

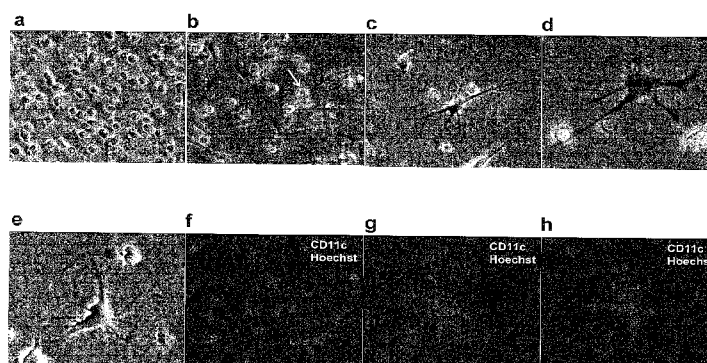
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(54) **Title:** METHODS AND COMPOSITIONS FOR PRODUCING ACTIVATED NATURAL KILLER CELLS AND RELATED USES**FIG. 1**

(57) **Abstract:** The invention provides methods for generating activated natural killer (NK) cells from acute myeloid leukemia (AML) cells. The methods involve contacting the AML cells with a TpoR agonist antibody, and culturing the cell mixture under conditions to allow conversion of the AML cells into NK cells. Also provided in the invention are therapeutic methods of using a pharmaceutical composition containing a TpoR agonist antibody or NK cells described herein for treating various diseases or disorders, e.g., tumors or pathogenic infections. The invention further provides TpoR agonist antibodies that can be employed in these methods.

Methods and Compositions for Producing Activated Natural Killer Cells and Related Uses

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The subject patent application claims the benefit of priority to U.S. Provisional Patent Application Numbers 62/182,386 (filed June 19, 2015) and 62/243,314 (filed October 19, 2015). The full disclosure of the priority applications is incorporated herein by reference in its entirety and for all purposes.

BACKGROUND OF THE INVENTION

[0002] Natural killer cells (NK) cells are cytotoxic lymphocytes and play a role in an innate immune system. NK cells also play a role in adaptive immune response. For example, NK cells can respond to virally infected cells and play a role in the host rejection of tumors. NK cells normally differentiate and mature in the bone marrow, lymph node, spleen, tonsils, and thymus. NK cells contain small granules in their cytoplasm which harbor special proteins such as perforin and proteases known as granzymes. Upon release in close proximity to a cell targeted for killing, perforin forms pores in the cell membrane of the target cell through which the granzymes and associated molecules can enter, inducing apoptosis. One granzyme, granzyme B (also known as granzyme 2 and cytotoxic T-lymphocyte-associated serine esterase 1), is a serine protease crucial for rapid induction of target cell apoptosis in the cell-mediated immune response.

[0003] There is a need in the art for better means for generating activated natural killer cells for direct use in patients. The present invention is directed to this and other unmet needs.

SUMMARY OF THE INVENTION

[0004] In one aspect, the invention provides methods for inducing formation of natural killer (NK) cells from acute myeloid leukemia (AML) cells. The methods entail contacting a population of AML cells with a TpoR agonist antibody under appropriate conditions to

convert the AML cells into NK cells. The TpoR agonist antibody used in the methods has the same binding specificity as that of a TpoR agonist antibody having heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) respectively shown in (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22; (2) SEQ ID NOs:4-7, 25 and 8; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 17; (4) SEQ ID NOs:4, 5, 16, 7, 25 and 18; (5) SEQ ID NOs:4, 5, 19, 7, 25 and 20; or (6) SEQ ID NOs:4, 5, 23, 7, 25 and 24. Also provided by the invention are populations of natural killer (NK) cells generated with these methods.

[0005] In some methods of the invention, the AML cells are cells isolated from a bone marrow sample or a peripheral blood sample of a human AML patient. The AML cells can be from newly diagnosed AML patients or relapsed patients. Some of these methods are directed to generating NK cells from human CD34+ leukemia cells, e.g., human CD34+/CD33+ leukemia cells or CD34+/CD38- leukemia cells. In some methods, the TpoR agonist antibody comprises heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) that are substantially identical to (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22, respectively; (2) SEQ ID NOs:4-7, 25 and 8, respectively; (3) SEQ ID NOs:4, 5, 16, 7, 25, and 17, respectively; (4) SEQ ID NOs:4, 5, 16, 7, 25 and 18, respectively; (5) SEQ ID NOs:4, 5, 19, 7, 25 and 20, respectively; or (6) SEQ ID NOs:4, 5, 23, 7, 25 and 24, respectively. In some methods, the employed TpoR antibody comprises heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) that are respectively identical to (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22; (2) SEQ ID NOs:4-7, 25 and 8; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 17; (4) SEQ ID NOs:4, 5, 16, 7, 25 and 18; (5) SEQ ID NOs:4, 5, 19, 7, 25 and 20; or (6) SEQ ID NOs:4, 5, 23, 7, 25 and 24. In some embodiments, the employed TpoR agonist antibody comprises heavy chain and light chain variable region sequences show in (1) SEQ ID NO:14 and SEQ ID NO:15, respectively; or (2) SEQ ID NO:2 and SEQ ID NO:3, respectively. In some embodiments, the employed TpoR agonist antibody comprises the scFv 3D9 antibody fragment shown in SEQ ID NO:12 or SEQ ID NO:1.

[0006] In some embodiments, contacting of the population of AML cells with the TpoR antibody occurs in vitro by culturing the cells in the presence of the TpoR agonist antibody. In some embodiments, the cells are cultured in the presence of the antibody for a period from about 4 days to about 20 days. In some embodiments, the methods of inducing NK cells from AML cells can include an additional step of detecting at least one cellular marker expressed by NK cells. For example, the methods can include detection of the dendritic cell

and NK cell marker CD11c, as well as one or more molecules specifically expressed by NK cells (e.g., perforin, granzyme B or interferon γ). In some other embodiments, the contacting of AML cells with the TpoR antibody occurs in vivo in a subject afflicted with acute myeloid leukemia. In some of these embodiments, the TpoR antibody can be engineered to be conjugated to an entity that specifically recognizes a surface antigen of AML cells. In some methods of the invention, the NK cell population induced from AML cells can be further enriched for NK cells to provide a homogenous population of NK cells.

[0007] In another aspect, the invention provides methods for treating acute myeloid leukemia (AML) in a subject. These methods involve administering to the subject a pharmaceutical composition that contains a therapeutic amount of a TpoR agonist antibody having the same binding specificity as that of a TpoR agonist antibody noted above. The subject suitable for treatment with these therapeutic methods can be one that has any subtype of AML or is at various stages of AML. For example, the subject can be afflicted with undifferentiated acute myeloblastic leukemia (M0), acute myeloblastic leukemia with minimal maturation (M1), acute myeloblastic leukemia with maturation (M2), acute promyelocytic leukemia (APL) (M3), acute myelomonocytic leukemia (M4), acute myelomonocytic leukemia with eosinophilia (M4 eos), acute monocytic leukemia (M5), acute erythroid leukemia (M6), or acute megakaryoblastic leukemia (M7).

[0008] In another aspect, the invention provides methods for treating a cancer or an infection in a subject. The methods entail administering to the subject a pharmaceutical composition that contains a therapeutic amount of a TpoR agonist antibody. The TpoR antibody used in these methods typically has the same binding specificity as that of a TpoR agonist antibody noted above. Some of the methods are directed to treating subjects suffering from blood tumors (e.g., AML). Some of the methods are directed to treating subjects suffering from solid tumors. Some other methods are directed to treating subjects suffering viral infections.

[0009] In another aspect, the invention provides methods for killing leukemia cells (e.g., AML cells or lymphoid tumor cells) in a subject. These methods entail first isolating a population of leukemia cells from the subject. This is followed by treating the isolated cells with a TpoR agonist antibody under conditions sufficient to convert the leukemia cells into NK cells. The TpoR antibody used in these methods typically has the same binding specificity as that of a TpoR agonist antibody noted above. Upon converting the leukemia

cells into NK cells, the NK cells are then administered back to the subject. Some of these methods are directed to killing AML cells in subjects afflicted with AML. In some of these methods, the antibody treated cells are enriched for NK cells prior to being administered back into the subject. Some of these methods further include examining the converted cells for the expression of one or more NK cellular markers, e.g., CD11c, perforin, granzyme B and interferon γ .

[0010] In still another aspect, the invention provides novel agonist antibodies for human thrombopoietin receptor (TpoR). Some of these antibodies have the same binding specificity as that of an antibody comprising heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) respectively shown in (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22; (2) SEQ ID NOs:4, 5, 16, 7, 25 and 17; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 18; (4) SEQ ID NOs:4, 5, 19, 7, 25 and 20; or (5) SEQ ID NOs:4, 5, 23, 7, 25 and 24. Some antibodies of the invention have the same binding specificity as that of a scFv antibody fragment shown in any one of SEQ ID NOs:9-13. Some of these antibodies have heavy chain and light chain CDR sequences that are the same as or substantially identical to (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22, respectively; (2) SEQ ID NOs:4, 5, 16, 7, 25 and 17, respectively; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 18, respectively; (4) SEQ ID NOs:4, 5, 19, 7, 25 and 20, respectively; or (5) SEQ ID NOs:4, 5, 23, 7, 25 and 24, respectively. Some of the antibodies have heavy chain and light chain variable region sequences show in SEQ ID NO:14 and SEQ ID NO:15, respectively.

[0011] A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification and claims.

DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 shows antibody induced differentiation of AML BM cells. a, b show microscopic images of AML BM cells after treatment of PBS or antibody (10 μ g/ml) for 4 days. The arrow in the insert box of panel b indicates a cell captured by the differentiated cell. c, d and e are the magnified images of fully differentiated cells in panel b. f shows the differentiated cells by confocal microscopy. The differentiated cells were stained for CD11c. Nuclei were stained by Hoechst 33342. g and h are enlarged images of the CD11c positive differentiated cells.

[0013] Figure 2 shows the immunocytochemistry of early-staged differentiated AML BM cells. a, b and c show the early-staged differentiated cells visualized by confocal microscopy. The differentiated cells were stained for Perforin (green), Interferon γ (green) and Granzyme B in a, b and c respectively. F-actin is labeled by rhodamin-phalloidin. Nuclei were stained by Hoechst 33342.

[0014] Figure 3 shows the immunocytochemistry of late-staged differentiated AML BM cells. a, b and c show the late-staged differentiated cells visualized by confocal microscopy. The differentiated cells were stained for Perforin (green), Interferon γ (green) and Granzyme B in a, b and c respectively. F-actin is labeled by rhodamin-phalloidin. Nuclei were stained by Hoechst 33342.

[0015] Figure 4 shows SEM analysis of target cell capturing by NK cells. a and c depict the representative SEM images of NK cells which captured AML cells after antibody treatment for 4 days. b is enlarged images of the area boxed in white in a.

[0016] Figure 5 shows AML cells after 4 days culture with PBS, antibody (10 $\mu\text{g/ml}$) and TPO (10 ng/ml). The inset of panel b is enlarged images of a differentiated cell in b. d, e and f show the of normal bone marrow CD34⁺ cells after 4 days culture with PBS, antibody (10 $\mu\text{g/ml}$) and TPO (10 ng/ml).

[0017] Figure 6 shows de-convoluted images of Figure 2.

[0018] Figure 7 shows de-convoluted images of Figure 3.

[0019] Figure 8 shows activation of signal transduction cascades in AML and normal BM cells after treatment with various doses of antibody or TPO for 1 hr. The phosphorylation of STAT-3, Akt and Erk was analyzed by western blotting using anti-p-STAT-3, p-Akt and p-ERK antibodies.

[0020] Figure 9 shows cytotoxic activity of the antibody induced differentiated cells. The AML cells were labeled with Calcein-AM and co-cultured with NK cells or undifferentiated cells. Cells were co-cultured for 4 hrs at 37°C. At the end of the incubation, dead cells were labeled with PI. The percentage of AML cell death was analyzed by a flow cytometer. (A) and (B) indicate different co-culture conditions; AML with NK cells or AML with undifferentiated cells.

[0021] Figure 10 shows antibody induced the differentiation of natural killer (NK) cells from AML cells of relapsed patients. (A) and (B) represent untreated AML cells and antibody induced NK cells. (C) to (E) show the magnified induced NK cells. The

differentiated cells were stained for perforin, interferon γ or granzyme B in (A), (B) or (C) respectively. Nuclei were stained by Hoechst 33342. F-actin is labeled by rhodamin-phalloidin.

[0022] Figure 11 shows antibody induced NK cells killed AML cells. (A) and (B) describes the NK cells capturing target cells (white arrow). The differentiated cells were stained for perforin, interferon γ or granzyme B in (A), (B). Nuclei were stained by Hoechst 33342. F-actin is labeled by rhodamin-phalloidin.

[0023] Figure 12 shows TPOR signal activation by antibody in relapsed AML cells. (A). After treatment with various doses of antibody or 10 ng/ml of TPO for 1 hr, the phosphorylation of AKT and ERK was analyzed by western blotting using anti-p-AKT and p-ERK antibodies. (B). The TPOR signaling was tested at various times after antibody or TPO treatment.

[0024] Figure 13 shows cytotoxic activity of the antibody induced NK cells from both newly diagnosed AML cells and relapsed AML cells. NK cells were respectively induced from relapsed AML cells and newly diagnosed AML cells with the 3D9 TpoR antibody for 4 days. Then undifferentiated AML cells were added and incubated for 24 hrs. To measure the cell viability, MTS solution was added to each well and absorbance were measured.

[0025] Figure 14 shows comparison of the efficacy of antibody with 5 affinity matured variants. The wild type 3D9 antibody and 5 variants were applied to relapsed AML cells to induce the differentiation of NK cells. The box in the upper left panel shows the magnified NK cells after differentiation.

DETAILED DESCRIPTION OF THE INVENTION

I. Overview

[0026] The recent discovery of many agonist antibodies that govern cell fates has opened the way to induce selectively a large variety of specific cells of the immune system from normal or malignant bone marrow or blood. Often these agonist antibodies induce cell differentiation along lineages expected from the known function of the receptor to which they bind. In other cases, they activate differentiation or trans-differentiation pathways that are different from what would be expected from the nature of the receptor with which they interact.

[0027] The present invention is predicated in part on the discovery by the present inventors that a thrombopoietin receptor (TpoR) agonist antibody induces efficiently highly activated NK cells from malignant human bone marrow (BM) and peripheral blood of several different AML patients. Specifically, primary human AML cells were exposed to the TpoR agonist antibody 3D9 (which was described in WO2014/035693) for about 4 days. Approximately 80% of the cells attached to the well and adopted a morphology characterized by extension of long dendrites (Figure 1). The morphology was similar to that of dendritic cells (DC) or NK cells. The inventors then analyzed cell markers using antibodies against marker molecules such as CD11c, Perforin, Interferon- γ and Granzyme B. CD11c is highly expressed in both DC and NK cells, and the other markers are mainly expressed in NK cells. As detailed herein, it was found that the induced cells were positively stained for all of these markers. These results strongly indicate that the differentiated cells are NK cells. In addition, it was observed that the antibody stimulated canonical TpoR signal transduction cascades, namely the phosphorylation of STAT-5, Akt and Erk in the TpoR Ab-treated AML cells (Figure 8). Furthermore, the inventors observed that the induced NK-like cells made contact with sister AML cells via dendritic processes, resulting in destruction of non-differentiated AML cells. Other than the 3D9 TpoR agonist antibody, the inventors also generated several variants from 3D9. These variants were found to also possess the ability in inducing NK formation from AML cells.

[0028] The invention accordingly provides methods of using the 3D9 TpoR agonist antibody and derivatives thereof for inducing formation of NK cells from AML cells. The invention also provides therapeutic applications of the TpoR agonist antibodies described herein for treating various diseases or conditions, e.g., cancers such as AML. From a therapeutic standpoint, the ability to induce activated NK cells from the readily accessible peripheral blood and bone marrow cellular compartments opens new routes to cancer therapy that are much simpler than those currently available in the art. Also provided in the invention are TpoR agonist antibodies that can be employed in the practice of the various methods described herein.

II. Definitions

[0029] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this

invention pertains. The following references provide one of skill with a general definition of many of the terms used in this invention: *Academic Press Dictionary of Science and Technology*, Morris (Ed.), Academic Press (1st ed., 1992); *Oxford Dictionary of Biochemistry and Molecular Biology*, Smith et al. (Eds.), Oxford University Press (revised ed., 2000); *Encyclopaedic Dictionary of Chemistry*, Kumar (Ed.), Anmol Publications Pvt. Ltd. (2002); *Dictionary of Microbiology and Molecular Biology*, Singleton et al. (Eds.), John Wiley & Sons (3rd ed., 2002); *Dictionary of Chemistry*, Hunt (Ed.), Routledge (1st ed., 1999); *Dictionary of Pharmaceutical Medicine*, Nahler (Ed.), Springer-Verlag Telos (1994); *Dictionary of Organic Chemistry*, Kumar and Anandand (Eds.), Anmol Publications Pvt. Ltd. (2002); and *A Dictionary of Biology (Oxford Paperback Reference)*, Martin and Hine (Eds.), Oxford University Press (4th ed., 2000). In addition, the following definitions are provided to assist the reader in the practice of the invention.

