 9, wherein the FcR-expressing NK-02 cells are further modified to express a suicide gene.

14. The method of claim 13, wherein the suicide gene is iCas9.

15. The method of any one of claims 1 to 13, wherein the cancer is multiple myeloma, leukemia, non-Hodgkin's lymphoma, metastatic breast cancer or gastric carcinoma.

16. The method of any one of claims 1 to 15, wherein the monoclonal antibody is a naked monoclonal antibody, a conjugated monoclonal antibody, or a bispecific monoclonal antibody.

17. The method of claim 16, wherein the monoclonal antibody is alemtuzumab, rituxumab, trastuzumab, ibritumomab, brentuximab, gemtuzumab, adotranstuzumab, blinatunomab, avelumamab, daratumumab or elotuzumab.

18. The method of any one of claims 1 to 17, wherein the monoclonal antibody and the FcR-expressing NK-92 cells are administered simultaneously to the subject.

19. The method of any one of claims 1 to 17, wherein the subject is administered the monoclonal antibody and subsequently treated with the FcR-expressing NK-92 cells.

20. The method of claim 18 or 19, wherein the monoclonal antibody is injected intravenously into the subject.

21. The method of claim 18, 19, or 20, wherein the genetically modified NK-92 cells are injected into the bone marrow.

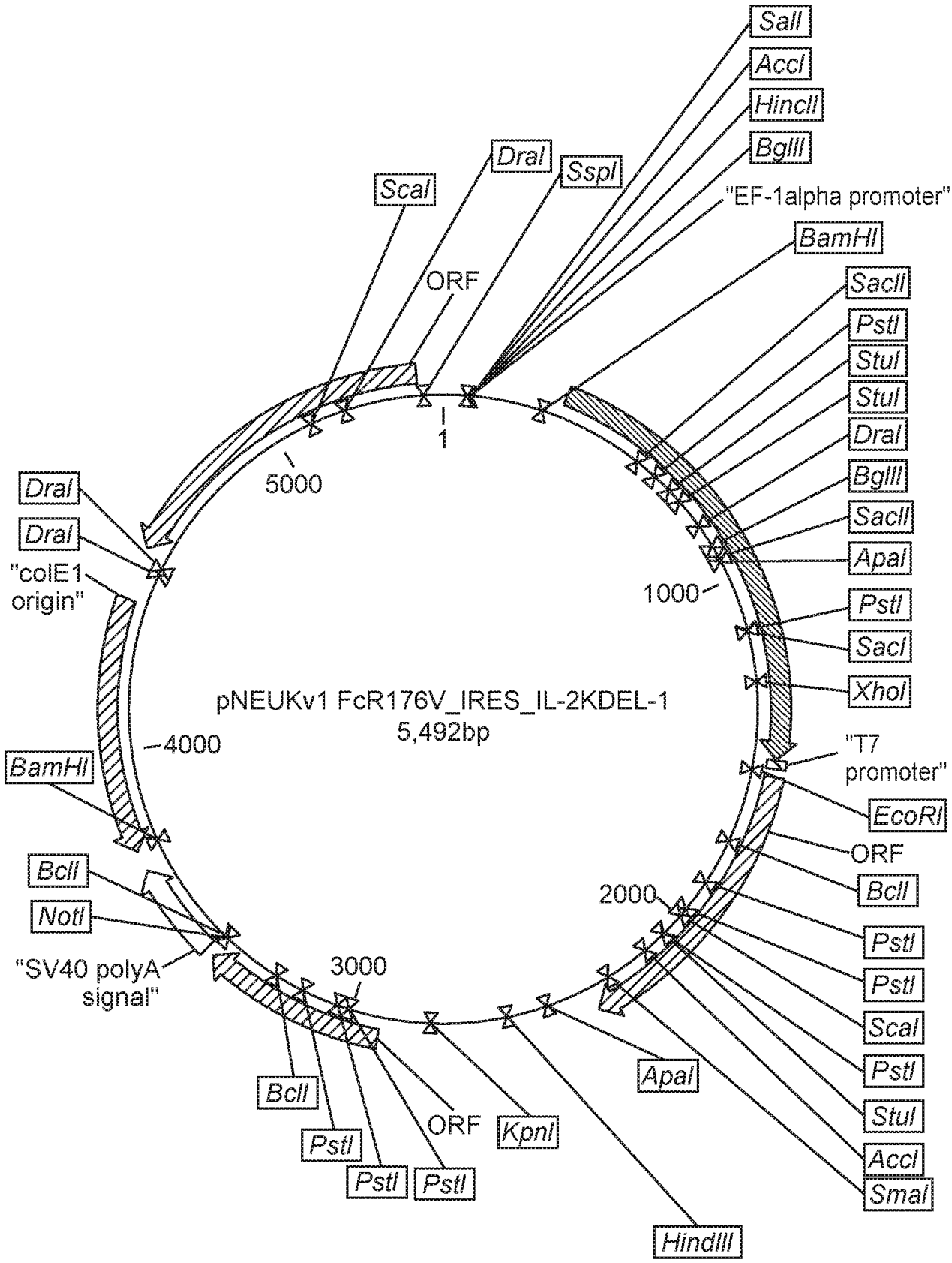
SalI
AccI
HincII
BglII
"EF-1alpha promoter"
BamHI
SacII
PstI
StuI
StuI
DraI
BglII
SacII
ApaI
PstI
SacI
XhoI
"T7 promoter"
EcoRI
ORF
BclI
PstI
PstI
ScaI
PstI
StuI
AccI
SmaI
HindIII
ApaI
KpnI
ORF
PstI
PstI
PstI
BclI
BclI
NotI
"SV40 polyA signal"
BamHI
"colE1 origin"
DraI
DraI
ScaI
DraI
SspI
ORF
1
1000
2000
3000
4000
5000
pNEUKv1 FcR176V_IRES_IL-2KDEL-1
5,492bp

FIG. 1

	Sample Name	Subset Name	Count
☐	NK-92 WCB CD16-APC Cy7.fcs	Cells	23128
☒	NK-92 FcR-IL2 CD16-APC Cy7.fcs	Cells	21703

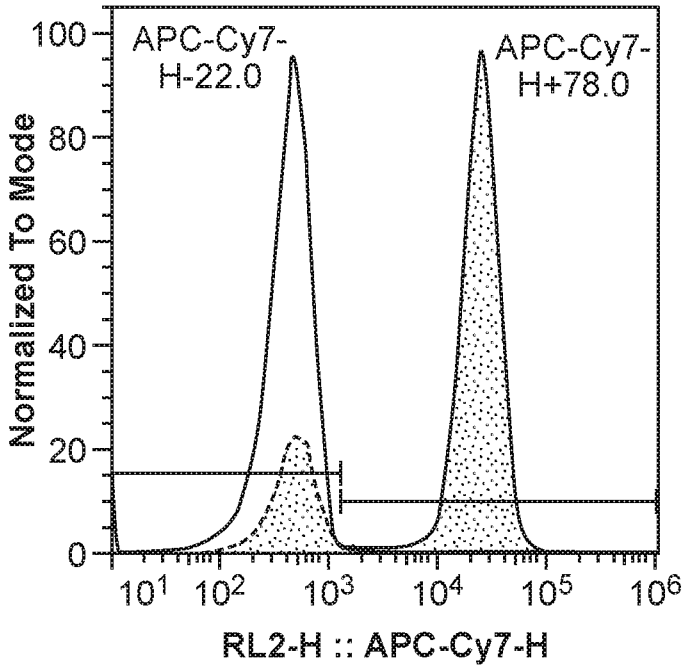

FIG. 2a

	Sample Name	Subset Name	Count
☐	NK-92 wcb CD16-APC Cy7.fcs	NK-92	30151
☒	NK-92 FcRIL CD16-APC Cy7.fcs	NK-92	30226

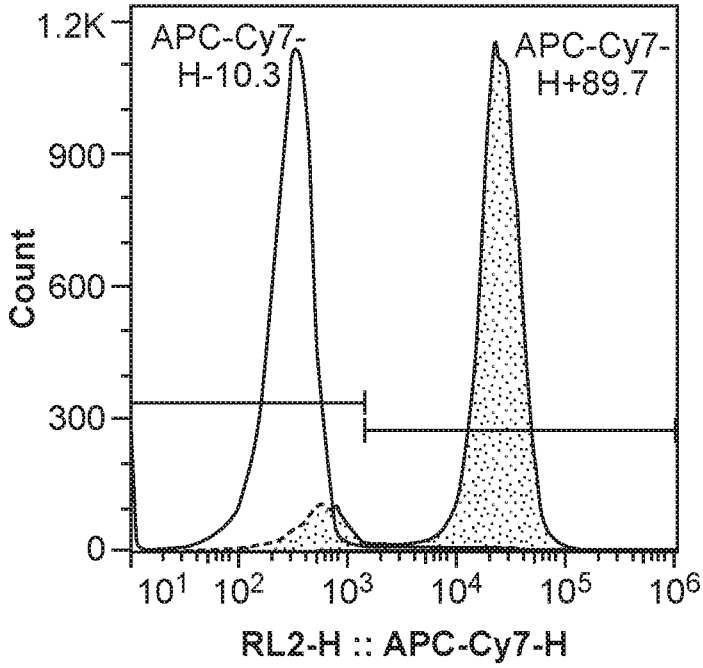

FIG. 2b

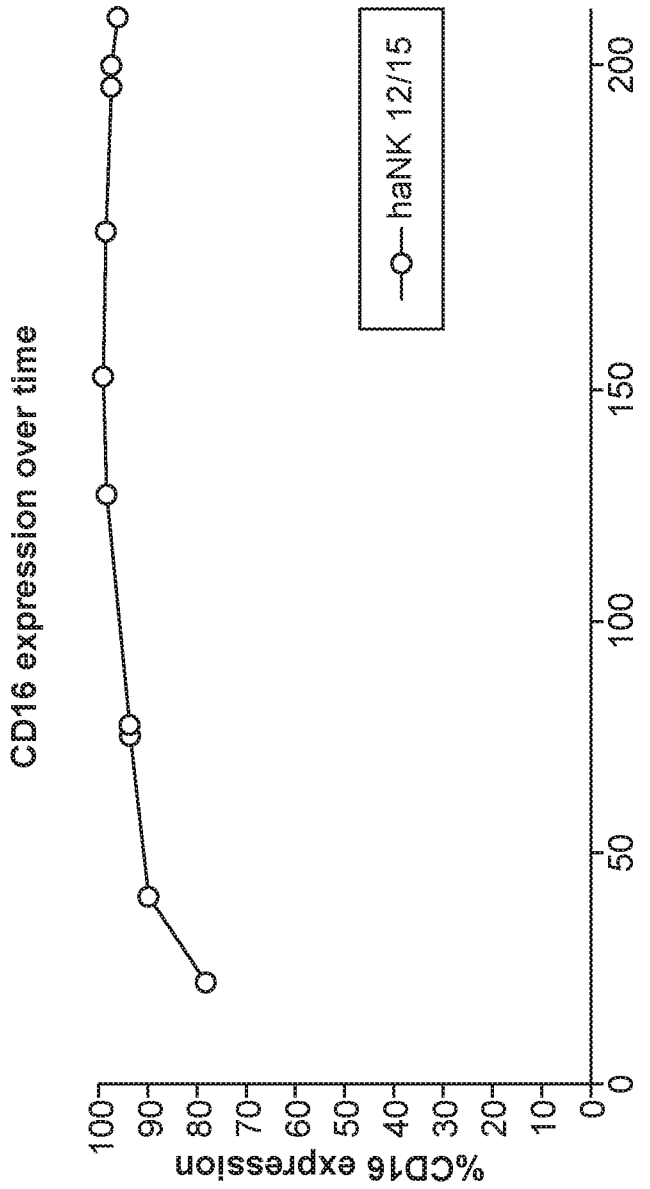
CD16 expression over time
haNK 12/15
%CD16 expression
Days
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200

FIG. 3

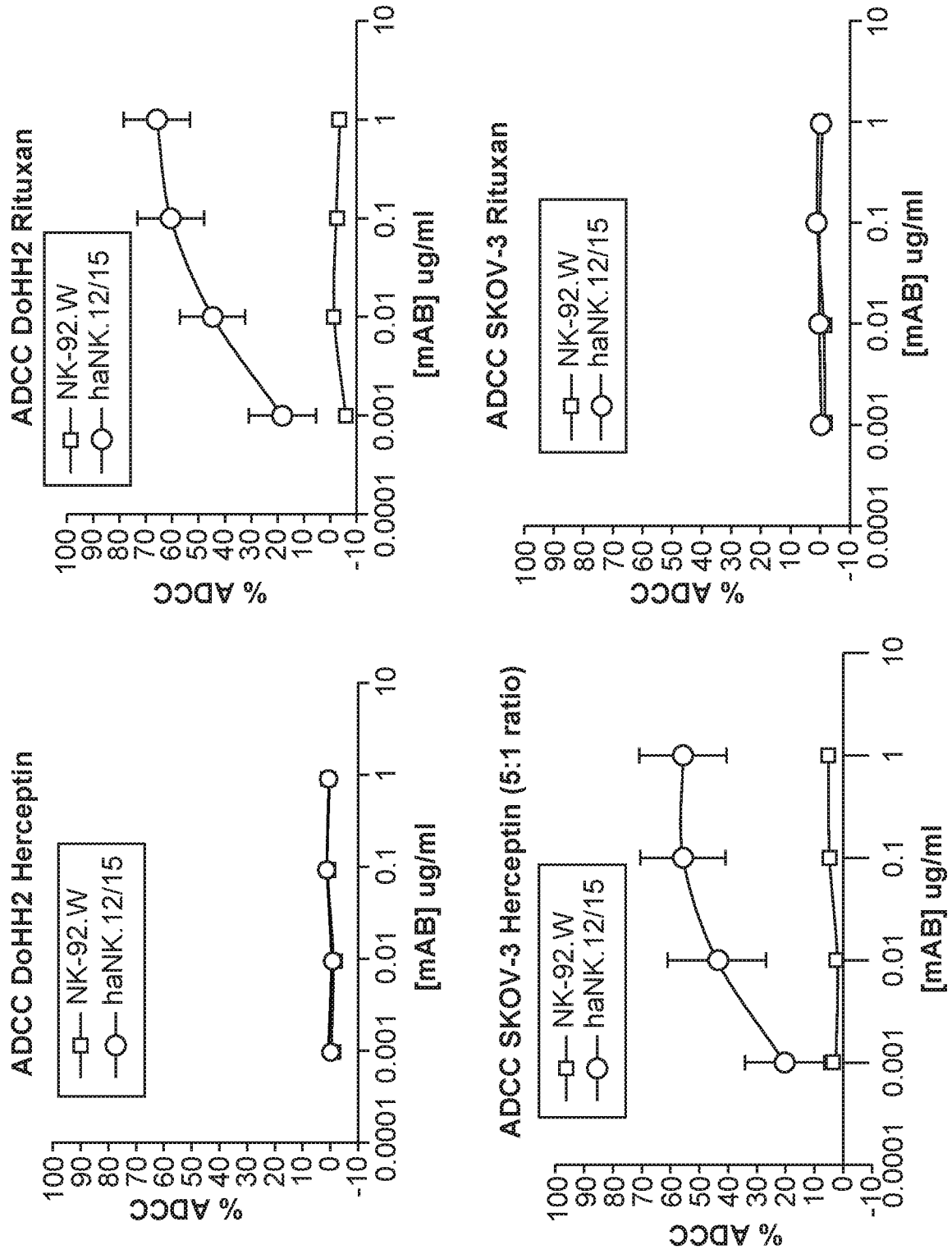
ADCC DoHH2 Rituxan
NK-92.W
haNK.12/15
% ADCC
[mAB] ug/ml
ADCC SKOV-3 Rituxan
ADCC DoHH2 Herceptin
ADCC SKOV-3 Herceptin (5:1 ratio)

FIG. 4

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PATENT COOPERATION TREATY

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

(PCT Article 17(2)(a), Rules 13*ter*.1(c) and (d) and 39)

Applicant's or agent's file reference 1006318	**IMPORTANT DECLARATION**	Date of mailing (*day/month/year*) 21 September 2016 (21.09.2016)
International application No. **PCT/US2016/024318**	International filing date (*day/month/year*) **25 March 2016 (25.03.2016)**	(Earliest) Priority date (*day/month/year*) 27 March 2015 (27.03.2015)

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

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Applicant

NANTKWEST, INC.