[0030] Acute myeloid leukemia (AML), also known as acute myelogenous leukemia, acute myeloblastic leukemia, or acute nonlymphocytic leukemia (ANLL), is a cancer of the myeloid line of blood cells, characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells. AML is the most common acute leukemia affecting adults, and its incidence increases with age. Although AML is a relatively rare disease, accounting for approximately 1.2% of cancer deaths in the United States, its incidence is expected to increase as the population ages.

[0031] The symptoms of AML are caused by replacement of normal bone marrow with leukemic cells, which causes a drop in red blood cells, platelets, and normal white blood cells. These symptoms include fatigue, shortness of breath, easy bruising and bleeding, and increased risk of infection. Several risk factors and chromosomal abnormalities have been identified, but the specific cause is not clear. As an acute leukemia, AML progresses rapidly and is typically fatal within weeks or months if left untreated.

[0032] AML has several subtypes; treatment and prognosis varies among subtypes. Five-year survival varies from 15–70%, and relapse rate varies from 33–78%, depending on subtype. AML is treated initially with chemotherapy aimed at inducing a remission; patients may go on to receive additional chemotherapy or a hematopoietic stem cell transplant. Recent research into the genetics of AML has resulted in the availability of tests that can predict which drug or drugs may work best for a particular patient, as well as how long that patient is likely to survive.

[0033] The term "antibody" or "antigen-binding fragment" refers to polypeptide chain(s) which exhibit a strong monovalent, bivalent or polyvalent binding to a given antigen, epitope or epitopes. Unless otherwise noted, antibodies or antigen-binding fragments used in the invention can have sequences derived from any vertebrate, camelid, avian or pisces species. They can be generated using any suitable technology, e.g., hybridoma technology, ribosome display, phage display, gene shuffling libraries, semi-synthetic or fully synthetic libraries or combinations thereof. Unless otherwise noted, the term "antibody" as used in the present invention includes intact antibodies, antigen-binding polypeptide fragments and other designer antibodies that are described below or well known in the art (see, e.g., Serafini, J Nucl. Med. 34:533-6, 1993).

[0034] An intact "antibody" typically comprises at least two heavy (H) chains (about 50-70 kD) and two light (L) chains (about 25 kD) inter-connected by disulfide bonds. The recognized immunoglobulin genes encoding antibody chains include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

[0035] Each heavy chain of an antibody is comprised of a heavy chain variable region (V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C_{H1} , C_{H2} and C_{H3} . Each light chain is comprised of a light chain variable region (V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L . The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system and the first component (C1q) of the classical complement system.

[0036] The V_H and V_L regions of an antibody can be further subdivided into regions of hypervariability, also termed complementarity determining regions (CDRs), which are interspersed with the more conserved framework regions (FRs). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxyl-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The locations of CDR and FR regions and a numbering system have been defined by, e.g., Kabat *et al.*, *Sequences*

of Proteins of Immunological Interest, U.S. Department of Health and Human Services, U.S. Government Printing Office (1987 and 1991).

[0037] Antibodies to be used in the invention also include antibody fragments or antigen-binding fragments which contain the antigen-binding portions of an intact antibody that retain capacity to bind the cognate antigen. Examples of such antibody fragments include (i) a Fab fragment, a monovalent fragment consisting of the V_L , V_H , C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an intact antibody; (v) disulfide stabilized Fvs (dsFvs) which have an interchain disulfide bond engineered between structurally conserved framework regions; (vi) a single domain antibody (dAb) which consists of a V_H domain (see, e.g., Ward et al., *Nature* 341:544-546, 1989); and (vii) an isolated complementarity determining region (CDR).

[0038] Antibodies suitable for practicing the present invention also encompass single chain antibodies. The term "single chain antibody" refers to a polypeptide comprising a V_H domain and a V_L domain in polypeptide linkage, generally linked via a spacer peptide, and which may comprise additional domains or amino acid sequences at the amino- and/or carboxyl-termini. For example, a single-chain antibody may comprise a tether segment for linking to the encoding polynucleotide. As an example, a single chain variable region fragment (scFv) is a single-chain antibody. Compared to the V_L and V_H domains of the Fv fragment which are coded for by separate genes, a scFv has the two domains joined (e.g., via recombinant methods) by a synthetic linker. This enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules.

[0039] Antibodies that can be used in the practice of the present invention also encompass single domain antigen-binding units which have a camelid scaffold. Animals in the camelid family include camels, llamas, and alpacas. Camelids produce functional antibodies devoid of light chains. The heavy chain variable (V_H) domain folds autonomously and functions independently as an antigen-binding unit. Its binding surface involves only three CDRs as compared to the six CDRs in classical antigen-binding molecules (Fabs) or single chain variable fragments (scFvs). Camelid antibodies are capable of attaining binding affinities comparable to those of conventional antibodies.

[0040] The various antibodies or antigen-binding fragments described herein can be produced by enzymatic or chemical modification of the intact antibodies, or synthesized de novo using recombinant DNA methodologies, or identified using phage display libraries. Methods for generating these antibodies or antigen-binding molecules are all well known in the art. For example, single chain antibodies can be identified using phage display libraries or ribosome display libraries, gene shuffled libraries (see, e.g., McCafferty et al., *Nature* 348:552-554, 1990; and U.S. Pat. No. 4,946,778). In particular, scFv antibodies can be obtained using methods described in, e.g., Bird et al., *Science* 242:423-426, 1988; and Huston et al., *Proc. Natl. Acad. Sci. USA* 85:5879-5883, 1988. Fv antibody fragments can be generated as described in Skerra and Plückthun, *Science* 240:1038-41, 1988. Disulfide-stabilized Fv fragments (dsFvs) can be made using methods described in, e.g., Reiter et al., *Int. J. Cancer* 67:113-23, 1996. Similarly, single domain antibodies (dAbs) can be produced by a variety of methods described in, e.g., Ward et al., *Nature* 341:544-546, 1989; and Cai and Garen, *Proc. Natl. Acad. Sci. USA* 93:6280-85, 1996. Camelid single domain antibodies can be produced using methods well known in the art, e.g., Dumoulin et al., *Nature Struct. Biol.* 11:500-515, 2002; Ghahroudi et al., *FEBS Letters* 414:521-526, 1997; and Bond et al., *J Mol Biol.* 332:643-55, 2003. Other types of antigen-binding fragments (e.g., Fab, F(ab')₂ or Fd fragments) can also be readily produced with routinely practiced immunology methods. See, e.g., Harlow & Lane, *Using Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1998.

[0041] The term "contacting" has its normal meaning and refers to combining two or more agents (e.g., polypeptides or phage), combining agents and cells, or combining two populations of different cells. Contacting can occur in vitro, e.g., mixing two polypeptides or mixing a population of antibodies with a population of cells in a test tube or growth medium. Contacting can also occur in a cell or in situ, e.g., contacting two polypeptides in a cell by coexpression in the cell of recombinant polynucleotides encoding the two polypeptides, or in a cell lysate.

[0042] The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (*i.e.*, 60% identity, optionally 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identity over a specified region,

or, when not specified, over the entire sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length.

[0043] Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.* 2:482c, 1970; by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48:443, 1970; by the search for similarity method of Pearson and Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444, 1988; by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, Madison, WI); or by manual alignment and visual inspection (see, *e.g.*, Brent et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (ringbou ed., 2003)). Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., *Nuc. Acids Res.* 25:3389-3402, 1977; and Altschul et al., *J. Mol. Biol.* 215:403-410, 1990, respectively.

[0044] A "ligand" is a molecule that is recognized by a particular antigen, receptor or target molecule. Examples of ligands that can be employed in the practice of the present invention include, but are not restricted to, agonists and antagonists for cell membrane receptors, toxins and venoms, viral epitopes, hormones, hormone receptors, polypeptides, peptides, enzymes, enzyme substrates, cofactors, drugs (*e.g.* opiates, steroids, etc.), lectins, sugars, polynucleotides, nucleic acids, oligosaccharides, proteins, and monoclonal antibodies.

[0045] Natural killer cells, or NK cells, are a type of cytotoxic lymphocyte critical to the innate immune system. The role NK cells play is analogous to that of cytotoxic T cells in the vertebrate adaptive immune response. NK cells provide rapid responses to viral-infected cells and respond to tumor formation, acting at around 3 days after infection. Typically, immune cells detect major histocompatibility complex (MHC) presented on infected cell surfaces, triggering cytokine release, causing lysis or apoptosis. NK cells are unique,

however, as they have the ability to recognize stressed cells in the absence of antibodies and MHC, allowing for a much faster immune reaction. They were named “natural killers” because of the initial notion that they do not require activation to kill cells that are missing “self” markers of MHC class I. This role is especially important because harmful cells that are missing MHC I markers cannot be detected and destroyed by other immune cells, such as T lymphocyte cells.

[0046] NK cells of the invention contain cytoplasmic markers such as CD11c, Perforin, Interferon- γ and Granzyme B. NK cells differ from natural killer T cells (NKTs) phenotypically, by origin and by respective effector functions. NKT cell activity promotes NK cell activity by secreting IFN γ . In contrast to NKT cells, NK cells do not express T-cell antigen receptors (TCR) or pan T marker CD3 or surface immunoglobulins (Ig) B cell receptors, but they usually express other specific surface markers such as CD16 (Fc γ RIII) and CD56 in humans.

[0047] Unless otherwise noted, the term "receptor" broadly refers to a molecule that has an affinity for a given ligand. Receptors may-be naturally-occurring or manmade molecules. Also, they can be employed in their unaltered state or as aggregates with other species. Receptors may be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. A typical example of receptors which can be employed in the practice of the invention is cell surface signaling receptor.

[0048] The term "subject" refers to human and non-human animals (especially non-human mammals). In addition to human, it also encompasses other non-human animals such as cows, horses, sheep, pigs, cats, dogs, mice, rats, rabbits, guinea pigs, monkeys.

[0049] The term "target," "target molecule," or "target cell" refers to a molecule or biological cell of interest that is to be analyzed or detected, e.g., a ligand such as a cytokine or hormone, a polypeptide, a cellular receptor or a cell.

[0050] A cell has been "transformed" by exogenous or heterologous polynucleotide when such polynucleotide has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming polynucleotide may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming polynucleotide has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This

stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming polynucleotide. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

III. Thrombopoietin receptor (TpoR) agonist antibodies

[0051] The invention provides novel methods that allow one to convert AML cells into natural killer cells via the use of a thrombopoietin receptor (TpoR) agonist antibody. The TpoR agonist antibody used in the methods has the same binding specificity as that of a specific TpoR antibody known in the art. The structural information of this TpoR antibody, 3D9, was described in detail in the literature, e.g., Zhang et al., Chem. Biol., 20: 734-741, 2013; and WO2014/035693. This scFv antibody has an amino acid sequence shown in SEQ ID NO:1. The sequences of the heavy chain and the light chain portions of the scFv are respectively shown in SEQ ID NOs:2 and 3. The CDR sequences of the heavy chain variable region of this antibody are RDTFNTYG (CDR1; SEQ ID NO:4), IIPFGTA (CDR2; SEQ ID NO:5), and CARDRLGGSDYW (CDR3; SEQ ID NO:6). The CDR sequences of its light chain variable region are QGLGRW (CDR1; SEQ ID NO:7), AAS (CDR2; SEQ ID NO:25), and QQSNSFPWT (CDR3; SEQ ID NO:8).

[0052] TpoR agonist antibodies used in the invention are preferably monoclonal antibodies like the antibodies exemplified in the Examples below. In general, the antibodies have the same binding specificity as that of the 3D9 TpoR agonist antibody. These antibodies should compete with the 3D9 antibody for binding to the TpoR receptor. The TpoR agonist antibodies suitable for the invention need not possess exactly the same functional properties as that of the 3D9 antibody, e.g., activities in activating the TpoR signaling pathway. In addition to containing variable regions sequences derived from the 3D9 antibody, some agonist antibodies of the invention can also contain other antibody sequences fused to the variable region sequences. For example, the antibodies can contain an Fc portion of IgG. The antibodies can also be conjugated, covalently or noncovalently, to another entity that specifically targets a surface antigen or marker on AML cells. The TpoR agonist antibody can also be part of a bispecific antibody that recognizes both the TpoR and a specific target antigen or epitope on the AML cells.

[0053] Some TpoR agonist antibodies for practicing the invention harbor variable region sequences that are substantially identical to that of the 3D9 antibody. Some other TpoR agonist antibodies have all CDR sequences in their variable regions of the heavy chain and light chain respectively identical to the corresponding CDR sequences of the 3D9 TpoR agonist antibody. In still some other embodiments, the TpoR antibody has its entire heavy chain and light chain variable region sequences respectively identical to the corresponding variable region sequences of the 3D9 antibody. In some other embodiments, other than the identical CDR sequences, the antibodies contain amino acid residues in the framework portions of the variable regions that are different from the corresponding amino acid residues of the 3D9 antibody. Relative to the 3D9 antibody, the agonist TpoR antibodies suitable for the invention can undergo non-critical amino-acid substitutions, additions or deletions in the variable region without loss of binding specificity or effector functions, or intolerable reduction of binding affinity or receptor agonizing activities. Usually, antibodies incorporating such alterations exhibit substantial sequence identity to the 3D9 TpoR antibody. For example, the mature light chain variable regions of some of the TpoR agonist antibodies for use in the invention have at least 75% or at least 85% sequence identity to the sequence of the mature light chain variable region of the 3D9 antibody. Similarly, the mature heavy chain variable regions of the antibodies typically show at least 75% or at least 85% sequence identity to the sequence of the mature heavy chain variable region of the 3D9 antibody. In various embodiments, the antibodies typically have their entire variable region sequences that are substantial identical (e.g., 75%, 85%, 90%, 95%, or 99%) to the corresponding variable region sequences of the 3D9 antibody. Some TpoR agonist antibodies for use in the invention have the same specificity but improved affinity or receptor-agonizing activities if compared with the 3D9 antibody.

[0054] General methods for preparation of monoclonal or polyclonal antibodies are well known in the art. See, e.g., Harlow & Lane, *Using Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1998; Kohler & Milstein, *Nature* 256:495-497, 1975; Kozbor et al., *Immunology Today* 4:72, 1983; and Cole et al., pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy*, 1985.

IV. Converting AML cells into natural killer cells

[0055] The TpoR agonist antibodies described above can induce AML or other leukemia cells to become NK cells. Acute myeloid leukemia (AML) is a malignant disease characterized by a disruption in normal hematopoietic differentiation and the accumulation of abnormal, immature myeloid cells in the bone marrow and peripheral blood. In general, determination and classification of AML can be based on the two well-known systems that have been used to classify AML into subtypes, the French-American-British (FAB) classification and the newer World Health Organization (WHO) classification. For example, under the FAB classification, AML subtypes include undifferentiated acute myeloblastic leukemia (M0), acute myeloblastic leukemia with minimal maturation (M1), acute myeloblastic leukemia with maturation (M2), acute promyelocytic leukemia (APL) (M3), acute myelomonocytic leukemia (M4), acute myelomonocytic leukemia with eosinophilia (M4 eos), acute monocytic leukemia (M5), acute erythroid leukemia (M6), and acute megakaryoblastic leukemia (M7). Subtypes M0 through M5 all start in immature forms of white blood cells. M6 AML starts in very immature forms of red blood cells, while M7 AML starts in immature forms of cells that make platelets. In addition to AML cells, other blood tumor cells (e.g., lymphoid tumor cells) may also be used in the practice of the invention.

[0056] Some embodiments of the invention are directed to converting AML cells into NK cells. AML cells of any subtypes can be used in the practice of the invention. Molecular markers known to be present on the different subtypes of AML cells are useful for their isolation and evaluation. A non-exhaustive list of antigen markers for AML cells include CD34, CD117, CD38, and HLA-DR (for progenitor AML cells); CD13, CD33, CD15, and MPO (for myeloid AML cells); CD33, CD14, CD64, and CD36 (for monocytic AML cells); CD235a and CD71 (for erythroid AML cells); CD41 and CD61 (for megakaryocytic AML cells); and CD19, CD79a, CD10, and cytoplasmic CD3 (for lymphoid AML cells). Advances in molecular genetics have identified additional markers that may be useful for diagnosing AML and identifying leukemic stem cells. Thus, increased expression of cell surface markers such as TIM-3, IL-3 R alpha (CD123), and CD44 can be used to distinguish normal CD34+CD38- hematopoietic stem cells and the subpopulation of leukemic stem cells. In addition, monoclonal antibodies targeted against leukemic stem cell

surface antigens such as CD44, CD47, and IL-3 R alpha, have demonstrated efficacy in the treatment of AML in animal models.

[0057] AML cells used in the practice of the invention can be prepared from either a BM sample or a blood sample of the AML patients. These include samples from peripheral blood, bone marrow fluid, umbilical blood, and etc. Leukemic cells from patient blood samples can be obtained by separating plasma from blood, by a known method, for example, a density gradient centrifugation method. Some methods of the invention utilize CD34+ leukemic cells. In addition to CD34+, the AML cells typically harbor one or more other surface markers of myeloid cells. For example, CD13 and CD33 are markers for cells of myeloid origin. In some embodiments, the AML cells used in the methods can include human CD34+/CD33+ leukemia cells (e.g., CD34+/CD33+/CD13+ cells). In some embodiments, the cell population for inducing NK cells includes CD34+/CD38- cells. As noted above, increased expression of cell surface markers such as TIM-3, IL-3 R alpha (CD123), and CD44 can be used to distinguish normal CD34+CD38- hematopoietic stem cells and the subpopulation of leukemic stem cells.