This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below.

1. ☒ The subject matter of the international application relates to:
   - a. ☐ scientific theories
   - b. ☐ mathematical theories
   - c. ☐ plant varieties
   - d. ☐ animal varieties
   - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes
   - f. ☐ schemes, rules or methods of doing business
   - g. ☐ schemes, rules or methods of performing purely mental acts
   - h. ☐ schemes, rules or methods of playing games
   - i. ☒ methods for treatment of the human body by surgery or therapy
   - j. ☐ methods for treatment of the animal body by surgery or therapy
   - k. ☐ diagnostic methods practised on the human or animal body
   - l. ☐ mere presentation of information
   - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art
2. ☒ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:
   ☐ the description ☒ the claims ☐ the drawings
3. ☐ A meaningful search could not be carried out without the sequence listing; the applicant did not, within the prescribed time limit:
   - ☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in a form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
   - ☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.
   - ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).
4. Further coments:

Name and mailing address of ISA/KR

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Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon, 35208, Republic of Korea

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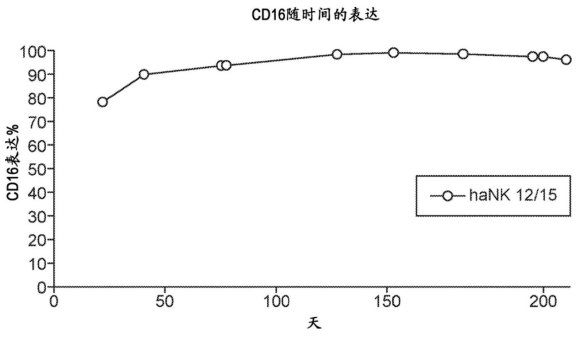
权利要求书1页 说明书25页 附图5页

(54)发明名称

用于治疗癌症的被遗传修饰的NK-92细胞和单克隆抗体

(57)摘要

本发明涉及患有或怀疑患有癌症的受试者的治疗,其包括向受试者施用单克隆抗体和表达Fc受体的NK-92。



CN 107427578 A

1.一种用于在有需要的受试者中治疗癌症的方法,其包括向所述受试者施用具有细胞毒性作用的单克隆抗体和被遗传修饰以表达FcR的NK-92细胞。

2.根据权利要求1所述的方法,其中所述Fc受体是CD16多肽,其在所述CD16的成熟形式的第158位具有缬氨酸。

3.根据权利要求1所述的方法,其中所述Fc受体包含编码与SEQ ID NO:2的氨基酸序列具有至少90%序列同一性并在第158位包含缬氨酸的多肽的多核苷酸序列。

4.根据权利要求1所述的方法,其中所述Fc受体包含SEQ ID NO:2的氨基酸序列。

5.根据权利要求1至4中任一项所述的方法,其中所述表达FcR的NK-92细胞被遗传修饰以表达细胞因子。

6.根据权利要求5所述的方法,其中所述细胞因子是白细胞介素-2。

7.根据权利要求8所述的方法,其中所述白细胞介素-2靶向内质网。

8.根据权利要求5所述的方法,其中所述Fc受体和至少一种细胞因子由不同的载体编码。

9.根据权利要求5所述的方法,其中所述Fc受体和至少一种细胞因子由相同的载体编码。

10.根据权利要求1所述的方法,其中所述Fc受体包含包含SEQ ID NO:2的氨基酸序列的CD16多肽,并且所述NK-92细胞被进一步遗传修饰以表达人白细胞介素-2,其中所述白细胞介素2靶向内质网。

11.根据权利要求10所述的方法,其中所述表达FcR的NK-92细胞被进一步修饰以表达自杀基因。

12.根据权利要求11所述的方法,其中所述自杀基因是iCas9。

13.根据权利要求1至9中任一项所述的方法,其中所述表达FcR的NK-02细胞被进一步修饰以表达自杀基因。

14.根据权利要求13所述的方法,其中所述自杀基因是iCas9。

15.根据权利要求1至13中任一项所述的方法,其中所述癌症是多发性骨髓瘤,白血病,非霍奇金淋巴瘤,转移性乳腺癌或胃癌。

16.根据权利要求1至15中任一项所述的方法,其中所述单克隆抗体是裸单克隆抗体,缀合单克隆抗体或双特异性单克隆抗体。

17.根据权利要求16所述的方法,其中所述单克隆抗体是阿仑单抗,利妥昔单抗,曲妥珠单抗,替伊莫单抗,本妥昔单抗,吉妥珠单抗,阿曲坦单抗,blinatunomab,avelumamab,daratumumab或埃罗妥珠单抗。

18.根据权利要求1至17中任一项所述的方法,其中所述单克隆抗体和所述表达FcR的NK-92细胞被同时施用于所述受试者。

19.根据权利要求1至17中任一项所述的方法,其中所述受试者被施用所述单克隆抗体,并随后被用所述表达FcR的NK-92细胞治疗。

20.根据权利要求18或19所述的方法,其中所述单克隆抗体被静脉内注射到所述受试者。

21.根据权利要求18、19或20所述的方法,其中所述被遗传修饰的NK-92细胞被注射到骨髓中。

用于治疗癌症的被遗传修饰的NK-92细胞和单克隆抗体

[0001] 相关申请的交叉引用

[0002] 本申请要求2015年3月27日提交的美国临时申请NO.62/139,258的优先权权益,其通过引用并入本文。

背景技术

[0003] 单克隆抗体(mAbs)的抗癌治疗已经显著改善癌症患者的临床结果,特别是当与化学疗法组合时。然而,患者经常最终复发。自然杀伤细胞也可以用作细胞毒性效应细胞用于基于细胞的免疫治疗。

[0004] NK-92是在患有非霍奇金淋巴瘤的受试者的血液中发现,且然后离体永生化的细胞溶解性癌细胞系。NK-92细胞来源于NK细胞,但缺乏正常NK细胞展示的主要抑制性受体,同时保留大部分活化受体。然而,NK-92细胞不攻击正常细胞,也不在人类中引起不可接受的免疫排斥反应。NK-92细胞系的表征在WO 1998/49268和美国专利申请公布No.2002-0068044中公开了。NK-92细胞也被评估为治疗某些癌症的潜在治疗剂。

[0005] 尽管NK-92细胞保留了与NK细胞相关的几乎所有活化受体和细胞溶解途径,但它们在其细胞表面上不表达CD16。CD16是Fc受体,其识别并结合抗体的Fc部分以激活NK细胞用于抗体依赖性细胞的细胞毒性(ADCC)。由于没有CD16受体,NK-92细胞不能通过ADCC机制裂解靶细胞。

[0006] 本发明通过向需要抗癌治疗的受试者同时或随后施用表达Fc受体的NK-92细胞,增强一些分子抗体的细胞毒性作用,提供了上述问题的解决方案。

发明内容

[0007] 在一个方面,本发明包括向需要抗癌治疗的受试者共同施用对靶癌细胞具有细胞毒性作用的单克隆抗体和被工程化以表达Fc受体的NK-92细胞。这种组合协同NK细胞的抗癌作用与治疗性抗体的抗癌作用。

[0008] 因此,在一个实施方式中,本发明提供了用于在有需要的受试者中治疗癌症的方法,其包括向所述受试者施用对靶癌细胞具有细胞毒性作用的单克隆抗体和表达FcR的NK-92细胞。在一些实施方式中,FcR是CD16。在本发明的一个方面,NK-92细胞被遗传修饰以表达编码与SEQ ID NO:1具有至少90%序列同一性的多肽的Fc受体(在第158位具有苯丙氨酸的FCγRIII-A或CD16(F-158);或与SEQ ID NO:2具有至少90%序列同一性的多肽的Fc受体(在第158位具有缬氨酸的CD16(F158V),更高亲和力形式)。在典型的实施方式中,CD16多肽在158位具有缬氨酸。

[0009] 在另外的实施方式中,NK-92细胞被另外地修饰以表达细胞因子,例如IL-2。在一些实施方式中,细胞因子靶向内质网。在具体实施方式中,细胞因子是靶向内质网的白细胞介素-2或其变体。在一些实施方式中,NK-92细胞被修饰以表达具有SEQ ID NO:7的序列的多肽。

[0010] 在其它实施方式中,NK-92细胞被进一步修饰以表达自杀基因。在一个方面,自杀

基因是iCas9。

[0011] 本发明的组合物可用于治疗癌症，包括但不限于癌症如多发性骨髓瘤，白血病，淋巴瘤，转移性乳腺癌或胃癌。

[0012] 被施用于患者的单克隆抗体可以是裸单克隆抗体，缀合单克隆抗体或双特异性单克隆抗体。在一些实施方式中，单克隆抗体是阿仑单抗，利妥昔单抗，曲妥珠单抗，替伊莫单抗(ibritumomab)，吉妥珠单抗(gemtuzumab)，本妥昔单抗(brentuximab)，阿曲坦单抗(adotranstuzumab)，blinatunomab，daratumumab或埃罗妥珠单抗(elotuzumab)。

[0013] 在一些实施方式中，单克隆抗体和表达FcR的NK-92细胞被同时施用于受试者。在其它实施方式中，受试者被施用单克隆抗体，并随后在施用单克隆抗体后，例如在24小时内，或在24至72小时内，被施用表达FcR的NK-92细胞。

[0014] 在一些方面，本发明涉及被遗传修饰以表达FcR(例如CD16)的NK-92细胞与细胞毒性单克隆抗体一起治疗癌症的用途。因此，在一些实施方式中，本发明提供被遗传修饰以表达CD16的NK-92细胞与细胞毒性单克隆抗体一起用于患有癌症的患者的用途。在一些实施方式中，Fc受体是在CD16的成熟形式的第158位具有缬氨酸的CD16。在一些实施方式中，Fc受体包含编码与SEQ ID NO:1或SEQ ID NO:2具有至少90％序列同一性的多肽的多核苷酸序列或编码SEQ ID NO:1或SEQ ID NO:2的多核苷酸。在一些实施方式中，表达FcR的NK-92细胞被遗传修饰以表达细胞因子，例如白细胞介素-2或其变体。在一些实施方式中，白细胞介素-2靶向内质网。在一些实施方式中，表达FcR的NK-92细胞被修饰以表达如SEQ ID NO:7所示的白细胞介素-2序列。在一些实施方式中，Fc受体和至少一种细胞因子由不同的载体编码。或者，Fc受体和至少一种细胞因子由相同的载体编码。在一些实施方式中，Fc受体包含在第158位具有V的CD16多肽，并且NK-92细胞被进一步遗传修饰以表达人白细胞介素-2，其中白细胞介素2靶向内质网。表达FcR的NK-92细胞还可以被进一步修饰以表达自杀基因，例如iCas9。在一些实施方式中，癌症是白血病，非霍奇金淋巴瘤，转移性乳腺癌或胃癌。单克隆抗体可以是裸单克隆抗体，缀合单克隆抗体或双特异性单克隆抗体。在一些实施方式中，单克隆抗体是阿仑单抗，利妥昔单抗，曲妥珠单抗，替伊莫单抗，本妥昔单抗，吉妥珠单抗，阿曲坦单抗，blinatunomab，avelumamab，daratumumab或埃罗妥珠单抗。在一些实施方式中，单克隆抗体和表达FcR的NK-92细胞被同时施用于受试者。在一些实施方式中，受试者被施用单克隆抗体，并随后被用被遗传修饰的表达FcR的NK-92细胞治疗。在一些实施方式中，单克隆抗体被静脉内注射到受试者。在其他实施方式中，被遗传修饰的表达FcR的NK-92细胞被注射到骨髓中。

[0015] 前述一般描述和以下详细描述是示例性和说明性的，并且旨在提供所要求保护的本发明的进一步解释。从本发明的以下详细描述，其他目的、优点和新颖特征对于本领域技术人员将是显而易见的。

附图说明

[0016] 在结合附图考虑时，参考下列公开内容，本发明的目的、特征和优点将更容易理解。

[0017] 图1显示了表达IL-2的修饰形式与ERRS(内质网保留信号)和CD16的质粒的示意图。

[0018] 图2a和2b提供了说明性数据，其显示在约2周(图2a)和约4周(图2b)时，使用图1所示的质粒载体，在被修饰以表达CD16的NK-92细胞中的CD16表达。

[0019] 图3提供了说明性数据，其显示被修饰的NK-92细胞中的CD16表达，所述NK-92细胞被冷冻储存，然后解冻培养。

[0020] 图4提供了说明性数据，其显示与单克隆抗体组合使用的表达CD16的NK-92细胞的ADCC活性。

具体实施方式

[0021] 在一个方面，本公开涉及被修饰以表达FcR的NK-92细胞和单克隆抗体在有需要的受试者中治疗癌症的用途。恶性细胞能够发展出逃避先天免疫细胞(如树突状细胞和天然杀伤细胞)和适应性免疫细胞(如T细胞和B细胞)提供的免疫保护的机制。因此，迫切需要减少患有癌症或怀疑患有癌症的受试者的肿瘤复发的发生率。

[0022] NK-92细胞呈现出有吸引力的特征，即它们可以容易地在体外繁殖和扩增。然而，它们不表达IgG Fc受体Fcγ RIII，因此这些细胞不能通过抗体依赖性细胞介导的细胞毒性(ADCC)起作用。本发明是基于使NK-92细胞表达IgG Fc受体Fcγ RIII的遗传转化将增强NK-肿瘤细胞相互作用，并允许NK细胞与通过ADCC杀死靶细胞的单克隆抗体一起工作的情况。因此，当单克隆抗体和NK-92细胞被同时地或以紧密的时间关系施用于患有癌症或在其他方面需要癌症治疗的受试者时，可以增加NK-92细胞和单克隆抗体单独的细胞毒性作用。

[0023] 因此，本发明提供了被遗传修饰以表达跨膜免疫球蛋白γ Fc区受体III-A的高亲和力形式(其中在多肽的成熟形式的第158位存在缬氨酸的Fcγ RIII-A或CD16)的NK-92细胞的用途。

[0024] 在一些实施方式中，表达FcR的NK-92细胞可以被进一步修饰以表达IL-2。在这样的细胞中，IL-2在细胞中的表达通常指向内质网。该特征防止全身性施用IL-2的不期望的作用，例如影响心血管，胃肠道，呼吸系统和神经系统的毒性。在一些实施方式中，当表达FcR的NK-92细胞被进一步修饰以表达IL-2时，也可将自杀基因插入到这些细胞中，以防止IL-2的未经调节的内源性表达，其可以导致具有自主生长的突变体的潜在发展。在一些实施方式中，自杀基因是iCas9。

[0025] 将根据本发明产生的表达FcR的NK-92细胞与靶向癌细胞的单克隆抗体组合施用于患有或怀疑患有癌症的受试者以有效治疗癌性疾病。

[0026] 施用表达FcR的NK-92细胞可以与施用单克隆抗体同时进行，或以顺序方式进行。在一些实施方式中，在已经用单克隆抗体治疗受试者后的24小时内，向受试者施用表达FcR的NK-92细胞。

[0027] 术语

[0028] 除非另有定义，本文使用的所有技术和科学术语具有与本发明所属领域的普通技术人员通常理解的相同的含义。

[0029] 在本说明书和下面的权利要求书中，将提及应被定义为具有以下含义的多个术语：

[0030] 本文使用的术语仅用于描述特定实施方式的目的，而不意在限制本发明。如本文所使用的，单数形式“一个”，“一种”和“该”也旨在包括复数形式，除非上下文另有明确指

示。

[0031] 包括范围在内的所有数字标示，例如pH，温度，时间，浓度，量，分子量是近似值，其在适合的地方通过0.1或1.0的增量而变化(+)或(-)。应当理解，虽然并不总是明确说明，但是所有的数字标示之前都可以是术语“约”。还应当理解，虽然并不总是明确说明，但是本文所述的试剂仅仅是示例性的，并且这些的等价物是本领域已知的。

[0032] “任选的”或“任选地”是指随后描述的事件或情况可以发生或可以不发生，并且该描述包括事件或情况发生的例子以及不发生的例子。

[0033] 术语“包含”旨在表示组合物和方法包括所记载的要素，但不排除其他要素。用于限定组合物和方法时，“基本上由...组成”是指所规定的材料或步骤以及不实质上影响要求保护的发明的基本和新颖特征的材料或步骤。“由……组成”应意味着排除超过痕量的其他成分和所记载的实质性方法步骤。由这些过渡术语中的每一个限定的实施方式都在本发明的范围内。

[0034] 如用于描述本发明的，“免疫疗法”是指与抗体(天然存在或被修饰的NK细胞或T细胞)(无论是单独还是组合使用)组合使用被修饰或未修饰的NK-92细胞，并且当与靶细胞接触时其能够诱导细胞毒性。

[0035] 如用于描述本发明的，“天然杀伤(NK)细胞”是在特异性抗原刺激不存在的情况下杀死靶细胞，并且根据MHC类没有限制的免疫系统的细胞。靶细胞可以是肿瘤细胞或携带病毒的细胞。NK细胞的特征在于存在CD56和不存在CD3表面标志物。

[0036] 术语“内源性NK细胞”用于指源自供体(或患者)的NK细胞，与NK-92细胞系不同。内源性NK细胞通常是异种细胞群，其中NK细胞已被富集。内源性NK细胞可以用于患者的自体或同种异体治疗。

[0037] “NK-92细胞”是指最初从患有非霍奇金淋巴瘤的患者获得的永生NK细胞系NK-92。为了本发明的目的，除非另有说明，术语“NK-92”是指原始的NK-92细胞系以及已被修饰的NK-92细胞系(例如通过引入外源基因)。NK-92细胞及其示例性和非限制性修饰在美国专利号7,618,817;8,034,332;和8,313,943中描述，其全部内容通过引用并入本文。

[0038] “被修饰的NK-92细胞”是指进一步包含编码转基因(包括CD16)的载体的NK-92细胞。在一些实施方式中，被修饰的表达FcR的NK-92细胞可以被进一步修饰以表达细胞因子如IL-2，和/或自杀基因。

[0039] 如本文所用，“未照射的NK-92细胞”是尚未被照射的NK-92细胞。照射使细胞不能生长和增殖。在一些实施方式中，设想用于施用的NK-92细胞将在治疗设施处或在治疗患者之前的某个其他点被照射，因为照射和输注之间的时间不应超过4小时，以保持最佳活性。或者，NK-92细胞可以通过另外的机制失活。

[0040] 如用于描述本发明的，NK-92细胞的“失活”使得它们不能生长。失活也可能与NK-92细胞的死亡有关。设想NK-92细胞在其已经有效地清除与治疗应用中的病理学相关的细胞的离体样品之后，或者在它们已经在哺乳动物体内驻留足以有效地杀死体内驻留的许多或所有靶细胞的时间段之后被失活。作为非限制性实例，可以通过施用NK-92细胞对其敏感的失活剂诱导失活。

[0041] 如用于描述本发明的，术语“细胞毒性的”和“细胞溶解的”当用于描述效应细胞例如NK细胞的活性时，旨在是同义的。通常，细胞毒性活性涉及通过各种生物、生物化学或生

物物理机制中的任一种杀死靶细胞。细胞溶解更具体地涉及效应物裂解靶细胞的质膜，从而破坏其物理完整性的活性。这导致杀死靶细胞。不希望被理论束缚，据信NK细胞的细胞毒性作用是由于细胞溶解。

[0042] 关于细胞/细胞群体的术语“杀死”旨在包括将导致该细胞/细胞群体死亡的任何类型的操作。

[0043] 术语“Fc受体”是指通过结合到称为Fc区的抗体的一部分而有助于免疫细胞的保护功能的某些细胞(例如天然杀伤细胞)的表面上发现的蛋白质。抗体的Fc区与细胞的Fc受体(FcR)的结合通过抗体介导的吞噬作用或抗体依赖性细胞介导的细胞毒性(ADCC)刺激细胞的吞噬或细胞毒性活性。对FcR根据其识别的抗体类型进行分类。例如，Fc-γ受体(FCγR)结合IgG类抗体。FCγRIII-A(也称为CD16)是结合IgG抗体并激活ADCC的低亲和性Fc受体。通常在NK细胞上发现FCγRIII-A。编码CD16的天然形式的代表性多核苷酸序列显示在SEQ ID NO:5中。

[0044] 术语“多核苷酸”、“核酸”和“寡核苷酸”可互换使用，并且是指任何长度的核苷酸的聚合形式：脱氧核糖核苷酸或核糖核苷酸或其类似物。多核苷酸可以具有任何三维结构并且可以执行已知或未知的任何功能。以下是多核苷酸的非限制性实例：基因或基因片段(例如，探针，引物，EST或SAGE标签)，外显子，内含子，信使RNA(mRNA)，转移RNA，核糖体RNA，核酶，cDNA，重组多核苷酸，支链多核苷酸，质粒，载体，任何序列的分离的DNA，任何序列的分离的RNA，核酸探针和引物。多核苷酸可以包含修饰的核苷酸，例如甲基化核苷酸和核苷酸类似物。如果存在，则可以在多核苷酸组装之前或之后赋予核苷酸结构以修饰。核苷酸序列可以被非核苷酸成分中断。聚合后可以进一步修饰多核苷酸，例如通过与标记组分缀合。该术语还指双链和单链分子二者。除非另有说明或要求，否则作为多核苷酸的本发明的任何实施方式都包括双链形式和已知或预期构成双链形式的两种互补单链形式的任一种。

[0045] 多核苷酸由四种核苷酸碱基(腺嘌呤(A)；胞嘧啶(C)；鸟嘌呤(G)；胸腺嘧啶(T)；和当多核苷酸是RNA时针对胸腺嘧啶的尿嘧啶(U))的特定序列组成：。因此，术语“多核苷酸序列”是多核苷酸分子的字母表示。

[0046] 如本文所用，“同一性百分比”是指两个肽之间或两个核酸分子之间的序列同一性。同一性百分比可以通过比较可以为比较目的而比对的每个序列中的位置而测定。当比较序列中的位置被相同的碱基或氨基酸占据时，则分子在该位置是相同的。如本文所用，短语“同源”或“变体”核苷酸序列，或“同源”或“变体”氨基酸序列是指特征为在核苷酸水平或氨基酸水平具有至少规定百分比的同一性的序列。同源核苷酸序列包括编码本文所述的核苷酸序列的天然存在的等位基因变体和突变的那些序列，同源核苷酸序列包括编码除人以外的哺乳动物物种的蛋白质的核苷酸序列。同源氨基酸序列包括含有保守氨基酸取代和其多肽具有相同结合和/或活性的那些氨基酸序列。在一些实施方式中，同源核苷酸或氨基酸序列与比较序列具有至少60％或更大，例如至少70％，或至少80％，或至少85％或更大。在一些实施方式中，同源核苷酸或氨基酸序列与比较序列具有至少90％，91％，92％，93％，94％，95％，96％，97％，98％或99％的同一性。在一些实施方式中，同源氨基酸序列具有不超过15个，不超过10个，不超过5个或不超过3个保守氨基酸取代。同一性百分比可以通过例如使用默认设置的Gap程序(Wisconsin Sequence Analysis Package，UNIX第8版，Genetics Computer Group，University Research Park，Madison Wis.)测定，其使用

Smith和Waterman算法Adv.Appl.Math.,1981,2482-489)。

[0047] 术语“表达”是指基因产物的产生。当涉及表达时,术语“瞬时(transient)”是指多核苷酸不被引入到细胞的基因组中。

[0048] 术语“细胞因子”或“细胞因子”是指影响免疫系统细胞的一般类别的生物分子。用于实施本发明的示例性细胞因子包括但不限于干扰素和白细胞介素(IL),特别是IL-2,IL-12,IL-15,IL-18和IL-21。在优选的实施方式中,细胞因子是IL-2。

[0049] 如本文所用,术语“载体”是指包含完整复制子,使得当被放置在允许的细胞内时,可以例如通过转化过程复制载体的非染色体核酸。载体可以在一种细胞类型例如细菌中复制,但是在另外的细胞例如哺乳动物细胞中复制能力有限。载体可以是病毒的或非病毒的。用于递送核酸的示例性非病毒载体包括裸DNA;与阳离子脂质复合的DNA,单独或与阳离子聚合物组合;阴离子和阳离子脂质体;DNA-蛋白复合物和包含与阳离子聚合物(如异源聚赖氨酸、限定长度的寡肽和聚乙烯亚胺)缩合的DNA的颗粒,在一些情况下被包含在脂质体中;以及使用包含病毒和聚赖氨酸-DNA的三元复合物。

[0050] 如本文所用,术语“靶向(targeted)”旨在包括但不限于将蛋白质或多肽引导到在细胞中或其外部的适当目的地。靶向通常通过信号肽或靶向肽(其是多肽链中的氨基酸残基段)而实现。这些信号肽可位于多肽序列内的任何位置,但通常位于N端。多肽也可以被工程化成在C端具有信号肽。信号肽可以引导多肽到胞外部分,质膜,高尔基体,内体,内质网和其它细胞区室的位置。例如,在其C端具有特定氨基酸序列的多肽(例如KDEL)被保留在ER腔中或者被运输回ER腔。

[0051] 术语“自杀基因”是允许细胞的阴性选择的基因。使用自杀基因作为安全系统,允许通过引入选择剂使表达该基因的细胞被杀死。在重组基因导致致使不受控制的细胞生长的突变的情况下,这是期望的。已经鉴定了许多自杀基因系统,包括单纯疱疹病毒胸苷激酶(TK)基因,胞嘧啶脱氨酶基因,水痘带状疱疹病毒胸苷激酶基因,硝基还原酶基因,大肠杆菌gpt基因和大肠杆菌Deo基因(还参见,例如,Yazawa K,Fisher W E,Brunicardi F C:Current progress in suicide gene therapy for cancer.World J.Surg.2002,7月;26(7):783-9)。在一个实施方式中,自杀基因是可诱导性半胱天冬酶9(iCas9)(Di Stasi,(2011)“Inducible apoptosis as a safety switch for adoptive cell therapy”,N Engl J Med 365:1673-1683。还参见Morgan,“Live and Let Die:A New Suicide Gene Therapy Moves to the Clinic”Molecular Therapy(2012);20:11-13)。TK基因可以是野生型或突变TK基因(例如,tk30,tk75,sr39tk)。使用更昔洛韦可以杀死表达TK蛋白的细胞。

[0052] 本文所用的术语“单克隆抗体”是指从在培养物中生长的细胞的单个克隆产生且能够无限增殖的纯的靶特异性抗体。根据本发明可以使用的单克隆抗体包括附着于癌细胞并阻断抗原的裸抗体。在一个实施方式中,裸单克隆抗体是阿伦单抗,其结合到淋巴细胞中的CD52抗原。还包括在根据本发明可以使用的单克隆抗体中的是缀合单克隆抗体,例如加标签的、标记的或加载的抗体。具体地,抗体可以被加标签或加载药物或毒素,或被放射性标记。这样的抗体的实例包括但不限于靶向CD20抗原的替伊莫单抗,靶向CD30抗原的brentuximab和靶向HER2蛋白的曲妥珠单抗。根据本发明可以使用的其它单克隆抗体是双特异性单克隆抗体,例如靶向淋巴瘤细胞中的CD19和T细胞中的CD3的blinatunomab。