[0058] The AML cell containing samples can be maintained and cultured in any physiologically-acceptable solution suitable for the collection and/or culture of the cells. These include, e.g., a saline solution (e.g., phosphate-buffered saline, Krebs's solution, modified Krebs's solution, Eagle's solution, 0.9% NaCl, etc.), and a culture medium (e.g., DMEM, H.DMEM, etc.), and the like. The solution can contain one or more tissue-degrading enzymes, e.g., a metalloprotease, a serine protease, a neutral protease, a hyaluronidase, an RNase, or a DNase, or the like. Such enzymes include, but are not limited to, collagenases (e.g., collagenase I, II, III or IV, a collagenase from *Clostridium histolyticum*, etc.); dispase, thermolysin, elastase, trypsin, LIBERASE, hyaluronidase, and the like. The solution can further include a bacteriocidally or bacteriostatically effective amount of an antibiotic. Examples of suitable antibiotics include a macrolide (e.g., tobramycin), a cephalosporin (e.g., cephalexin, cephadrine, cefuroxime, cefprozil, cefaclor, cefixime or cefadroxil), a clarithromycin, an erythromycin, a penicillin (e.g., penicillin V) or a quinolone (e.g., ofloxacin, ciprofloxacin or norfloxacin), a tetracycline, a streptomycin, and etc.

[0059] The culture of a cell population in the presence of the TpoR agonist antibody can be performed in accordance with standard cell culturing protocols well known in the art.

Some specific procedures for generating NK cells from an AML cell population are exemplified herein. Typically, the isolated AML cells are contacted with an effective amount of an antibody having the same binding specificity as that of 3D9 TpoR agonist antibody under appropriate conditions to facilitate the conversion. In some embodiments, the antibody is contacted with the cells in vitro. In these embodiments, the cell population can be cultured at a concentration of about 1×10^2 , 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 , 1×10^8 cells/ml or higher. As exemplified herein, the AML cells can be cultured in the presence of an effective amount of the antibody, e.g., at a concentration of 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100 $\mu\text{g/ml}$ or higher. The contacting can last for a sufficient period of time, e.g., at least 12 hours, 1 day, 2 days, 4 days, 6 days, 10 days, 25 days, 50 days, 75 days, 100 days or longer. In some of these embodiments, the cells can be examined along the process for molecular markers and/or morphology indicative of the presence of NK cells. As demonstrated herein, the cells can be analyzed for the presence of CD11c, interleukin γ , perforin and granzyme B. This can be achieved, e.g., by staining with the appropriate antibodies and visual inspection via confocal microscopy as exemplified herein.

[0060] The presence of NK cells in the induced cell population can also be analyzed by detecting many other NK specific markers well known in the art. In some embodiments, properties of the induced cells can be assessed by detecting the presence of other NK cell-specific markers, e.g., CD56, CD94, CD117 and NKp46. This can be accomplished by routinely practiced techniques, e.g., by flow cytometry. In some other embodiments, NK cell properties of the induced cells can be examined by the morphological characteristics of NK cells, e.g., large size, high protein synthesis activity in the abundant endoplasmic reticulum (ER), and/or preformed granules. In some embodiments, maturation of NK cells can be assessed by detecting one or more functionally relevant markers, for example, CD94, CD161, NKp44, DNAM-1, 2B4, NKp46, CD94, KIR, and the NKG2 family of activating receptors (e.g., NKG2D). Maturation of NK cells can also be assessed by detecting specific markers during different developmental stages. In some embodiments, the induced cells contain pro-NK cells that are CD34+, CD45RA+, CD10+, CD117-, and/or CD161- cells. In some embodiments, the induced cells contain pre-NK cells that are CD34+, CD45RA+, CD10-, CD117+, and/or CD161- cells. In other embodiments, the induced cells contain immature NK cells that are CD34-, CD117+, CD161+, NKp46-, and/or CD94/NKG2A- cells. In still some other embodiments, the induced cells contain NK cells that are CD117+,

NKp46+, CD94/NKG2A+, CD16-, and/or KIR+/- . In some embodiments, the induced cells contain NK cells that are CD117-, NKp46+, CD94/NKG2A+/-, CD16+, and/or KIR+. In some other embodiments, maturation of NK cells can be determined by the percentage of NK cells that are CD161-, CD94+ and/or NKp46+. In some embodiments, the induced cell population contains at least 10%, 20%, 25%, 30%, 35%, 40%, 50%, 55%, 60%, 65% or 70% of mature NKp46+ NK cells, mature CD94+ NK cells, or mature CD161- cells. In various embodiments, expression of these markers can be detected and quantified via standard immunological assays using, e.g., antibodies to one or more of these cell markers. The antibodies can be conjugated to a detectable label, e.g., a fluorescent label, to facilitate detection and quantitation.

[0061] In some embodiments, the TpoR agonist antibody is contacted with the AML cells or other tumor cells (e.g., lymphoid tumor cells) *in vivo*. For example, as detailed herein, a therapeutically effective amount of the antibody can be administered to a subject afflicted with AML or another type of tumor (e.g., another blood tumor). In some other embodiments, subjects with AML or other tumors can be treated *ex vivo*. In these methods, AML cells are first isolated from the subjects and contacted with the TpoR agonist antibody to generate NK cells. The induced NK cells are then reintroduced into the subjects. Preferably, the AML cells to be treated with the TpoR agonist antibody are obtained from the same subject into whom the resulting NK cell population will be administered.

[0062] In some embodiments, the AML cells isolated from AML patients can be treated with one or more stimulating cytokines such as GM-CSF, IL-2, IL-4 and TNF α prior to, simultaneously with, or subsequent to being contacted with the TpoR agonist antibody. The concentration of each of the cytokines in the culture medium can be in the range of, e.g., about 0.01 to 1×10^5 U/ml. The culture medium can further contain serum or plasma. The amount of serum or plasma can be present at concentration of, e.g., about 0 to 20% by volume, preferably more than 0 to 10% by volume.

[0063] The cell culture may be performed under known culture conditions, and the conditions which are used in normal cell culture can be applied. For example, culture can be performed under the conditions of 37°C and 5% CO₂. Cells can be diluted by adding a fresh medium to a cell culture liquid at a suitable time interval, a medium can be exchanged with a fresh medium, or a cell culture instrument can be exchanged. The culturing period for converting the AML cells into NK cells can be, e.g., from 1 to 100 days, 2 to 75 days, 3-50

days, or 4-25 days. In some embodiments, the AML cells are cultured in the presence of the TpoR antibody and other agents for a period of around 4 days, 5 days, 6 days, 7 days, or 8 days. Any cell culture instrument can be used in the invention. These include, e.g., a petri dish, a flask, a bag, a bioreactor etc. can be used. For cell culture bags, a CO₂ gas permeable bag for cell culture can be used. When a large amount of cells are being treated, use of a bioreactor is advantageous. Although the cell culturing can be performed in either an open system or a closed system, it is preferable to perform the culturing in a closed system from a view point of safety of the resulting NK cells.

V. Therapeutic applications

[0064] The invention provides methods of treating cancer and other diseases via the use of TpoR agonist antibodies described herein to induce formation of NK cells. The ability to induce some members of an AML cell population to become NK cells which in turn can kill other members of the AML cell population is particularly beneficial for therapeutic applications. It is a dynamic process at the population level where a uniform population of cancer cells is converted to a mixture of targets and killers. In effect, the cancer cells kill one another and since any member of the clonal population can be converted to a killer cell. With sufficient time, the entire malignant cell population or clone can be eliminated. Accordingly, in some embodiments of the invention, a pharmaceutical composition containing one of the TpoR agonist antibodies described herein is administered to patients so that the converted NK cells can recognize and kill the neighboring diseased cells. In some of these embodiments, a recognition element such as an antibody can be expressed on the cell surface to provide more specific and/or stronger cytotoxicity. In some other embodiments, NK cell populations induced with the TpoR antibody and further enriched in vitro can be administered to subjects suffering from cancers or other diseases, e.g., AML, other blood tumors, solid tumors and viral infections. The pharmaceutical composition of the invention can be used alone or in combination with other known agents in the art for treating cancer or pathogenic infections.

[0065] NK cells produced by the method of the present invention and a composition containing the same may be used for treatment of cancers and infectious diseases. NK cells produced by the method of the present invention may be applied to all types of cancers, including solid cancer and blood cancer. As used herein, the term "solid cancer" refers to

mass-type cancer formed in an organ, unlike blood cancer. Cancers developed in most of organs correspond to solid cancers. In addition to blood cancer such as AML, cancers which can be treated using the TpoR antibody- or NK cell-containing compositions of the present invention also include solid cancer such as stomach cancer, liver cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, cervical cancer, thyroid cancer, laryngeal cancer, acute myeloid leukemia, brain cancer, neuroblastoma, retinoblastoma, head and neck cancer, salivary gland cancer, lymphoma and so on. In some other embodiments, the therapeutic compositions of the invention are used for treating infectious diseases, e.g., viral infections. As used herein, the term "infectious diseases" is means to include all diseases which are caused by infection with viruses or pathogenic bacteria and can be infected through respiratory organ, blood or skin contact. Non-limiting examples of such infectious diseases include, but are not limited to, hepatitis B, hepatitis C, human papilloma virus (HPV) infection, cytomegalovirus infection, viral respiratory disease, influenza and so on.

[0066] In some embodiments, the methods of the invention are used for treating blood tumors such as leukemia. Leukemia is cancer of the blood cells, usually affecting the white blood cells. Leukemia can occur in either the lymphoid or myeloid white blood cells. Cancer that develops in the lymphoid cells is called lymphocytic leukemia. Cancer that develops in the myeloid cells is called myelogenous leukemia. The disease can be either acute (begins abruptly and is usually short lived) or chronic (persists for a long period of time). Based on these findings, leukemia is then classified into one of the four main types of leukemia: acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), or chronic lymphocytic leukemia (CLL). The methods described herein can be used to convert any of the leukemia cells into NK cells and to treat subjects afflicted with any of these blood tumors.

[0067] In one aspect, the invention provides therapeutic applications of the TpoR agonist antibodies described herein for inducing NK cells in subjects suffering from various diseases or disorders. In some embodiments, the TpoR agonist antibody can be employed to treat subjects afflicted with a condition that is manifested by the presence of AML cells or lymphoid tumor cells. In these therapeutic methods, the TpoR antibody, preferably in a pharmaceutical composition, is directly administered to subjects in need of treatment. Upon contacting the antibody with AML cells in vivo, NK cells can be induced which in turn can

exert cytotoxic activities against neighboring non-induced AML cells and other diseased cells. In some embodiments, the antibody can be conjugated to a moiety that specifically recognizes a surface marker on the AML cells to facilitate targeted delivery of the TpoR antibody, e.g., a ligand for an AML specific surface marker described herein. In some embodiments, a bispecific antibody comprised of the antigen-binding site of the TpoR agonist antibody and also the antigen-binding site of a second antibody recognizing an AML specific surface marker can be used. Such immune conjugates or bispecific antibodies can be generated using standard procedures routinely practiced in the art.

[0068] As described above, some embodiments of the invention are directed to ex vivo treatment of subjects suffering from cancers or other diseases. For example, a subject afflicted with a blood tumor (e.g., AML or lymphoid tumors) can be treated by administering NK cell population generated in vitro from tumor cells that are isolated from the same subject. This is followed by treating the isolated cells with a TpoR agonist antibody described herein that can convert the tumor cells into NK cells. Thereafter, the NK cells generated in vitro are reintroduced into the subject to kill leukemia cells (e.g., AML cells) that remain in the subject. In these embodiments, a population of leukemia cells (e.g., AML cells) is first isolated from the subject. The population should contain tumor cells for converting into NK cells that are sufficient for subsequent treatment. In various embodiments, the isolated cell population should contain at least 1×10^2 , 1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 , 1×10^7 or more leukemia cells. In some embodiments, the cell population treated with the antibody is further enriched for NK cells. This allows the generation of a more homogenous population of NK cells to enhance therapeutic efficacy. In some of these embodiments, the treated cell population can be examined for expression of one or more cellular markers indicative of NK cells. For example, the cells can be analyzed for the expression of NK marker CD11c, as well as additional markers such perforin, granzyme B and interferon γ .

[0069] Also provided in the invention are NK cell populations generated in vitro by the methods described herein and therapeutic applications of such NK cell populations. For therapeutic applications of the NK cell populations, the induced cell population is preferably enriched for NK cells. NK cells can be first isolated from the induced cell population noted above, and then administered to a subject in need of treatment of a disease or disorder (e.g., tumor or viral infection). Isolation and enrichment of natural killer cells from the induced

cell population can be readily carried out with methods well known in the art. For example, activated NK cells can be isolated or selected for cells expressing NK specific markers exemplified herein, e.g., perforin, interferon gamma, and granzyme B. Enrichment can also include staining cells from the induced cell population or previously NK selected cells with antibodies recognizing NK specific markers, e.g., CD56 and CD3, and selecting for CD56+ CD3- cells. Commercially available kit, e.g., the NK Cell Isolation Kit (Miltenyi Biotec) can be employed. The NK cells can also be isolated or enriched by removal of cells other than NK cells in the induced cell population. For example, NK cells may be isolated or enriched by depletion of cells displaying non-NK cell markers using, e.g., antibodies to one or more of CD3, CD4, CD14, CD19, CD20, CD36, CD66b, CD123, HLA DR and/or CD235a (glycophorin A). Negative isolation can be carried out using a commercially available kit, e.g., the NK Cell Negative Isolation Kit (DynaL Biotech). Cells isolated by these methods may be additionally sorted, e.g., to separate CD16+ and CD16- cells. Cell separation can be accomplished by, e.g., flow cytometry, fluorescence-activated cell sorting (FACS) or magnetic cell sorting using microbeads conjugated with specific antibodies. Upon enrichment, the NK cell populations of the invention typically contain at least 50%, 60%, 70%, 80%, 90%, 92%, 94%, 96%, 98% of homogeneous or heterogeneous NK cells that express the NK specific markers described herein.

[0070] The NK populations of the invention can be administered allogeneically or autologously to subjects for enhanced cytotoxicity. In some embodiments, the cytotoxic activity of the cells can be first examined in vitro prior to administration to subjects. This can be performed by, e.g., cytotoxicity assays using a target cell such as a cultured tumor cell (AML cell or other types of tumor cells). The cell population can then be administered to subjects with conditions that can benefit from enhanced cytotoxic activity provided by the administered NK cells, e.g., solid tumors and viral infections. In some embodiments, the cytotoxicity of the administered NK cell population can be enhanced by treatment or co-administration of the cell population with a suitable cytokine, e.g., IFN- α . Such treated cell population can exert enhanced cytotoxicity against various cancer cells. In some embodiments, the administered NK cells can be engineered to express a molecule having affinity for a target molecule (e.g., T cell receptor, antibody, receptor etc.), a cell surface antigen, an enzyme, a signal transduction molecule (e.g. cytokine), or a functional domain thereof (e.g. a variable region of an antibody or TCR, a single-chain antibody, a signaling

domain of a receptor). For example, for targeted delivery of the NK cells to tumors or pathogens, the isolated NK cells can be further modified by expressing a moiety (e.g., an antibody) on the cell surface that specifically recognizes an antigen of a tumor cell or a pathogen.

[0071] The invention additionally provides kits or pharmaceutical combinations for converting AML cells into NK cells. The kits typically contain one or more TpoR agonist antibodies described herein, tools and materials for isolating AML cells from a subject, and reagents for co-culturing AML cells with the TpoR antibody. In some embodiments, the kits can contain the TpoR antibody and a cultured AML cell population for generating NK cells that can be applied allogeneically to subjects afflicted with tumors or pathogenic infections.

[0072] The pharmaceutical compositions containing a TpoR agonist antibody or a NK cell population described herein can be administered to subjects in need of treatment in accordance with standard procedures of pharmacology. Methods of administering the therapeutic compositions to a subject can be accomplished based on procedures routinely practiced in the art. See, e.g., Remington: *The Science and Practice of Pharmacy*, Mack Publishing Co., 20th ed., 2000; Ritter et al., *J. Clin. Invest.* 116:3266-76, 2006; Iwasaki et al., *Jpn. J. Cancer Res.* 88:861-6, 1997; Jespersen et al., *Eur. Heart J.* 11:269-74, 1990; and Martens, *Resuscitation* 27:177, 1994. For example, a composition containing the induced NK cells are typically administered (e.g., via injection) in a physiologically tolerable medium, such as phosphate buffered saline (PBS). The isolated cells, or their engineered form as disclosed herein, should be administered to the subject in a number sufficient to inhibit the development of the disease (e.g., growth of a tumor) in the subject. In some embodiments, administration of therapeutic composition is carried out by local or central injection of the cells into the subject. In some other embodiments, the administration is via a systemic route such as peripheral administration. Additional guidance for preparation and administration of the pharmaceutical compositions of the invention are described in the art. See, e.g., *Goodman & Gilman's The Pharmacological Bases of Therapeutics*, Hardman et al., eds., McGraw-Hill Professional (10th ed., 2001); *Remington: The Science and Practice of Pharmacy*, Gennaro, ed., Lippincott Williams & Wilkins (20th ed., 2003); and *Pharmaceutical Dosage Forms and Drug Delivery Systems*, Ansel et al. (eds.), Lippincott Williams & Wilkins (7th ed., 1999).