[0053] 术语“患者”、“受试者”、“个体”等在本文中可互换使用,并且是指体外或原位可适

用于本文所述的方法的任何动物或其细胞。在某些非限制性实施方式中，患者、受试者或个体是人。

[0054] 术语“治疗”或“治疗”涵盖在受试者例如人中治疗如本文所述的疾病或紊乱，并且包括：(i)抑制疾病或紊乱，即阻止其发展；(ii)减轻疾病或紊乱，即导致紊乱消退；(iii)减缓紊乱进展；和/或(iv)抑制、减轻或减缓疾病或紊乱的一种或多种症状的进展。术语向受试者“施用”或“施用”单克隆抗体或天然杀伤细胞包括引入或递送抗体或细胞以执行预期功能的任何途径。施用可以通过适合于递送细胞或单克隆抗体的任何途径进行。因此，递送途径可以包括静脉内，肌肉内，腹膜内或皮下递送。在一些实施方式中，单克隆抗体和/或NK-92细胞被直接施用于肿瘤，例如通过注射到肿瘤中。施用包括自我管理和通过另外的人施用。

[0055] NK-92细胞

[0056] NK-92细胞系是被发现在白细胞介素2(IL-2)存在的情况下增殖的唯一细胞系。Gong等，Leukemia 8:652-658(1994)。这些细胞对各种癌症具有高细胞溶解活性。NK-92细胞系是具有广泛的抗肿瘤细胞毒性、扩增后具有可预测的收率的同质癌性NK细胞群。I期临床试验已证实其安全性。

[0057] 发现NK-92细胞系展示$CD56^{bright}$，CD2，CD7，CD11a，CD28，CD45和CD54表面标志物。此外，它不展示CD1，CD3，CD4，CD5，CD8，CD10，CD14，CD16，CD19，CD20，CD23和CD34标志物。NK-92细胞在培养物中的生长取决于重组白介素2(rIL-2)的存在，剂量低至1IU/mL足以维持增殖。IL-7和IL-12不支持长期生长，所测试的其他细胞因子(包括IL-1α，IL-6，肿瘤坏死因子α，干扰素α和干扰素γ)也不支持。NK-92即使在1:1的低效应物:靶(E:T)比率下也具有高细胞毒性。Gong等，同上。NK-92细胞被保藏在美国典型培养物保藏中心(ATCC)，名称为CRL-2407。

[0058] 迄今为止，关于内源性NK细胞的研究已经表明，IL-2(1000IU/mL)对于运输过程中的NK细胞激活至关重要，但是细胞不需要被保持在37℃和5％二氧化碳下。Koepsell等，Transfusion 53:398-403(2013)。

[0059] 自杀基因

[0060] 术语“自杀基因”是允许细胞的阴性选择的基因。使用自杀基因作为安全系统，允许通过引入选择剂使表达该基因的细胞被杀死。在重组基因导致致使不受控制的细胞生长的突变的情况下，这是期望的。已经鉴定了许多自杀基因系统，包括单纯疱疹病毒胸苷激酶(TK)基因，胞嘧啶脱氨酶基因，水痘带状疱疹病毒胸苷激酶基因，硝基还原酶基因，大肠杆菌gpt基因和大肠杆菌Deo基因(还参见，例如，Yazawa K,Fisher W E,Brunicardi F C:Current progress in suicide gene therapy for cancer.World J.Surg.2002,7月;26(7):783-9)。如本文所用，自杀基因在NK-92细胞中是有活性的。通常，自杀基因编码对细胞没有不良影响，但在特定化合物存在的情况下将杀死细胞的蛋白质。因此，自杀基因通常是系统的一部分。

[0061] 在一个实施方式中，自杀基因是胸苷激酶(TK)基因。TK基因可以是野生型或突变TK基因(例如，tk30，tk75，sr39tk)。使用更昔洛韦可以杀死表达TK蛋白的细胞。

[0062] 在另一个实施方式中，自杀基因是在5-氟胞嘧啶存在的情况下对细胞具有毒性的胞嘧啶脱氨酶。Garcia-Sanchez等，“Cytosine deaminase adenoviral vector and 5-

fluorocytosine selectively reduce breast cancer cells 1million-fold when they contaminate hematopoietic cells:a potential purging method for autologous transplantation"Blood1998年7月15日;92(2):672-82。

[0063] 在另一个实施方式中,自杀基因是在异环磷酰胺或环磷酰胺存在的情况下是具有毒性的细胞色素P450。参见,例如,Touati等,"A suicide gene therapy combining the improvement of cyclophosphamide tumor cytotoxicity and the development of an anti-tumor immune response"Curr Gene Ther.2014;14(3):236-46。

[0064] 在另一个实施方式中,自杀基因是iCas9。Di Stasi,(2011)"Inducible apoptosis as a safety switch for adoptive cell therapy",N Engl J Med 365:1673-1683。还参见,Morgan,"Live and Let Die:A New Suicide Gene Therapy Moves to the Clinic"Molecular"Molecular Therapy(2012);20:11-13。在小分子AP1903存在的情况下,iCas9蛋白诱导凋亡。AP1903是生物学惰性的小分子,其已经在临床研究中表现出被良好耐受,并且已经在过继细胞治疗的情况下使用。

[0065] Fc受体

[0066] Fc受体结合到抗体的Fc部分。多种Fc受体是已知,并且根据其优选配体,亲和力,表达和结合到抗体后的效果而不同。

[0067] 表1.说明性的Fc受体

[0068]

受体名称	主要抗体配体	对抗体的亲和力	细胞分布	结合到抗体后的效果
FcγRI（CD64）	IgG1 和 IgG3	高（Kd ~ 10^{-9} M）	巨噬细胞 嗜中性粒细胞 嗜酸性粒细胞 树突状细胞	吞噬作用 细胞激活 呼吸爆发激活 诱导杀死微生物
FcγRIIA（CD32）	IgG	低（Kd > 10^{-7} M）	巨噬细胞 嗜中性粒细胞 嗜酸性粒细胞 血小板 朗格汉斯细胞	吞噬作用 脱粒（嗜酸性粒细胞）

[0069]

FcγRIIB1（CD32）	IgG	低（Kd > 10^{-7} M）	B 细胞 肥大细胞	无吞噬作用 抑制细胞活性
FcγRIIB2（CD32）	IgG	低（Kd > 10^{-7} M）	巨噬细胞 嗜中性粒细胞 嗜酸性粒细胞	吞噬作用 抑制细胞活性
FcγRIIIA（CD16a）	IgG	低（Kd > 10^{-6} M）	NK 细胞 巨噬细胞（某些组织）	诱导抗体依赖性细胞介导的细胞毒性（ADCC） 诱导巨噬细胞释放细胞因子
FcγRIIIB（CD16b）	IgG	低（Kd > 10^{-6} M）	嗜酸性粒细胞 巨噬细胞 嗜中性粒细胞 肥大细胞 滤泡树突状细胞	诱导杀死微生物
FcεRI	IgE	High（Kd ~ 10^{-10} M）	肥大细胞 嗜酸性粒细胞 嗜碱性粒细胞 朗格汉斯细胞 单核细胞	脱粒 吞噬作用
FcεRII（CD23）	IgE	低（Kd > 10^{-7} M）	B 细胞 嗜酸性粒细胞 朗格汉斯细胞	可能的粘附分子 IgE 转运穿过人肠上皮 增强过敏敏化作用的正反馈机制（B 细胞）
FcαRI（CD89）	IgA	低（Kd > 10^{-6} M）	单核细胞 巨噬细胞 嗜中性粒细胞 嗜酸性粒细胞	吞噬作用 诱导杀死微生物
Fcα/μR	IgA 和 IgM	对于 IgM 高，对于 IgA 中等	B 细胞 肾系膜细胞 巨噬细胞	胞吞作用 诱导杀死微生物

[0070]

FcRn	IgG		单核细胞 巨噬细胞 树突状细胞 上皮细胞 内皮细胞 肝细胞	IgG 从母亲经过胎盘转移到胎儿 IgG 在母乳中从母亲转移到婴儿 保护 IgG 免于降解

[0071] 在一些实施方式中,NK-92细胞被修饰以在细胞表面上表达Fc受体蛋白。

[0072] 在一些实施方式中,Fc受体是CD16。为了本公开的目的,CD16的特定氨基酸残基参考SEQ ID NO:2或相对于SEQ ID NO:2在一个位置上不同的SEQ ID NO:1命名。因此,当CD16多肽和SEQ ID NO:2最大程度地比对时,根据本发明的CD16多肽的“第158位”氨基酸残基是对应于SEQ ID NO:2(或SEQ ID NO:1)的第158位氨基酸残基。在一些实施方式中,NK-92细胞被修饰以表达在蛋白质的成熟形式的第158位具有苯丙氨酸的人CD16,例如SEQ ID NO:1。在典型的实施方式中,NK-92细胞被修饰以表达在蛋白质的成熟形式的第158位具有缬氨酸的人CD16的高亲和力形式,例如SEQ ID NO:2。成熟蛋白质的第158位对应于包含天然信号肽的CD16序列的第176位。在一些实施方式中,CD16多肽由编码SEQ ID NO:3或SEQ ID NO:4的前体(即具有天然信号肽)多肽序列的多核苷酸编码。

[0073] 在一些实施方式中,编码CD16多肽的多核苷酸与编码全长(包括信号肽)天然存在的CD16的多核苷酸序列具有至少约70%的多核苷酸序列同一性,所述全长天然存在的CD16在全长CD16的第176位(其对应于成熟CD16蛋白的第158位)具有苯丙氨酸。在一些实施方式中,编码CD16多肽的多核苷酸与编码全长(包括信号肽)天然存在的CD16的多核苷酸序列具有至少约70%的多核苷酸序列同一性,所述全长天然存在的CD16在第176位(其对应于成熟CD16蛋白的第158位)具有缬氨酸。在一些实施方式中,编码CD16的多核苷酸与SEQ ID NO:5具有至少70%的同一性,并且包含编码在编码全长(包含信号肽)CD16多肽的第176位的多核苷酸位置处的缬氨酸的密码子。在一些实施方式中,编码CD16的多核苷酸与SEQ ID NO:5具有至少90%的同一性,并且包含编码全长CD16的第176位的缬氨酸的密码子。在一些实施方式中,编码CD16的多核苷酸包含SEQ ID NO:5,但具有编码全长CD16的第176位的缬氨酸的密码子。

[0074] 在一些实施方式中,CD16多核苷酸编码与SEQ ID NO:1或SEQ ID NO:2具有至少70%,80%,90%或95%同一性的多肽。在一些实施方式中,多核苷酸编码与SEQ ID NO:2具有至少70%同一性或至少80%同一性,并且如参考SEQ ID NO:2确定的,包含第158位的缬氨酸的多肽。在一些实施方式中,多核苷酸编码与SEQ ID NO:2具有至少90%同一性,并且如参考SEQ ID NO:2确定的,包含第158位的缬氨酸的多肽。在一些实施方式中,多核苷酸编码与SEQ ID NO:2具有至少95%同一性,并且如参考SEQ ID NO:2确定的,包含第2位的缬氨酸的多肽。在一些实施方式中,多核苷酸编码SEQ ID NO:2。在一些实施方式中,CD16多核苷酸编码具有或不具有信号序列,或全长CD16的任何其它片段,或包含与另外的蛋白质的氨基酸序列融合的CD16的至少部分序列的嵌合受体的CD16的细胞外结构域。在其他实施方式中,可以将表位标签肽(如FLAG,myc,多组氨酸或V5)加入到成熟多肽的氨基末端结构域中,

以通过使用抗表位标签肽单克隆或多克隆抗体辅助细胞表面检测。

[0075] 在一些实施方式中，尽管具有多于700至800个多核苷酸的CD16变体在本公开的范围内，但同源CD16多核苷酸的长度可以为约150至约700，约750，或约800个多核苷酸。

[0076] 同源多核苷酸序列包含编码多肽序列的那些，所述多肽序列编码CD16的变体。同源多核苷酸序列还包括与SEQ ID NO:5相关的天然存在的等位基因变异。用编码具有SEQ ID NO:1或SEQ ID NO:2所示氨基酸序列，其天然存在的变体，或与SEQ ID NO:1或SEQ ID NO:2至少70％相同，或至少80％，90％或95％相同的序列的多肽的任何多核苷酸转染NK-92细胞是在本公开的范围内。在一些实施方式中，同源多核苷酸序列编码SEQ ID NO:1或SEQ ID NO:2中的保守氨基酸取代。在一些实施方式中，使用与天然多核苷酸序列不同，但编码相同多肽的简并同源CD16多核苷酸序列转染NK-92细胞。

[0077] 在其他实例中，使用具有改变CD16氨基酸序列的多态性的cDNA序列，例如在CD16基因中显示遗传多态性的个体中的等位基因变化，修饰NK-92细胞。在其他实例中，使用来自具有不同于SEQ ID NO:5的序列的多核苷酸序列的其他物种的CD16基因修饰NK-92细胞。

[0078] 在实例中，使用本领域已知的方法制备变体多肽，例如寡核苷酸介导的(定点诱变)诱变，丙氨酸扫描和PCR诱变。可以在克隆的DNA上进行定点诱变(Carter，1986；Zoller和Smith，1987)，盒式诱变，限制性选择诱变(Wells等，1985)或其他已知技术以产生CD16变体(Ausubel，2002；Sambrook和Russell，2001)。

[0079] 在一些实施方式中，使编码CD16的多核苷酸突变以改变编码CD16的氨基酸序列而不改变CD16的功能。例如，可以在SEQ ID NO:1或SEQ ID NO:2中在“非必需”氨基酸残基处进行导致氨基酸取代的多核苷酸取代。

[0080] SEQ ID NO:NO:1或SEQ ID NO:2中的保守取代，借此一种类别的氨基酸被同一类别的另一氨基酸取代，落入所公开的CD16变体的范围内，只要该取代不实质上改变多肽活性。保守取代是本领域技术人员熟知的。影响(1)多肽骨架的结构，例如β-折叠或α-螺旋构象，(2)电荷，(3)疏水性，或(4)靶点的侧链的体积的非保守取代可以修饰CD16多肽的功能或免疫学身份。非保守取代需要将这些类别中的一个的成员交换为另一个类别。取代可以被引入到保守取代位点或更优选被引入到非保守位点。

[0081] 在一些实施方式中，CD16多肽变体的长度为至少200个氨基酸，并且与SEQ ID NO:1或SEQ ID NO:2具有至少70％氨基酸序列同一性，或至少80％，或至少90％同一性。在一些实施方式中，CD16多肽变体的长度为至少225个氨基酸，并且与SEQ ID NO:1或SEQ ID NO:2具有至少70％氨基酸序列同一性，或至少80％，或至少90％同一性。在一些实施方式中，如参考SEQ ID NO:2确定的，CD16多肽变体在第158位具有缬氨酸。

[0082] 在一些实施方式中，编码CD16多肽的核酸可编码CD16融合蛋白。CD16融合多肽包括与非CD16多肽融合的CD16的任何部分或整个CD16。使用重组方法方便地产生融合多肽。例如，编码CD16多肽的多核苷酸(例如SEQ ID NO:1或SEQ ID NO:2)与编码非CD16的多核苷酸(例如编码异源蛋白质的信号肽的多核苷酸序列)框内(in-frame)融合。在一些实施方式中，可以产生融合多肽，其中异源多肽序列与CD16的C端融合或被内部地设置在CD16中。通常，可以更换至多约30％的CD16细胞质结构域。这样的修饰可以增强表达或增强细胞毒性(例如ADCC响应性)。在其它实例中，嵌合蛋白(例如来自其它淋巴细胞激活受体(包括但不限于Ig-a，Ig-B，CD3-e，CD3-d，DAP-12和DAP-10)的结构域)替代CD16细胞质结构域的部分。

[0083] 融合基因可以通过常规技术合成，包括使用锚引物的自动化DNA合成仪和PCR扩增，所述锚引物造成两个连续基因片段之间的互补突出端，其随后可以被退火并重新扩增以产生嵌合基因序列(Ausubel，2002)。许多载体是可商购的，其促进将CD16框内亚克隆到融合部分。

[0084] 细胞因子

[0085] NK-92细胞的细胞毒性取决于细胞因子(例如白细胞介素-2(IL-2))的存在。使用外源性添加的、在商业规模培养中保持和扩增NK-92细胞所需要的IL-2的成本是显著的。向人受试者以足以继续激活NK92细胞的量施用IL-2将引起不良副作用。

[0086] 在一些实施方式中，表达FcR的NK-92细胞被进一步修饰以表达至少一种细胞因子和自杀基因。在具体实施方式中，至少一种细胞因子是IL-2，IL-12，IL-15，IL-18，IL-21或其变体。在优选的实施方式中，细胞因子是IL-2。在某些实施方式中，IL-2是靶向内质网的变体，并且自杀基因是iCas9。

[0087] 在一个实施方式中，IL-2与将IL-2引导至内质网的信号序列一起表达。在一些实施方式中，编码IL-2的多核苷酸编码具有SEQ ID NO:7序列的多肽。不受理论束缚，但是将IL-2引导到内质网允许IL-2以足以进行自分泌激活的水平表达，但不细胞外地释放IL-2。参见Konstantinidis等，“Targeting IL-2to the endoplasmic reticulum confines autocrine growth stimulation to NK-92cells”Exp Hematol.2005年2月;33(2):159-64。可以例如通过自杀基因的存在防止连续激活表达FcR的NK-92细胞。

[0088] 免疫治疗

[0089] 抗体可以用于靶向被感染或表达癌症相关标志物的细胞。许多抗体已经被批准用于单独治疗癌症。

[0090] 表2.说明性的治疗性单克隆抗体

[0091]

FDA 批准的治疗性单克隆抗体的实例				
抗体	**商标名**	**公司**	**靶标**	**适应症（目标疾病）**
阿伦单抗	Campath®	Genzyme	CD52	慢性淋巴细胞性白血病
本妥昔单抗（Brentuximab vedotin）	Adcetris®		CD30	间变性大细胞淋巴瘤（ALCL）和霍奇金淋巴瘤
西妥昔单抗	Erbitux®	Bristol-Myers Squibb/Eli Lilly/Merck KGaA	表皮生长因子受体	结肠直肠癌，头颈癌
吉妥珠单抗	Mylotarg®	Wyeth	CD33	急性骨髓性白血病（使用卡

[0092]

FDA 批准的治疗性单克隆抗体的实例				
抗体	商标名	公司	靶标	适应症（目标疾病）
				奇霉素）
替伊莫单抗（Ibritumomab tiuxetan）	Zevalin®	Spectrum Pharmaceuticals,Inc.	CD20	非霍奇金淋巴瘤（使用钇-90或铟-111）
伊匹单抗(MDX-101)	Yervoy®		阻断 CTLA-4	黑色素瘤
奥法木单抗	Arzerra®		CD20	慢性淋巴细胞性白血病
帕利珠单抗	Synagis®	MedImmune	RSV F 蛋白的表位	呼吸道合胞病毒
帕尼单抗	Vectibix®	Amgen	表皮生长因子受体	结肠直肠癌
利妥昔单抗	Rituxan®,Mabthera®	Biogen Idec/Genentech	CD20	非霍奇金淋巴瘤
托西莫单抗	Bexxar®	GlaxoSmithKline	CD20	非霍奇金淋巴瘤
曲妥珠单抗	Herceptin®	Genentech	ErbB2	乳腺癌
Blinatunomab			双特异性CD19-引导CD3 T-细胞衔接体	费城染色体阴性复发或难治B 细胞前体急性淋巴细胞白血病（ALL）
Avelumamab			抗-PD-L1	非小细胞肺癌，转移性Merkel 细胞癌；胃癌，乳腺癌，卵巢癌，膀胱癌，黑色素瘤，间皮瘤，

[0093]

FDA批准的治疗性单克隆抗体的实例				
抗体	商标名	公司	靶标	适应症（目标疾病）
				包括转移性或局部晚期实体肿瘤
Daratumumab			CD38	多发性骨髓瘤
埃罗妥珠单抗			SLAMF7-引导(也称为CD319)免疫刺激性抗体	多发性骨髓瘤

[0094] 抗体可以通过多种机制治疗癌症。当免疫细胞如NK细胞通过Fc受体如CD16与结合到靶细胞的抗体结合时，发生抗体依赖性细胞的细胞毒性(ADCC)。

[0095] 因此，在一些实施方式中，表达CD16的NK-92细胞与针对特定癌症相关蛋白引导的抗体一起被施用于患者。

[0096] 施用表达FcR的NK-92细胞可以与施用单克隆抗体同时进行，或以顺序方式进行。使NK-92细胞表达FcR的遗传修饰能够使细胞识别被Ab包被的靶细胞并触发NK细胞介导的ADCC，从而导致快速NK细胞激活。在一些实施方式中，在已经用单克隆抗体治疗受试者之后，向受试者施用表达FcR的NK-92细胞。在一些实施方式中，在施用单克隆抗体的24小时内，或在18小时内，或在12小时内，或在8小时内或在6,5,4,3,2或1小时内施用表达FcR的NK-92细胞。在一些实施方式中，在施用抗体后24至72小时施用表达FcR的NK-92细胞。在一些实施方式中，在施用抗体的1,2,3或4天或更长时间内施用表达FcR的NK-92细胞。

[0097] 在一些实施方式中，表达FcR的NK-92细胞和单克隆抗体被静脉内施用。在一些实施方式中，表达FcR的NK-92细胞被直接输注到骨髓中。

[0098] 在本发明的一个方面，将表达FcR的NK-92细胞与治疗性单克隆抗体(例如阿仑单抗)组合施用于患有白血病的患者。在一些实施方式中，表达FcR的NK-92细胞与阿仑单抗被同时施用。在一些实施方式中，在已经用阿仑单抗治疗受试者后施用表达FcR的NK-92细胞。在一些实施方式中，在施用阿伦单抗的24小时内，或在18小时内，或在12小时内，或在8小时内或在6,5,4,3,2或1小时内施用表达FcR的NK-92细胞。在一些实施方式中，在施用阿仑单抗后24至72小时或更长时间施用表达FcR的NK-92细胞。

[0099] 在另一方面，将表达FcR的NK-92细胞与曲妥珠单抗组合施用于患有癌症(如乳腺癌或胃癌)的患者。在一些实施方式中，表达FcR的NK-92细胞与曲妥珠单抗被同时施用。在一些实施方式中，在曲妥珠单抗之后施用表达FcR的NK-92细胞。在一些实施方式中，在施用曲妥珠单抗的24小时内，或在18小时内，或在12小时内，或在8小时内或在6,5,4,3,2或1小时内施用表达FcR的NK-92细胞。在一些实施方式中，在施用曲妥珠单抗后24至72小时或更长时间施用表达FcR的NK-92细胞。

[0100] 在另外的方面，将表达FcR的NK-92细胞与brentuximab组合施用于患有霍奇金淋巴瘤的患者。在一些实施方式中，表达FcR的NK-92细胞与brentuximab被同时施用。在一些实施方式中，在brentuximab之后施用表达FcR的NK-92细胞。在一些实施方式中，在施用brentuximab的24小时内，或在18小时内，或在12小时内，或在8小时内或在6,5,4,3,2或1小时内施用表达FcR的NK-92细胞。在一些实施方式中，在施用brentuximab后24至72小时或更长时间施用表达FcR的NK-92细胞。

[0101] 在另外的方面，将表达FcR的NK-92细胞与daratumumab组合施用于患有多发性骨髓瘤的患者。在一些实施方式中，表达FcR的NK-92细胞与daratumumab被同时施用。在一些实施方式中，在daratumumab之后施用表达FcR的NK-92细胞。在一些实施方式中，在施用daratumumab的24小时内，或在18小时内，或在12小时内，或在8小时内或在6,5,4,3,2或1小时内施用表达FcR的NK-92细胞。在一些实施方式中，在施用daratumumab后24至72小时或更长时间施用表达FcR的NK-92细胞。

[0102] 转基因表达

[0103] 可以通过本领域技术人员已知的任何机制将转基因(例如CD16和IL-2)工程化到表达质粒中。转基因可以被工程化到相同或不同的表达质粒中。在优选的实施方式中，转基因在相同质粒上表达。

[0104] 可以使用本领域已知的任何瞬时转染方法将转基因引入NK-92细胞，包括例如电穿孔，脂质转染，核转染或“基因枪”。

[0105] 可以使用任何数量的载体表达CD16和IL-2。在一些实施方式中，载体是逆转录病毒载体。在一些实施方式中，载体是质粒载体。可以使用的其他病毒载体包括腺病毒载体，腺相关病毒载体，单纯疱疹病毒载体，痘病毒载体等。

[0106] 可以通过绝对数量的细胞向这样的个体施用NK-92细胞，例如，所述个体可以被施用约1000个细胞/注射到至多约100亿个细胞/注射，例如约，至少约，或最多约1×10^8,1×10^7,5×10^7,1×10^6,5×10^6,1×10^5,5×10^5,1×10^4,5×10^4,1×10^3,5×10^3(等等)个NK-92细胞/注射，或数字中的任何两个之间的任何范围，包括端点。在其它实施方式中，可以通过相对数量的细胞向这样的个体施用NK-92细胞，例如，所述个体可以被施用约1000个细胞到至多约100亿个细胞/千克个体，例如约，至少约，或最多约1×10^8,1×10^7,5×10^7,1×10^6,5×10^6,1×10^5,5×10^5,1×10^4,5×10^4,1×10^3,5×10^3(等等)个NK-92细胞/千克个体，或数字中的任何两个之间的任何范围，包括端点。在一些实施方式中，向患者施用约10亿至约30亿个NK-92细胞。在其他实施方式中，可以基于体表面积的m^2计算总剂量，包括11×10^{11},1×10^{10},1×10^9,1×10^8,$1\times10^7/m^2$。一般人为1.6-1.8m^2。

[0107] 如下所述的NK-92细胞，单克隆抗体和/或其它抗癌剂可以被施用于患有癌症或感染病毒的患者一次，或者可以被施用多次，例如治疗过程中每1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22或23小时一次，或每1,2,3,4,5,6或7天一次，或每1,2,3,4,5,6,7,8,9,10或更多周一次，或数字中的任何两个之间的任何范围，包括端点。

[0108] 实施例

[0109] 以下实施例仅用于说明目的，而不应被解释为所要求保护的本发明的限制。本领域技术人员可以使用各种替代技术和程序，其类似地允许本领域技术人员成功实施预期发明。

[0110] 实施例1:CD16重组逆转录病毒制备

[0111] 根据标准方法,使用BamHI和NotI限制性位点,将编码跨膜免疫球蛋白γFc区受体III-A(FcγRIII-A或CD16)[苯丙氨酸-158(F158)),完整序列:SwissProt P08637(SEQ ID NO:3)]的低亲和力成熟形式(SEQ ID NO:1)的CD16cDNA X52645.1或编码CD16受体的更高亲和力成熟形式[缬氨酸-158(F158V)(SEQ ID NO:2)),完整序列:SwissProt VAR_008801(SEQ ID NO:4)]的多态性变体亚克隆到双顺反子逆转录病毒表达载体pBMN-IRES-EGFP(得自G.Nolan,Stanford University,Stanford,Calif.)。

[0112] 将重组载体与10μL的PLUS™试剂(Invitrogen;Carlsbad,Calif.)混合;用预加温的无血清Opti-MEM®(Invitrogen;MEM,最小必需培养基)稀释至100μL;通过将8μL Lipofectamine™(Invitrogen)加入到100μL预加温的无血清Opti-MEM®中而进一步稀释;并在室温下温育15分钟。然后通过加入预加温的无血清Opti-MEM®使该混合物达到的总体积为1mL。使Phoenix-Amphotropic包装细胞(得自G.Nolan,Stanford University,Stanford,Calif.;(Kinsella和Nolan,1996))在6孔板中生长至70-80%汇合,并用6mL预加温的无血清Opti-MEM®培养基(Invitrogen)洗涤。除去培养基后,向每个孔中加入1mL重组载体在Lipofectamine™PLUS™试剂中的溶液,并将细胞在37℃,7%CO_2/平衡空气气氛下孵育至少3小时。将含有10%胎牛血清(FBS)的4mL预加温RPMI培养基加入到每个孔中,并将细胞在37℃,7%CO_2/平衡空气气氛下孵育过夜。然后除去培养基;用6mL预加温的无血清Opti-MEM®洗涤细胞;加入2mL无血清Opti-MEM®;并将细胞在37℃,7%CO_2/平衡空气气氛下再孵育48小时。

[0113] 将含病毒的上清液收集到15mL塑料离心管中;以1300rpm离心5分钟以除去细胞和细胞碎片;并将上清液转移到另一个15mL塑料离心管中。使用前立即将20μL PLUS™试剂加入病毒悬浮液中;将混合物在室温下温育15分钟;向混合物中加入8μL Lipofectamine™;并将混合物在室温下再温育15分钟。

[0114] 实施例2:将IL-2基因和TK自杀基因克隆到CD16重组逆转录病毒中

[0115] 使用胸苷激酶(TK)基因和产生ER-驻留性IL-2的KDEL标记的构建体(Konstantinidis等,2005Experimental Hematology 33:159-64)制备重组逆转录病毒,其包含用于表达IL-2并将相应的cDNA连接到CD16pBMN-IRES-EGFP载体中的基因(Miah和Campbell 2010Methods Mol.Biol.612:199-208)。然后将pBMN-IRES-EGFP载体在Lipofectamine™Plus存在下转染到Phoenix-Amphotropic包装细胞系中。