VI. TpoR agonist antibodies

[0073] The invention further provides TpoR agonist antibodies that can be employed in the methods of the invention. As described in the Examples, the inventor performed affinity maturation of the 3D9 antibody via yeast display, and obtained several variants that can also induce NK formation from AML cells. Accordingly, the invention provides antibodies or antigen-binding molecules that specifically bind to TpoR with the same binding specificity as that of the 3D9 variants (SEQ ID Nos:9-13). The TpoR agonist antibodies of the invention are preferably monoclonal antibodies like the antibodies exemplified in the Examples below. Preferably, they have the same binding specificities as that of the exemplified functional antibodies shown in Table 1. These antibodies typically harbor variable region sequences that are the same or substantially identical to that of the exemplified antibodies. General methods for preparation of monoclonal or polyclonal antibodies are well known in the art. See, e.g., Harlow & Lane, *Using Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1998; Kohler & Milstein, *Nature* 256:495-497, 1975; Kozbor et al., *Immunology Today* 4:72, 1983; and Cole et al., pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy*, 1985.

[0074] Some of the anti-TpoR agonist antibodies of the invention are derived from the 3D9-4 scFv variant antibody. This scFv antibody has an amino acid sequence shown in SEQ ID NO:12. The sequences of the heavy chain and the light chain portions of the scFv are respectively shown in SEQ ID NOs:14 and 15. The CDR sequences of the heavy chain variable region of this antibody are RDTFNTYG (HCDR1; SEQ ID NO:4), IIPIFGTA (HCDR2; SEQ ID NO:5), and CARDRRSGGSDYW (HCDR3; SEQ ID NO:21). The CDR sequences of its light chain variable region are QGLGRW (LCDR1; SEQ ID NO:7), AAS (LCDR2; SEQ ID NO:25), and QQTRRRPWT (LCDR3; SEQ ID NO:22). Some other TpoR agonist antibodies of the invention are derived from the 3D9-1, 3D9-2, 3D9-3, and 3D9-5 variants shown in Table 1. Heavy chain and light chain CDR sequences of these variants are shown in Table 2.

[0075] A typical intact antibody interacts with target antigen predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining regions (CDR's). The TpoR agonist antibodies of the invention encompass antibodies or antigen-binding fragments having at least one of their heavy chain CDR sequences and light

chain CDR sequences that is the same as or substantially identical to the corresponding CDR sequence of the 3D9 antibody or its variant exemplified herein (e.g., antibody 3D9-4). Some of the agonist antibodies of the invention have the same binding specificity as that of the exemplified antibodies shown in Table 1. These antibodies can compete with the exemplified antibodies for binding to the TpoR receptor. The antibodies can additionally possess the same or similar functional properties as that of the exemplified antibodies, e.g., activating TpoR signaling pathway and/or induce NK cells from AML cells. Some agonist antibodies of the invention are homodimers having all CDR sequences in their variable regions of the heavy chain and light chain respectively identical to the corresponding CDR sequences of the exemplified TpoR agonist antibodies.

[0076] In addition to having CDR sequences respectively identical to the corresponding CDR sequences of an exemplified antibody (e.g., the 3D9-4 antibody), some of the TpoR agonist antibodies of the invention have their entire heavy chain and light chain variable region sequences respectively identical to the corresponding variable region sequences of the exemplified antibodies. In some other embodiments, other than the identical CDR sequences, the antibodies contain amino acid residues in the framework portions of the variable regions that are different from the corresponding amino acid residues of the exemplified antibodies. Relative to the exemplified antibodies, the agonist antibodies of the invention can undergo non-critical amino-acid substitutions, additions or deletions in the variable region without loss of binding specificity or effector functions, or intolerable reduction of binding affinity or receptor agonizing activities. Usually, antibodies incorporating such alterations exhibit substantial sequence identity to a reference antibody (e.g., the 3D9-4 variant antibody) from which they were derived. For example, the mature light chain variable regions of some of the agonist antibodies of the invention have at least 75% or at least 85% sequence identity to the sequence of the mature light chain variable region of the exemplified antibodies. Similarly, the mature heavy chain variable regions of the antibodies typically show at least 75% or at least 85% sequence identity to the sequence of the mature heavy chain variable region of the exemplified agonist antibodies. In various embodiments, the antibodies typically have their entire variable region sequences that are substantial identical (e.g., 75%, 85%, 90%, 95%, or 99%) to the corresponding variable region sequences of the exemplified antibodies. Some agonist antibodies of the invention

have the same specificity but improved affinity or receptor-agonizing activities if compared with the exemplified antibodies.

EXAMPLES

[0077] The following examples are provided to further illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims.

Example 1. AML cell differentiation induced by TpoR agonist antibody

[0078] Because signal transduction pathways, especially for cytokines, are degenerate, we tested whether our panel of agonist antibodies with their different mode of binding could induce stem cell-like acute myeloblastic leukemia (AML) cells to differentiate. We studied bone marrow or peripheral blood samples from 7 patients where the differentiation status of their AML according to the French-American-British (FAB) cooperative group was known. Six were classified as minimal maturation (M1) and the other one was acute monocytic leukemia (M5). The one patient with M5 differentiated cells was treated for 4 months with Ara-C prior to collection of cells whereas the other 6 patients were untreated. Samples of leukemic cells were obtained by BM aspiration or from peripheral blood.

[0079] Initially, the leukemic cells from the bone marrow of an 83-year-old white female who had progressed from myelodysplastic syndrome to frank AML were studied in the greatest detail because they were the most stem cell like (M1) (minimal maturation). A smear of the BM and FACS analysis showed that her BM was almost totally replaced by AML cells (greater than 93%) with a myeloid to erythroid ratio greater than 10:1. The myeloid cells had round or slightly indented nuclei and were of normal female karyotype (Figs. 1a-c). According to FACS analysis, the CD34⁺/CD13⁺/CD33⁺ population of cells represented 2.4% of the BM whereas the CD34⁺/CD38⁻ cell population represented 2.02 % of the BM. Based on the morphology and the cell surface phenotype, the leukemic cells were classified as M1 subtype.

[0080] The effect of two of the antibodies on this sentinel patient's AML cells was the most interesting. The agonist antibody to the granulocyte colony stimulating factor receptor (GCSFR) induced the AML cells to adopt a neural cell phenotype and express neural markers, as seen previously by us for normal CD34⁺ cells using this antibody. By contrast,

in these same cells, an agonist antibody that is highly specific for the thrombopoietin receptor (TPOR) induced a dendritic cell-like phenotype that was accompanied by expression of the dendritic cell marker CD11c (Figure 1). Differentiation begins on day two and appears to be complete by day four at which time about 80% of the cells were converted. This 3D9 TpoR agonist antibody was also able to induce formation of similar cells from bone marrow and peripheral blood from five out of seven other AML patients including the one classified as M5. By contrast natural TPO did not induce a dendritic like phenotype in AML cells (Figure 5C). Similar effects were observed for five out of seven other AML patients when their bone marrow or peripheral blood was studied, indicating that the NK induction can be generalizable to a majority of AML patients.

[0081] Importantly, this antibody agonist to the TPO receptor gave a totally different phenotype when normal CD34+ bone marrow cells instead of leukemic cells were studied. Here, like natural TPO, the antibody induced large numbers of megakaryocytes that is in keeping with the expected lineage conversion after activating the TpoR. No megakaryocytes were seen when either peripheral or bone marrow-derived AML cells were treated with the TpoR agonist antibody, indicative of the fact that the nature of the phenotype induced by antibody agonists may be cell context dependent.

[0082] In the course of the many experiments we carried out, we noticed that the induced cells could have an “early” or “late” phenotype” depending on the time in culture and somewhat on whether the culture dishes were collagen coated. When grown for short periods of time on collagen coated dishes, the induced cells are rounded and have extensive “needle like” projections and make large amounts of perforin, interferon gamma, and granzyme B (Figure 2, Figure 5). However, when grown for longer periods on glass surfaces, the induced cells have a more dendritic like phenotype (Fig. 1). Some of the projections from the induced cells appear to interact with the target cells that begin to bleb at their surface (Fig. 1B). Given that the induced leukemic cells with an “early” phenotype make large amounts of molecules known to be associated with killer cells, we refer to them simply as NK cells. We further examined whether the NK cells with dendritic like phenotypes also produce molecules associated with killing. Like the more rounded cells, we found that these cells also produced large amounts of perforin, interferon γ , and granzyme B (Fig.3, and Fig. 7). All these data indicate that the TpoR agonist antibody 3D9 can convert the AML cells into NK cells.

Example 2. Tumor cell killing

[0083] Based on serial qualitative observations at the level of light microscopy, the induced cells appeared to insert cytoplasmic extensions into the neighboring, noninduced AML cells and kill them (Figure 4). To gain further information about the mechanism of killing, we studied the process by scanning electron microscopy, real time imaging, and quantitated the killing process. Multiple fine filopodia from the dendritic extensions of the killer cells attach to the target cells. Some of these filopodia actually appear to enter the interior of the target cell through very obvious holes that presumably are generated at the site of attachment by molecules in these filopodia such as perforin. Presumably perforin itself, granzyme B, and IFN- γ now access the cytoplasm of the target cells via the cytoplasmic extensions of the killer cells that have penetrated into the target cell interior. As observed by electron microscopy, the target cells now contain canyon-like fractures at their surface. These images support a killing mechanism similar to that studied in detail for NK cells (Cheng et al., Cell Mol. Immunol. 10:230–252, 2013).

[0084] We quantitated the killing potential of the AML cells that were induced by the antibody into NK cells. To do so, we used a FACS- based cytotoxicity assay in which target cells in suspension that were fluorescently labeled with calcein-AM were cocultured with antibody-induced NK cells that were attached to the dish. The calcein-AM-labeled target AML cells were incubated with NK cells or control cultures for 24 h. Propidium iodide (PI) was used to identify the dead cells. Thus, cells that both stain for calcein- AM and are PI+ represent dead target cells. The FACS analysis showed that the induced NK cells specifically killed 13-16% the target cells per 24 h (Fig. 9). This degree of killing occurs even though the required format for these experiments afforded less than the optimal potential for cell-cell interaction between attached killer cells and target cells in suspension. As a test of specificity, we studied whether the induced NK cells would kill breast cancer cells. The breast cancer cell line MDA-MB-231 was cocultured with induced NK cells or undifferentiated AML cells as described above. Unlike the killing seen when AML cells are the targets, the induced NK cells did not specifically kill breast cancer cells, indicating that the requirement for killing goes beyond simple oncogenic transformation and may include a like-like recognition component.

Example 3. Different signal transduction activation kinetics

[0085] We analyzed the signal transduction pattern in AML cells after treatment with several doses of agonist antibody. In a previous study, we showed that the antibody induces rapid and strong STAT-3, AKT and ERK phosphorylation in normal CD34⁺ hematopoietic stem cells. Similarly, in AML cells the antibody induces efficient STAT-3, AKT and ERK phosphorylation. We tested the activation of phosphorylation at several time points using either the antibody or TPO. Both the antibody and TPO activated the phosphorylation of STAT-3 and ERK with similar kinetics but AKT was different. While the antibody increased the phosphorylation of AKT gradually, TPO showed different kinetics in that the activation of AKT phosphorylation was immediate and bi-phasic. To determine which signaling pathway(s) is necessary for NK cell differentiation by antibody, AML cells were co-treated with antibody and specific inhibitors of each signal pathway individually. Interestingly, antibody-induced NK cell differentiation was significantly blocked by the inhibitors of STAT-3 or PI 3-kinase that are upstream of AKT. However, the inhibitor of MEK that is upstream of ERK did not affect the differentiation of AML cells. These observations suggest that two of the three main signaling pathways of the TPOR (STAT-3 and PI 3-kinase) are required for antibody induction of NK cell differentiation from AML cells.

Example 4. Quantitative gene expression analysis

[0086] To gain more quantitative information about the gene expression that accompanies the differentiation of AML cells into NK cells and to further characterize the nature of the induced cells, whole transcriptome shotgun sequencing (RNAseq) was carried out. Untreated cells were compared to those treated with either TPO or agonist antibody. The overall gene expression profile of the groups treated with either the antibody or TPO were very different. The number of differently expressed transcripts was 3506 between the untreated and antibody treated cells versus 1902 between the untreated and TPO treated cells with a false discovery rate < 0.1 and average log₂ (count per million) > 4 (Dataset S1). The whole transcriptome gene set enrichment analysis (GSEA) and ingenuity pathway analysis (IPA) showed that there were large increases in gene expression for molecules that encode genes associated with both developing and mature NK cells including those associated with signal transduction. These changes include large increases in the DC markers such as CD80, CD83, CD86, CD123 and CCR7 and many NK markers of death receptor pathway such as

FAS, FAS ligand and TNF α .

[0087] The consequences of having both dendritic and NK cell markers is interesting in that the IPA analysis showed that there is extensive “cross talk” between dendritic cells and NK cells in the gene set induced by our antibody. Moreover, after antibody treatment but not in control experiments, both the GSEA and IPA revealed up-regulation of molecules associated with cytotoxicity such as perforin and granzyme B, the interferon γ response, inflammation, and death receptor and TPOR signaling. Interestingly, many genes related to oxidative phosphorylation in mitochondria were also up regulated in the antibody-treated cells when compared to untreated cells. Probably, the killer cells have been reprogrammed to generate the large amounts of energy needed to synthesize killing molecules and deliver them. For instance, in going from AML to NK cells there is massive remodeling of the plasma membrane. *In toto*, the RNAseq analysis confirmed and added to the data obtained by morphological and immunocytochemical analysis.

Example 5. IPA Analysis of the mechanistic network

[0088] Aside from understanding the initiating events and identification of effector molecules, we were interested in the mechanism of the antibody-induced conversion of AML cells to NK cells. This conversion doubtless involves differential activation of signal transduction pathways (see above). To predict a mechanistic network, we have utilized the Upstream Analysis feature in Ingenuity Pathway Analysis (IPA) software. Upstream Analysis is based on prior knowledge of expected effects between transcriptional regulators and their target genes collected in the Ingenuity® Knowledge Base. This database includes Expression, Transcription and Protein-DNA binding relationships. Activation Z-score and Overlap p-value are two statistical measures to identify and rank significant regulators. The list of differentially expressed genes between the untreated and antibody treated cells with a false discovery rate < 0.1 and average $\log_2$ (count per million) > 4 was analyzed in IPA (~3500 DE genes). Interestingly, the Upstream Analysis ranked STAT1 among top five upstream transcription regulators based on activation Z-score and STAT3 was ranked 3rd based on Overlap p-value. In fact, there are 98 targets of STAT1 (overlap p-value $< 8.58E-20$) and 153 targets of STAT3 (overlap p-value $< 2.02E-24$) in the list of differentially expressed genes. The mechanistic network derived from the STAT1 analysis includes upstream regulators such as STAT3, RELA, IFNG, IL1B, IRF1, IRF8, IRF9, JUN, MYC, NFKB1,

NFKBIA, and SPI1 which are predicted to be activated/inhibited based on a computationally generated directional network. However, this analysis doesn't require all the upstream regulators to be significantly changed. The mechanistic network derived from the STAT3 analysis, which shares many genes with STAT1 network, includes upstream regulators such as STAT1, RELA, IFNG, IL1B, IRF1, IRF7, JUN, NFKB1, NFKBIA, IL6, TNF, NR3C1, and Interferon alpha.

[0089] From a mechanistic point of view, the presence of STAT1 and STAT3 will allow formation of the heterodimer that binds to the interferon-gamma-activated sequence promoter element. Interestingly, we were also able to identify the V\$STAT transcription factor binding site family as the second highest over-represented binding site family in the promoter regions of the top 500 up-regulated genes in the antibody-treated cells using Genomatix software (<http://www.genomatix.de/>).

Example 6. Additional studies on NK cells generated from AML cells.

[0090] Further experiments were performed to study NK cell differentiation from AML cells and their activities. First, a critical problem related to AML therapy is a high probability of recurrence of AML after chemotherapy. To examine if the 3D9 TpoR antibody also induces NK cells from relapsed AML cells (like newly diagnosed AML cells), relapsed AML cells were incubated with the antibody. NK cells markers including Perforin, Granzyme B and Interferon γ were then analyzed by confocal microscopy. The results are shown in Figure 10. As indicated in the figure, the antibody potentially increased the differentiation of relapsed AML cells as well.

[0091] The killing process by the antibody-induced NK cells was examined with confocal microscopy. As shown in Figure 11, it was found that the antibody-induced NK cells captured target AML cells using the tip of dendrites. An additional test was performed to determine if the TpoR antibody affects the TPO receptor signaling. It was observed that, like the Tpo ligand, the antibody activated the phosphorylation of Akt and Erk over various range of concentrations and time periods (Figure 12). Further, cytotoxic activities of NK cells induced from both newly diagnosed AML cells and relapsed AML cells by the TpoR antibody were demonstrated in vitro with undifferentiated AML cells (Figure 13). As shown in the figure, cytotoxic activity induced by the 3D9 antibody from relapsed AML cells is stronger than that induced from newly diagnosed AML cells.