[0116] 实施例3:将TK,CD16和IL-2逆转录病毒转导到NK-92细胞中

[0117] 通过1300rpm离心5分钟收集在补充有12.5%FBS,12.5%胎马血清(FHS)和500IU rhIL-2/mL(Chiron;Emeryville,Calif.)的A-MEM(Sigma;St.Louis,Mo.)中培养的NK-92细胞。并将细胞沉淀重悬于10mL无血清Opti-MEM®培养基中。将含有5×10^4个细胞的细胞悬浮液的等分试样以1300rpm沉降5分钟;将细胞沉淀重悬于实施例1中所述的2mL逆转录病毒悬浮液中,并将细胞接种到12孔培养板中。将板在1800rpm下离心30分钟,并在37℃,7%CO_2/平衡空气气氛下温育3小时。第二次重复这个离心和孵育循环。将细胞用8mL的α-MEM稀释,转移到T-25烧瓶中,并在37℃,7%CO_2/平衡空气下孵育直至细胞汇合。收集转导的细

胞，重悬于无血清Opti-MEM®培养基中，并使用荧光激活细胞分选仪(FACS)基于其EGFP表达水平分选，EGFP与针对CD16的替代标志物共同表达。CD16的细胞表面表达通过用抗CD16抗体对转导的细胞进行免疫染色而证实。IL-2的细胞表面表达通过用纯化的大鼠抗人IL-2抗体进行免疫染色而测定，并且IL-2细胞内定位通过用兔抗钙网蛋白ER-Marker进行免疫染色而证实。在使用前，分析转导的细胞(命名为NK-92-TK-CD16-IL2)的CD16细胞表面表达和IL-2细胞内表达。通过测试对gangcylovir的敏感性，分析细胞的TK表达。

[0118] 实施例4：在外源性IL-2存在或不存在的情况下，NK-92-TK-CD16-IL-2和未修饰的NK-92细胞的生长

[0119] NK-92-TK-CD16-IL-2和未修饰的NK-92细胞首先在外源性IL-2(1,200IU/mL)存在的情况下培养4至5周，然后被转移到无IL-2培养基，并在外源性IL-2不存在的情况下培养。然后评估这些细胞的增殖。

[0120] 通过流式细胞术测量CD16和IL-2的表面表达。NK-92-TK-CD16-IL-2和未修饰的NK-92细胞在不存在外源性IL-2的情况下孵育24小时后进行的流式细胞分析显示在NK-92-TK-CD16-IL-2和未修饰的NK-92细胞中具有相似的细胞毒性作用，与未修饰的NK-92细胞相比，NK-92-TK-CD16-IL-2细胞呈现增加的CD16表面表达和低得多的IL-2表面表达。

[0121] 这些结果通过确定NK-92-TK-CD16-IL-2细胞是否支持旁观者未修饰的NK-92细胞的生长的实验得以证实，其中未修饰的NK-92细胞与NK-92-TK-CD16-IL-2混合且在外源性IL-2不存在的情况下共培养。这些实验表明，NK-92-TK-CD16-IL-2不支持未修饰的NK-92细胞的生长，因为IL-2最小释放到培养基中。事实上，未修饰的NK-92细胞在在外源性IL-2不存在的情况下孵育48小时后停止增殖。相比之下，NK-92-TK-CD16-IL-2细胞的增殖在72小时孵育后仍然可见。

[0122] 总之，这些结果表明，当这些细胞被保持在不含外源性IL-2的环境中时，ER-IL-2刺激NK-92-TK-CD16-IL-2细胞的生长。

[0123] 实施例5：NK-92-TK-CD16-IL-2细胞的全身性毒性和扩增被自杀基因有效地消除

[0124] IL-2的内源性表达可能导致具有自主生长的杀伤细胞突变体的潜在发展。因此，评估NK-92-TK-CD16-IL-2细胞，NK-92-TK-CD16-IL-2细胞和非修饰的NK-92细胞的体内扩增。SCID小鼠被亚致死照射(250rad)并分成两组。15-20天后，当肿瘤可触及(直径0.5-0.8cm)时，将NK-92-TK-CD16-IL-2细胞静脉内注射到第一组经照射的小鼠中，将未修饰的NK-92细胞静脉内注射到第二组小鼠中。没有向小鼠施用外源性细胞因子。用荧光激活细胞分选仪(FACS)检测EGFP表达，用于监测定位和扩增。两组小鼠在注射后24小时都显示出靶向定位和扩增。24小时后，对照小鼠组中的未修饰的NK-92细胞停止扩增，而NK-92-TK-CD16-IL-2细胞继续显著扩增。注射后48小时，对照小鼠中未修饰的NK细胞的凋亡和小鼠测试组中NK-92-TK-CD16-IL-2细胞的指数扩增都是可见的。在对照小鼠中，肿瘤快速达到直径等于或大于1.2cm的大小，并将小鼠安乐死。

[0125] 将来自具有较小肿瘤或完全肿瘤消退的测试组的小鼠隔离成两组以评估自杀基因的功能。每隔一天用两剂或三剂更昔洛韦(50μg)腹膜内治疗第一组中的小鼠。用安慰剂治疗第二组的小鼠。将更昔洛韦施用于小鼠导致NK-92-TK-CD16-IL-2细胞在24小时至72小时内显著降低，细胞恢复到预扩增水平。在用安慰剂治疗的小鼠中，NK-92-TK-CD16-IL-2细胞的扩增随时间继续增加。

[0126] 这些结果表明,TK基因的存在确保NK-92-TK-CD16-IL-2保持对更昔洛韦敏感,并阻止NK-92-TK-CD16-IL-2细胞指数扩增。被引入到NK92细胞染色体中的在相同逆转录病毒载体上的TK和IL-2的组合提供了增强的生物学安全性。因为细胞依赖于IL-2,所以对于保留TK-CD16-IL-2序列有着很强的选择。因此,细胞对更昔洛韦敏感。那些失去TK基因并且变得对更昔洛韦有抗性的细胞也会失去它们生长所必需的IL-2基因。

[0127] 实施例6:NK-92-TK-CD16-IL-2对不同白血病细胞系的细胞毒性活性

[0128] NK-92-TK-CD16-IL-2效应细胞通过悬浮在α-MEM(不含IL-2)中洗涤,并以1300rpm沉降5分钟。将细胞沉淀悬浮在α-MEM中,计数细胞,并以1×10^5/mL(效应物与靶细胞的比率(E:T)=1:1),5×10^5/mL(E:T=5:1),1×10^6/mL(E:T=10:1),2×10^6/mL(E:T=20:1)或适合于进行的测定的细胞浓度制备等分试样。

[0129] 测定NK-92-TK-CD16-IL-2效应细胞对K562,Daudi,TF-1,AML-193和SR-91细胞的细胞毒性活性(Gong等(1994))。K562(红白血病)和Daudi(Burkitt)淋巴瘤细胞系获自ATCC。它们在补充有10%胎牛血清(FCS)的RPMI 1640培养基中保持连续悬浮培养。TF-1是需要存在含有2ng/mL人GM-CSF的培养基的骨髓单核细胞系(Kitamura等,J.Cell Physiol.140:323-334(1989))。AML-193是在10%5637条件培养基存在的情况下保持的骨髓细胞系(Lange等,Blood 70:192-199(1987))。TF-1和AML-193细胞均获自不列颠哥伦比亚省温哥华市不列颠哥伦比亚大学特里福克斯实验室D.Hogge博士。SR-91是由Gong等(1994)从急性淋巴细胞白血病(ALL)患者(Klingemann等,Leuk.Lymphoma,12,463-470(1994))建立的具有早期祖细胞特征的细胞系。它对NK和活化NK(A-NK)细胞的细胞毒性均有抗性。SR-91也保存在RPMI 1640/10%FCS中,该细胞系可以通过用细胞因子治疗而使得对NK-92杀灭敏感。

[0130] 在标准的4小时51Cr释放试验中一式三份地测量NK-92-TK-CD16-IL-2效应细胞对这些靶细胞的细胞毒性活性。简言之,用100μL 51Cr(比活度为1mCi/mL)标记1×10^6个NK-92-TK-CD16-IL-2细胞,并在37℃孵育1小时。使用台盼蓝染料排斥法计数效应细胞,并将其与靶细胞混合以获得10:1,3:1,1:1和0.3:1的效应物:靶比率。CellGro培养基用作阴性对照,而对于阳性对照,将细胞与1%Triton X一起孵育。在V底部形状的96孔板中于37℃孵育4小时后,从各个孔吸取70μL上清液,并使用Packard Cobra Auto-Gamma 5000系列计数系统(Meriden,CT,USA)计数。自发释放的百分比由以下公式计算:%特异性51Cr释放=(样品释放-自发释放)/(最大释放-自发释放)×100。

[0131] NK-92-TK-CD16-IL-2细胞对K562和Daudi细胞的细胞毒性活性明显高于未修饰的NK细胞-92的细胞毒性活性。NK-92-TK-CD16-IL-2细胞对TF-1细胞和AML-193细胞的细胞溶解活性不太有效,但仍高于未修饰的NK-92细胞的细胞溶解活性。SR-91细胞对NK-92-TK-CD16-IL-2细胞和未修饰的NK-92细胞的细胞毒性作用均具有抗性。这种对SR-91细胞的细胞毒性活性的缺乏与SR-91细胞中缺乏介导与NK-92细胞初始结合所必需的粘附分子一致。

[0132] 实施例7:NK-92-TK-CD16-IL-2细胞对人原代白血病细胞的细胞溶解

[0133] 在患有新诊断的或复发性的白血病的患者的常规诊断性血液研究过程中或骨髓(BM)抽吸物中,在知情同意的情况下,获得样品。富集母细胞(blast-enriched)的单核细胞通过Ficoll Hypaque(Pharmacia,Piscataway,N.J.)密度梯度分离而分离,并在RPMI 1640培养基中洗涤。将NK-92-TK-CD16-IL-2细胞和未修饰的NK-92细胞培养并保持在补充有

12.5％FCS和12.5％马血清的α-MEM培养基中。然后使用标准的4小时铬释放试验，比较NK-92-TK-CD16-IL-2细胞和未修饰的NK-92细胞对白血病样品的细胞毒性活性。

[0134] NK-92-TK-CD16-IL-2细胞对白血病靶标的细胞溶解活性明显高于未修饰的NK-92细胞。本发明的NK-92-TK-CD16-IL-2细胞在裂解患者来源的肿瘤细胞方面令人惊奇地且显著地更有效，并且在比未修饰的NK-92细胞更短的时间内发挥其作用。

[0135] 实施例8：NK-92-TK-CD16-IL-2细胞在人白血病异种移植SCID小鼠模型中的抗白血病作用

[0136] 为了研究NK-92-TK-CD16-IL-2细胞的体内杀肿瘤能力，将来自T谱系急性淋巴细胞性白血病(ALL)患者、急性骨髓性白血病(AML)患者和前B-ALL患者的白血病细胞通过S.C.接种在SCID小鼠中过继生长和扩增。在这些实验中使用从小鼠中的白血病结节回收的白血病细胞(第一次传代)。对每个组中的SCID小鼠I.P.接种在0.2mL PBS中来自第一次传代的5×10^6个白血病细胞，并在24小时后通过LP.注射施用0.4ml PBS中的2×10^7个NK-92-TK-CD16-IL-2细胞。在具有和不具有外源性IL-2的情况下，动物接受1剂或一系列5剂NK-92-TK-CD16-IL-2细胞，其在第1,3,5,7和9天施用。

[0137] 所有人类白血病在SCID小鼠中侵袭性地生长。来源于T-ALL患者、AML患者和前B-ALL患者的白血病细胞在体外对NK-92-TK-CD16-IL-2细胞和未修饰的NK-92细胞高度敏感。

[0138] 与用未修饰的NK-92细胞治疗相比，用NK-92-TK-CD16-IL-2细胞治疗显著延长小鼠寿命并延长存活。接受5剂NK-92-TK-CD16-IL-2细胞注射的多只动物在接种后6个月存活，没有任何白血病发展迹象。用NK-92治疗的小鼠显示初步改善，但在6个月时在少数小鼠中观察到白血病细胞。

[0139] 这些结果表明，使用NK-92-CD16-IL-2细胞体内治疗白血病肿瘤非常有效，并且导致寿命延长和健康改善。

[0140] 实施例9：ADCC介导的细胞裂解

[0141] 作为高度选择性和有效的抗肿瘤剂的多种抗体的活性至少部分地依赖于天然杀伤细胞与抗体的Fc(恒定)部分的结合，使得肿瘤细胞的裂解通过抗体依赖性细胞的细胞毒性(ADCC)机制发生。尽管NK-92细胞几乎保留了与NK细胞相关的几乎所有激活受体和细胞溶解通路，但它们不表达CD16受体，因此不可以通过ADCC机制裂解靶细胞。如果细胞对有效抗体具有足够的结合亲和力，则将CD16表达转基因地插入到NK-92细胞中允许NK-92细胞通过ADCC机制起作用。

[0142] 在被施用于患有白血病的受试者的表达FcR的NK-92细胞中，在已经用阿伦单抗治疗所述受试者后24至72小时，评价结合不同抗体的效果。将表达FcR的NK-92细胞和结合靶癌细胞的抗体的细胞毒性作用与未修饰的NK-92细胞的细胞毒性作用进行比较。根据表2选择和分析抗体和相应的靶癌细胞。

[0143] 所选择的靶细胞用Na[^{51}Cr]铬酸盐标记。将^{51}[Cr]标记的靶细胞的等分试样与选定的抗体在0.01μg和5μg/mL之间的多种浓度下在室温下进一步孵育15分钟，用α-MEM洗涤，并在使用前调节至1×10^5个细胞/mL浓度。将细胞浓度为1×10^5个细胞/mL(E:T＝1:1)，5×10^5个细胞/mL(E:T＝5:1)的100μL选定类型的靶细胞和100μL效应细胞，1)，1×10^6个细胞/mL(E:T＝10:1)，2×10^6个细胞/mL(E:T＝20:1)或适合于进行的测定的100μL选定类型的靶细胞和100μL效应细胞加入到96孔V底部板的每个孔中。在每个E:T比率下制备三至六个重

复孔以进行评估。将至少6个孔分配给每个自发裂解对照(效应细胞用100μL的α-MEM替代)和总释放控制(效应细胞用100μL在α-MEM中的2％Triton X-100洗涤剂替代)。将每个E:T比率的另外三个孔分配给“非ADCC”对照,其中靶细胞不暴露于抗体。将另外6个或更多个孔分配给使用不表达CD16的未修饰的NK-92效应细胞作为程序控制和内标。然后将板在500rpm离心3分钟,并在37℃,7％CO_2/平衡空气气氛下孵育4小时。在孵育期结束时,将板在1500rpm下离心8分钟,并从每个孔中收集100mL上清液以在γ计数器中计数,作为由于细胞毒性而导致的^{51}[Cr]释放的量度。然后计算特异性裂解的百分数。

[0144] 用表达不同表面水平的CD16的表达FcR的NK-92细胞重复这些试验。

[0145] 在选定抗体存在的情况下,表达FcR的NK-92细胞显示出对靶癌细胞的高细胞毒性活性。未修饰的NK-92细胞显示出对靶癌细胞的较低细胞毒活性。这些结果证实,表达FcR的NK-92细胞具有通过ADCC机制起作用的能力,并因此在抗体存在的情况下提供对肿瘤细胞的增强的治疗作用。

[0146] 实施例10:表达FcR的NK-92细胞和吉妥珠单抗在人白血病异种移植SCID小鼠模型中的组合抗白血病效应

[0147] 为了研究表达FcR的NK-92细胞的体内杀肿瘤能力,将来自急性骨髓性白血病(AML)患者的白血病细胞通过S.C.接种在SCID小鼠中过继生长并扩增。在这些实验中使用从小鼠中的白血病结节回收的白血病细胞(第一次传代)。对每组中的SCID小鼠I.P.接种在0.2mL PBS中来自第一次传代的5×10^6个白血病细胞,并在24小时后通过LP.注射施用在0.4mL PBS和吉妥珠单抗中的2×10^7个FcR表达的NK-92细胞。在具有或不具有吉妥珠单抗的情况下,动物接受1剂或一系列5剂表达FcR的NK-92细胞,其在第1,3,5,7和9天施用。在具有或不具有吉妥珠单抗的情况下,对照动物用未修饰的NK-92细胞治疗。

[0148] 人白血病在SCID小鼠中侵袭性地生长。用表达FcR的NK-92细胞与吉妥珠单抗联合治疗的小鼠显示肿瘤消退,并且抗肿瘤作用比在不具有吉妥珠单抗的情况下仅使用表达FcR的NK-92细胞治疗的小鼠更高,而且比用NK-92治疗的小鼠更高。

[0149] 这些结果表明,用表达FcR的NK-92细胞与单克隆抗体(例如吉妥珠单抗)组合体内治疗白血病肿瘤非常有效,并且导致寿命延长和健康改善。

[0150] 实施例11:表达CD16和内质网靶向性IL-2的质粒表达载体的构建

[0151] 使用GeneArt(Life Technologies)的Gene String程序从头设计质粒骨架。其最小结构包括colE1细菌复制起点,氨苄青霉素抗性盒和由位于多克隆位点(MCS)侧翼的EF1α启动子和SV40聚腺苷酸化位点组成的哺乳动物表达盒。

[0152] 哺乳动物表达盒的侧翼是BamHI位点,其不仅允许质粒的线性化,而且还允许去除所有非真核生物序列。

[0153] 所表达的转基因是人CD16 158V序列,随后是IRES序列本身,随后是ERIL-2序列(IL-2KDEL),使得IL-2靶向内质网。通过GeneArt对CD16和ERIL-2序列二者进行密码子优化,以使在人中的表达最大化。可以使用EcoRI和NotI切除转基因。所得mRNA是在EF1α启动子控制下的双顺反子转录物,ERIL-2在IRES序列的控制下独立地从CD16翻译。图1中提供了质粒的示意图。该质粒用于转染NK-92细胞。

[0154] 实施例12:使用质粒表达载体转染NK-92.W细胞

[0155] NK-92.W细胞是迄今大多数临床试验的亲本系。将一瓶Bioreliance工作细胞库

(WCB,p15 11/30/00)解冻到具有12ml X-Vivo10 5%HS+500IU/ml rhIL-2的T25烧瓶中,并且每2-4天传代(×2至×4稀释到新鲜X-vivo10 5%HS+IL-2中,总共18-20次分离/传代)。

[0156] 将用于转染的NK-92.W细胞在500g下旋转10分钟。弃去上清液,并将细胞沉淀重悬于15ml D-PBS 1×中,并以500g离心10分钟。将沉淀以10^7个细胞/ml的细胞密度重悬于缓冲液R(Neon kit,Invitrogen)中。使用Neon电穿孔机(5ug DNA对100ul缓冲液R中的10^6个细胞;1250V/10ms/3脉冲,电穿孔管中含有3ml缓冲液E2),用pNEUKv1CD16(158V)-ERIL2质粒电穿孔NK-92.W细胞。将经电穿孔的细胞在培养基+IL-2(在6孔板中,4ml培养基/孔)中孵育过夜,并在10/16/14(一次PBS旋转/洗涤)转移到不含IL-2的培养基中。使用抗CD16抗体(克隆3G8,小鼠IgG1k)APC-Cy7缀合的(Bd Pharmingen),分析CD16表达。在短短两周内,约78%的细胞是CD16阳性(右峰,图2a)。约90%的细胞在约4周时呈阳性(右峰,图2b)。

[0157] 将NK-92.W CD16(158V)-ERIL2细胞冷冻(5个小瓶,1×10^6个细胞/小瓶),12/15/14(5个小瓶,1×10^6个细胞/小瓶)。冷冻培养基为10%DMSO,50%HS,40%

[0158] 评估冷冻的NK-92.W FcR-ERIL2细胞。将细胞解冻并在T25烧瓶中不含IL-2的X-Vivo10 5%HS中培养。使用与APC-Cy7缀合的抗-CD16抗体克隆3G8,通过流式细胞术(Attune)随时间跟踪CD16(158V)的表达,使用相同设置以允许在试验之间比较MFI。CD16表达随时间稳定(图3)。

[0159] 实施例13:ADCC活性的评估

[0160] 首先针对与利妥昔单抗组合的CD20+细胞系DoHH2测试ADCC活性。随时间(n=9),以及针对与赫赛汀组合的Her2/Neu+细胞系SKOV3(n=5)重复测试。结果显示在图4中。表达CD16和内质网靶向性IL-2的被修饰的NK-92.W细胞(在图4中称为HaNK.12/15)显示出在用赫赛汀一起使用时对SKOV-3细胞增强的ADCC活性,和在与利妥昔单抗一起使用时对DoHH2细胞增强的ADCC活性。ThehaNK.12/15细胞在对照(DoHH2细胞,赫赛汀抗体;SKOV-3细胞/利妥昔单抗)中未显示ADCC活性。当与抗体一起施用时,未修饰的NK-92.2细胞也不显示ADCC活性。

[0161] 实施例11-13因此证明当与单克隆抗体组合使用时,使用质粒载体修饰以表达CD16和IL-2的NK-92细胞表现出增强的ADCC活性。

[0162] 应当理解,本文描述的实施例和实施方式仅用于说明目的,并且鉴于其的各种修改或改变将被提议给本领域技术人员,且要被包括在本申请的精神和视界内和随附权利要求的范围内,本文引用的所有出版物、序列登录号、专利和专利申请出于所有目的通过引用以其全文并入本文。

[0163] 说明性序列表

[0164] SEQ ID NO:1:低亲和力免疫球蛋白γFc区受体III-A氨基酸序列(成熟形式)。第158位的苯丙氨酸标上下划线。

[0165] Arg Thr Glu Asp Leu Pro Lys Ala Val Val Phe Leu Glu Pro Gln Trp Tyr Arg Val Leu Glu Lys Asp Ser Val Thr Leu Lys Cys Gln Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp Phe His Asn Glu Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile Asp Ala Ala Thr Val Asp Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn Leu Ser Thr Leu Ser Asp Pro Val Gln Leu Glu Val His Ile Gly Trp Leu Leu Leu Gln Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His Leu Arg Cys His Ser

Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn Gly Lys Gly Arg Lys Tyr Phe His His Asn Ser Asp Phe Tyr Ile Pro Lys Ala Thr Leu Lys Asp Ser Gly Ser Tyr Phe Cys Arg Gly Leu Phe Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile Thr Ile Thr Gln Gly Leu Ala Val Ser Thr Ile Ser Ser Phe Phe Pro Pro Gly Tyr Gln Val Ser Phe Cys Leu Val Met Val Leu Leu Phe Ala Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile Arg Ser Ser Thr Arg Asp Trp Lys Asp His Lys Phe Lys Trp Arg Lys Asp Pro Gln Asp Lys

[0166] SEQ ID NO:2:高亲和力变体F158V免疫球蛋白γFc区受体III-A氨基酸序列(成熟形式)。第158位的缬氨酸标上下划线。

[0167] Arg Thr Glu Asp Leu Pro Lys Ala Val Val Phe Leu Glu Pro Gln Trp Tyr Arg Val Leu Glu Lys Asp Ser Val Thr Leu Lys Cys Gln Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp Phe His Asn Glu Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile Asp Ala Ala Thr Val Asp Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn Leu Ser Thr Leu Ser Asp Pro Val Gln Leu Glu Val His Ile Gly Trp Leu Leu Leu Gln Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His Leu Arg Cys His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn Gly Lys Gly Arg Lys Tyr Phe His His Asn Ser Asp Phe Tyr Ile Pro Lys Ala Thr Leu Lys Asp Ser Gly Ser Tyr Phe Cys Arg Gly Leu Val Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile Thr Ile Thr Gln Gly Leu Ala Val Ser Thr Ile Ser Ser Phe Phe Pro Pro Gly Tyr Gln Val Ser Phe Cys Leu Val Met Val Leu Leu Phe Ala Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile Arg Ser Ser Thr Arg Asp Trp Lys Asp His Lys Phe Lys Trp Arg Lys Asp Pro Gln Asp Lys

[0168] SEQ ID NO:3:低亲和力免疫球蛋白γFc区受体III-A氨基酸序列(前体形式)。该前体形式的第176位对应于成熟形式的第158位。第176位的Phe标上下划线。

[0169] Met Trp Gln Leu Leu Leu Pro Thr Ala Leu Leu Leu Leu Val Ser Ala Gly Met Arg Thr Glu Asp Leu Pro Lys Ala Val Val Phe Leu Glu Pro Gln Trp Tyr Arg Val Leu Glu Lys Asp Ser Val Thr Leu Lys Cys Gln Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp Phe His Asn Glu Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile Asp Ala Ala Thr Val Asp Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn Leu Ser Thr Leu Ser Asp Pro Val Gln Leu Glu Val His Ile Gly Trp Leu Leu Leu Gln Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His Leu Arg Cys His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn Gly Lys Gly Arg Lys Tyr Phe His His Asn Ser Asp Phe Tyr Ile Pro Lys Ala Thr Leu Lys Asp Ser Gly Ser Tyr Phe Cys Arg Gly Leu Phe Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile Thr Ile Thr Gln Gly Leu Ala Val Ser Thr Ile Ser Ser Phe Phe Pro Pro Gly Tyr Gln Val Ser Phe Cys Leu Val Met Val Leu Leu Phe Ala Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile Arg Ser Ser Thr Arg Asp Trp Lys Asp His Lys Phe Lys Trp Arg Lys Asp Pro Gln Asp Lys

[0170] SEQ ID NO:4:高亲和力变体免疫球蛋白γFc区受体III-A氨基酸序列(前体形

式)。该前体形式的第176位对应于成熟形式的第158位。第176位的Val标上下划线。

[0171] Met Trp Gln Leu Leu Leu Pro Thr Ala Leu Leu Leu Leu Val Ser Ala Gly Met Arg Thr Glu Asp Leu Pro Lys Ala Val Val Phe Leu Glu Pro Gln Trp Tyr Arg Val Leu Glu Lys Asp Ser Val Thr Leu Lys Cys Gln Gly Ala Tyr Ser Pro Glu Asp Asn Ser Thr Gln Trp Phe His Asn Glu Ser Leu Ile Ser Ser Gln Ala Ser Ser Tyr Phe Ile Asp Ala Ala Thr Val Asp Asp Ser Gly Glu Tyr Arg Cys Gln Thr Asn Leu Ser Thr Leu Ser Asp Pro Val Gln Leu Glu Val His Ile Gly Trp Leu Leu Leu Gln Ala Pro Arg Trp Val Phe Lys Glu Glu Asp Pro Ile His Leu Arg Cys His Ser Trp Lys Asn Thr Ala Leu His Lys Val Thr Tyr Leu Gln Asn Gly Lys Gly Arg Lys Tyr Phe His His Asn Ser Asp Phe Tyr Ile Pro Lys Ala Thr Leu Lys Asp Ser Gly Ser Tyr Phe Cys Arg Gly Leu Val Gly Ser Lys Asn Val Ser Ser Glu Thr Val Asn Ile Thr Ile Thr Gln Gly Leu Ala Val Ser Thr Ile Ser Ser Phe Phe Pro Pro Gly Tyr Gln Val Ser Phe Cys Leu Val Met Val Leu Leu Phe Ala Val Asp Thr Gly Leu Tyr Phe Ser Val Lys Thr Asn Ile Arg Ser Ser Thr Arg Asp Trp Lys Asp His Lys Phe Lys Trp Arg Lys Asp Pro Gln Asp Lys

[0172] SEQ ID NO:5:编码低亲和力免疫球蛋白γFc区受体III-A(前体)的多核苷酸(编码第158位的苯丙氨酸)