[0092] Finally, we performed affinity maturation of the 3D9 antibody using yeast display and found 5 variants antibodies. Sequences of these 3D9 variants are shown in Tables 1 and 2. These 5 variants were tested for activity in induce MK differentiation from relapsed AML cells. Compared to the activity of 3D9, the variant antibodies showed varying activities in converting AML cells into NK cells (Figure 14). In particular, variant 4 showed higher potency than 3D9 in inducing differentiation of relapsed AML cells.

Example 7. Materials and methods

[0093] This example describes some of the material and methods that were employed in the exemplified embodiments herein.

[0094] Expression and purification of antibodies. The expression vector containing the antibody gene was transfected into 293F cells. Antibodies from the pooled supernatants were purified using HiTrap Protein G HP columns with ÄKTAexpress purifier. The buffer was exchanged to Dulbecco's PBS (pH 7.4) and the purified antibodies were stored at 4°C.

[0095] Culture of AML cells. Human AML BM and PB cells were isolated from patients and frozen at -80°C (AllCells). AML cells were cultured in StemSpan serum-free media (SFEM) from Stemcell technologies supplemented with streptomycin and penicillin.

[0096] Immunocytochemistry. Cells were fixed with 4% paraformaldehyde at room temperature (RT) for 15 min, blocked and stained with specific antibodies, followed by fluorophore conjugated secondary antibody staining. Hoechst 3342 (Cell signaling) and rhodamine-phalloidin (Life technologies). Incubation with all antibodies was carried out for 30 min at RT. After washing 3 times for 5 min, images were collected using a confocal microscope.

[0097] Flow cytometry. After co-culture, killer cells and Calcein-AM labeled target cells were stained with PI (Life technologies) to identify dead cells and washed with PBS. For quantification, Stained cells were sorted with a LSRII flow cytometer (Becton Dickinson).

[0098] Western blotting. To prepare total cell lysates, AML BM cells were washed with PBS and then lysed in lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 50 mM NaF, 1 mM Na₃VO₄, 10% glycerol, 1% Triton X-100). The lysates were then centrifuged at 15,000 rpm for 15 min at 4°C to remove aggregates, and the soluble proteins were denatured in Laemmli sample buffer (5 min at 95°C) and then separated by SDS-PAGE. The proteins were transferred to nitrocellulose membranes using the iBlot blotting system from Invitrogen

and blocked in PBST containing 5% BSA for 1 hr before being incubated with antibodies overnight at 4°C. After washing the membranes several times with PBST, the blots were incubated with horseradish peroxidase-conjugated anti-human or anti-rabbit antibody for 1 hr. The membranes were then washed with PBST and developed by ECL. The anti-STAT-3, anti-p-STAT-3 (Tyr-705), anti-AKT, anti-p-AKT (Thr-308), anti-ERK1/2 and anti-p-ERK1/2 (Thr-202/Tyr-204) antibodies were purchased from Cell signaling.

[0099] Electron microscopy. The cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and a small volume was placed on a 13 mm Whatman PC filter. The cells were rinsed on the filter with PBS. The entire double filter was then clamped between nylon washers and the unit placed in 1% osmium tetroxide. After a PBS and water wash, the entire unit was dehydrated in an ethanol and placed in a critical point dryer. The nylon washers were then dismantled, the filters separated, and both filters mounted onto SEM stubs with carbon tape. The stubs with attached filters were then sputter coated with Iridium for subsequent examination and documentation on a Hitachi S-4800 SEM (Hitachi High Technologies).

[00100] RNAseq and bioinformatic analysis. We prepared 3 replicas of RNA from AML cells after treatment with PBS, antibody or TPO. RNAseq libraries were prepared with NuGEN Ovation RNA-Seq System V2. RNA was sequenced using an Illumina HiSeq Analyzer 2000, Casava v1.8.2 genome analyzer pipeline, TopHat v1.4.1/Bowtie2 genome-alignment and Partek v6.6 mRNA annotation software. Statistical analyses were done with edgeR (Bioconductor), excluding genes with false discovery rates > 0.10, log₂ (counts per million) > 4. GSEA was performed with gene set permutation, using gene sets from MSigDB (www.broadinstitute.org/gsea/msigdb/index.jsp) or manually curated from excluding genes without HUGO approved symbols. We examined the molecular interactions to confirm the GSEA data that were captured previously. The differentially expressed genes were further analyzed using the IPA software (Ingenuity Systems, Redwood City, CA; <http://www.ingenuity.com>). This all-in-one web-based software that makes use of the ingenuity pathways knowledge base (IPKB) to generate interaction networks of focus genes based on manually curated information reported in the literature.

[00101] Statistical analysis. The data are expressed as the means ± S.E. Statistical analysis was performed using the Student's *t* test or by one-way analysis of variance and the post hoc test. *p*-values of <0.05 were considered significant.

[00102] 3D9 TpoR agonist antibody amino acid sequence**[00103]** scFv sequence (SEQ ID NO:1)

[00104] MAQVQLVQSGAEVRKVGSSVKVSKASRDTFNTYGISWVRQAPGQGLE
 WMGGIPIFGTADYAQKFRGRVTITADESTSTAYMELSSLRSED AVYYCARDRLG
 GSDYWGGQGLTVTVSSGGGGSGGGGSGGGGSDIVMTQSPSSVSASVGDKVTITCRAS
 QGLGRWLAWYQQEPGKAPKLLIYAASLTQRGVPSRFSGSGSGTDFLTISLQPEDF
 ATYYCQQSNSFPWTFGQGTKLEIKR

[00105] Heavy chain variable region sequence (SEQ ID NO:2)

[00106] MAQVQLVQSGAEVRKVGSSVKVSKASRDTFNTYGISWVRQAPGQGLE
 WMGGIPIFGTADYAQKFRGRVTITADESTSTAYMELSSLRSED AVYYCARDRLG
 GSDYWGGQGLTVTVSS

[00107] Light chain variable region sequence (SEQ ID NO:3)

[00108] DIVMTQSPSSVSASVGDKVTITCRASQGLGRWLAWYQQEPGKAPKLLIY
 AASLTQRGVPSRFSGSGSGTDFLTISLQPEDFATYYCQQSNSFPWTFGQGTKLEIK
 R

Table 1. Variants of 3D9 TpoR agonist antibody

Designa tion	Sequence	SEQ ID NO:
3D9-1	MAQVQLVQSGAEVRKVGSSVKVSKASRDTFNTYGISWVRQAPGQGLEWMGGIIP IFGTADYAQKFRGRVTITADESTSTAYMELSSLRSED AVYYCARDRRAGGSDYWGGQGL VTVSSGGGGSGGGGSGGGGSDIVMTQSPSSVSASVGDKVTITCRASQGLGRWLAWYQQEP GKAPKLLIYAASLTQRGVPSRFSGSGSGTDFLTISLQPEDFATYYCQQSRRNPWTFGQ GTKLEIKRGLGGLEQKLISEEDL	9
3D9-2	AAQPAMAQVQLVQSGAEVRKVGSSVKVSKASRDTFNTYGISWVRQAPGQGLEWMGGIIP IFGTADYAQKFRGRVTITADESTSTAYMELSSLRSED AVYYCARDRRAGGSDYWGGQGL VTVSSGGGGSGGGGSGGGGSDIVMTQSPSSVSASVGDKVTITCRASQGLGRWLAWYQQEP GKAPKLLIYAASLTQRGVPSRFSGSGSGTDFLTISLQPEDFATYYCQQNNHNPWTFGQ GTKLEIKRGLGGLEQKLISEEDL	10
3D9-3	AAQPAMAQVQLVQSGAEVRKVGSSVKVSKASRDTFNTYGISWVRQAPGQGLEWMGGIIP IFGTADYAQKFRGRVTITADESTSTAYMELSSLRSED AVYYCARDRALGGSDYWGGQGL VTVSSGGGGSGGGGSGGGGSDIVMTQSPSSVSASVGDKVTITCRASQGLGRWLAWYQQEP GKAPKLLIYAASLTQRGVPSRFSGSGSGTDFLTISLQPEDFATYYCQQAMHRPWTFGQ GTKLEIKRGLGGLEQKLISEEDL	11
3D9-4	AAQPAMAQVQLVQSGAEVRKVGSSVKVSKASRDTFNTYGISWVRQAPGQGLEWMGGIIP IFGTADYAQKFRGRVTITADESTSTAYMELSSLRSED AVYYCARDRRSGGSDYWGGQGL VTVSSGGGGSGGGGSGGGGSDIVMTQSPSSVSASVGDKVTITCRASQGLGRWLAWYQQEP GKAPKLLIYAASLTQRGVPSRFSGSGSGTDFLTISLQPEDFATYYCQQTRRRPWTFGQ GTKLEIKRGLGGLEQKLISEEDL	12
3D9-5	AAQPAMAQVQLVQSGAEVRKVGSSVKVSKASRDTFNTYGISWVRQAPGQGLEWMGGIIP IFGTADYAQKFRGRVTITADESTSTAYMELSSLRSED AVYYCARDRRTGSDYWGGQGL VTVSSGGGGSGGGGSGGGGSEIVLTQSPSSVSASVGDKVTITCRASQGLGRWLAWYQQEP GKAPKLLIYAASLTQRGVPSRFSGSGSGTDFLTISLQPEDFATYYCQQTKWPPWTFGQ	13

	GTKLEIKRGLGGLEQKLISEEDL	
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[00109] 3D9-4 variant antibody heavy chain variable region sequence (SEQ ID NO:14)

[00110] MAQVQLVQSGAEVRKVGSSVKVSCASRDTFNTYGISWVRQAPGQGLE
WMGGIPIFGTADYAQKFRGRVTITADESTSTAYMELSSLRSEDNAVYYCARDRRSG
GSDYWGGQTLVTVSS

[00111] 3D9-4 variant antibody light chain variable region sequence (SEQ ID NO:15)

[00112] DIVMTQSPSSVSASVGDKVTITCRASQGLGRWLAWYQQEPGKAPKLLIY
AASTLQRGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQTRRRPWTFGQGTKLEIK
R

Table 2 CDR sequences of 3D9 and variant antibodies

	HCDR1	HCDR2	HCDR3	LCDR1	LCDR2	LCDR3
3D9 CDR Sequences	RDTFNTYG	IPIFGTA	CARDRKLGGSDYW	QGLGRW	AAS	QQSNSFPWT
3D9 CDR SEQ ID NO:	4	5	6	7	25	8
3D9-1 CDR Sequences	RDTFNTYG	IPIFGTA	CARDRRAGGSDYW	QGLGRW	AAS	QQSRRNPWT
3D9-1 CDR SEQ ID NO:	4	5	16	7	25	17
3D9-2 CDR Sequences	RDTFNTYG	IPIFGTA	CARDRRAGGSDYW	QGLGRW	AAS	QQNNHNPWT
3D9-2 CDR SEQ ID NO:	4	5	16	7	25	18
3D9-3 CDR Sequences	RDTFNTYG	IPIFGTA	CARDRALGGSDYW	QGLGRW	AAS	QQAMHRPW T
3D9-3 CDR SEQ ID NO:	4	5	19	7	25	20
3D9-4 CDR Sequences	RDTFNTYG	IPIFGTA	CARDRRSGGSDYW	QGLGRW	AAS	QQTRRRPWWT
3D9-4 CDR SEQ ID NO:	4	5	21	7	25	22
3D9-5 CDR Sequences	RDTFNTYG	IPIFGTA	CARDRRTGGSDYW	QGLGRW	AAS	QQTKWPPWT
3D9-5 CDR SEQ ID NO:	4	5	23	7	25	24

[00113] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

[00114] All publications, databases, GenBank sequences, patents, and patent applications cited in this specification are herein incorporated by reference as if each was specifically and individually indicated to be incorporated by reference.

WE CLAIM:

1. A method for inducing formation of natural killer (NK) cells from acute myeloid leukemia (AML) cells, comprising contacting a population of AML cells with a TpoR agonist antibody under conditions sufficient to convert the AML cells into NK cells, thereby inducing the AML cells into NK cells, wherein the TpoR agonist antibody has the same binding specificity as that of an antibody comprising heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) respectively shown in (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22; (2) SEQ ID NOs:4-7, 25 and 8; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 17; (4) SEQ ID NOs:4, 5, 16, 7, 25 and 18; (5) SEQ ID NOs:4, 5, 19, 7, 25 and 20; or (6) SEQ ID NOs:4, 5, 23, 7, 25 and 24.
2. The method of claim 1, wherein the AML cells are cells isolated from a bone marrow sample or a peripheral blood sample of a human AML patient.
3. The method of claim 1, wherein the AML cells are human CD34+ leukemia cells.
4. The method of claim 3, wherein the human CD34+ leukemia cells are CD34+/CD33+ leukemia cells or CD34+/CD38- leukemia cells.
5. The method of claim 1, wherein the TpoR agonist antibody comprises heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) that are substantially identical to (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22, respectively; (2) SEQ ID NOs:4-7, 25 and 8, respectively; (3) SEQ ID NOs:4, 5, 16, 7, 25, and 17, respectively; (4) SEQ ID NOs:4, 5, 16, 7, 25 and 18, respectively; (5) SEQ ID NOs:4, 5, 19, 7, 25 and 20, respectively; or (6) SEQ ID NOs:4, 5, 23, 7, 25 and 24, respectively.
6. The method of claim 1, wherein the TpoR agonist antibody comprises heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) that are respectively identical to (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22; (2) SEQ ID NOs:4-7, 25 and 8; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 17; (4) SEQ ID NOs:4, 5, 16, 7, 25 and 18; (5) SEQ ID NOs:4, 5, 19, 7, 25 and 20; or (6) SEQ ID NOs:4, 5, 23, 7, 25 and 24.

7. The method of claim 1, wherein the TpoR agonist antibody comprises heavy chain and light chain variable region sequences respectively shown in (1) SEQ ID NO:14 and SEQ ID NO:15; or (2) SEQ ID NO:2 and SEQ ID NO:3.
8. The method of claim 1, wherein the TpoR agonist antibody comprises a scFv antibody fragment shown in SEQ ID NO:12 or SEQ ID NO:1.
9. The method of claim 1, wherein the contacting occurs in vitro by culturing the population of AML cells in the presence of the TpoR agonist antibody.
10. The method of claim 9, wherein culturing is for about 4 to 20 days.
11. The method of claim 1, further comprising detecting at least one cellular marker expressed by NK cells.
12. The method of claim 11, wherein the at least one cellular marker expressed by NK cells is CD11c and one additional marker selected from the group consisting of perforin, granzyme B and interferon γ .
13. The method of claim 1, wherein the contacting occurs in vivo in a subject afflicted with acute myeloid leukemia.
14. The method of claim 13, wherein the antibody is a bispecific antibody or is conjugated to an entity that specifically recognizes a surface antigen of AML cells.
15. The method of claim 1, further comprising enriching NK cells from the population of cells after contacting with the antibody to generate a homogenous population of NK cells.
16. A population natural killer (NK) cells produced by the method of claim 1.
17. A method for treating acute myeloid leukemia (AML) in a subject, comprising administering to the subject a pharmaceutical composition comprising a therapeutic amount of a TpoR agonist antibody, thereby treating AML in the subject, wherein the TpoR agonist antibody has the same binding specificity as that of an antibody

comprising heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) respectively shown in (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22; (2) SEQ ID NOs:4-7, 25 and 8; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 17; (4) SEQ ID NOs:4, 5, 16, 7, 25 and 18; (5) SEQ ID NOs:4, 5, 19, 7, 25 and 20; or (6) SEQ ID NOs:4, 5, 23, 7, 25 and 24.

18. The method of claim 17, wherein the subject is afflicted with undifferentiated acute myeloblastic leukemia (M0), acute myeloblastic leukemia with minimal maturation (M1), acute myeloblastic leukemia with maturation (M2), acute promyelocytic leukemia (APL) (M3), acute myelomonocytic leukemia (M4), acute myelomonocytic leukemia with eosinophilia (M4 eos), acute monocytic leukemia (M5), acute erythroid leukemia (M6), or acute megakaryoblastic leukemia (M7).

19. A method for treating a cancer or an infection in a subject, comprising administering to the subject a pharmaceutical composition comprising a therapeutic amount of a TpoR agonist antibody, thereby treating AML in the subject, wherein the TpoR agonist antibody has the same binding specificity as that of an antibody comprising heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) respectively shown in (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22; (2) SEQ ID NOs:4-7, 25 and 8; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 17; (4) SEQ ID NOs:4, 5, 16, 7, 25 and 18; (5) SEQ ID NOs:4, 5, 19, 7, 25 and 20; or (6) SEQ ID NOs:4, 5, 23, 7, 25 and 24.

20. The method of claim 19, wherein the subject is afflicted with a solid tumor.

21. The method of claim 19, wherein the subject is afflicted with a viral infection.

22. A method for killing leukemia cells in a subject, comprising (a) isolating a population of leukemia cells from the subject, (b) treating the isolated cells with a TpoR agonist antibody under conditions sufficient to convert the AML cells into NK cells, and (c) reintroducing the treated cells into the subject, thereby killing leukemia or lymphoid tumor cells in the subject; wherein the TpoR agonist antibody has the same binding specificity as that of an antibody comprising heavy chain and light chain CDR sequences (HCDR1-3 and

LCDR1-3) respectively shown in (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22; (2) SEQ ID NOs:4, 7, 25 and 8; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 17; (4) SEQ ID NOs:4, 5, 16, 7, 25 and 18; (5) SEQ ID NOs:4, 5, 19, 7, 25 and 20; or (6) SEQ ID NOs:4, 5, 23, 7, 25 and 24.