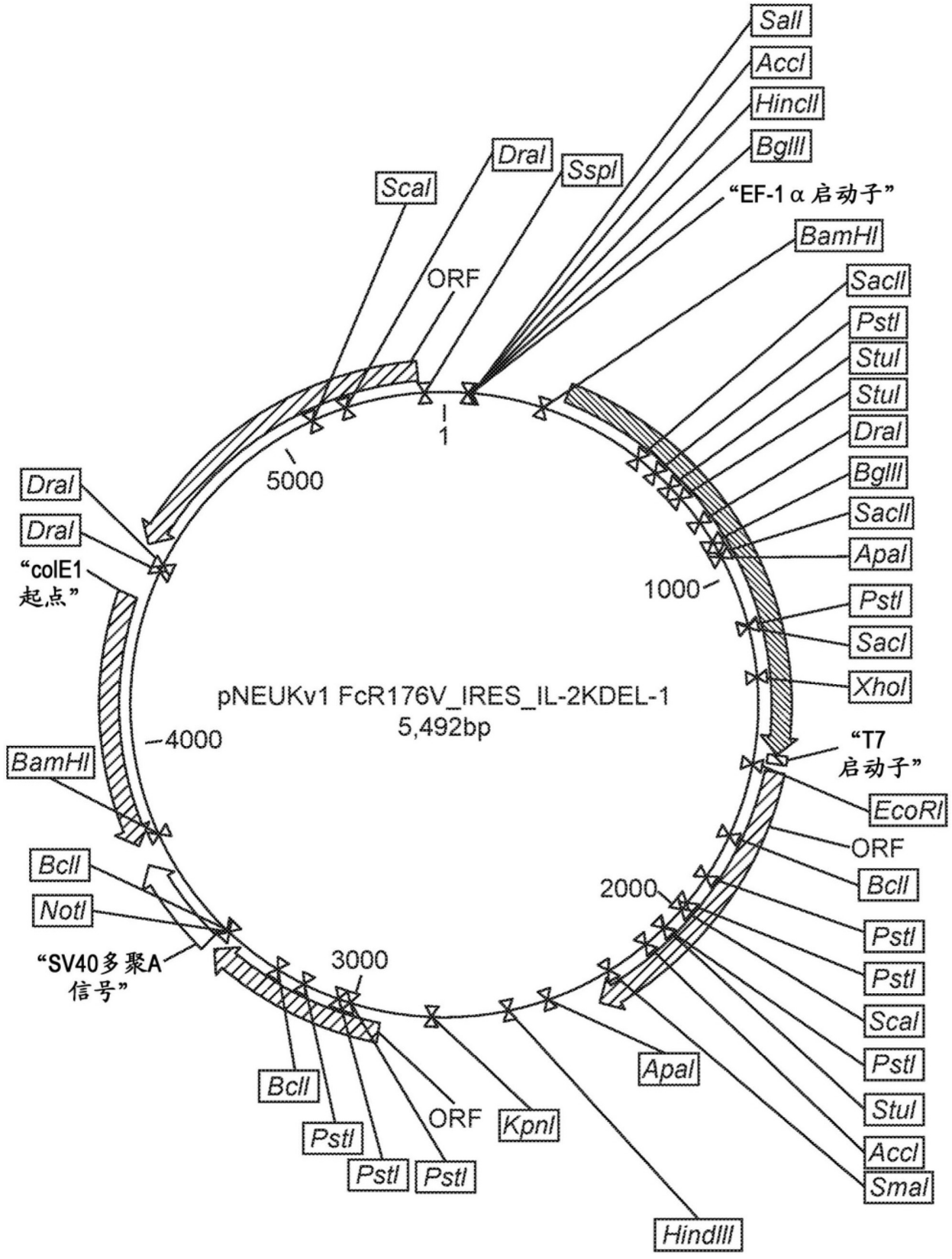
[0173] atgtggcagc tgctcctccc aactgctctg ctacttctag tttcagctgg catgcggact gaagatctcc caaaggctgt ggtgttcctg gagcctcaat ggtacagggt gctcgagaag gacagtgtga ctctgaagtg ccagggagcc tactcccctg aggacaattc cacacagtgg tttcacaatg agagcctcat ctcaagccag gcctcgagct acttcattga cgctgccaca gtcgacgaca gtggagagta caggtgccag acaaacctct ccaccctcag tgacccggtg cagctagaag tccatatcgg ctggctgttg ctccaggccc ctcggtgggt gttcaaggag gaagacccta ttcacctgag gtgtcacagc tggaagaaca ctgctctgca taaggtcaca tatttacaga atggcaaagg caggaagtat tttcatcata attctgactt ctacattcca aaagccacac tcaaagacag cggctcctac ttctgcaggg ggctttttgg gagtaaaaat gtgtcttcag agactgtgaa catcaccatc actcaaggtt tggcagtgtc aaccatctca tcattctttc cacctgggta ccaagtctct ttctgcttgg tgatggtact ccttttgca gtggacacag gactatattt ctctgtgaag acaaacattc gaagctcaac aagagactgg aaggaccata aatttaaatg gagaaaggac cctcaagaca aatga

[0174] SEQ ID NO:6:野生型IL-2

[0175] Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu Val Thr Asn Ser Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr

[0176] SEQ ID NO:7:IL-2-ER

[0177] Met Tyr Arg Met Gln Leu Leu Ser Cys Ile Ala Leu Ser Leu Ala Leu Val Thr Asn Ser Ala Pro Thr Ser Ser Ser Thr Lys Lys Thr Gln Leu Gln Leu Glu His Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Asn Asn Tyr Lys Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Lys Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys Asn Phe His Leu Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile Val Leu Glu Leu Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Cys Gln Ser Ile Ile Ser Thr Leu Thr Gly Ser Glu Lys Asp Glu Leu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AccI
HincII
BglII
"EF-1α启动子"
BamHI
SacII
PstI
StuI
StuI
DraI
BglII
SacII
ApaI
PstI
SacI
XhoI
"T7
启动子"
EcoRI
ORF
BclI
PstI
PstI
ScaI
PstI
StuI
AccI
SmaI
HindIII
ApaI
KpnI
ORF
PstI
PstI
PstI
BclI
"SV40多聚A
信号"
NotI
BclI
BamHI
"colE1
起点"
DraI
DraI
ScaI
DraI
SspI
ORF
1
5000
1000
2000
3000
4000
pNEUKv1 FcR176V_IRES_IL-2KDEL-1
5,492bp

图1

	样品名	亚组名	计数
□	NK-92 WCB CD16-APC Cy7.fcs	Cells	23128
⊡	NK-92 FcR-IL2 CD16-APC Cy7.fcs	Cells	21703

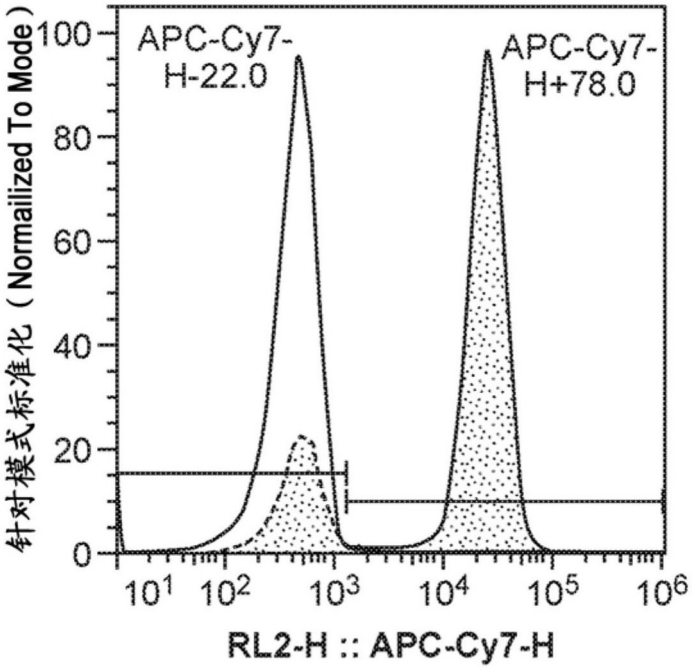

图2a

	样品名	亚组名	计数
□	NK-92 wcb CD16-APC Cy7.fcs	NK-92	30151
⊡	NK-92 FcRIL CD16-APC Cy7.fcs	NK-92	30226

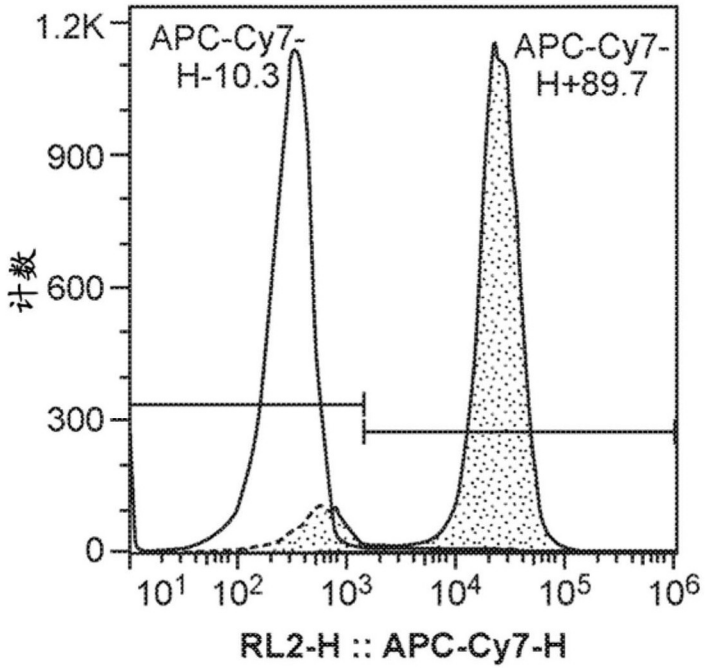

图2b

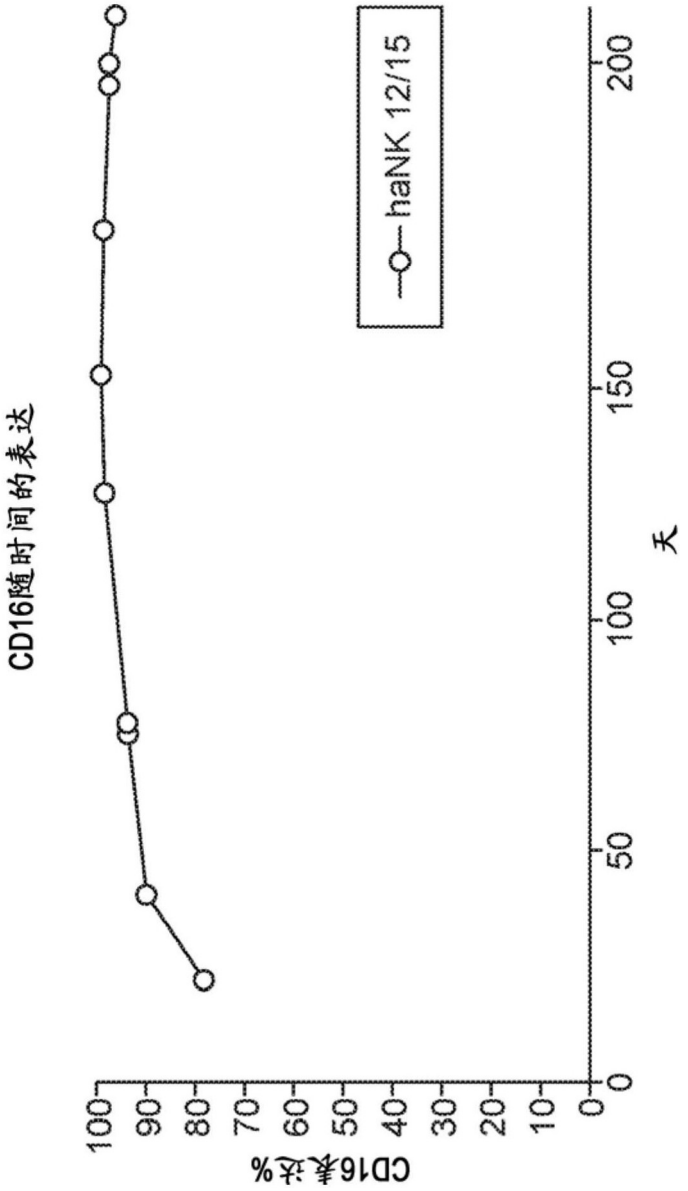
CD16随时间的表达
haNK 12/15
CD16表达%
天
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200

图3

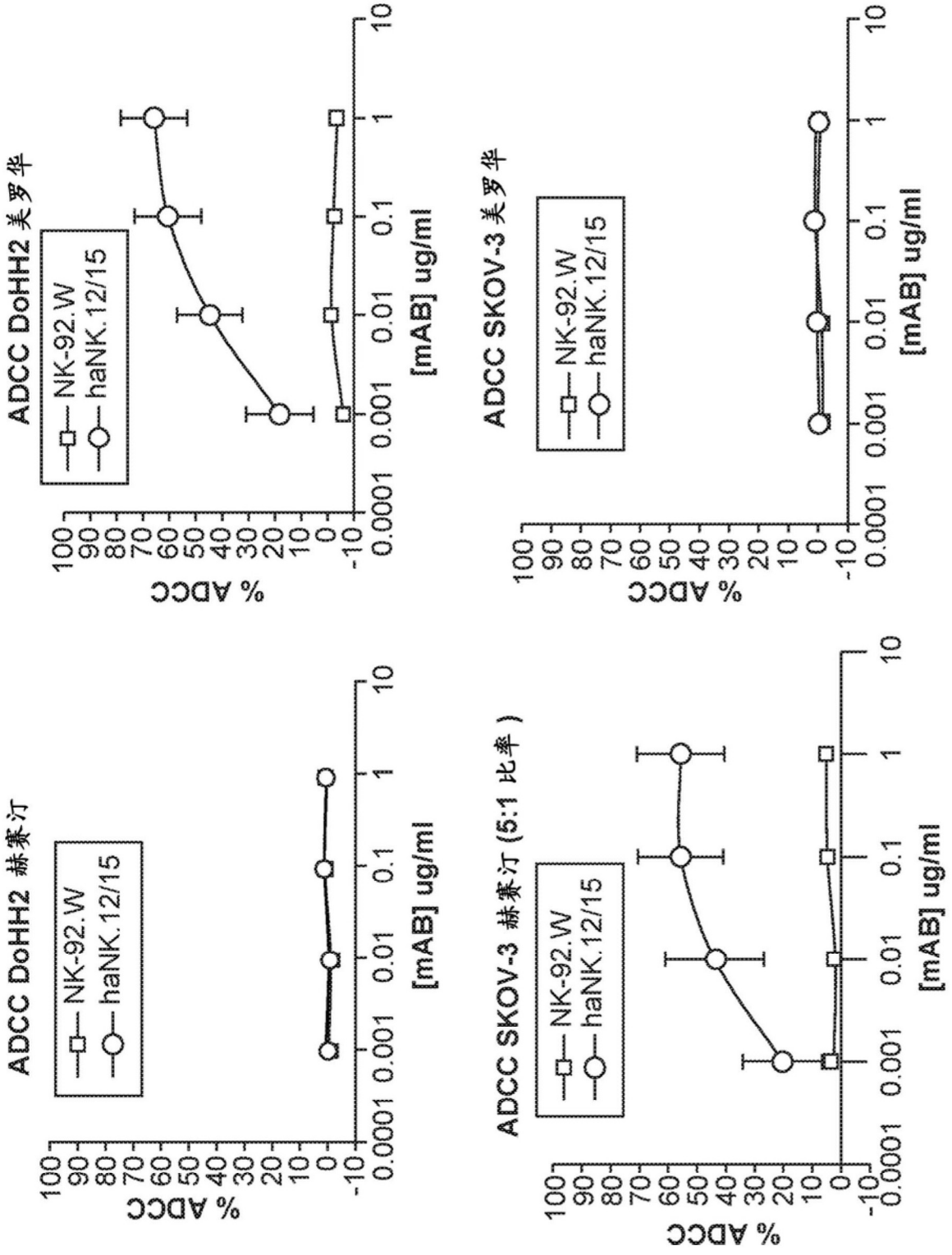
ADCC DoHH2 美罗华
NK-92.W
haNK.12/15
% ADCC
[mAB] ug/ml
ADCC SKOV-3 美罗华
ADCC DoHH2 赫赛汀
ADCC SKOV-3 赫赛汀 (5:1 比率)

图4