23. The method of claim 22, wherein the subject is afflicted with acute myeloid leukemia (AML), and the isolated cells are AML cells.

24. The method of claim 22, wherein the isolated cell population comprises at least 1×10^2 , 1×10^3 , 1×10^4 , 1×10^5 , or 1×10^6 leukemia cells.

25. The method of claim 22, further comprising enriching NK cells from the isolated population of cells after being treated with the antibody to generate a homogenous population of NK cells.

26. The method of claim 22, further comprising detecting in the treated cells at least one cellular marker expressed by NK cells.

27. The method of claim 26, wherein the at least one cellular marker expressed by NK cells is CD11c and one additional marker selected from the group consisting of perforin, granzyme B and interferon γ .

28. An antibody or antigen-binding fragment thereof which has the same binding specificity as that of an antibody comprising heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) respectively shown in (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22; (2) SEQ ID NOs:4, 5, 16, 7, 25 and 17; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 18; (4) SEQ ID NOs:4, 5, 19, 7, 25 and 20; or (5) SEQ ID NOs:4, 5, 23, 7, 25 and 24.

29. The antibody or antigen-binding fragment thereof of claim 28, comprising heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) that are substantially identical to (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22, respectively; (2) SEQ ID NOs:4, 5, 16, 7, 25, and 17, respectively; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 18, respectively; (4) SEQ ID NOs:4, 5, 19, 7, 25 and 20, respectively; or (5) SEQ ID NOs:4, 5, 23, 7, 25 and 24, respectively.

30. The antibody or antigen-binding fragment thereof of claim 28, comprising heavy chain and light chain CDR sequences (HCDR1-3 and LCDR1-3) that are respectively identical to (1) SEQ ID NOs:4, 5, 21, 7, 25 and 22; (2) SEQ ID NOs:4, 5, 16, 7, 25 and 17; (3) SEQ ID NOs:4, 5, 16, 7, 25 and 18; (4) SEQ ID NOs:4, 5, 19, 7, 25 and 20; or (5) SEQ ID NOs:4, 5, 23, 7, 25 and 24.

31. The antibody or antigen-binding fragment thereof of claim 28, comprising heavy chain and light chain variable region sequences respectively shown in SEQ ID NO:14 and SEQ ID NO:15.

32. The antibody or antigen-binding fragment thereof of claim 28, comprising a scFv antibody fragment shown in SEQ ID NO:12.

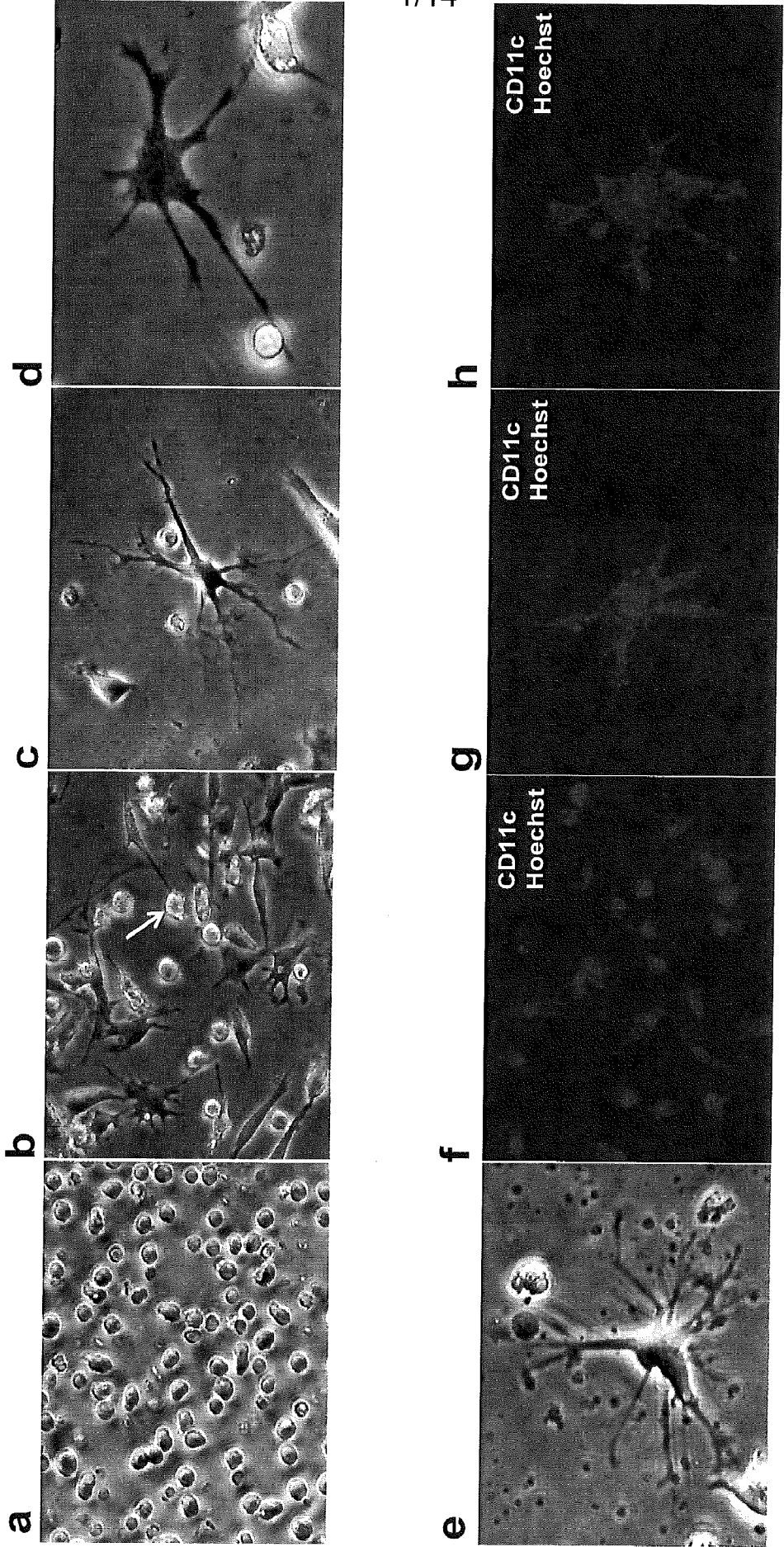


FIG. 1

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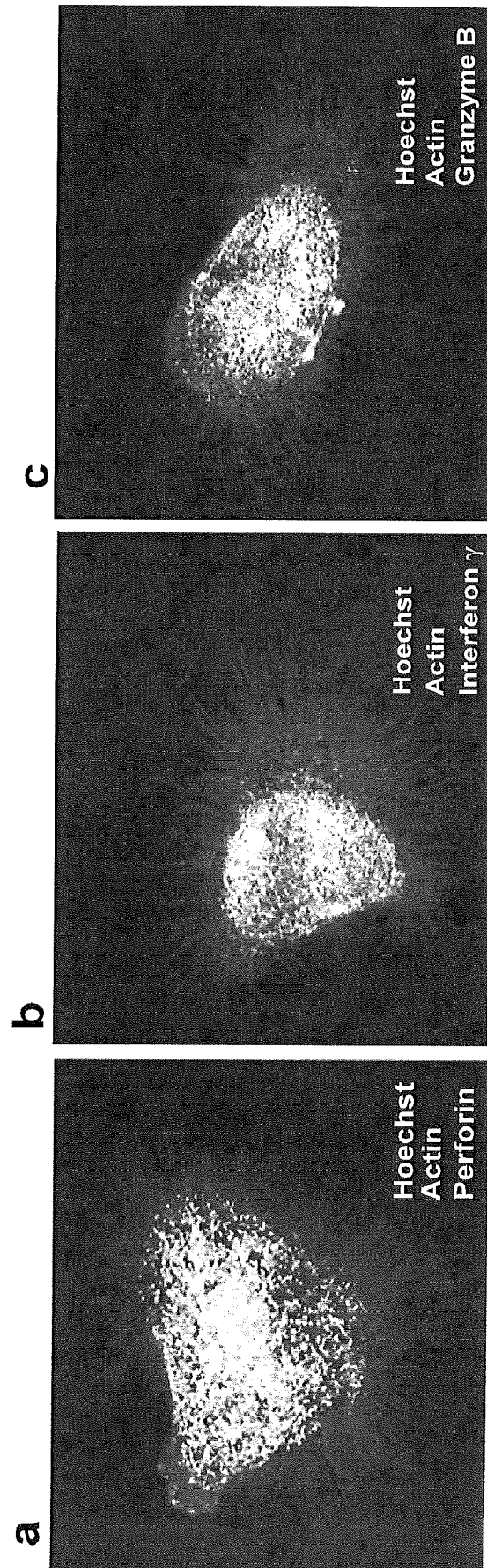


FIG. 2

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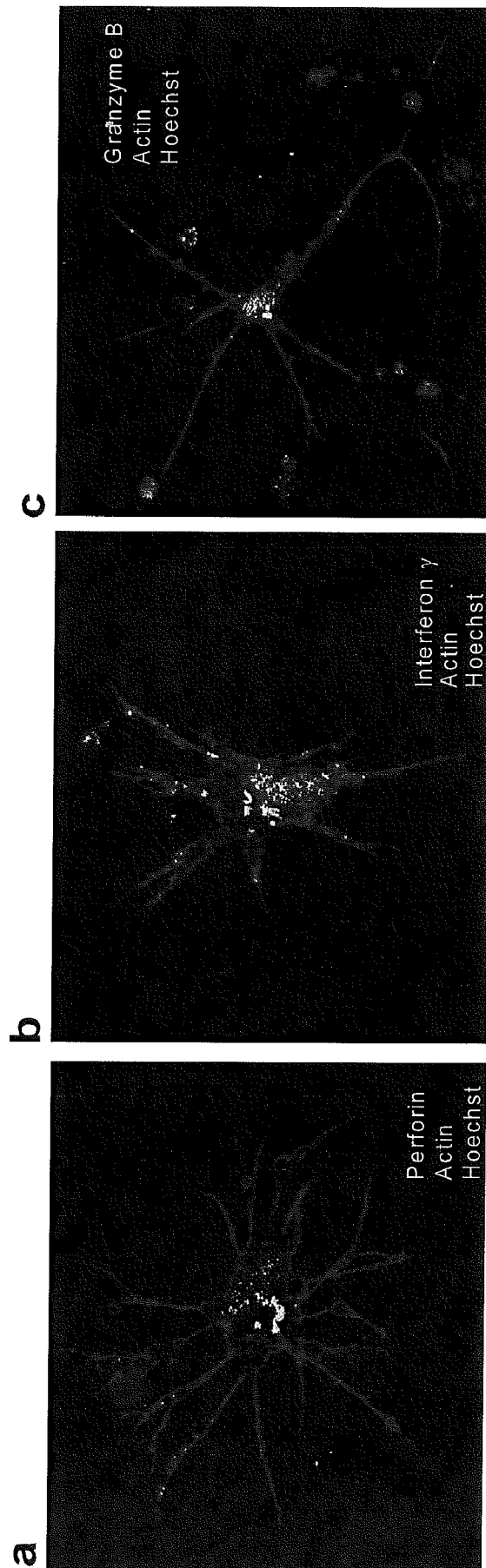


FIG. 3

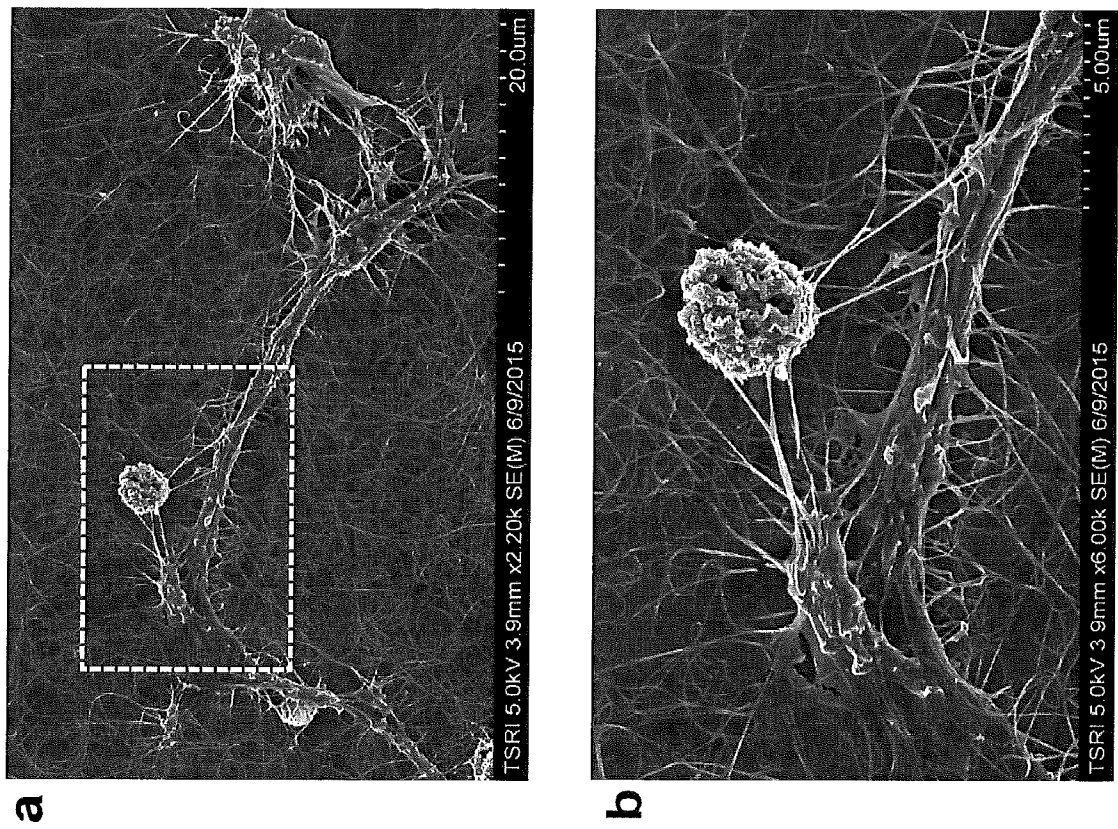


FIG. 4

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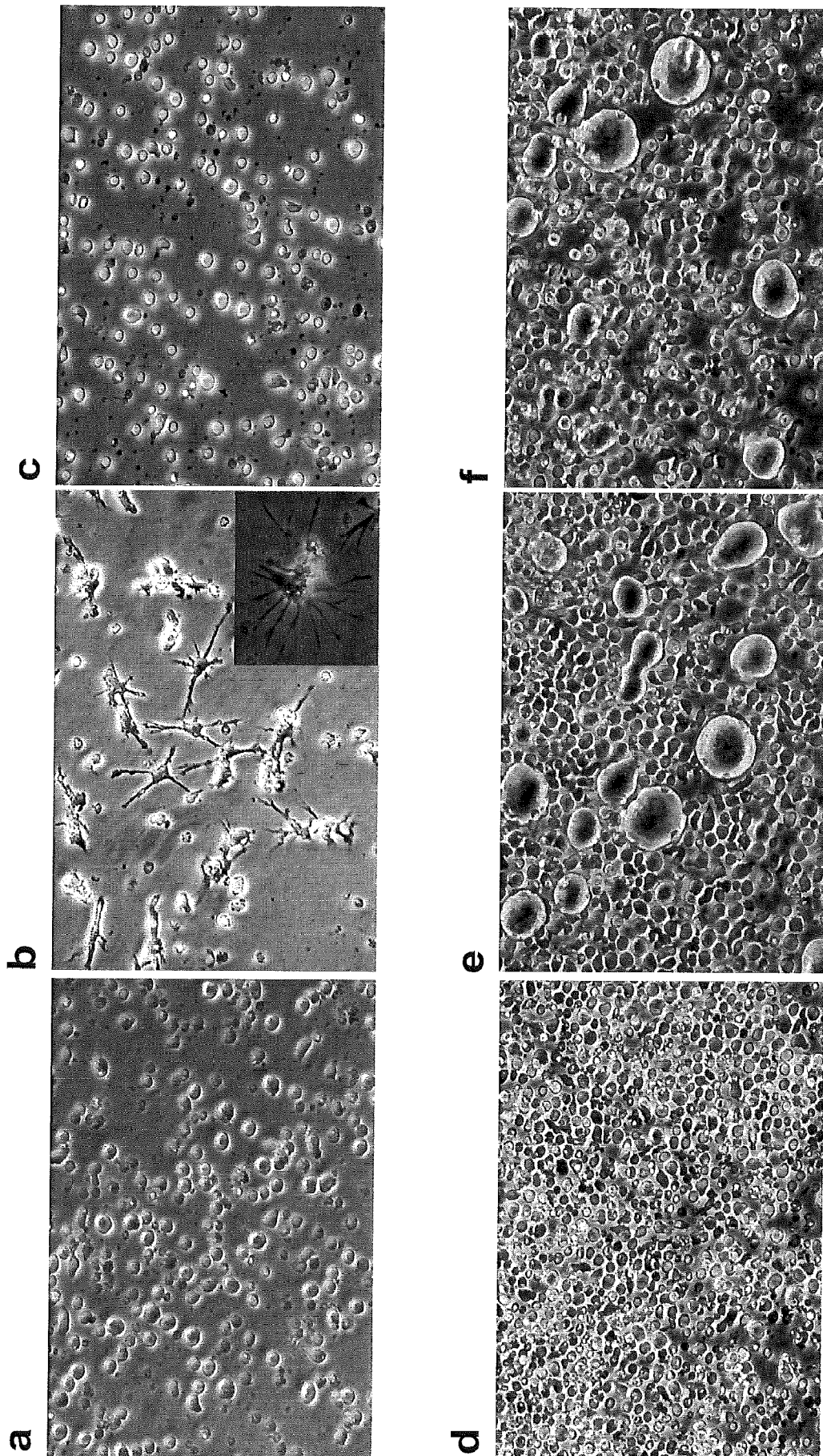


FIG. 5

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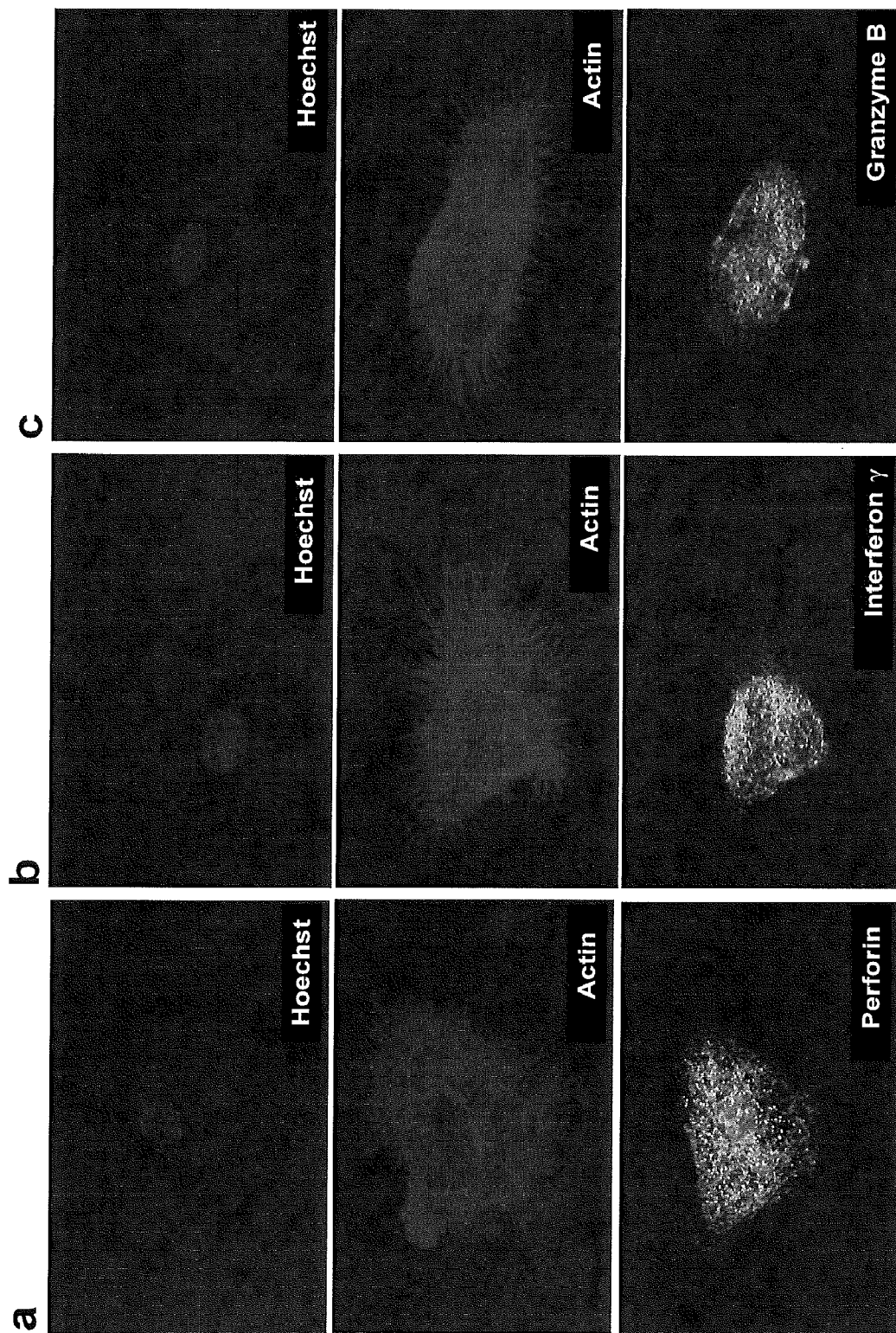


FIG. 6

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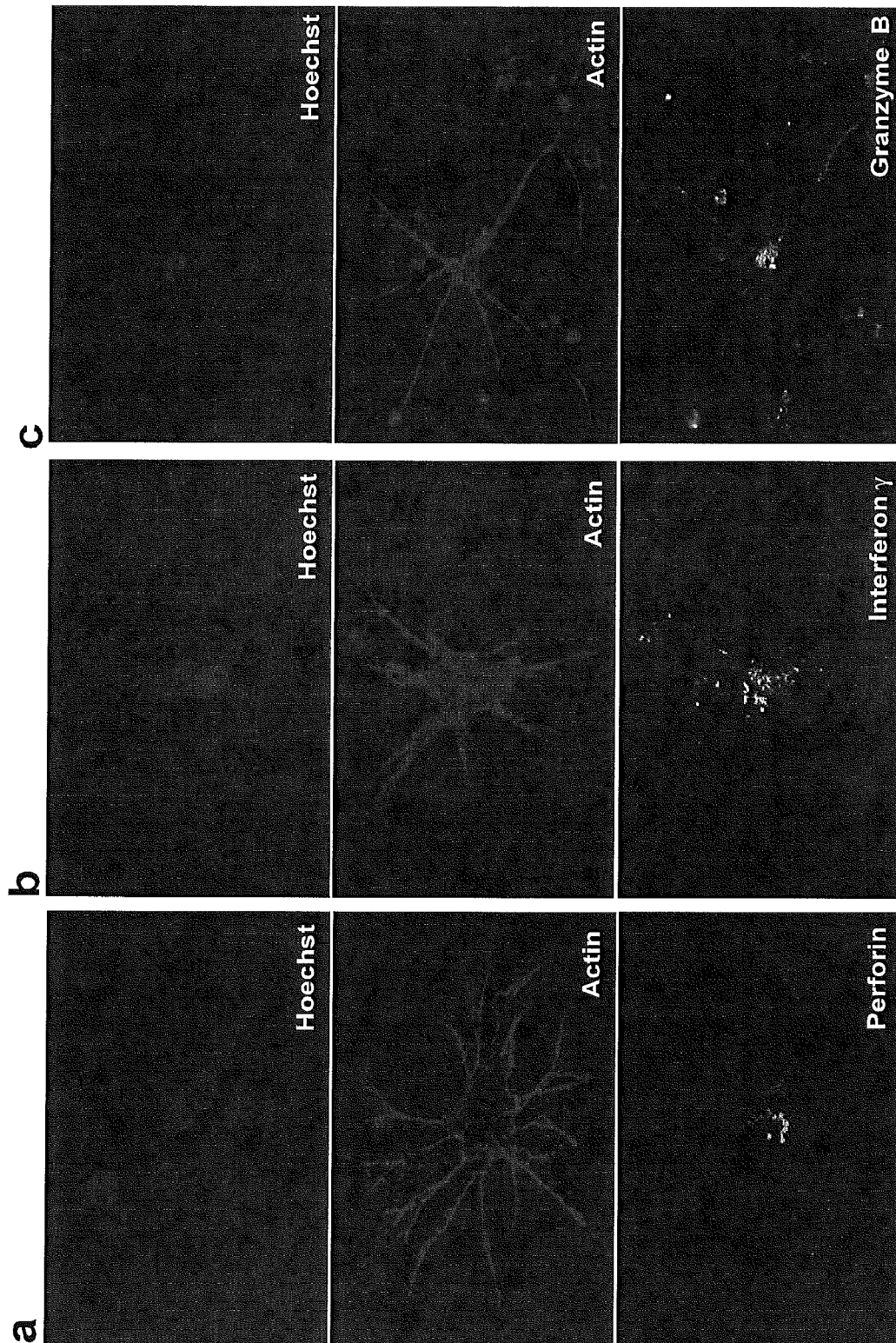


FIG. 7

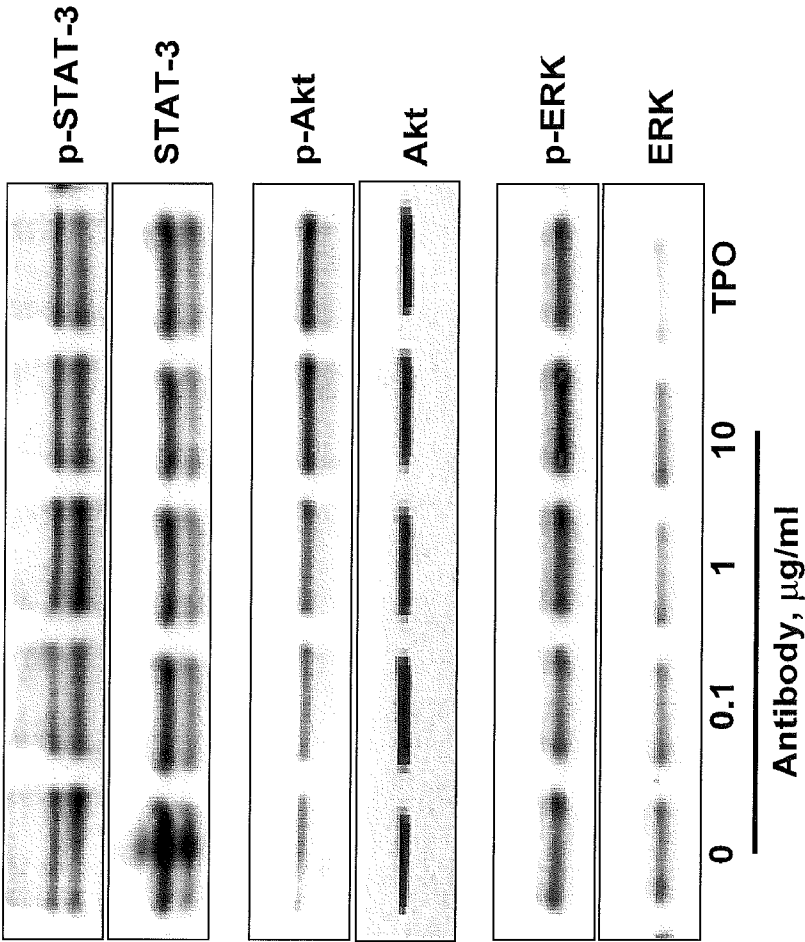


FIG. 8

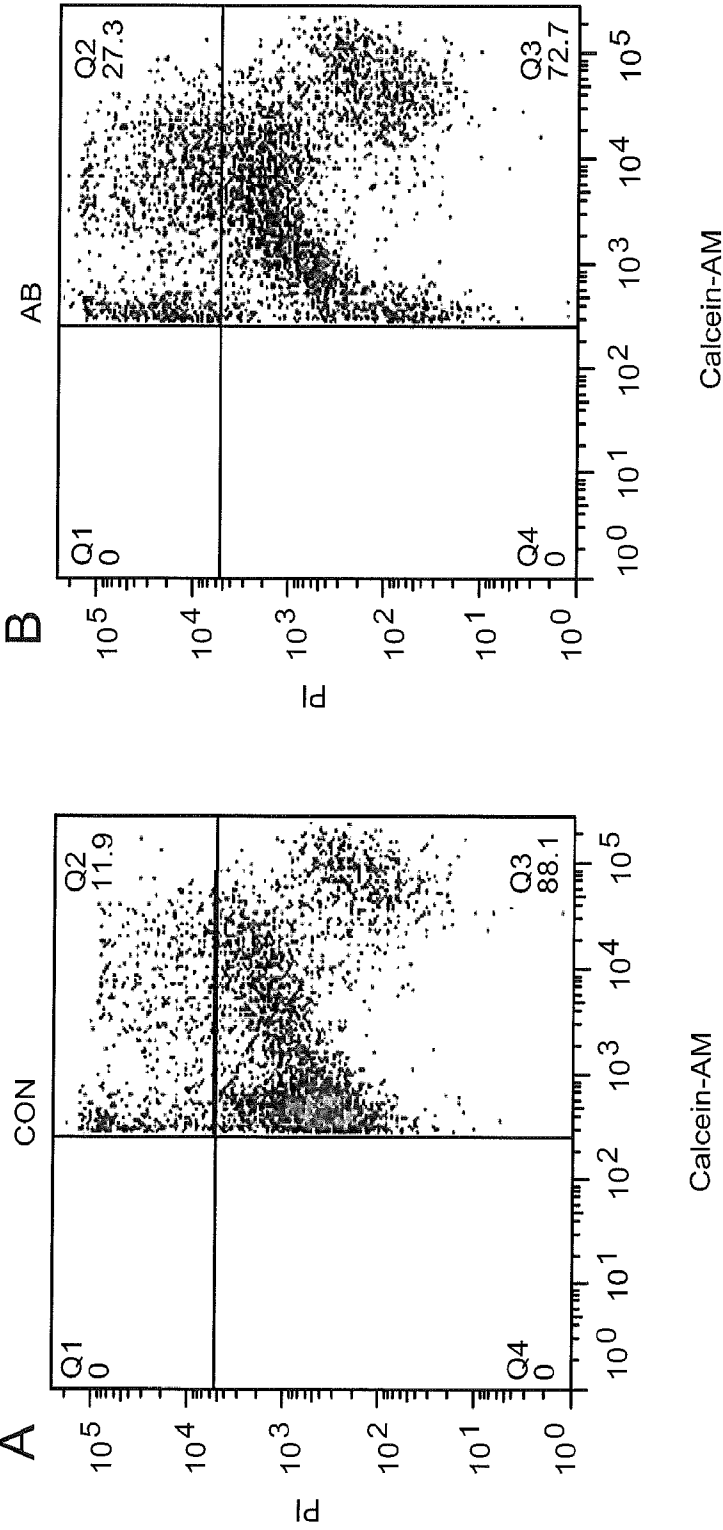


FIG. 9

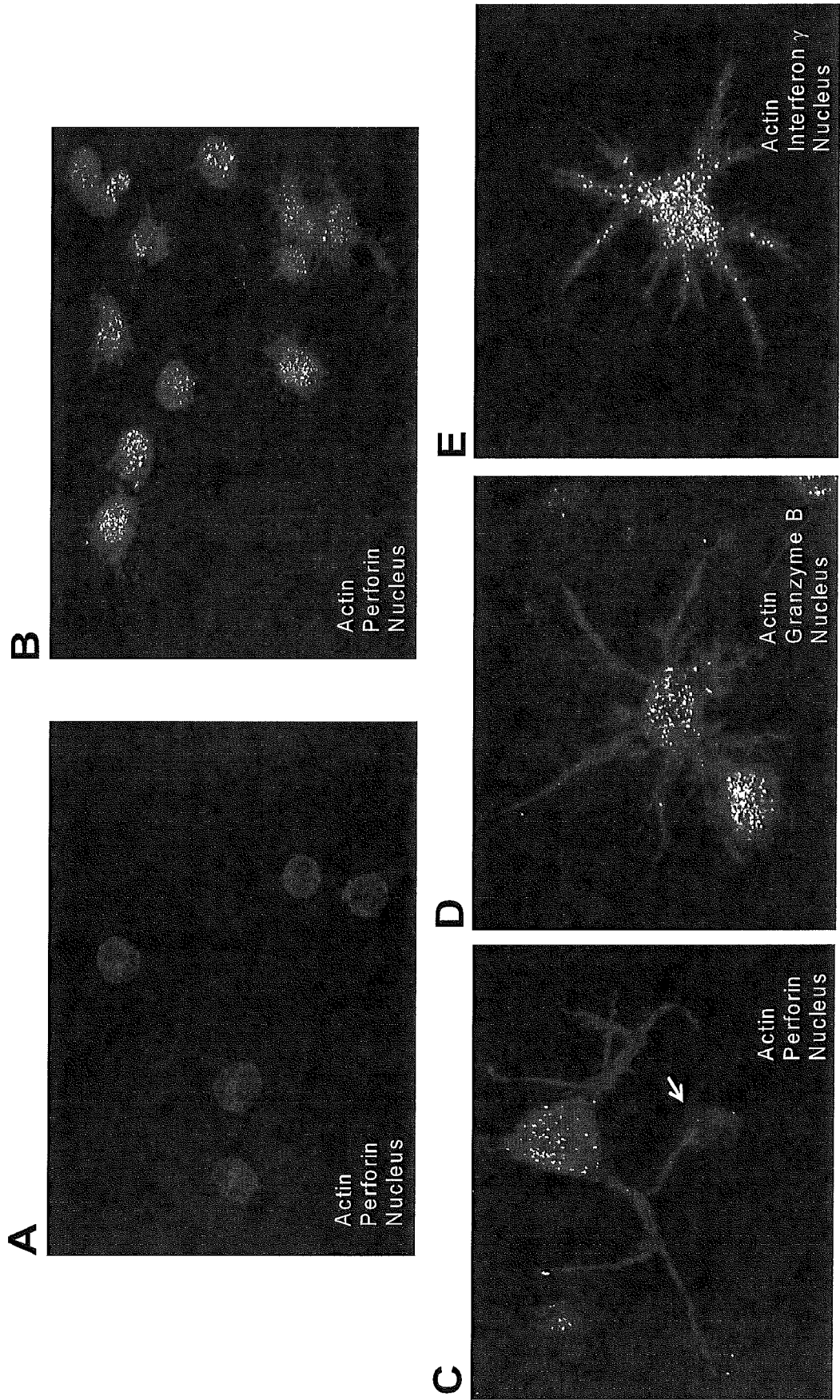


FIG. 10

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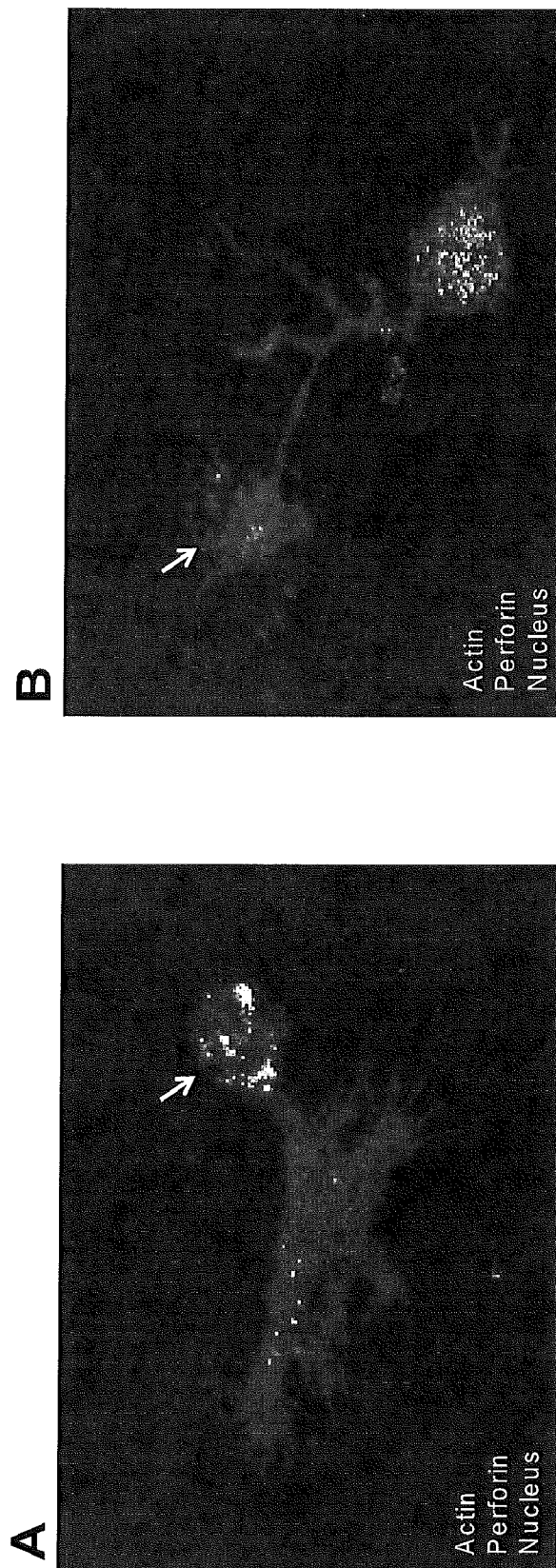


FIG. 11

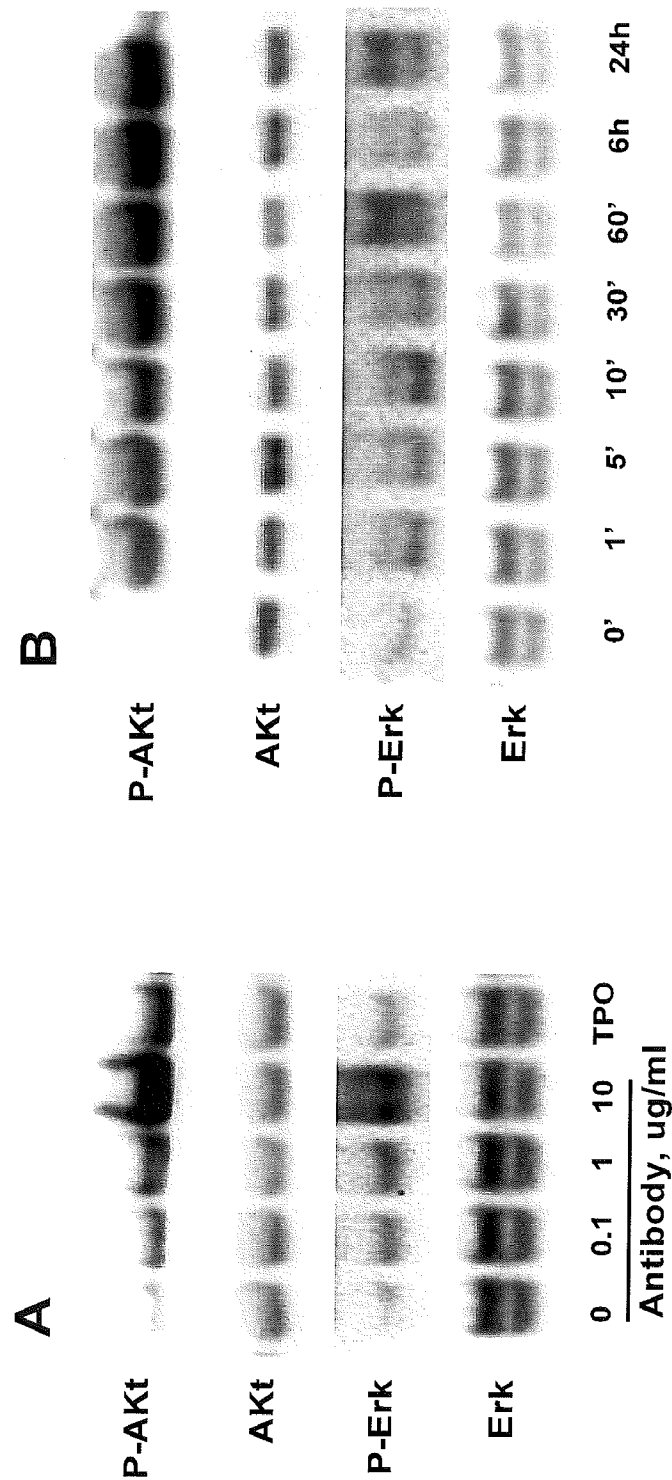


FIG. 12

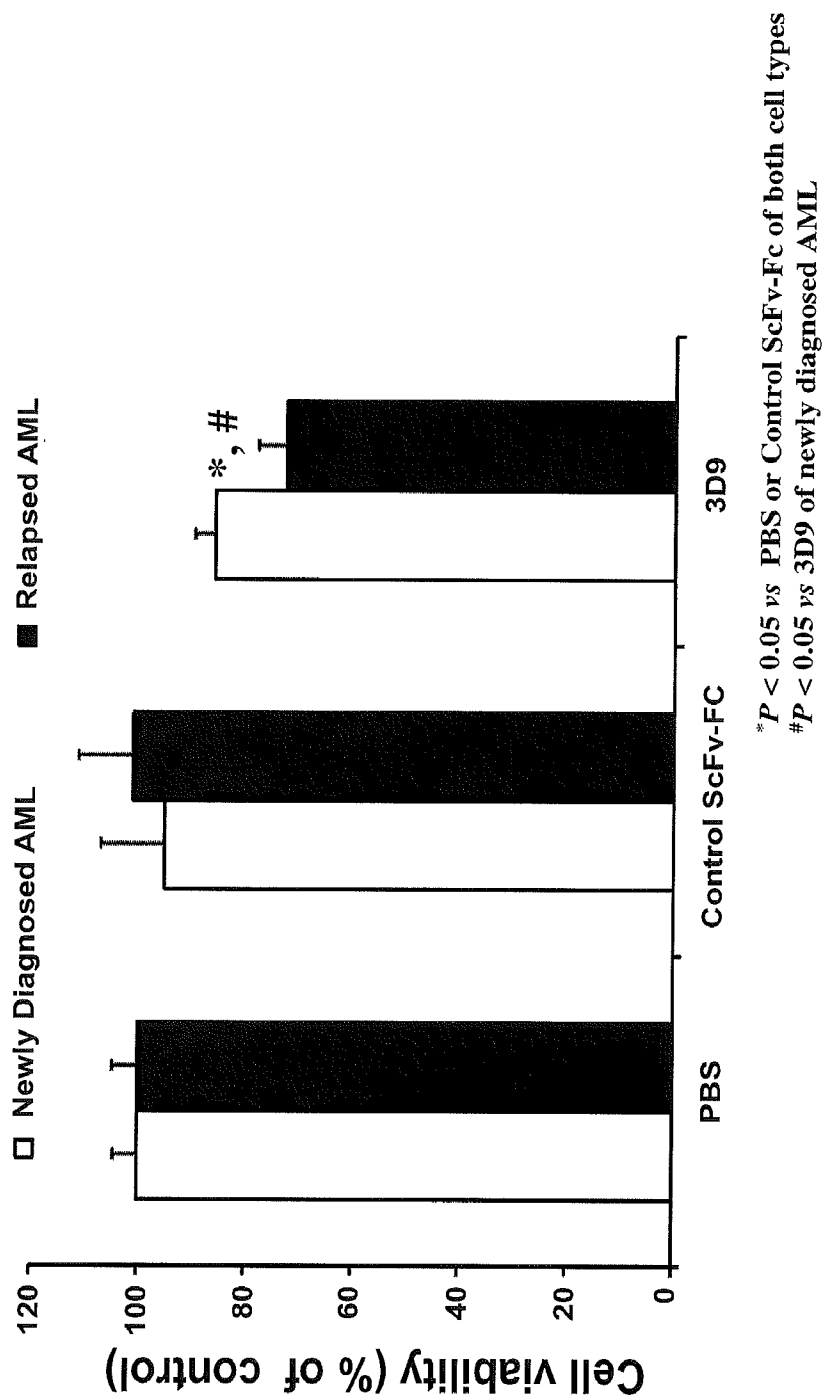


FIG. 13

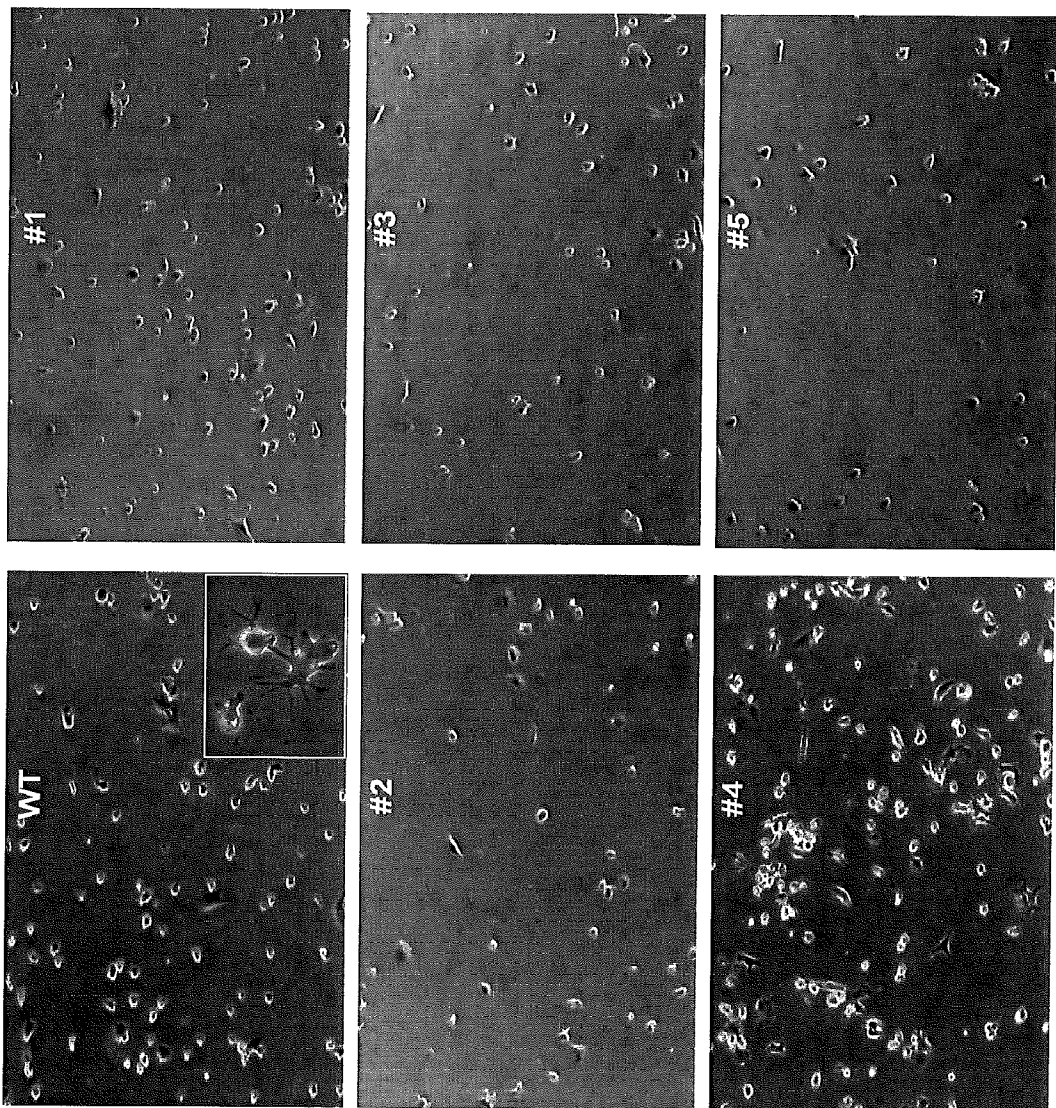


FIG. 14

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/38338

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A61K 39/395, C40B 30/06 (2016.01) CPC - C07K 14/524, C07K 14/715, C07K 2317/56, C07K 2317/52, C07K 14/71, C07K 14/7153, C07K 16/2839 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC(8) - A61K 39/395, C40B 30/06 (2016.01) CPC - C07K 14/524, C07K 14/715, C07K 2317/56, C07K 2317/52, C07K 14/71, C07K 14/7153, C07K 16/2839 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched CPC - C07K 16/2863, C07K 16/2866, C07K 2317/21, C07K 2317/31, C07K 2317/35, C07K 2317/622, C07K 2317/74, C07K 2317/75, C07K 2317/92, C07K 2319/30, A61K 2039/505, Y10S 424/801, Y10S 424/809, G01N 33/6845, G01N 33/6854, G01N 33/746 Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PubWEST(PGPB,USPT,USOC,EPAB,JPAB); PatBase, Google/Scholar: Thrombopoietin receptor, TpoR, induced AML cells, activated NK, acute myeloid leukemia, AML, natural killer cells, 3D9, CR2-9 Cells, receptor pleiotropism, pleiotropy, fratricidin, romiplostim, Eltrombopag, SB-497115; GenCore 6.4.1: SEQ ID NO: 4-8, 12, 14-25		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y -- A	US 2010/0040600 A1 (Yoshikubo, et al.) 18 February 2010 (18.02.2010) Abstract, claim 12; para [0029], [0074], [0145]	17-21 ----- 1-16, 22-27
Y -- A	WO 2014/035693 A2 (The Scripps Research Institute) 06 March 2014 (06.03.2014) pg 77, 78, SEQ ID NO:32; para [00104]; claim 45, SEQ ID NO:37	17-21 ----- 1-16, 22-32
Y	~ Seo, et al. Gene expression profiling of cancer stem cell in human lung adenocarcinoma A549 cells. Mol Cancer. 2007, 6:75; pg 2 [according to the posted document], col 1	18, 20
A	US 2010/0298398 A1 (Gewirtz, et al.) 25 November 2010 (25.11.2010) Abstract, claim 1	1-16, 22-27
A	~ UniProtKB/TrEMBL Submission I4ER67_9ACTN (2012) [Retrieved from the Internet 17 September 2016: <http://www.uniprot.org/uniprot/I4ER67.txt?version=1>]; amino acids 21-29, 92.5% identity to SEQ ID NO:22	28-32
A	US 2010/0081796 A1 (Brinkmann, et al.) 01 April 2010 (01.04.2010) SEQ ID NO 14, amino acids 2-10, 86.9% identity to SEQ ID NO:24	28-32
☐ Further documents are listed in the continuation of Box C. ☐		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 09 September 2016 (09.09.2016)		Date of mailing of the international search report 20 OCT 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300		Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/38338

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

- a. ☐ forming part of the international application as filed:
☐ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13*ter*. 1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*. 1(a)).
☒ on paper or in the form of an image file (Rule 13*ter*. 1(b) and Administrative Instructions, Section 713).
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: