(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 August 2013 (08.08.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/116505 A1

(51) International Patent Classification:
C12N 5/071 (2010.01) *A61K 35/12* (2006.01)
C12N 5/09 (2010.01) *A61P 35/00* (2006.01)

(21) International Application Number:
PCT/US2013/024123

(22) International Filing Date:
31 January 2013 (31.01.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/594,304 2 February 2012 (02.02.2012) US

(71) Applicant: **CALIFORNIA STEM CELL, INC.** [US/US]; 10803 Von Karman Avenue, Suite 103, Irvine, California 92612 (US).

(72) Inventors; and
(71) Applicants *(for US only)*: **CORNFORTH, Andrew** [US/US]; 18301 Von Karman Avenue, Suite 130, Irvine, California 92612 (US). **DILLMAN, Robert** [US/US]; 10803 Von Karman Avenue, Suite 103, Irvine, California 92612 (US).

(74) Agent: **KRIETZMAN, Mark**; Washington Square, Suite 1100, 1050 Connecticut Avenue NW, Suite 1100, Washington, District of Columbia 20036 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(54) Title: PLURIPOTENT GERM LAYER ORIGIN ANTIGEN PRESENTING CANCER VACCINE

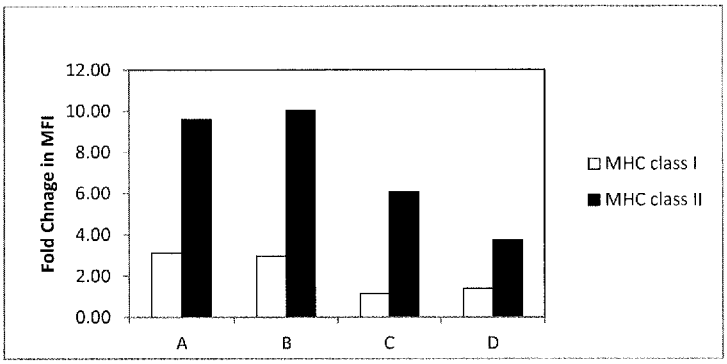


Figure 15

(57) Abstract: The disclosure provides reagents, methods, and kits, for treating or preventing cancers derived from each of the germ layers (endoderm, mesoderm, ectoderm, neural crest). The reagent encompasses interferon-gamma (IFN-gamma) responsive cancer cells, where the cells are autophagic and non apoptotic cancer cells, and where the cells express MHC Class II.

WO 2013/116505 A1

PLURIPOTENT GERM LAYER ORIGIN ANTIGEN PRESENTING CANCER VACCINE

Related Applications

[0001] This application claims the full Paris Convention priority to and benefit of U.S. Provisional Application no. 61/594,304, filed on February 2, 2012, the contents of which is incorporated by this reference, as if fully set forth in its entirety herein.

Field

[0002] The present disclosure relates to treating cancers derived from each of the embryonic germ layers, screening subjects suitable for treatment, compositions of matter, methods and kits.

Background

[0003] Cancer is distinguished by the lack of effective immune response against the cancer. Lack of immune response can result, for example, from the fact that many tumor antigens are "self-antigens," from lack of expression of MHC by the tumor cells and consequent lack of presentation of tumor antigens by the tumor cells, from the association of macrophages with tumors where the macrophages express cytokines that reduce immune response, and from the immunosuppressive activity of T regulatory cells (Tregs). Lack of immune response against tumors also results from the fact that tumor cells tend not to express molecules that stimulate innate immune response, that is, molecules that stimulate toll-like receptors (TLRs) or nucleotide-binding oligomerization domain (NOD)-like receptors). Cancer encompasses solid tumors as well as the hematological cancers, such as the leukemias and the myelodysplastic syndromes.

[0004] The embryonic germ layers are endoderm, mesoderm, ectoderm, and neural crest. Neural crest is derived from ectoderm, but is sometimes considered to be a fourth germ layer. Neoplastic cells can be identified as being derived from one of these germ layers. For example, breast cancer, sarcoma, and colon cancer, arise in tissues derived from embryonic ectoderm, mesoderm, and endoderm, respectively (Singer et al (1997) J. Clin. Endocrinol. Metabol. 82:1917-1922). The present disclosure provides reagents that stimulate immune response against cancers

derived from tissues arising from each of the embryonic germ layers, as well as against tumors of mixed embryological origin.

[0005] The immune system encompasses cellular immunity, humoral immunity, and complement response. Cellular immunity includes a network of cells and events involving dendritic cells, $CD8^+$ T cells (cytotoxic T cells; cytotoxic lymphocytes), and $CD4^+$ T cells (helper T cells). Dendritic cells (DCs) acquire polypeptide antigens, where these antigens can be acquired from outside of the DC, or biosynthesized inside of the DC by an infecting organism. The DC processes the polypeptide, resulting in peptides of about ten amino acids in length, transfers the peptides to either MHC class I or MHC class II to form a complex, and shuttles the complex to the surface of the DC. When a DC bearing a MHC class I/peptide complex contacts a $CD8^+$ T cell, the result is activation and proliferation of the $CD8^+$ T cell. Regarding the role of MHC class II, when a DC bearing a MHC class II/peptide complex contacts a $CD4^+$ T cell, the outcome is activation and proliferation of the $CD4^+$ T cell (Munz, et al. (2010) Curr. Opin. Immunol. 22:89-93; Monaco (1995) J. Leukocyte Biol. 57:543-547; Robinson, et al (2002) Immunology 105:252-262). Although dendritic cells presenting antigen to a T cell can "activate" that T cell, the activated T cell might not be capable of mounting an effective immune response. Effective immune response by the $CD8^+$ T cell often requires prior stimulation of the DC by one or more of a number of interactions. These interactions include direct contact of a $CD4^+$ T cell to the DC (by way of contact the $CD4^+$ T cell's CD40 ligand to the DC's CD40 receptor), or direct contact of a TLR agonist to one of the dendritic cell's toll-like receptors (TLRs).

[0006] Humoral immunity refers to B cells and antibodies. B cells become transformed to plasma cells, and the plasma cells express and secrete antibodies. Naïve B cells are distinguished in that they do not express the marker CD27, while antigen-specific B cells do express CD27 (Perez-Andres, et al. (2010) Cytometry Part B 78B (Suppl. 1) S47-S60). The secreted antibodies can subsequently bind to tumor antigens residing on the surface of tumor cells. The result is that the infected cells or tumor cells become tagged with the antibody. With binding of the antibody to the infected cell or tumor cell, the bound antibody mediates killing of the infected cell or tumor cell, where killing is by NK cells. Although NK cells are not configured to recognize specific target antigens, in the way that T cells are configured to recognize

target antigens, the ability of NK cells to bind to the constant region of antibodies, enables NK cells to specifically kill the cells that are tagged with antibodies. The NK cell's recognition of the antibodies is mediated by Fc receptor (of the NK cell) binding to the Fc portion of the antibody. This type of killing is called, antibody-dependent cell cytotoxicity (ADCC). NK cells can also kill cells independent of the mechanism of ADCC, where this killing requires expression of MHC class I to be lost or deficient in the target cell (see, e.g., Caligiuri (2008) Blood 112:461-469).

[0007] The technique of "delayed type hypersensitivity response" can be used to distinguish between immune responses that mainly involve cellular immunity or mainly involve humoral immunity. A positive signal from the delayed type hypersensitivity response indicates a cellular response (see, e.g., Roychowdhury, et al. (2005) AAPS J. E834-E846).

[0008] Autophagy is a homeostatic process by which cytosolic components and organelles are delivered to the lysosome for degradation. Autophagy is also associated with innate and adaptive immune responses to intracellular pathogens whereby cytosolic antigens are loaded onto major histocompatibility complex (MHC) class II molecules for $CD4^+$ T-cell recognition.

Description

[0009] As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the" include their corresponding plural references unless the context clearly dictates otherwise. All references cited herein are incorporated by reference to the same extent as if each individual publication, patent, published patent application, and sequence listing, as well as figures and drawings in said publications and patent documents, was specifically and individually indicated to be incorporated by reference.

DISCLOSURE

[0010] The present disclosure provides a population of mammalian dendritic cells comprising: cancer-specific peptides from cancer cells taken from a subject that has a cancer; wherein the cancer-specific peptides are acquired in vitro by dendritic cells from said cancer cells; wherein the cancer cells are not treated in vitro with interferon-gamma (IFN-gamma) or with IFN-gamma mimetic; wherein the majority of the cancer cells are not treated in vitro with a interferon-gamma (IFN-gamma) or

with IFN-gamma mimetic; wherein less than about 40 percent of the cancer cells are treated in vitro with interferon-gamma (IFN-gamma) or with IFN-gamma mimetic; wherein less than about 30 percent of the cancer cells are treated in vitro with interferon-gamma (IFN-gamma) or with IFN-gamma mimetic; wherein less than about 20 percent of the cancer cells are treated in vitro with interferon-gamma (IFN-gamma) or with IFN-gamma mimetic; wherein less than about 10 percent of the cancer cells are treated in vitro with interferon-gamma (IFN-gamma) or with IFN-gamma mimetic; wherein less than about 5 percent of the cancer cells are treated in vitro with interferon-gamma (IFN-gamma) or with IFN-gamma mimetic; wherein less than about 1% percent of the cancer cells are treated in vitro with interferon-gamma (IFN-gamma) or with IFN-gamma mimetic; wherein greater than 60 percent (%) of said cancer cells that are not treated in vitro with IFN-gamma or IFN-gamma mimetic, are autophagic and non-apoptotic, and wherein the dendritic cells and cancer cells are from the same subject; wherein greater than 70 percent (%) of said cancer cells that are not treated in vitro with IFN-gamma or IFN-gamma mimetic, are autophagic and non-apoptotic, and wherein the dendritic cells and cancer cells are from the same subject; wherein greater than 80 percent (%) of said cancer cells that are not treated in vitro with IFN-gamma or IFN-gamma mimetic, are autophagic and non-apoptotic, and wherein the dendritic cells and cancer cells are from the same subject; wherein greater than 90 percent (%) of said cancer cells that are not treated in vitro with IFN-gamma or IFN-gamma mimetic, are autophagic and non-apoptotic, and wherein the dendritic cells and cancer cells are from the same subject.

[0011] In some exemplars wherein IFN-gamma is required to not be added the disclosure refers to significant or substantial amounts. The requirement, stated in a different way, is that at least 60% of the cancer cells are not treated (or exposed to) IFN-gamma. In some instances, at least 70% of the cancer cells are not treated (or exposed to) IFN-gamma. In some instances, at least 80% of the cancer cells are not treated (or exposed to) IFN-gamma. In some instances, at least 90% of the cancer cells are not treated (or exposed to) IFN-gamma. In some instances, at least 95 % of the cancer cells are not treated (or exposed to) IFN-gamma. In some instances, at least 98% of the cancer cells are not treated (or exposed to) IFN-gamma. In some instances, at least 99% of the cancer cells are not treated (or exposed to) IFN-

gamma. In some instances, at least 100% of the cancer cells are not treated (or exposed to) IFN-gamma. In the above context, "treated" and "exposed" refer to exogenously added IFN-gamma or IFN-gamma mimetic. Thus, where an instruction or protocol mandates that at least 60% of the cancer cells are not treated, a user who follows that protocol and who treats only 30% of the cells in IFN-gamma, will have complied successfully with the requirements of the protocol. However, where the user who follows the protocol makes a mistake and treats 62% of the cells with IFN-gamma, the user will have failed to meet the requirements of the protocol. The present disclosure does not set forth any particular concentration of the added IFN-gamma or IFN-gamma mimetic or the amount of cancer cells treated or not treated. The present disclosure does not set forth any particular time frame of exposure, where the added IFN-gamma or IFN-gamma mimetic contact cancer cells. The present disclosure does not set forth any requirement for efficacy of the added IFN-gamma or IFN-gamma mimetic.

[0012] The present disclosure provides a population of mammalian dendritic cells comprising: cancer-specific peptides from cancer cells taken from a subject that has a cancer; wherein the cancer-specific peptides are acquired in vitro by dendritic cells from said cancer cells; wherein the cancer cells are not treated in vitro with interferon-gamma (IFN-gamma) or with IFN-gamma mimetic; wherein greater than 60 percent (%), or greater than 70%, or greater than 80%, or greater than 90%, or greater than 95%, or greater than 98%, or greater than 99%, of said cancer cells that are not treated in vitro with IFN-gamma or IFN-gamma mimetic, are autophagic and non-apoptotic, and wherein the dendritic cells and cancer cells are from the same subject.

[0013] In those instances where IFN-gamma is required to be added such as a population of dendritic cells from a subject comprising: cancer-specific peptides from cancer cells taken from a subject that has a cancer, wherein said cancer-specific peptides are acquired in vitro by dendritic cells from said cancer cells that are treated in vitro with IFN-gamma or IFN-gamma mimetic, wherein greater than 60 percent (%) of said cancer cells that are treated in vitro with IFN-gamma or IFN-gamma mimetic are autophagic and non-apoptotic, and wherein the dendritic cells and cancer cells are from the same subject. In other embodiments, what can be required is greater than 65%, greater than 70%, greater than 75%, greater than 80%, greater than 90%,

greater than 95%, greater than 98%, greater than 99%, or 100%, of the cancer cells are autophagic and non-apoptotic. While the above exemplary protocol (where IFN-gamma is required to be added) does not expressly set forth a requirement for the percentage of the total cancer cells that are treated with IFN-gamma or IFN-gamma mimetic, does not set forth any requirement for concentration of IFN-gamma or IFN-gamma mimetic, does not set forth any requirement that added IFN-gamma or IFN-gamma mimetic have any particular level of efficacy on the cancer cells. However, in alternate exemplary embodiments (where IFN-gamma is required to be added), what can be required is that at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or 100%, of the cancer cells be exposed to (or be contacted with) the exogenously added IFN-gamma or IFN-gamma mimetic. The "population" can be defined in terms of residence in a flask, beaker, bioreactor, well of a titer plate, all wells of a given titer plate, bottle, lot number, particular tumor biopsy where the cancer cells originated, date and time at which a particular batch of cancer cells was first subjected to maintenance culture or to growth culture, and the like. In exclusionary embodiments, the present disclosure can exclude cells, cancer cells, dendritic cells, or dendritic cells treated with tumor antigen, that are not from the desired flask, beaker, bioreactor, specific tumor biopsy, maintenance culture batch, growth culture batch, and the like.

[0014] Also provided is the above population of dendritic cells, wherein the cancer is from a tissue of one or more of endodermal origin, mesodermal origin, or ectodermal origin. Also provided is the above population of dendritic cells, wherein the cancer is from a tissue of neural crest origin, and wherein the neural crest is of ectodermal origin. Also provided is the above population of dendritic cells, wherein the cancer is from a tissue of one or more of endodermal origin, mesodermal origin, or ectodermal origin, and where the cancer is melanoma of neural crest origin, colon cancer of endoderm origin, renal cancer of mesoderm origin, glioblastoma of ectoderm origin, or ovarian cancer of mixed mesoderm plus extra-embryonic origin.

[0015] Also provided is the above population of dendritic cells, wherein greater than 80% of said cancer cells that are not treated in vitro with IFN-gamma or IFN-gamma mimetic, are autophagic and non-apoptotic. Also provided is a vaccine for the above subject, comprising the above population of dendritic cells. Also provided

is the above population of dendritic cells of, wherein essentially all of the cancer cells that are not treated with IFN-gamma or IFN-gamma mimetic are incapable of cell division. Also provided is the above population of dendritic cells, wherein essentially all of the cancer cells that are not treated with IFN-gamma or IFN-gamma mimetic are irradiated and incapable of cell division. Also provided is the above population of dendritic cells, wherein at least 80% of the cancer cells that are not treated with IFN-gamma or IFN-gamma mimetic are irradiated and incapable of cell division. Also provided is the above population of dendritic cells of, wherein at least 80% of the cancer cells that are not treated with IFN-gamma or IFN-gamma mimetic are treated with a nucleic acid cross-linker and are incapable of cell division.

[0016] Also provided is the above population of dendritic cells of claim 1 that comprise one or more peptides derived from a melanoma-specific antigen that is S-100, HMB-45, Mel-2, Melan-A, Mel-5, MAGE-1, MART-1, or Tyrosinase, and wherein the cancer is melanoma. Also provided is the above population of dendritic cells, wherein the given subject is a human subject. Also provided is the above population of dendritic cells, wherein the given subject is a mammal that is not human. In a vaccine embodiment, what is provided is a cancer vaccine comprising: at least one mature dendritic cell from a subject that has cancer; wherein the at least one mature dendritic cell had been contacted with at least one cancer tumor cell from the same subject, wherein the at least cancer tumor cell that is contacted with the at least one mature dendritic cell is non-dividing, autophagic, and non-apoptotic. Also provided is a method for stimulating immune response against a cancer-specific antigen, comprising administering an immune-stimulatory amount of the above population of dendritic cells to the subject.

[0017] Also provided is the above method, wherein the immune response that is stimulated comprises one or more of $CD4^+$ T cell response, $CD8^+$ T cell response, and B cell response. Also provided is the above method, wherein the $CD4^+$ T cell response, $CD8^+$ T cell response, or B cell response, can be measured by ELISPOT assays, by intracellular cytokine staining assays, by tetramer assays, or by detecting antigen-specific antibody production. Also provided is the above method, wherein the immune response comprises a survival time that comprises 2-year overall survival (OS), and where the 2-year overall survival is at least 60%. Also provided is the above method, wherein the administration comprises subcutaneous injections of

the vaccine. Also provided is the above method, wherein the administration comprises injections of the vaccine given weekly for three months and then monthly for five months. Also provided is the a method for preparing a dendritic cell vaccine, involving cancer cells and dendritic cells from the same subject, the method comprising: one or more cancer cells is treated with an agent that prevents cell division; the one or more cancer cells are not treated in vitro with interferon-gamma (IFN-gamma) or with an IFN-gamma mimetic; cancer cells that are autophagic and non-apoptotic are selected; cancer cells that are non-autophagic and apoptotic are rejected; and, wherein the cancer cells that are autophagic and non-apoptotic are provided to one or more autologous dendritic cells, or, wherein peptides derived from the cancer cells that are autophagic and non-apoptotic are provided to one or more autologous dendritic cells.

[0018] The following provides a composition embodiment. What is provided is a composition comprising: at least one cancer cell that is not treated with interferon-gamma (IFN-gamma) from a first subject, and at least one antigen presenting cell (APC) from the same first subject, wherein the cancer cell is: autophagic; and non-apoptotic. Also provided is the above composition, wherein the cancer cell is MHC class II-expressing. Also provided is the above composition, wherein the APC is a dendritic cell, a macrophage, or a B cell. Also provided is the above composition, wherein the at least one cancer cell comprises cancer-specific peptides, and wherein the cancer specific-peptides are substantially not contained in said APCs and are substantially not processed by said APCs. Also provided is the above composition, wherein the cancer cells comprise cancer-specific peptides, and wherein the cancer specific-peptides are substantially contained in said APCs and are partially or substantially processed in said APCs.

[0019] Also provided is the above composition, wherein the cancer cell is loaded into the APC. Also provided is the above composition, wherein the cancer cell is not loaded into the APC. Also provided is the above composition, wherein autophagy is demonstrated by a test that assays microtubule-associated protein light chain 3 (LC3). Also provided is the above composition, wherein the cells are demonstrated to be non-apoptotic using at least one of the reagent, 7-aminoactinomycin D (7-ADD), or the reagent, annexin. In a methods embodiment, what is provided is a method of stimulating immune response in a subject having a cancer and

comprising cancer cells, wherein the subject is the same subject as the first subject, comprising administering an immunology effective amount of the above composition. Also provided is the above composition, wherein at least 90% of the cancer cells are not treated in vitro with IFN-gamma, and less than 10% of the cancer cells are treated in vitro with IFN-gamma.

[0020] The following provides a method of manufacturing embodiment. What is provided is a method for manufacturing the above vaccine or the above composition, comprising contacting at least one cancer tumor cell to at least one antigen presenting cell (APC), wherein the at least one cancer tumor cell is from a first human subject, and wherein the at least one APC is from the same first human subject. What is also provided, is a method for preparing a dendritic cell vaccine, comprising: treating cancer cells acquired from a first subject with an agent that prevents cell division; wherein the cancer cells are not treated in vitro with IFN-gamma or an IFN-gamma mimetic; selecting cancer cells that are autophagic and non-apoptotic; and, contacting the selected cancer cells with autologous dendritic cells from the same first subject. What is also provided, is a composition that comprises a dendritic cell vaccine, as prepared by the above method. Also, what is provided is a method for stimulating immune response against a cancer-specific antigen, comprising administering the above composition to a subject that has cancer.

[0021] Disclosed is a population of mammalian dendritic cells comprising melanoma-specific peptides from a given subject that has melanoma and comprises melanoma cells; wherein said melanoma-specific peptides are acquired in vitro by dendritic cells from said melanoma cells that are not treated in vitro with IFN-gamma or IFN-gamma mimetic, wherein greater than 60 percent (%) of said melanoma cells that are not treated in vitro with IFN-gamma or IFN-gamma mimetic are autophagic and non-apoptotic, and wherein the dendritic cells and melanoma cells are from the same subject.

[0022] Also disclosed is the above population of mammalian dendritic cells, wherein: greater than 80% of said melanoma cells are autophagic and non-apoptotic.

[0023] What is disclosed are the above dendritic cells, wherein essentially all of the melanoma cells that are not treated with IFN-gamma or IFN-gamma mimetic are incapable of cell division; as well as the above dendritic cells wherein essentially all of the melanoma cells that are not treated with IFN-gamma or IFN-gamma mimetic are irradiated and incapable of cell division; as well as the above dendritic cells, wherein at least 80% of the melanoma cells that are not treated with IFN-gamma or IFN-gamma mimetic are irradiated and incapable of cell division; as well as the above dendritic cells wherein at least 80% of the melanoma cells that are not treated with IFN-gamma or IFN-gamma mimetic are treated with a nucleic acid cross-linker and are incapable of cell division.

[0024] Also disclosed is a vaccine comprising the above population of the above mammalian dendritic cells.

[0025] Also disclosed, are the above dendritic cells, wherein essentially all of the melanoma-specific peptides are from melanoma cells that are incapable of cell division

[0026] Furthermore, what is disclosed are the above dendritic cells, wherein essentially all of the melanoma-specific peptides are from melanoma cells that are incapable of cell division because the melanoma cells are irradiated.

[0027] Disclosed are the above dendritic cells, wherein essentially all of the melanoma-specific peptides are from melanoma cells that are incapable of cell division because the chromosomes of the melanoma cells are cross-linked by a nucleic acid cross-linking agent.

[0028] Also disclosed are the above dendritic cells, comprising melanoma-specific peptides that are from melanoma cells that are treated with radiation.

[0029] Disclosed are the above dendritic cells, comprising melanoma-specific peptides, wherein all of said peptides are from melanoma cells that are treated with radiation.

[0030] Also disclosed are the above dendritic cells, that comprise one or more peptides derived from a melanoma-specific antigen that S-100, HMB-45, Mel-2, Melan-A, Mel-5, MAGE-1, MART-1, or Tyrosinase.

[0031] What is disclosed, are the above dendritic cells, wherein essentially all of the melanoma-specific peptides are from melanoma cells that are treated in vitro to be incapable of cell division.

[0032] Also disclosed, are the above dendritic cells, wherein the given subject is a human subject.

[0033] Disclosed are the above dendritic cells, wherein the given subject is a mammal that is not human.

[0034] Disclosed is a melanoma vaccine comprising at least one mature dendritic cell from a subject that has melanoma, wherein the at least one mature dendritic cell had been contacted with at least one melanoma tumor cell from the same subject, wherein the at least melanoma tumor cell that is contacted with the at least one mature dendritic cell is non-dividing, autophagic, and non-apoptotic.

[0035] Also disclosed is a method for stimulating immune response against a melanoma-specific antigen, comprising administering an immune-stimulatory amount of the dendritic cells of claim 1 to a subject.

[0036] What is disclosed is wherein the subject has melanoma and does comprise melanoma cells.

[0037] What is disclosed is the above method, wherein the immune response that is stimulated comprises one or more of $CD4^+$ T cell response, $CD8^+$ T cell response, and B cell response.

[0038] What is disclosed is the above method, wherein the $CD4^+$ T cell response, $CD8^+$ T cell response, or B cell response, can be measured by ELISPOT assays, by intracellular cytokine staining assays, by tetramer assays, or by detecting antigen-specific antibody production.

[0039] Also disclosed is the above method, wherein the immune response comprises a survival time that comprises 2-year overall survival (OS), and where the 2-year overall survival is at least 60%.

[0040] What is disclosed is the above method, wherein the administration comprises subcutaneous injections of the vaccine.

[0041] What is disclosed is the above method, wherein the administration comprises injections of the vaccine given weekly for three months and then monthly for five months.

[0042] Also disclosed is the above method for preparing a dendritic cell vaccine, involving melanoma cells and dendritic cells from the same subject, the method comprising: one or more melanoma cells is treated with an agent that prevents cell division; the one or more melanoma cells are not treated in vitro with interferon-gamma (IFN-gamma) or with an IFN-gamma mimetic; melanoma cells that are autophagic and non-apoptotic are selected; melanoma cells that are non-autophagic and apoptotic are rejected; and, wherein the melanoma cells that are autophagic and non-apoptotic are provided to one or more autologous dendritic cells, or, wherein peptides derived from the melanoma cells that are autophagic and non-apoptotic are provided to one or more autologous dendritic cells.

[0043] What is disclosed is a composition comprising: at least one melanoma cell that is not treated with interferon-gamma (IFN-gamma) from a first subject, and at least one antigen presenting cell (APC) from the same first subject, wherein the melanoma cell is: autophagic; and non-apoptotic.

[0044] Also, what is disclosed is the above composition, wherein the melanoma cell is MHC class II-expressing.

[0045] Also disclosed is the above composition, wherein the APC is a dendritic cell, a macrophage, or a B cell.

[0046] Disclosed is the above composition, wherein the at least one melanoma cell comprises melanoma-specific peptides, and wherein the melanoma specific-peptides are substantially not contained in said APCs and are substantially not processed by said APCs.

[0047] Also disclosed is the above composition, where the melanoma cells comprise melanoma-specific peptides, and wherein the melanoma specific-peptides are substantially contained in said APCs and are partially or substantially processed in said APCs. Also disclosed is the above composition, wherein the melanoma cell is loaded into the APC. What is disclosed is the above composition wherein the melanoma cells are not loaded into the APC.

[0048] Disclosed is the above composition, wherein autophagy is demonstrated by a test that assays microtubule-associated protein light chain 3 (LC3).

[0049] Disclosed is the above composition, wherein the cells are demonstrated to be non-apoptotic using at least one of the reagent, 7-aminoactinomycin D (7-ADD), or the reagent, annexin.

[0050] What is disclosed is a method of stimulating immune response in a subject having melanoma and comprising melanoma cells, wherein the subject is the same subject as the first subject, comprising administering an immunology effective amount of the above composition.

[0051] What is disclosed is the above composition, wherein at least 90% of the melanoma cells are not treated in vitro with IFN-gamma, and less than 10% of the melanoma cells are treated in vitro with IFN-gamma.

[0052] What is disclosed is a method for manufacturing the above vaccine or the above composition, comprising contacting at least one melanoma tumor cell to at least one antigen presenting cell (APC), wherein the at least one melanoma tumor cell is from a first human subject, and wherein the at least one APC is from the same first human subject.

[0053] What is disclosed is a method for preparing a dendritic cell vaccine, comprising: treating melanoma cells acquired from a first subject with an agent that prevents cell division; wherein the melanoma cells are not treated in vitro with IFN-gamma or an IFN-gamma mimetic; selecting melanoma cells that are autophagic and non-apoptotic; and, contacting the selected melanoma cells with autologous dendritic cells from the same first subject.

[0054] What is disclosed is a composition that comprises a dendritic cell vaccine, as prepared by the above method.

[0055] Disclosed is a method for stimulating immune response against a melanoma-specific antigen, comprising administering the above composition to a subject that has melanoma.

[0056] Disclosed is a composition comprising at least one melanoma cell from a first subject, and at least one antigen presenting cell (APCs) from the same first subject, wherein the melanoma cell is: autophagic; non-apoptotic; and MHC class II-

expressing. In the present disclosure an IFN-gamma-treated melanoma cell is not loaded into the APC, and wherein an IFN-gamma treated melanoma cell is not loaded into the APC. In another aspect, what is embraced is the above composition wherein the melanoma cell is from a subject with Stage I, Stage II, Stage III, or Stage IV melanoma. Additionally, what is contemplated is the above composition, related kits, and related methods, wherein the APC comprises at least one dendritic cell.

[0057] In one aspect, the pharmaceutical composition, reagent, and related methods, of the present disclosure uses a preparation of cancer cells that, is 7-AAD negative and annexin V negative. This population can be, e.g., about 99% 7-AAD negative and about 99% annexin V negative, or at least 95% 7-AAD negative and at least 95% annexin V negative, or at least 90% 7-AAD negative and at least 90% annexin V negative, to provide non-limiting examples.

[0058] Furthermore, what is embraced is the above composition, wherein autophagy is demonstrated by a test that assays microtubule-associated protein light chain 3 (LC3); and the above composition, wherein the cells are demonstrated to be non-apoptotic using at least one of the reagent, 7-aminoactinomycin D (7-AAD), or the reagent, annexin.

[0059] In methods aspects, what is provided a method of manufacturing the above-disclosed composition, comprising removing at least one melanoma cell from the first subject, removing at least one APC from the first subject, and allowing the melanoma cell to contact the APC; as well as a method for stimulating immune response against a melanoma in a subject or patient, comprising administering the above composition of to a subject.

[0060] In a kit aspect, the present disclosure provides a kit for testing immune response against a tumor antigen in a subject, wherein the subject is treated by one or more of the above methods, and wherein the kit comprises a reagent that detects humoral immune response, cellular immune response, or innate immune response.

FIGURES

[0061] Figure 1. Graphic showing method for separating floating cells from adherent cells.

[0062] Figure 2. Histograms and marker expression.

[0063] Figure 3. Flow cytometry showing MHC class II versus marker expression.

[0064] Figure 4. Flow cytometry and marker expression.

[0065] Figure 5. Phase-contrast photomicrographs of cells.

[0066] Figure 6. Flow cytometry.

[0067] Figure 7. Histograms.

[0068] Figure 8. Flow cytometry showing MHC class II versus annexin-V.

[0069] Figure 9. Histograms.

[0070] Figure 10. Plot of MHC class Ii expression versus percent annexin-V positive.

[0071] Figure 11. Survival plot.

[0072] Figure 12. Survival plot.

[0073] Figure 13. Survival plot.

[0074] Figure 14. Flow cytometry showing expression of MHC.

[0075] Figure 15. Expression of MHC class I and of MHC class II.

[0076] Figure 16. Flow cytometry showing 7-AAD versus annexin V.

[0077] Figure 17. Flow cytometry showing MHC expression versus annexin V.

[0078] Figure 18. Expression of MHC class II.

[0079] Figure 19. Flow cytometry showing MHC expression versus annexin V.

Definitions

[0080] Immune-stimulatory amount, without limitation, can be an amount that increases ELISPOT assay results by a measurable amount, that increases ICS assay results by a measurable amount, that increases tetramer assay results by a measurable amount, that increases the blood population of antigen-specific $CD4^+$ T cells by a measurable amount, that increases the blood population of antigen-specific $CD8^+$ T cells by a measurable amount, or where the increase is by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 1.5-fold, 2.0-fold, 3.0-fold, and the like, when compared to a suitable control. A suitable control can

be a control vaccine, where dendritic cells are not loaded with melanoma cells, or are not loaded with peptide derived from melanoma cells.

[0081] Where a particular sentence repeatedly refers to a "cancer," it will be assumed, unless specified otherwise, that the cancer is the same type of cancer. For example, where it is recited that, "cancer-specific peptides from cancer cells taken from a subject that has a cancer," it means the following. If the subject with the cancer has melanoma, the peptides are melanoma-specific peptides, and that the cancer cells are melanoma cells.

[0082] The term "melanoma-specific antigen," to provide the non-limiting example of melanoma, encompasses antigens that are frequently associated with melanoma, and where the antigen is considered to be unique to melanoma, as opposed to being associated with other cancers, and in addition, the term "melanoma-specific antigens" encompasses antigens that are frequently associated with melanoma, and where the antigen is also associated with other types of cancer, such as breast cancer, colorectal cancer, and the like.

[0083] "Irradiated," in the context of irradiating melanoma cells for the present disclosure, is preferably by gamma-irradiation, but also encompasses irradiation by x-rays, electrons, neutrons, protons, electromagnetic irradiation, visible light, ultraviolet light, and so on. In one aspect, the irradiation functions to prevent cell division of the melanoma cells. In another aspect, the irradiation prevents cell division, but also denatures cellular proteins. As an alternative to irradiation, the present disclosure prevents cell division of melanoma cells by way of physical disruption, e.g., sonication, cavitation, dehydration, ion depletion, or by toxicity from exposure to one or more salts.

[0084] The term "percent," as in "greater than 60% of the melanoma-specific peptides," refers to the number of peptide molecules, and not to the number of different antigenically distinct peptides. The term "percent," as in "greater than 80% of the melanoma-specific peptides," refers to the number of peptide molecules, and not to the number of different antigenically distinct peptides. The term "percent," as in "less than 40% of the melanoma-specific peptides," refers to the number of peptide molecules, and not to the number of antigenically distinct peptides. The term "percent," as in "less than 20% of the melanoma-specific peptides," refers to the

number of peptide molecules, and not to the number of antigenically distinct peptides, and the like.

[0085] The term “peptides,” as in “greater than 60% of the melanoma-specific peptides,” refers to the sum of the number of peptide molecules, oligopeptides molecules, and polypeptide molecules. The term “peptides,” as in “greater than 80% of the melanoma-specific peptides,” refers to the sum of the number of peptide molecules, oligopeptides molecules, and polypeptide molecules. The term, “peptides,” as in “less than 40% of the melanoma-specific peptides,” refers to the sum of the number of peptide molecules, oligopeptides molecules, and polypeptide molecules. The term, “peptides,” as in “less than 20% of the melanoma-specific peptides,” refers to the sum of the number of peptide molecules, oligopeptides molecules, and polypeptide molecules, and the like.

[0086] “Derived from,” in the context of peptides derived from one or more cancer cells, encompasses the following. The cancer cell can be broken, for example, by a homogenizer or by osmotic bursting, resulting in a crude extract. Peptides, oligopeptides, and polypeptides of the crude extract can be exposed to dendritic cells, followed by processing of the peptides by the dendritic cells. Derived from also encompasses providing dendritic cells with intact cancer cells, where the cancer cells are living, or where the cancer cells have been treated with irradiation but are still metabolically active, or where the cancer cells have been treated with a nucleic acid cross-linking agent but are still metabolically active. “Derived from” includes mixtures of cancer cell debris, free cancer cell proteins, and irradiated cancer cells, that are taken up by dendritic cells, and therefore are derived from the cancer cells.

[0087] "Administration" as it applies to a human, mammal, mammalian subject, animal, veterinary subject, placebo subject, research subject, experimental subject, cell, tissue, organ, or biological fluid, refers without limitation to contact of an exogenous ligand, reagent, placebo, small molecule, pharmaceutical agent, therapeutic agent, diagnostic agent, or composition to the subject, cell, tissue, organ, or biological fluid, and the like. "Administration" can refer, e.g., to therapeutic, pharmacokinetic, diagnostic, research, placebo, and experimental methods. Treatment of a cell encompasses contact of a reagent to the cell, as well as contact of a reagent to a fluid, where the fluid is in contact with the cell. "Administration" also

encompasses in vitro and ex vivo treatments, e.g., of a cell, by a reagent, diagnostic, binding composition, or by another cell.

[0088] An "agonist," as it relates to a ligand and receptor, comprises a molecule, combination of molecules, a complex, or a combination of reagents, that stimulates the receptor. For example, an agonist of granulocyte-macrophage colony stimulating factor (GM-CSF) can encompass GM-CSF, a mutein or derivative of GM-CSF, a peptide mimetic of GM-CSF, a small molecule that mimics the biological function of GM-CSF, or an antibody that stimulates GM-CSF receptor. An antagonist, as it relates to a ligand and receptor, comprises a molecule, combination of molecules, or a complex, that inhibits, counteracts, downregulates, and/or desensitizes the receptor. "Antagonist" encompasses any reagent that inhibits a constitutive activity of the receptor. A constitutive activity is one that is manifest in the absence of a ligand/receptor interaction. "Antagonist" also encompasses any reagent that inhibits or prevents a stimulated (or regulated) activity of a receptor. By way of example, an antagonist of GM-CSF receptor includes, without implying any limitation, an antibody that binds to the ligand (GM-CSF) and prevents it from binding to the receptor, or an antibody that binds to the receptor and prevents the ligand from binding to the receptor, or where the antibody locks the receptor in an inactive conformation.

[0089] Unless expressly stated otherwise, or dictated otherwise by the context, the term “expression” encompasses the following. Expression encompasses the biosynthesis of mRNA, polypeptide biosynthesis, polypeptide activation, e.g., by post-translational modification, or an activation of expression by changing the subcellular location or by recruitment to chromatin. In other words, “increased expression” encompasses increased biosynthesis, or increased activity that is caused by phosphorylation, or an increased activity that is caused by migration from the cytosol to the nucleus.

[0090] Antigen presenting cells (APCs) are cells of the immune system used for presenting antigen to T cells. APCs include dendritic cells, monocytes, macrophages, marginal zone Kupffer cells, microglia, Langerhans cells, T cells, and B cells (see, e.g., Rodriguez-Pinto and Moreno (2005) Eur. J. Immunol. 35:1097-1105). Dendritic cells occur in at least two lineages. The first lineage encompasses pre-DC1, myeloid DC1, and mature DC1. The second lineage encompasses $CD34^{++}CD45RA$- early progenitor multipotent cells, $CD34^{++}CD45RA^{+}$ cells,

$CD34^{++}CD45RA^{++}$ $CD4^{+}$ IL-3Ralpha^{++} pro-DC2 cells, $CD4^{+}CD11c^{-}$ plasmacytoid pre-DC2 cells, lymphoid human DC2 plasmacytoid-derived DC2s, and mature DC2s (see, e.g., Gilliet and Liu (2002) J. Exp. Med. 195:695-704; Bauer, et al. (2001) J. Immunol. 166:5000-5007; Arpinati, et al. (2000) Blood 95:2484-2490; Kadowaki, et al. (2001) J. Exp. Med. 194:863-869; Liu (2002) Human Immunology 63:1067-1071; McKenna, et al. (2005) J. Virol. 79:17-27; O'Neill, et al. (2004) Blood 104:2235-2246; Rossi and Young (2005) J. Immunol. 175:1373-1381; Banchereau and Palucka (2005) Nat. Rev. Immunol. 5:296-306).

[0091] "Effective amount" encompasses, without limitation, an amount that can ameliorate, reverse, mitigate, prevent, or diagnose a symptom or sign of a medical condition or disorder. Unless dictated otherwise, explicitly or by context, an "effective amount" is not limited to a minimal amount sufficient to ameliorate a condition. The severity of a disease or disorder, as well as the ability of a treatment to prevent, treat, or mitigate, the disease or disorder can be measured, without implying any limitation, by a biomarker or by a clinical parameter. Biomarkers include blood counts, metabolite levels in serum, urine, or cerebrospinal fluid, tumor cell counts, cancer stem cell counts, tumor levels. Tumor levels can be determined by the RECIST criteria (Eisenhauer, et al. (2009) Eur. J. Cancer. 45:228-247). Expression markers encompass genetic expression of mRNA or gene amplification, expression of an antigen, and expression of a polypeptide. Clinical parameters include progression-free survival (PFS), 6-month PFS, disease-free survival (DFS), time to progression (TTP), time to distant metastasis (TDM), and overall survival, without implying any limitation.

[0092] A composition that is "labeled" is detectable, either directly or indirectly, by spectroscopic, photochemical, biochemical, immunochemical, isotopic, or chemical methods. For example, useful labels include ^{32}P, ^{33}P, ^{35}S, ^{14}C, ^{3}H, ^{125}I, stable isotopes, epitope tags fluorescent dyes, electron-dense reagents, substrates, or enzymes, e.g., as used in enzyme-linked immunoassays, or fluorettes (see, e.g., Rozinov and Nolan (1998) Chem. Biol. 5:713-728).

/ / /

Immune system modulating agents

[0093] The following generally concerns toll-like receptor (TLR) agonists, agents that deplete T regulatory cells (Tregs), agents that can enhance activity of $CD8^+$ T cells or $CD4^+$ T cells, and other agents that modulate the immune system. The present disclosure provides reagents, methods of administration, methods of diagnosis, and methods for stimulating immune response against tumor antigens, that use one or more, as well as combinations thereof, of the following reagents.

[0094] The present disclosure provides immune adjuvants and other immune system modulating agents for use with dendritic cell vaccine. What is provided are toll-like receptor (TLR) agonists, such as the following. Imidazoquinolines, such as imiquimod and resiquimod, are TLR7 or TLR8 agonists. Stimulation of TLR7 of plasmacytoid dendritic cells results in expression of IFN-alpha. Stimulation of TLR7 of myeloid dendritic cells results in expression of interleukin-12, and the consequent Th1-type immune response. CpG oligonucleotides (CpG ODNs) are TLR9 agonists. CpG ODNs occur in three classes, CpG-A, CpG-B, and CpG-C. CpG-A stimulates NK cells, owing to its IFN-alpha producing effect on plasmacytoid DCs. CpG-B induces IFN-alpha, and upregulates co-stimulatory markers on pDCs and B cells. TLR3 agonists include polyriboinosinic-polyribocytidylic acid (poly I:C), which is an analogue of viral double stranded RNA (dsRNA). TLR4 agonists include monophosphoryl lipid A (MPL), which is a derivative of *Salmonella minnesota* lipopolysaccharide, and which is used as part of a vaccine against human papillomavirus. Attenuated bacteria are used in anti-cancer therapy. *Mycobacterium bovis* stimulates TLR2, and TLR4, and TLR9. *Listeria monocytogenes* stimulates various TLRs (US 2007/0207171 of Dubensky et al, which is incorporated herein by reference, in its entirety). See also, Galluzzi et al (2012) OncoImmunology. 1:699-716; Adams (2009) Immunotherapy. 1:949-964.

[0095] Alpha-galactosylceramide (alpha-GalCer) is also provided (Schwaab and Ernstoff (2011) Therapy. 4:369-377). Alpha-GalCer provokes the activation of NKT cells that express certain T cell receptors (Lopez-Sagaseta et al (2012) PLoS Biol. 10:e1001412 (11 pages)). What is also provided are agonists of NOD receptors. NOD receptors include NOD1 and NOD2. NOD agonists include N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyldipeptide (MDP)), which binds to NOD 2. NOD agonists include gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP), which binds to NOD1. NOD agonists include desmuramylpeptides (DMP), which binds to

NOD1. See, e.g., Uehara et al (2006) J. Immunol. 177:1796-1804). NOD agonists are derived from fragments of peptidoglycan.

[0096] Also provided are agents that inhibit T regulatory cells (Tregs). These agents include cyclophosphamide, gemcitabine, and antibodies that deplete Tregs (see, e.g., Le and Jaffee (2012) Cancer Res. 72:3439-3444; Klages et al (2010) Cancer Res. 70:7788-7799). Gemcitabine reduces the immunosuppressive activity of myeloid-derived suppressor cells (MDSCs) (Le et al (2009) Int. Immunopharmacol. 9:900-909). Anti-CD25 antibodies can deplete Tregs, and have been used in the treatment of cancer (Klages et al (2010) Cancer Res. 70:7788-7799). Daclizumab is an anti-CD25 antibody, which blocks binding of interleukin-2 (IL-2) to CD25, thereby blocking a signal needed for Treg maintenance. Some Tregs express high levels of CTLA4 ($CD45RA^+$ Tregs). Anti-CTLA4 antibodies, such as ipilimumab, target CTLA4. Ipilimumab is used for treating melanoma (Rech et al (2012) Immunotherapy. 4:1103-1105).

[0097] Lymphocyte activation gene-3 (LAG-3) blocking agents, such as anti-LAG-3 antibodies or soluble LAG-3 (e.g., LAG-3 Ig), can enhance immune response to cancers or infections. Anti-LAG-3 antibodies reduce the activity of Tregs (see, e.g., Huang, et al. (2004) Immunity 21:503-513; Triebel (2003) Trends Immunol. 24:619-622).

[0098] Antibodies are available that directly target $CD8^+$ T cells or $CD4^+$ T cells. These antibodies include those that target costimulatory receptors (4-1BB, OX40 and GITR) or the blockade of co-inhibitory receptors (CTLA-4, PD-1 and PD-L1). GITR is glucocorticoid-induced TNFR-related protein. See, e.g., Schaer et al (2010) Curr. Opin. Investig. Drugs. 11:1378-1386). Anti-GITR can stop the suppressive function of Tregs, thus explaining anti-GITR's efficacy in anti-cancer therapy (Coe et al (2010) Cancer Immunol. Immunother. 59:1367-1377). DTA-1 is an anti-GITR antibody.

Methods for assessing immune response

[0099] The present disclosure also provides ELISPOT assays, intracellular cytokine staining (ICS), and tetramer assays, for characterizing immune response (see, e.g., of US 2007/0190029 of Pardoll; Chattopadhyay (2008) Cytometry A. 2008 73:1001-1009; Vollers (2008) Immunology. 123:305-313; Lalvani, et al. (1997) J.

Exp. Med. 186:859-865; Waldrop (1997) J. Clin. Invest. 99:1739-1750; Hudgens (2004) J. Immunol. Methods 288:19-34; Goulder (2001) J. Virol. 75:1339-1347; Goulder (2000) J. Exp. Med. 192:1819-1831; Anthony (2003) Methods 29:260-269; Badovinac and Harty (2000) J. Immunol. Methods 238:107-117). Immune response in a patient can be assessed by endpoints that are used in oncology clinical trials, including objective response (RECIST criteria), overall survival, progression-free survival (PFS), disease-free survival, time to distant metastasis, 6-month PFS, 12-month PFS, and so on.

Antigens

[00100] Glioblastoma-specific antigens include PTPRZ1; EGFR; SEC61G; TNC; HER2; TRP-2; gp100; MAGE-1; IL13Ralpha2; AIM-2 (Phuphanich et al (2013) Cancer Immunol. Immunother. 62:125-135; Neidert et al (2012) J. Neurooncol.). Colorectal cancer-specific antigens include SPARC, CEA, Cep55/c10orf3 (Inoue et al (2010) Int. J. Cancer. 127:1393-1403, Parkhurst et al (2011) Mol. Ther. 19:620-626; Inoda et al (2011) Exp. Mol. Pathol. 90:55-60). Renal cancer-specific antigens include carbonic anhydrase IX (CA-IX), MUC-1, and NYESO-1, and 5T4 (Tykodi et al (2012) J. Immunother. 35:523-533). Ovarian cancer-specific antigens include WT1, mesothelin, NY-ESO-1, p53, p53 carrying specific mutations, HER-2/neu, folate receptor-alpha, IGFBP; MUC1, MUC4, MUC16, EpCAM; CTA (Dohi et al (2011) Anticancer Res. 31:2441-2445; Vermeij et al (2012) Curr. Pharm. Des. 18:3804-3811; Preston et al (2011) Immunother. 3:539-556). Specificity can arise from the amino acid sequence of the antigen, from the degree of expression of that antigen by the tumor cell, from post-translational modification of the antigen, and the like. Specificity to a certain type of cancer cell can also arise from a particular fingerprint of a plurality of tumor antigens. Specificity can also arise, from the fact that a particular antigen, while expressed by a wide variety of tumor cells, has particular use in immunotherapy against a smaller number of tumor types. Specificity can also arise from the fact that a particular collection of MHC class I presentable and MHC class II presentable epitopes exist, on a particular polypeptide or polypeptide fragment. Also, specificity in an administered antigen can arise by omitting one or more peptides that can provoke immunotolerance. The skilled artisan can easily locate the relevant nucleic acid and polypeptide sequences, e.g., on the U.S. government's web site, at ncbi.nlm.nih.

Vaccines

[00101] A vaccine of the present disclosure can be purely preventative, it can be used for treating an existing cancer or pre-neoplastic disorder, or it can be used for preventing recurrence of a previously treated cancer. Dendritic cell vaccine of the present disclosure can be administered by intradermal, intranodal, mucosal, or subcutaneous routes, or any combination of the above. Each dose can comprise about 10×10^3 dendritic cells, 20×10^3 cells, 50×10^3 cells, 100×10^3 cells, 200×10^3 cells, 500×10^3 cells, 1×10^6 cells, 2×10^6 cells, 20×10^6 cells, 50×10^6 cells, 100×10^6 cells, 200×10^6, 500×10^6, 1×10^9 cells, 2×10^9 cells, 5×10^9 cells, 10×10^9 cells, and the like. Administration frequency can be, e.g., once per week, twice per week, once every two weeks, once every three weeks, once every four weeks, once per month, once every two months, once every three months, once every four months, once every five months, once every six months, and so on. The total number of days where administration occurs can be one day, on 2 days, or on 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 days, and so on. It is understood that any given administration might involve two or more injections on the same day. In one aspect, the disclosure involves loading dendritic cells with whole tumor cells, where at least 10%, where at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, or at least 99%, of the melanoma cell-derived protein that is loaded into the dendritic cells resides in whole tumor cells. In non-limiting embodiments, dendritic cell vaccine is held in a flask, in a vial, in a bottle, in a syringe, in a catheter, in a cannula, and so on. For administration, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 99%, of the dendritic cells that are administered are mature dendritic cells.

Vaccine homogeneity

[00102] In embodiments, the disclosure provides a vaccine comprising dendritic cells that contain melanoma peptides derived from in vitro loading, where the vaccine comprises dendritic cells (sum of DCs containing melanoma peptide, and DCs not containing melanoma peptides) at a ratio of dendritic cells/melanoma cells of at least 5/95, 10/90, 20/80, 30/70, 40/60, 50/50, 60/40, 70/30, 80/20, 90/10, 95/5, 98/2, 99/1, and the like. Also provided, is a vaccine comprising dendritic cells that contain melanoma peptides derived from in vitro loading, where the vaccine

comprises dendritic cells (sum of DCs containing melanoma peptide, and DCs not containing melanoma peptides) at a ratio of [dendritic cells]/[cells that are neither DCs nor melanoma], of at least 5/95, 10/90, 20/80, 30/70, 40/60, 50/50, 60/40, 70/30, 80/20, 90/10, 95/5, 98/2, 99/1, and the like. The disclosure provides a compartmented container, where a first compartment contains melanoma cells, and a second compartment contains dendritic cells. The two compartments can be separated by a membrane, filter, valve, conduit, coupler, which prevents the melanoma cells from contacting the dendritic cells, but where manual transfer, or where removal of the membrane or opening of the valve allows the melanoma cells to contact the dendritic cells, allowing loading of melanoma cells, melanoma cell fragments, and/or melanoma peptides, on the dendritic cells.

Interferon-gamma mimetics

[00103] The present disclosure encompasses mimetics, for example, interferon-gamma mimetics, such as mimetic peptide 95-132 (Ahmed (2007) J. Immunol. 178:4576-4583; Fulcher (2008) FEBS Lett. 582:1569-1574). IFN-mimetic encompasses, e.g., an antibody that has the same agonist activity of interferon-gamma.

Inactivating melanoma cells

[00104] The present disclosure provides compositions and methods, where cancer cells are inactivated, for example, by radiation or by way of nucleic acid cross-linkers. Exemplary cross-linkers, have the ability to cross-link DNA but to leave proteins unmodified. A nucleic acid alkylator can be beta-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In some embodiments, the nucleic acid targeted compound is a psoralen compound activated by UVA irradiation. For instance, the nucleic acid targeting compound can be 4'-(4-amino-2-oxa) butyl-4,5',8-trimethylpsoralen (also referred to herein as "S-59"). Cells can be inactivated with 150 micromolar of psoralen S-59 and 3 J/cm^2 UVA light (FX 1019 irradiation device, Baxter Fenwal, Round Lake, IL). The inactivation with S-59 is referred to as photochemical treatment and results in complete inactivation of the cells. Various concentrations of nucleic acid cross-linked agent can be tested for efficacy in inactivating cells, for example, for efficacy in preventing cell division. S-59 is distinguished by its ability to cross-link nucleic acids, but to leave proteins intact an unmodified. Cells can be suspended in 5 mL of saline containing 0, 1, 10, 100, and

1000 nM of psoralen S-59. Each sample can be irradiated as follows. The S-59 can be added at a concentration of 100 nM. Samples can be UVA irradiated at a dose of approximately 2 J/cm^2 (FX1019 irradiation device, Baxter Fenwal, Round Lake, Ill.). Each sample can then transferred to a 15 mL tube, centrifuged, and the supernatant removed, and then washed with 5 mL saline, centrifuged and the supernatant removed and the final pellet suspended in 0.5 mL of saline (U.S. Pat. Nos. 7,833,775 of Dubensky and 7,691,393 of Dubensky).

Enriching for cancer cells that are non-apoptotic

[00105] A population of melanoma cells can be enriched in melanoma cells that are non-apoptotic, for example, by use of the technique that separates non-apoptotic and autophagic cells from cells that are non-autophagic and apoptotic, where separation is by the adhesion of the autophagic and non-apoptotic cells to a surface, where the other cells are floating. A population enriched in non-apoptotic melanoma cells can also be acquired by removing apoptotic cells by way of an antibody specific for phosphatidyl serine. Techniques for removing cells by way of immobilized antibodies are available (Onodera (1998) Ther. Apher. 2:37-42). Antibodies specific for phosphatidylserine are available (e.g., EMD Millipore, Billerca, MA). Also, bulk population of melanoma cells can be labeled with fluorescent anti-phosphatidylserine antibodies, where the tagged apoptotic melanoma cells are removed by flow cytometry, affinity chromatography, immunomagnetic separation (see, e.g., Hoeppener (2012) Recent Results Cancer Res. 195:43-58; Dainiak (2007) Adv. Biochem. Eng. Biotechnol. 106:1-18). This technique is generally applicable to enriching in cancer cells that are melanoma cells, and also for cancer cells that are not melanoma cells.

Inhibitors of apoptosis

[00106] Z-VAD (Z-VAD-fmk), an inhibitor of apoptosis, can be acquired from, e.g., Enzo Life Sciences (Exeter, UK), R & D Systems (Minneapolis, MN), Tocris Biosciences (Bristol, UK), BioMol (Plymouth Meeting, PA), and EMD Chemicals (Gibbstown, NJ). Z-VAD-fmk is a synthetic peptide, Z-Val-Ala-Asp(OMe)-CH_2F. Caspases are cysteine-aspartic acid-specific members of the protease family. Caspases are activated by a death receptor ligation, e.g., TRAIL, FAS, by DNA damage, stress, serum starvation and in some cell types, interferons. Caspases play a critical role in the highly regulated process of apoptosis that

includes nuclear fragmentation, chromatin condensation, and loss of cytoplasmic integrity. The pan-capase inhibitor, z-VAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone) irreversibly binds the catalytic site of caspase proteases and inhibits their function in inducing apoptosis. Inhibiting the ability of cells to undergo apoptosis in response to IFN-gamma can be a means by which cells that are non-apoptotic but autophagic and be generated without the steps of selection by the washing to remove floating apoptotic cells.

[00107] The disclosure provides pharmaceuticals, reagents, kits including diagnostic kits, that wherein the pharmaceuticals, reagents, and kits, comprise dendritic cells, antibodies, or antigens. What is also provided are methods for administering compositions that comprise at least one dendritic cell and at least one antigen, methods for stimulating antibody formation, methods for stimulating ADCC, methods for stimulating complement-dependent cytotoxicity, and methods and kits for determining patient suitability, for determining patient inclusion/exclusion criteria in the context of a clinical trial or ordinary medical treatment, and for predicting response to the pharmaceutical or reagent. Complement-dependent cytotoxicity is described (see, e.g., Goodman, et al. (1990) J. Clin. Oncol. 8:1083-1092; Cheson (2010) J. Clin. Oncol. 28:3525-3530). The pharmaceutical compositions, reagents, and related methods, of the disclosure encompass CD83 positive dendritic cells, where CD83 is induced by loading with IFN-gamma-treated cancer cells. In a CD83 aspect of the disclosure, the CD83 is induced by at least 2%, at least 3%, at least 4%, 6%, 7%, 8%, 9%, 10%, and the like.

[00108] **Figure 1** reveals a graphic of cultured tumor cells before treatment with IFN-gamma (left) and after treating with IFN-gamma for 72 hours (right). After treatment, the cultured tumor cells are either floating, non-autophagic, and apoptotic, or adherent, autophagic, and non-apoptotic. The floating cells are shown expressing the apoptotic marker, phosphatidyl serine. The floating cells are shown with relatively few expressed MHC class II, while the adherent cells are shown with over-expressed MHC class II.

[00109] **Figures 2A-D** show characterization of IFN-gamma treated autologous tumor cells used for loading dendritic cells. Autologous melanoma tumor cells were treated with or without 1000 IU/mL IFN-gamma for 72 hours in 15% FBS/ECS in RPMI, harvested and irradiated with 100Gy and cryopreserved. Cells were then

thawed in AIMV and a sample taken for flow cytometry and for preparation of cell lysates for immunoblotting prior to antigen loading of DCs. An example of four separate autologous melanoma cell lines is shown (Fig.2A, Fig.2B and Fig.2C). Induction major histocompatibility complexes by IFN-gamma treatment of autologous tumor cells (Fig.2D). Tumor cells were harvested after being treated with or without 1000 IU/mL IFN-gamma for 72 hours and then assayed for MHC class I and class II. Control isotype antibodies were used to identify positive populations. Dark data points indicate median mean fluorescent plus/minus 95% confidence interval. N = 65. After irradiation, melanoma cells are checked by assays to ensure that there is not any mitosis.

[00110] In one aspect, the disclosure excludes non-autologous tumor cells for loading dendritic cells, and excludes methods of using non-autologous tumor cells for loading dendritic cells.

[00111] **Figures 3A and 3B** describe phenotype of dendritic cells loaded with autologous melanoma cell lines treated with or without interferon-gamma. A set of four autologous melanoma cell lines were treated with or without 1000 IU/mL of IFN-gamma for 72 hours, irradiated and cryopreserved. The cells were then thawed in AIMV and combined with autologous dendritic cells for approximately 24 hours prior to harvest and assaying by flow cytometry for the expression of CD80, CD83, CD86 and MHC class II (Figure 3A). The data is summarized in Figure 3B. Averages ± SD are shown, n = 4.

[00112] **Figures 4A and 4B** show phenotype of dendritic cells used for dose preparation. Samples of DC prior to loading (Pre-ATC Load DC, N = 53) and after loading (Post-ATC Load DC, N = 65) with IFN-gamma treated, irradiated autologous tumor cells were accessed by flow cytometry for the expression of CD80, CD83, CD86 and MHC class II. FACS Caliber® beads were used to set the initial flow cytometer instrument settings which were then held constant throughout the collection of data (Figure 4A). Values of percent expression and mean fluorescence intensity (MFI) ± SD are compared in Figure 4B for Pre-ATC and Post-ATC loading. * $p = 0.019$ and ** $p = 0.0009$.

[00113] **Figures 5A to 5C** show interferon-gamma treated melanoma cells undergo autophagy. A selection of commercially-available melanoma cell lines were

incubated with 1000 IU/mL IFN-gamma for 72 hours in 5%FBS/RMPI. Phase-contrast photomicrographs of SK-5-Mel cell cultures were taken at the end of the incubation period (Figure 5A) showing enlarged cells with vacuoles reminiscent of autophagosomes. Confirmation of the formation of autophagosomes was demonstrated by transfection with GFP-LC3B constructs prior to treatment with IFN-gamma (Figure 5B). Autophagy induction after IFN-gamma treated was confirmed by western blotting using an antibody for LC3B (Figure 5C) which identifies a faster migrating form of LC3 that has been shown to be associated with autophagic vessel formation.

[00114] **Figures 6A and 6B** reveal apoptosis and autophagy induced in response to interferon-gamma. SK-5-Mel cells were incubated with 1000 IU/mL of IFN-gamma for 72 hours after which non-adherent and adherent populations were collected and assayed for apoptosis and autophagy by flow cytometry using 7-AAD and Annexin-V (Figure 6A). Enzo Cyto-ID Autophagy Detection Dye was used to measure autophagy by flow cytometry by measuring the mean intensity peak shift of dye provided by the manufacturer (Figure 6B). Fold changes in the peak shift in comparison to 5% FBS/RPMI are shown in Figure 6C with serum-free as positive control for the induction of autophagy.

[00115] **Figure 7** discloses autophagy induction after blocking of caspase activity did not affect the induction of autophagy in response to IFN-gamma in melanoma cells. SK-5-Mel cells were treated with 1000 IU/mL of IFN-gamma in the presence of 20uM of the pan-caspase inhibitor z-VAD or its control compound, z-FA for 72 hours. The cells were harvested and assayed for autophagy by flow cytometry as in Figure 6C.

[00116] **Figure 8** shows SK-5-Mel cells which were incubated with 1000 IU/mL of IFN-gamma in the presence of 10 uM of the autophagy inhibitor 3-methyladenine (3-MA) for 72 hours. The cells were then harvested and assayed for apoptosis and MHC class II (HLA-DR) expression by flow cytometry.

[00117] **Figure 9** shows IFN-gamma treated cells from tumor cell lines generated from patient tumor specimens (N = 36) were assayed for changes in MHC class II or apoptosis. The data shown are averages of mean fluorescent intensity (MFI) ± SE.

[00118] **Figure 10** shows IFN-gamma treated cells that were assayed for MHC class II or apoptosis by flow cytometry from samples used for loading dendritic cells for a patient-specific vaccine immunotherapy (N = 54). Fold changes in MHC class II mean fluorescence intensity (MFI) and percent apoptotic cells (Annexin-V positive) are shown.

[00119] **Figure 11 and Figure 12** show a correlation between induction of MHC class II and the absence of apoptosis (Interferon-gamma resistant) is associated with better progression-free survival (Fig.11) and overall survival (Fig.12) in patients received dendritic cells loaded with autophagic, non-apoptotic interferon-gamma treated tumor cells.

[00120] **Figure 13** shows survival curves from three trials. The plot (Kaplan-Meier plot) is a stepwise curve showing the percent of study subjects surviving during the course of clinical trials. The groups are designated **DC-54** (solid circle); **TC-74** (solid square); **TC-24** (solid triangles); and **DC-18** (line). Poorest survival occurred with **TC-24**. The next poorest survival was with **TC-74**. **TC-24** refers to a vaccine of tumor cells in a study involving 24 subjects.

[00121] **Figure 14** shows survival curves from three trials. The trials are the same clinical trials as those disclosed in Figure 13, but with additional data acquired from later time points.

FURTHER DISCLOSURE

Autologous dendritic cell generation

[00122] Dendritic cells were generated by plastic adherence method of ficoled apheresis products (Choi, et al. (1998) Clin. Cancer Res. 4:2709-2716; Luft, et al. (1998) Exp. Hematol. 26:489-500;Cornforth, et al. (2011) Cancer Immunol. Immunother. 60:123-131), in antibiotic-free AIM-V medium (Invitrogen, Grand Island, NY) supplemented with 1,000IU/mL each of IL-4 (CellGenix, Freisberg, Germany) and GM-CSF (Berlex, Seattle, WA) (DC medium). The flasks were then cultivated for 6 days prior to loading with IFN-gamma treated, irradiated autologous tumor cells.

IFN-gamma autologous tumor cell line generation and preparation of pharmaceutical

[00123] Pure tumor cells were generated according to Cornforth, et al. (Cornforth, et al. (2011) Cancer Immunol. Immunother. 60:123-131; Dillman, et al. (1993) J. Immunother. Emphasis Tumor Immunol. 14:65-69; Dillman, et al. (2000) Cancer Biother. Radiopharm. 15:161-168). The tumor cells were then incubated with 1,000U/mL of interferon-gamma (InterMune, Brisbane, CA) for 72h, irradiated with 100Gy from a cesium source and cryopreserved (Selvan, et al.(2007) Int. J. Cancer 122:1374-1383; Selvan, et al. (2010) Melanoma Res. 20:280-292). The IFN-gamma treated and irradiated tumor cells were recovered from cryopreservation, washed with phosphate buffered saline (PBS), and then added to the cultivated dendritic cells (DCs) and then incubated for about 24h. The antigen-loaded DCs were harvested by gentle scraping with a rubber policeman and cryopreserved. Aliquots of IFN-gamma treated or untreated tumor cells and loaded DCs were obtained for flow cytometry evaluation and trypan-blue exclusion assay.

Staging of cutaneous melanoma

[00124] The pharmaceutical or reagent of the disclosure can be administered to melanoma patients, where melanoma is diagnosed at Stage I, Stage II, Stage III, or Stage IV (Mohr, et al (2009) Ann. Oncology (Suppl. 6) vi14-vi21). Stage I, for example, refers to patients with primary melanomas without evidence of regional or distant metastasis. Stage II includes patients without evidence of lymphatic disease or distant metastases, where the patients are further characterized, e.g., by lesions greater than 1mm and less than or equal to 2mm thick with ulceration of the overlying epithelium, or by lesions greater than 2mm and less than or equal to 4mm thick with epithelial ulceration. Stage III melanoma includes lesions with pathologically documented involvement of regional lymph nodes or in-transit or satellite metastases, where patients may have, e.g., one, two, three, or four or more affected lymph nodes. Stage IV melanoma is defined by the presence of distant metastases, where the metastasis is located only in distant skin, subcutaneous tissues, or lymph nodes, where the metastasis involves lung metastases, or where the metastasis involves all other visceral sites.

[00125] The disclosure encompasses methods for administration that are preventative, that is, for use with subjects not yet or never diagnosed with a melanoma. What is encompassed are methods for administration where a subject had earlier been diagnosed with a melanoma, and had earlier been successfully

treated to eradicate the melanma (or had experienced a spontaneous complete remission), and where following eradication the administration is used preventatively.

Tumor antigens

[00126] Without implying any limitation, melanoma cells of the disclosure express one or more of Mage, Mart-1, Mel-5, HMB45, S100, or tyrosinase (Dillman, et al. (2011) Cancer Biotherapy Radiopharmaceuticals 26:407-415). In one aspect, detection of tumor antigen uses cells that were not exposed to IFN-gamma while, in another aspect, detection of tumor antigen is conducted on cells that were treated with IFN-gamma (see, e.g., Cornforth, et al. (2011) Cancer Biotherapy Radiopharmaceuticals 26:345-351). What is encompassed are melanoma cells expressing one or more melanoma antigens, or compositions comprising one or more isolated melanoma antigens, as disclosed by US2007/0207171 of Dubensky, et al, which is incorporated herein by reference in its entirety.

Measuring apoptosis

[00127] Apoptosis can be detected or measured with a number of reagents, e.g., fluorochrome-labeled annexin, by staining with dyes such as propidium iodide and 7-aminoactinomycin D (7-AAD), by determining loss of mitochondrial inner memebrane potential, by measuring activation or cleavage of caspases. See, e.g., George, et al. (2004) Cytometry Part A. 59A:237-245. An early event in apoptosis is exposure of phosphatidyl serine on the outer surface of the plasma membrane, which can be detected by fluorochrome-labeled annexin. The available methods can distinguish between live cells, necrotic cells, early apoptotic cells, and late apoptotic cells. The disclosure uses melanoma cells that are not apoptotic by 7-ADD assay, not apoptotic by annexin V assay, not apoptotic by an assay for apoptosis after IFN-gamma treatment (Dillman, et al. (2011) Cancer Biotherapy Radiopharmaceuticals 26:407-415), or not apoptotic by one or more of the biomarkers Bcl-2, caspase-3, P53, or survivin (Karam, et al. (2007) Lancet Oncol. 8:128-136). The pharmaceutical compositions, reagents, and related methods, of the disclosure exclude IFN-gamma-treated melanoma cells that are apoptotic, where apoptosis is determined, e.g., according to U.S. Pat. No. 7,544,465 issued to Herlyn, et al; U.S. Pat. No. 7,714,109 issued to Thorpe, et al, which are incorporated herein by reference.

Measuring autophagy

[00128] Autophagy is a naturally occurring process that is used for the degradation of many proteins and some organelles. Autophagy mediates protein and organelle turnover, starvation response, cell differentiation, cell death, and so on. Microtubule-associated protein light chain 3 (LC3) is to monitor autophagy. In one approach, autophagy can be detected by measuring the conversion of LC3, which involves conversion of LC3-I to LC3-II. The amount of LC3-II is correlated with the number of autophagosomes. In detail, LC3 is cytosolic and soluble, while LC3-II is present on membranes. LC3-II has a greater molecular weight because it is conjugated with a lipid. LC3 processing can be measured, e.g., by western blots, while autophagy, autophagic vesicles, and autophagosomes, can be measured by microscopy. Autophagy can be quantitated, e.g., by detecting processed LC3-II, by the ratio between early to late autophagic compartments, or by autophagic volume. See, (Mizushima and Yoshimori (2007) Autophagy 3:542-546:634-641; Tanida, et al. (2008) Methods Mol. Biol. 445:77-88; Eng, et al. (2010) Autophagy 6:634-641). In one aspect, the present disclosure uses autophagy as a screening tool, for selecting appropriate autophagic cancer cells, where the cells can be selected according to occurrence of autophagy in one or more particular stages. These autophagy stages include: (1) sequestering of cytosolic compartments by the autophagosome, (2) fusion of the autophagosome with the lysosome to form the autolysosome, and (3) degradation of the autophagosomal contents by proteases within the lysosome. In another aspect, the present disclosure includes mainly cells displaying the first stage, mainly the second stage, mainly the third stage, mainly the first and second stage, mainly the second and third stage, or mainly cells displaying all three stages. In yet another aspect, the disclosure comprises cells displaying the first stage, the second stage, the third stage, the first and second, the second and third stage, or cells displaying all three stages.

Interferon-gamma (IFN-gamma) signaling

[00129] IFN-gamma (type II interferon) signaling depends on expression of a number of genes, e.g., IFN-gamma receptor, STAT1, STAT2, STAT1 homodimers, STAT1/STAT2 heterodimers, IRF-1, GAS, and IRF-E. Studies have shown that IFN-gamma signaling is dependent on IFN-gamma receptor (IFNGR1 chain; IFNGR2 chain). Low expression of IFNGR on the cell surface can block some aspects of IFN-gamma signaling (Schroder, et al. (2004) J. Leukocyte boil. 75:163-189). In one

aspect, the present disclosure excludes using cancer cells that show low surface expression of IFNGR. In another aspect, the present disclosure screens cancer cells for those that express the STAT1 homodimer, uses these cells, and substantially excludes cells that do not express STAT1 homodimer. In yet another aspect, the disclosure contemplates screening cells for those with STAT1 phosphorylation (serine-727). What is also contemplated, is excluding cancer cells from patients having loss of function mutations in the STAT1 gene (see, e.g., Dupuis, et al. (2001) Science 293:300-303; Schroder, et al. (2004) J. Leukoc. Biol. 75:163-189). The following concerns the IRF gene family. IRF-1, IRF-2, and IRF-9, all participate in IFN-gamma signaling. The disclosure embraces using cancer cells that express one or more of these IRF gene family genes, or excluding cancer cells that do not express one or more of these genes.

IFN-gamma responsive genes

[00130] The present disclosure embraces biologic material, compositions, reagents, and methods that require using a melanoma cell, or pre-neoplastic melanoma cell, that responds to IFN-gamma. The melanoma cell can be identified, distinguished, and selected, by an assay for the expression of one or more IFN-gamma-responsive genes. A number of IFN-gamma-responsive genes have been identified (see, e.g., Halonen, et al. (2006) J. Neuroimmunol. 175:19-30; MacMicking (2004) 11:601-609; Boehm, et al. (1997) 15:749-795). Said assay can involve removing one or more melanoma cells from the patient, culturing the cell in the presence and absence of added IFN-gamma, and determining responsiveness to IFN-gamma. In the assay, IFN-gamma induced gene expression can be detected by assays sensitive to binding of a transcription factor to the promoter of an IFN-gamma induced gene, to expression of mRNA from an IFN-gamma induced gene, to expressed polypeptide, and the like. The IFN-gamma response gene can include, e.g., a gene used for immune response, encoding a transcription factor, a transport protein, an apoptosis gene, a gene used for cell growth or maintenance, a gene used for lipid metabolism, a gene that mediates endocytosis or exocytosis, an intracellular signaling gene, a glucose metabolism gene, a cell adhesion gene, as well as genes without an established function.

[00131] In one aspect, the disclosure excludes melanoma cells that, with IFN-gamma treatment, show reduced expression of MHC class II, show no detectable

change in expression of MHC class II, show an increase of MHC class II expression of 10% or less, show an increase in MHC class II expression of 15% or less, show an increase in MHC class II expression of 20% or less, 25% or less, 30% or less, 40% or less, 50% or less, and the like. In one aspect, the value for percentage refers to the average expression value for the population of melanoma cells, residing in a biopsy or part of a biopsy, from a given subject or patient.

Non-limiting lists of IFN-gamma inducible genes for use in screening for IFN-gamma responsive cancer cells

[00132] ab000677, JAB/SOCS1; m63961, IFN-gamma inducible protein (mag-1) ; m35590, Macrophage inflammatory protein 1-β; m19681, MCP-1 (JE); y07711, zyxin; M34815, Monokine induced by IFN-gamma (MIG); m33266, Interferon inducible protein 10 (IP-10); U44731 , Purine nucleotide binding protein ; U88328, Sup. of cytokine signalling-3 (SOCS-3); M21065 , Interferon regulatory factor 1; M63630, GTP binding protein (IRG-47) ; U19119 , G-protein-like LRG-47; L27990, Ro protein ; M31419, 204 interferon-activatable protein ; af022371, Interferon-inducible protein 203; U28404, MIP-1 alpha receptor; U43085, Glucocorticoid-attenuated response 39; x56123, Talin; m31419, 204 interferon-activatable protein ; U53219 , GTPase IGTP; I38444, T-cell specific protein; M31418, 202 interferon-activatable protein; d38417, Arylhydrocarbon receptor; m26071, Tissue factor (mtf); D13759, Cot proto-oncogene; M18194, Fibronectin; u59463, ICH-3; M13945, pim-1 proto-oncogene; L20450 , DNA-binding protein (see, Gil, et al. (2001) Proc. Natl. Acad. Sci 98:6680-6685). The disclosure encompasses use of the IFN-gamma induced gene, CIITA (see, e.g., Chan, et al. (2010) J. Leukocyte Biol. 88:303-311; Kwon, et al (2007) Mol. Immunol. 44:2841-2849).

[00133] The present disclosure embraces measuring expression of one or more of the following IFN-gamma inducible genes, as a screening procedure for qualifying or selecting patients for administering a pharmaceutical. The genes include, (gene 1) FCGR1A, (gene 2) IL6R, (gene 3) CXCL9, (gene 4) CLCSF14, (gene 5) UBD, (gene 6) C/EBPalpha, and (gene 7) MHC2TA (CIITA) (see, Waddell, et al. (2010) PLoS ONE 5:e9753). Also embraced are use of specific clusters of these genes, in the qualifying procedure, such as, genes 1 and 2, 2 and 3, 3 and 4, 4 and 5, 5 and 6, 6 and 7, 1 and 3, 1 and 4, 1 and 5, 1 and 6, 1 and 7, 2 and 4, 2 and 5, 2 and 6, 2 and 7, 3 and 5, 3 and 6, 3 and 7, 4 and 6, 4 and 7, 5, and 7, and well as

combinations of three genes, e.g., 1, 2, 3; or 3, 4, 5; or 4, 5, 6; or 5, 6, 7; or 1, 3, 4; or 1, 3, 5, or 1, 3, 6, or 1, 3, 7; or 1, 2, 4; or 1, 2, 5; or 1, 2, 6; or 1, 2, 7; and the like. (These gene numbers are arbitrary.)

[00134] What is excluded is a population of melanoma cells that is less than 90% are autophagic, less than 80% are autophagic, less than 70% are autophagic, less than 60% are autophagic, less than 50% are autophagic, less than 40% are autophagic, and the like.

[00135] What is excluded is a population of melanoma cells where, that is less than 90% are non-apoptotic, less than 80% are non-apoptotic, less than 70% are non-apoptotic, less than 60% are non-apoptotic, less than 50% are non-apoptotic, less than 40% are non-apoptotic, and the like.

[00136] What is excluded is a population of melanoma cells that is less than 90% are non-adherent, less than 80% are non-adherent, less than 70% are non-adherent, less than 60% are non-adherent, less than 50% are non-adherent, less than 40% are non-adherent, and the like.

Measuring expression of MHC class II

[00137] Expression of MHC class II can be measured, for example, using antibodies or nucleic acid probes that are specific for MHC class II gene products. These MHC class II gene products include HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB1, as well as HLA-DM and HLA-DO (see, e.g., Apostolopoulos, et al. (2008) Human Vaccines 4:400-409).

[00138] For example, the present disclosure encompasses reagents, methods of treatment, and methods of diagnosis, that require the melanoma cells to express STAT1 and STAT2, to have an active STAT1-signaling pathway, to have an active STAT2-signaling pathway, or to have active STAT1 and STAT2-signaling pathways.

[00139] The disclosure provides a pharmaceutical composition or pharmaceutical reagent, related methods of administration, and methods of treatment, that result in survival data with a hazard ratio (HR) of less than 1.0, HR less than 0.9, HR less than 0.8, HR less than 0.7, HR less than 0.6, HR less than 0.5, HR less than 0.4, HR less than 0.3, and the like. The disclosure results in overall survival data, progression-free survival data, time to progression data, and so on. What is also provided is 6-month PFS of at least 40%, at least 50%, at least 60%, at least 70%, at

least 75%, at least 80%, at least 85%, at least 90%, at least 95%, and so on. Moreover, what is provided is 6-month overall survival of at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, and so on. Additionally, what is provided is 1-year (or 2-year) PFS of at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, and so on. Moreover, what is provided is 1-year (or 2-year) overall survival of at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, and so on (see, e.g., U.S. Dept. of Health and Human Services. Food and Drug Administration. Guidance for Industry. Clinical trial endpoints for the approval of cancer drugs and biologics (April 2005)).

IFN-gamma and the induction of autophagy

[00140] Induction of autophagy after IFN-gamma treatment, as measured by increases in the expression of major histocompatibility class II complexes, can be used to determine response to systemic IFN-gamma treatment. If biopsied melanoma tumor cells, upon exposure to IFN-gamma in culture, undergo autophagy but not apoptosis, this indicates that these patients will respond favorably to systemic IFN-gamma treatment. Additionally, if successful cell lines are established from the biopsies, that patient would also benefit from cell-therapy products prepared from IFN-gamma treated purified tumor cells lines that are from autophagic but non-apoptotic adherent populations.

[00141] The disclosure embraces isolating and characterizing major histocompatiability complexes isolated from autophagic, non-apoptotic cells collected from tumor cell lines treated with interferon-gamma. Major histocompatibility complexes contain antigens specific for $CD4^+$ T cells and have been associated with antibody mediated immune responses. The complexes would represent a large repertoire of antigens would not be present in non-autophagic cells due to the action of lysosomal mediated antigen processing induced in autophagic cells.

[00142] Non-apoptotic, autophagic tumor cells generated from IFN-gamma treated cell lines can be fused with dendritic cells to enhance the antigen presentation due to the high levels of major histocompatability complexes on the surface of the

autophagic tumor cells. This process would yield a novel cellular product generated from the fusion of the two cell types.

[00143] The process of induction of autophagy in response to IFN-gamma may be induced in a manner that does not result in apoptosis. By combining the treatment of tumor cells with caspase inhibitors and interferon gamma, the process of cell death (and ultimately the formation of tolergeneic apoptotic cells) can be blocked without inhibiting the induction of autophagy or the increase in major histocompatibility class II complexes.

Procedure to eliminate apoptotic cells, while retaining viable autophagic cells

[00144] Studies of melanoma demonstrated a correlation between the presence of apoptotic cells and poor survival in a clinical trial (Cornforth, et al. (2011) Cancer Immunol. Immunother. 60:123-131; Dillman, et al. (2011) Cancer Biother. Radiopharmaceuticals 26:407-415). The following study investigated the induction of autophagy, apoptosis and MHC class II molecules after IFN-gamma treatment of melanoma tumor cells in vitro.

[00145] The methodology of the study was as follows. Autologous and model melanoma tumor cell lines were incubated with 1000 IU/mL of IFN-gamma for 72 hours prior to assaying for autophagy, apoptosis and MHC class II expression. Autophagy was detected by immunobloting with antibodies against LC3 II and by flow cytometry with Enzo's CytoID® Autophagy Detection Kit. Apoptosis and MHC class II induction were assayed by flow cytometry using 7-AAD and annexin-V staining and antibodies against MHC class II, respectively. 7-AAD intercalates in dsDNA. 7-AAD is excluded by viable cells, and thus it can be used to detect dead cells.

[00146] The results from the study demonstrated that IFN-gamma induces both autophagic and apoptotic cell populations in melanoma cell lines. The apoptotic population was predominantly found in the non-adherent population while the autophagic cells remained adherent to the flask. Blocking of autophagy with the inhibitor 3-methyladenine (3-MA) inhibits the induction of MHC class II positive cells in response to IFN-gamma (39.4% IFN-gamma vs. 10.0% IFN-gamma + 3-MA). Inhibition of caspase activity with the pan caspase inhibitor Z-VAD prevents apoptosis but does not perturb autophagy in IFN-gamma treated cells (2.75 ± 0.15

IFN-gamma vs. 3.04 ± 0.27 IFN-gamma + Z-VAD, fold change). To conclude, induction of apoptosis is associated with reduced levels of autophagy and MHC class II induction. This disclosure provides method or procedure to eliminate apoptotic cells while retaining viable autophagic cells after IFN-gamma treatment can enhance the effectiveness of this type of cell-based immunotherapy.

[00147] IFN-gamma has been associated with suppression of immune response against tumors (see, e.g., Hallermalm (2008) J. Immunol. 180:3766-3774; Romieu-Mourez (2010) Cancer Res. 70:7742-7747; Lee (2005) Clinical Cancer Res. 11:107-112).

[00148] A tumor can be a heterogeneous population of more or less differentiated cells. IFN-gamma treatment of melanoma cells of a tumor can act on some of the more differentiated cells that are more susceptible to apoptosis. By eliminating these cells from the antigen source, the result can be loss of some effect on the tumor bulk following vaccination, translated by slow or no apparent regression of tumor size. Studies have shown that apoptotic cells do not activate dendritic cells (Sauter (2000) J. Exp. Med. 191:423-434).

[00149] IFN-gamma may act to skew monocyte differentiation from DCs to macrophages. The amount of IFN-gamma in the preparation may influence the incomplete differentiation of DCs by skewing the phenotype to the less specialized macrophages.

[00150] IFN-gamma may be used to enhance the MHC Class II molecules, and have a direct presentation to the T cells. However, the co-induction of Ii protein (Calprotectin) with MHC Class II molecules prevents the presentation of endogenous tumor antigens from MHC Class II molecules.

Materials and methods from first study

Autologous dendritic cell generation

[00151] Dendritic cells were generated by plastic adherence method as previously described (Choi (1998) Clin. Cancer Res. 4:2709-2716; Luft (1998) Exp. Hematol. 26:489-500). Briefly, autologous apheresis product was subjected to ficoll-hypaque (GE Healthcare, Buckinghamshire, United Kingdom) density gradient separation. The resulting peripheral blood mononuclear cells were placed in antibiotic-free AIM-V medium (Invitrogen, Grand Island, NY) supplemented with 1,000 IU/mL each of IL-

4 (CellGenix, Freisberg, Germany) and GM-CSF (Berlex, Seattle, WA) (DC medium) at 15 x 10^6 cells/mL in cell cultivation flasks (Corning-Costar, Corning, NY). After one hour incubation, the non-adherent population was discarded and fresh DC medium was added to the flasks. The following morning, the non-adherent cells were discarded, the flasks were washed once with ambient temperature PBS, and fresh DC medium was added. The flasks were then cultivated for 6 days at which time flow cytometry evaluation is performed to determine the percentage and phenotype of DC generated by this approach (pre-load DC).

Autologous tumor cell line generation

[00152] Pure tumor cells generated and characterized as previously reported were expanded to 200 million cells and then incubated with 1000 IU/mL of IFN-gamma (InterMune, Brisbane, CA) for 72 hours in 15%FBS/ECS in RPMI (complete medium), irradiated with 100 Gy from a cesium source and cryopreserved as previously described (Choi (1998) Clin. Cancer Res. 4:2709-2716; Luft (1998) Exp. Hematol. 26:489-500; Dillman (1993) J. Immunother. Emphasis Tumor Immunol. 14:65-69). The IFN-gamma treated and irradiated tumor cells were recovered from cryopreservation, washed 3x with PBS, and then added to the *in vitro* cultivated DC and incubated for ~24 hours. The antigen loaded DC were harvested by gentle scraping with a rubber policeman and cryopreserved at equal amounts in 9-11 aliquots. An aliquot of cells was obtained for flow cytometry evaluation which represents the post-loaded DC cells.

/ / /

Flow cytometry

[00153] Phenotypic characterization of the dendritic cell populations were performed using monoclonal antibodies against surface markers obtained from BD Pharmingen San Diego, CA: anti-MHC class II conjugated to PerCp, anti CD11c conjugated to APC, anti-CD80, anti-CD83, anti-CD86 conjugated to PE. Isotype controls were used to determine percent positive cells. Flow cytometry of tumor cells was conducted using antibodies against MHC class I and II conjugated to FITC, annexin-V-PE and 7-amino-actinomycin D (7-AAD) from BD Pharmingen. CaliBRITE flow cytometry calibration (BD Pharmingen) was used prior to each run and the same instrument settings were used throughout the collection of flow cytometric data.

Immunoblot assays

[00154] Cytoplasmic cell lysates were prepared with Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL) plus protease inhibitor cocktail (Roche, Indianapolis, IN) at 10,000 cells/uL on ice. Approximately 25 uLs/lane of cell lysates were separated on 12.5% tris-glycine gels, transferred to PVDF membrane and probed with antibodies against the following: calreticulin (MBL, Woburn, MA), Hsp-60, Hsp-70, Hsp-90 (R&D Systems, Minneapolis, MN), HMBG-1 (Cell Signaling, Danvers, MA), ICAM-1 (Santa Cruz Biotech, Santa Cruz, CA), Mel-4, Mart-1 (Signet, Emeryville, CA), tyrosinase (Upstate, Lake Placid, NY) and GADPH (Calbiochem, Darmstadt, Germany).

Immunohistochemistry

[00155] Expression of a panel antigens by melanoma lines were determined using immunocytochemical procedure. Cells were cultured in 8-chamber culture slides (Thermo Fisher, Rochester, NY) in the presence or absence of 1000 IU/mL IFN-gamma. After 72 hours, the cells were washed 3 times with 1X Phosphate Buffered Saline (PBS) and fixed in cold acetone. After blocking endogenous peroxidase, cells were incubated with appropriate primary antibodies against the antigens listed. Immunohistochemistry was performed using biotinylated anitmouse or rabbit immunoglobulins, Super Sensitive enzyme-conjugated streptavidin labeling and horse radish peroxidase chromogen, and substrate kits (Biogenex, San Ramon, CA). The reactivity of the following anti-human polyclonal or monoclonal antibodies was investigated with isotype matched control antibody: S-100 and HMB-45 (Biogenex, San Ramon, CA), Mel-2, Mel-5, Mart-1 (Signet, Dedham, MA), Tyrosinase and Mage-1 (Thermo Scientific, Fremont, CA), Melan-A, HLA-class I and HLA-class II (Dako, Denmark).

Statistical analysis

[00156] Student t-test of two-tailed, two samples of equal variance. Significant differences were determined by p value ≤ 0.05.

Results from the first study

[00157] Cell death was differentially induced in the autologous melanoma tumor cells line in response to incubation with IFN-gamma for 72 hours in complete medium. Trypan-blue dye exclusion assay performed on cells either treated with

IFN-gamma or not, revealed a significant trend toward lower viability in the IFN-gamma treated cells (89.1 ± 6.8% vs. 84.9 ± 9.3%, p = 0.014, N = 47). Analysis of a sample of four autologous melanoma cell lines by flow cytometry for apoptosis induction (Figure 1A) revealed that melanoma cells are differentially sensitive to the effects of IFN-gamma induced apoptosis with some cells displaying more late apoptosis or 'dead' populations (7-AAD+/Annexin-V+) while others displayed signs of early apoptosis or 'dying' populations (7-AAD-/Annexin-V+). The resulting presence of apoptotic cells after IFN-gamma treatment was associated with significant decreases in progression-free and overall survival (Cornforth (2010) Cancer Immunol. Immunother. Resistance to the proapoptotic effects of interferon-gamma on melanoma cells used in patient-specific dendritic cell immunotherapy is associated with improved overall survival). A log-rank test revealed a significant association with lower viability upon IFN-gamma treatment of melanoma tumor cells and overall survival in patients under study.

[00158] Lysates from cells that were incubated in the presence or absence of IFN-gamma were subjected to immunoblotting for a variety of molecules that may be important mediators of immunity (Figure 1B). In the setting of melanoma cells treated with IFN-gamma, heat shock proteins appear to be differentially regulated but remain largely present in the cell preparations, especially in the case of hsp-70. The endoplasmic reticulum protein, calreticulin, and high-mobility group box-1 protein (HMGB-1), appear to be up-regulated in some cases upon treatment with IFN-gamma (Figure 1B). By contrast, common melanoma antigens (mel-4, Mart-1 and tyrosinase) generally appear to be down regulated by IFN-gamma while ICAM-1, a lymphocyte adhesion molecule associated with sensitivity to lymphocyte mediated cytotoxicity (Hamai (2008) Cancer Res. 68:9854-9864), is significantly up-regulated (Figure 1C). Indeed, IFN-gamma treated melanoma tumor cells were found to be more sensitive to cytotoxic T lymphocyte (CTL) activity. Additionally, immunohistochemistry of a panel of melanoma associated antigens revealed that IFN-gamma results in the down regulation of antigen expression in many of the antigens examined (Table 1).

[00159] The use of IFN-gamma results in the up-regulation of the major histocompatibility complexes, class I and class II (Bohn (1998) J. Immunol. 161:897-908). As shown in Figure 1D, the treatment of autologous melanoma cells with IFN-

gamma resulted in the near universal and significant up-regulation of MHC class I ($p = 2.8 \times 10^{-8}$) with a median fold induction of 2.91 ± 1.13 (95% C.I.). Additionally, the mean fluorescence intensity (MFI) of MHC class II was also significantly higher but less so ($p = 0.039$) with a median induction of 4.23 ± 2.66 (95% C.I.). The level of MHC class II molecules on the surface of the autologous melanoma cells was generally lower than that of the MHC class I molecules but in 70% of the cases the induction was greater than two fold in response to IFN-gamma treatment for the MHC class II molecules due to the low initial level of MHC class II expression. The presence of these molecules on the tumor cells during loading of antigens onto dendritic cells may provide an opportunity for "cross dressing" MHC complexes onto antigen presenting cells (Dolan (2006) J. Immunol. 277:6018-6024, Dolan (2006) J. Immunol. 176:1447-1455).

[00160] A set of four representative autologous melanoma cell lines were incubated with IFN-gamma and loaded in equal amounts onto dendritic cells which were then assayed by flow cytometry for the expression of CD80, CD83, CD86 and MHC-class II. The results indicated that a small but appreciable increase in the percent positive population of dendritic cells expressing CD83 was seen upon the loading of the IFN-gamma treated melanoma cells (Figure 2). Additionally, more unprocessed tumor cells are noted in the CD86 dot plot (upper left quadrant) which resulted in a discernible reduction in the percent CD86 positive population, indicating that IFN-gamma untreated tumor cells were still present. This effect is most likely due to the induction of apoptosis by IFN-gamma, as apoptotic cells are more likely to be phagocytosed by dendritic cells as previously reported.

[00161] As shown in Figure 3, a sample of pre-loaded DC showed that they expressed CD80 (39.0 ± 16.2%), CD83 (7.1 ± 6.9%), CD86 (73.6 ± 19.5%) and were MHC class II positive with a viability of 96.2 ± 5.0%. The loaded DC had a significantly higher percentage of CD83 (9.4 ± 7.1%, $p = 0.019$) with a significantly higher mean fluorescence intensity (MFI) (172.9 ± 79.0, $p = 0.0009$) indicating that loading the DC with irradiated, IFN-gamma treated tumor cells induces maturation in some dendritic cells (Figure 3B).

Discussion from first study

[00162] Protocols for antigen loading, maturation, and administration, in the context of anti-tumor immunity, and guidance on dendritic cell (DC)-based immune therapy are practiced by the skilled artisan. This type of therapy encompasses use of purified autologous tumor cells as the source of antigen, and contains a patient-specific repertoire of tumor-associated antigens (Selvan (2010) Melanoma Res. 20:280-292; Dillman (2007) Cancer Biother. Radiopharm. 22:309-321). Some clinical trials are using unpurified autologous bulk tumors. This source of antigen may have contaminating fibroblasts and necrotic tissue (O'Rourke (2007) Melanoma Res. 17:316-322). Tumor stem cell associated antigens may be present in the purified cell lines (Dillman (2006) New Engl. J. Med. 355:1179-1181). IFN-gamma treatment increases expression of MHC class II molecules. MHC class II molecules are important for response to dendritic cell-based therapy. Molecules present in phagocytosed material, such as calreticulin, HMGB-1, and heat shock proteins, may contribute to a maturation signal, where this contribution may be in addition to contributions by cytokine cocktails. The present preparation of DCs shows a trend toward maturation, which can be associated with the phagocytosis of late stage apoptotic cells (Ip (2004) J. Immunol. 173:189-196). Use of apoptotic cells has been correlated with the generation of dendritic cells that were more effective at stimulating lymphocyte IFN-gamma secretion versus dendritic cells loaded with either tumor cell lysates or necrotic cells suggesting that dendritic cells loaded with apoptotic cells may be more potent in vivo. Resistance to the proapoptotic effects of IFN-gamma may be associated with a better clinical outcome (Cornforth (2010) Cancer Immunol. Immunother. 60:123-131). Interleukin-12 (IL-12) secretion by mature DC can lead to robust cytotoxic lymphocyte (CTL; $CD8^+$ T cells) activity. The issue of whether ex vivo maturation leads to lasting tumor immunity, has been addressed. The risk of induction of regulatory T cells, which can suppress antigen specific CTLs, by immature DC has also been shown to occur with cytokine matured DC. A re-evaluation of the sequence of signaling events that leads to maturation is being investigated to improve DC maturation protocols. Thus, the use of irradiated whole tumor cells as the antigen source in this study, without the necessity of ex vivo cytokine maturation, may be a more preferable method of DC immunotherapy since the evidence presented here indicates that the DC have begun the process of maturation. Upon injection, these "maturing" DCs may complete the process of maturation by secreting chemokines which will attract licensing, antigen-specific

CD40L expressing $CD4^+$ T cells. Serum chemokines, like CCL17/TARC produced by dendritic cells in response to the adjuvant GM-CSF, have been associated with better progression-free survival rates. In some contexts, activation of lymphocytes by dendritic cells may require the expression of co-stimulatory molecules like CD80 and CD86. As a marker of maturation, CD83, is expressed on mature dendritic cells and may correspond to dendritic cells that can induce a more potent immune response (Prazma (2008) Immunol. Lett. 115:1-8). This represents a fraction of all the cells in the pharmaceutical preparation. The number of mature DCs alone, in any one pharmaceutical regiment, may or may not be correlated with a better patient response.

/ / /

Table from the first study

[00163] Table 1: Change in the expression level of common tumor associated antigens in response to interferon-gamma in melanoma cell lines used patient-specific cell based dendritic cell therapy.

Table 1				
Antigens	No basal expression	Basal expression	Change after IFN-gamma treatment	
			None	Down
S-100	74.1%	25.9%	42.9%	57.1%

HMB-45	18.5	81.5	54.5	45/5
Mel-2	3.7	96.3	46.2	53.8
Melan-A	11.1	88.9	29.2	70.8
Mel-5	18.5	81.5	72.7	27.3
MAGE-1	51.9	48.1	38.5	61.5
MART-1	11.1	88.9	14.8	85.2
Tyrosinase	25.9	74.1	40.0	60.0

[00164] N = 27 samples.

Materials and methods for the second study

Melanoma cell lines

[00165] The commercially available melanoma cell lines A375, SK-Mel-5 and SK-Mel-28 were purchased from American Type Culture Collection (Catalogue numbers: CRL-1619, HTB-70, and HTB-72). A375, SK-Mel-5, and SK-Mel-28 were maintained in 5% fetal bovine serum in RPMI-1640 (Invitrogen, catalogue number 11875-085). The pan-capase inhibitor, z-VAD-fmk and its control compound, z-FA-fmk, were purchased from BD Pharmingen (Catalogue numbers: 550377 and 550411). Transfections of GFP-LC3 were performed as per manufacturer instructions (InvivoGen, catalogue numbers psetz-gfplc3 and lyec-12) and photomicrograph were taken on an Olympus BX-51 microscope using a DP72 digital camera. Tumor cells lines were incubated with 1000 U/mL of IFN-γ (InterMune, Cat #) for 72 hours prior to assaying. Patient-specific cell lines were generated as described (Hamai (2008) Cancer Res. 68:9854-9864; Tyring (1984) J. Natl. Cancer Inst. 73:1067-1073) by enzymatic digestion of surgical tumor samples, cultivation in RPMI-1640 tissue culture media supplemented with fetal bovine and enriched calf serum (Omega Scientific, San Diego, CA) plus 1mM sodium pyruvate, 1 mM glutamine and HEPES buffer. Phase contrast photomicrographs were taken on a Olympus CK-2 microscope using a Nikon DS-L1 digital microscope camera.

Autologous dendritic cell generation

[00166] Dendritic cells were generated by plastic adherence method of ficoled apheresis products (Selvan (2007) Int. J. Cancer. 122:1374-1383; Cornforth (2010) Cancer Immunol. 60:123-131) in antibiotic-free AIM-V medium (Invitrogen, Cat#) supplemented with 1,000 IU/mL each of IL-4 (CellGenix, Cat#) and GM-CSF (Berlex,

Seattle, WA) (DC medium). The flasks were then cultivated for 6 days prior to loading with IFN-gamma treated, irradiated autologous tumor cells.

Flow cytometry

[00167] Analysis of tumor cell death and changes in major histocompatibility class II expression in response to IFN-gamma were conducted by use of antibodies directed against MHC class II, annexin-V and 7-amino-actinomycin D (7-AAD) and acquired on a Beckton-Dickenson FACS Calibur® flow cytometer.

Western blotting

[00168] Melanoma tumor cell lysates were resolved on 10-12.5% SDS-PAGE, transferred to nitrocellulose and probed with primary antibodies overnight prior to secondary antibody conjugation and development by Novex AP Chromogenic substrate (Invitrogen, Carlsbad, CA) to develop bands. Antibodies against LC3-B antibodies (Cell Signaling Technologies, Boston, MA) and GADPH (EMD biosciences, Germany) were used at manufacturers recommended dilutions of 1:100 and 1:10,000, respectively.

Description of the second study

[00169] What was investigated was the induction of autophagy, apoptosis and MHC class II molecules after IFN-gamma treatment of melanoma tumor cells in vitro. Autologous and model melanoma tumor cell lines were incubated with 1000 IU/mL of IFN-gamma for 72 hours prior to assaying for autophagy, apoptosis and MHC class II expression. Autophagy was detected by immunobloting with antibodies against LC3 II and by flow cytometry with Enzo's CytoID Autophagy Detection Kit. Apoptosis and MHC class II induction were assayed by flow cytometry using 7-AAD and annexin-V staining and antibodies against MHC class II, respectively.

Results of the second study

[00170] The results demonstrated that IFN-gamma induces both autophagic and apoptotic cell populations in melanoma cell lines. The apoptotic population is predominantly found in the non-adherent population while the autophagic cells remain adherent to the flask. Blocking of autophagy with the inhibitor 3-methyladenine (3-MA) inhibits the induction of MHC class II positive cells in response to IFN-gamma (39.4% IFN-gamma vs. 10.0% IFN-gamma + 3-MA).

Inhibition of caspase activity with the pan caspase inhibitor Z-VAD prevents apoptosis but does not perturb autophagy in IFN-gamma treated cells (2.75 ± 0.15 IFN-gamma vs. 3.04 ± 0.27 IFN-gamma + Z-VAD, fold change). Induction of apoptosis is associated with reduced levels of autophagy and MHC class II expression. Patients receiving autologous tumor cell loaded dendritic cells that are non-apoptotic autophagic cells derived from interferon-gamma treated purified tumor cell lines have improved progression-free and overall survival (p 0.003 and p 0.002, respectively). A procedure to eliminate apoptotic cells while retaining viable autophagic cells after IFN-gamma treatment may enhance the effectiveness of this type of cell-based immunotherapy.

Pooled Analysis of Studies

[00171] Autologous, proliferating, self-renewing tumor cells (putative tumor stem cells and/or early progenitor cells), are important to establishment of new depots of metastatic cancer, and may be excellent sources of antigen for vaccines. These studies addressed the impact on survival from immunizing with antigens from such cells.

Methods

[00172] Data was pooled from three successive phase II trials, all of which included patients with documented metastatic melanoma, who were treated in protocols that utilized antigens from cell cultures of autologous tumor cells. S.C. injections were given weekly for 3 weeks, then monthly for 5 months: 74 patients were injected with irradiated tumor cells (TC): 54 patients were injected with autologous dendritic cells (DC) that had been co-cultured with irradiated autologous tumor cells (NCI-V01-1646): in a randomized phase II trial, 24 patients were injected with TC, and 18 with DC.

Results

[00173] Table 2 summarizes overall survival (OS) in each trial. In the pooled analysis there were 98 TC and 72 DC patients. Characteristics were similar in terms of age (51, 52), male gender (62%, 62%), no evidence of disease at the time of treatment (46%, 47%), and presence of M1c visceral disease at the time of treatment (13%, 14%). OS was longer in patients treated with DC (median 63.1 vs 20.2

months, 5-year OS 51% vs 26%, p=0.0002 Mantle-Cox log-rank test). The difference in OS in the randomized trial is also significant (p=0.007).

[00174] Patient-specific DC vaccines primed with antigens from autologous proliferating, self-renewing tumor cells are associated with encouraging long-term survival rates, and are superior to patient-specific TC vaccines in populations of patients who have been diagnosed with metastatic melanoma.

Table 2.

Vaccine	# patients	# deaths	Median OS	2-yr OS	5-yr OS
TC	74	60	20.3 mos	45%	28%
DC (Use IFN-gamma treated melanoma cells)	54	31	58.4 mos	72%	50%
TC	24	16	15.9 mos	31%	---
DC (No IFN-gamma treatment of melanoma cells)	18	5	Not Reached	72%	---

[00175] The survival curves from the three trials of patient specific vaccines are shown in **Figure 13**. Consecutive phase I and II clinical trials were conducted using autologous tumor cells, in combination with autologous dendritic cells or without the dendritic cells, were conducted. Subcutaneous injections were given weekly for three (3) weeks, then monthly for five (5) months, 74 patients were injected with irradiated tumor cells without pretreatment with IFN-gamma (TC): 54 patients were injected with autologous dendritic cells (DC) that had been co-cultured with irradiated autologous tumor cells with pretreatment with IFN-gamma : in a randomized phase II trial, 24 patients were injected with TC without pretreatment without IFN-gamma, and 18 with DC plus TC without pretreatment with IFN-gamma .

[00176] **Figure 14** shows survival curves from three trials, where the trials are the same clinical trials as those disclosed in Figure 13, but with additional data acquired from later time points, as is evident from comparing the step plots in the two figures. The melanoma cells in the clinical trials, TC-24 and TC-74, did not receive IFN-gamma. The melanoma cells in the clinical trial, DC-TC-18, did not receive IFN-gamma. The melanoma cells in the clinical trial, DC-TC-54, did get IFN-gamma.

[00177] A non-limiting standard operating procedure for preparing dendritic cell vaccine includes the following (Table 3). Upon harvesting tumor cells after expansion, the following are to be made for each patient's tumor cell lot. What is needed is about 220 million cells to make the tumor cell vaccine lot. Any extra cells are to be cryopreserved as back up cells. Make stock cell suspension as 220 x 10^6 cells in 22 ml medium to distribute in the following manner (Table 3).

Table 3. Operating Procedure

Use	Total cell # needed	First action	Second action	Final disposition
TC Vaccine Doses or DC Loading Cells	150 million	15 ml from the stock to a 50 ml conical tube, add 25 ml AIM-V, and irradiate	Cryopreserve cells after irradiation in 10 small cryovials.	Store until needed for patient treatment.

[00178] Trial #2: DC 2000-2006 (NCI-V01-1646). Phase II Trial of Autologous Dendritic Cells Loaded with Antigens from Irradiated Autologous Tumor Cells as Patient Specific Vaccines (BB-IND 8554): Dendritic Cell (DC) Vaccine. In the production of the vaccine for this trial, autologous proliferating tumor cells were co-incubated with IFN-gamma, cryopreserved, and then subsequently co-incubated with autologous dendritic cells. Each aliquot of cells was suspended in 500 micrograms of GM-CSF for injection

[00179] Trial #3: DC vs. TC 2007-2011 (NCT00436930): Randomized Phase II Trial Of Autologous Vaccines Consisting Of Adjuvant GM-CSF plus Proliferating Tumor Cells Versus GM-CSF Plus Dendritic Cells Loaded With Proliferating Tumor Cells In Patients With Metastatic Melanoma (BB-IND 8554 and BB-IND 5838): 'MAC VAC.' The third trial was a randomized trial to determine whether there was a difference in the two approaches noted above. IFN-gamma was not used in the production of the tumor cells. As in the DC trial above, all patients were randomized to receive either TC or DC injected s.c. with 500 micrograms of GM-CSF, weekly for 3 weeks and then monthly for five months. The projected 72% 2-year survival rate for patients in the DC arm is comparable to the 71% observed 2-year survival observed in the previous 54-patient trial of DC in which the median survival was five years.

Information regarding colon cancer, ovarian cancer, glioblastoma, and renal cancer cells

[00180] The following provides flow cytometry data from studies of colon cancer cells (endoderm), ovarian cancer cells (mixed mesoderm plus extra-embryonic), glioblastoma cells (ectoderm), and renal cancer cells (mesoderm). The embryological points of origin of each of these types of cells is indicated in parenthesis. Melanoma cells are of neurocrest origin. Table 3 summarizes the tumor cell types, while Table 4 summarizes the results from experiments on these cells, where the cells were treated with interferon-gamma (IFN-gamma).

[00181] Tumor cells have various similarities with adult stem cells, such as potential to self-propagate and differentiate in more mature type of cells. The following data demonstrate that autophagia is phenomenon that can be induced to tumor cells derived from all embryonic layers. In addition to melanoma, which originated from cells derived from neurocrest, the present study involved tumors generated in tissue derived from endoderm (colon), mesoderm (kidney), ectoderm (glioblastoma) and mixed mesoderm plus extra-embryonic (ovarian).

Table 3. Figure legend to tumor types	
Sample	Tumor type
A	Colon
B	Ovarian
C	Glioblastoma
D	Renal cell carcinoma

[00182] Table 4 discloses the following results. Autophagy was shown to be induced in the tumor types examined based on the induction of MHC class II complexes in the absence of apoptosis (phosphatidylserine exposure) in the adherent cell populations upon interferon-gamma treatment.

Table 4		Response to interferon-gamma			
Sample	Tumor type	Induction of MHC class I	Induction of MHC class II	Induction of apoptosis	Induction of autophagy
A	Colon	Yes	Yes	No	Yes
B	Ovarian	Yes	Yes	No	Yes
C	Glioblastoma	No	Yes	Yes	Yes
D	Renal Cell Carcinoma	No	Yes	Yes	Yes

Materials and methods

[00183] Pure tumor cells were generated from colon, ovarian, glioblastoma, and renal cell carcinoma according to Dillman and Cornforth, et al. (Cornforth, et al. (2011) Cancer Immunol. Immunother. 60:123-131; Dillman, et al. (1993) J. Immunother. Emphasis Tumor Immunol. 14:65-69; Dillman, et al. (2000) Cancer Biother. Radiopharm. 15:161-168; Dillman, et al. (1999) 14:443-449; Dillman et al. (2000) 15:161-168).

[00184] Briefly, tumor biopsies from surgical samples were enzymatically processed into single cell suspensions and purified cell lines generated by differential attachment and serum starvation. The cell lines generated were cryopreserved in liquid nitrogen, thawed and treated with or without interferon-gamma in 5% fetal bovine serum/RPMI media for 72 hours. After exposure to interferon-gamma, the non-adherent cells were analyzed separately from the adherent cells by flow-cytometry. The markers investigated were MHC class I, MHC class II, Annexin V (apoptosis), and 7-ADD (viability).

[00185] Figure 14 discloses the following. The data are from bulk cells without any separation into adherent cells and non-adherent cells. Interferon-gamma treatment increases the expression of major histocompatibility complexes: Colon (A), Ovarian (B), Glioblastoma (C), and Renal Cell Carcinoma (D). Tumor cell lines were harvested after being treated with or without 1000 IU/mL IFN-gamma for 72 hours and then assayed for MHC class I and class II by flow-cytometry (Figure 1A). Figure 15 reveals the following. Control isotype antibodies were used to identify positive populations. The fold change in mean fluorescence intensity (MFI) for major histocompatibility complex class I and class II are summarized in Figure 15.

[00186] Figure 16 discloses the following. Apoptosis is induced by IFN-gamma treatment of tumor cells from a variety of sources. Colon (A), Ovarian (B), Glioblastoma (C), and Renal Cell Carcinoma (D). Tumor cell lines were harvested after being treated with or without 1000 IU/mL IFN-gamma for 72 hours and then assayed for phosphatidylserine exposure by annexin-V and cell viability by 7-AAD.

[00187] Figure 17 discloses the following. Non-apoptotic, autophagic, MHC class II expressing cells are induced by IFN-gamma treatment of tumor cells from a variety of sources in the adherent population of cells (but not in the non-adherent population). Colon cancer (A), Glioblastoma (C), and Renal Cell Carcinoma (D). The

non-adherent populations of tumor cells were removed by washing with HBSS and the adherent cells were harvested after being treated with or without 1000 IU/mL IFN-gamma for 72 hours and then assayed for phosphatidylserine exposure by annexin-V and major histocompatibility complex class II in the 7-AAD negative fractions (Figure 17).

[00188] Figure 18 discloses the following. Control isotype antibodies were used to identify positive populations. The fold change in mean fluorescence intensity (MFI) for major histocompatibility complex II is summarized in Figure 18 in the non-adherent cell population. Because MHC class II expression is a surrogate for autophagy, the Mean Fluorescence Intensity (MFI) data of this figure represents the fold-change, shown in the figures as changes that are increases in autophagy, for colon cancer cells (A), glioblastoma (C), and renal cell carcinoma cells (D). The cells used were from a non-apoptotic population. The changes in MFI were in response to IFN-gamma treatment.

[00189] Figure 19 discloses the following. The figure discloses induction of autophagy in the adherent, non-apoptotic cells (Annexin-V) from the four tumor types examined. After treatment with or without 1000 IU/mL IFN-gamma for 72 hours, the non-adherent populations were removed by washing with HBSS and the adherent cells were harvested and assayed for phosphatidylserine exposure by annexin-V and major histocompatibility complex class II in the 7-AAD negative fractions. Colon (A), Ovarian (B), Glioblastoma (C), and Renal Cell Carcinoma (D).

Results

[00190] MHC class II molecules are normally expressed exclusively on specialized cells such as dendritic cells, mononuclear phagocytes, B lymphocytes, some endothelial cells and thymus. As shown in Table 4, the expression of MHC class II was found clearly expressed by all the investigated samples as the result of the described in-vitro manipulations. Some of the samples were more sensitive to IFN-gamma and resulted in induction of apoptosis which may be a feature associated with the expression of apoptotic pathway proteins such as caspases and bcl family proteins.

[00191] MHC class II molecule expression on the tumor cells after the described in vitro manipulations is the result of lysosomal processing, thus the results indicate

that induction of autophagy occurs in the adherent, non-apoptotic population in tumor cell lines derived from all embryonic germ layers (ectoderm, mesoderm and endoderm) exemplified here by colon, ovarian, glioblastoma and renal cell carcinoma. This response to the interferon-gamma is identical with the response seen with melanoma cells and suggests that this feature could be used as means to generate non-apoptotic autophagic cells populations that we show to be the key component in cell-based immunotherapy for cancer. The MHC class II presenting tumor cells as the result of autophagy can interact directly with the helper T lymphocytes ($CD4^+$ T cells) and induce an immune response. The complex of dendritic cells and autophagic tumor cells induce a faster and more robust response in patients compared to other immune therapies that are based on dendritic cells alone, non-manipulated tumor cell population or cell lysate from tumors, as demonstrated by our clinical data.

[00192] The use of MHC class II expression as surrogate marker for autophagy in response to interferon-gamma treatment is well supported by the evidence presented here and in cited works that show that autophagy has been described to participate in the translocation of endogenous protein into the MHC class II loading compartment. This effect is revealed by the increase in the expression of MHC class II complexes on the surface of autophagic cells (see, e.g., Crotzer and Blum (2009) J. Immunol. 182:3335-3341). The present disclosure, or cited priority documents, demonstrates that the use of the autophagy inhibitor 3-methyl adenine resulted in the loss of MHC class II expression despite treatment with interferon-gamma. Thus, measurement of the induction of MHC class II can be used as a surrogate marker for autophagy.

[00193] Thus, while there have shown and described and pointed out fundamental novel features of the disclosure as applied to an exemplary implementation and/or aspects thereof, it will be understood that various omissions, reconfigurations and substitutions and changes in the form and details of the exemplary implementations, disclosure and aspects thereof may be made by those skilled in the art without departing from the spirit of the disclosure and/or claims. For example, it is expressly intended that all combinations of those elements and/or method steps which perform substantially the same function in substantially the same way to achieve the same results are within the scope of the disclosure. Moreover, it should be recognized that

structures and/or elements and/or method steps shown and/or described in connection with any disclosed form or implementation may be incorporated in any other disclosed or described or suggested form or implementation as a general matter of design choice. It is the intention, therefore, to not limit the scope of the disclosure. All such modifications are intended to be within the scope of the claims appended hereto.

[00194] All publications, patents, patent applications, references, and sequence listings, cited in this specification are herein incorporated by this reference as if fully set forth herein.

[00195] The Abstract is provided to comply with 37 CFR §1.72(b) to allow the reader to quickly ascertain the nature and gist of the technical disclosure. The Abstract is submitted with the understanding that it will not be used to interpret or limit the scope or meaning of the claims.

What is claimed is:

Claim 1. A population of mammalian dendritic cells comprising:

cancer-specific peptides from cancer cells taken from a subject that has a cancer;

wherein the cancer-specific peptides are acquired in vitro by dendritic cells from said cancer cells;

wherein the cancer cells are not treated in vitro with interferon-gamma (IFN-gamma) or with IFN-gamma mimetic;

wherein greater than about 60 percent (%) of said cancer cells that are not treated in vitro with IFN-gamma or IFN-gamma mimetic, are autophagic and non-apoptotic, and,

wherein the dendritic cells and cancer cells are from the same subject.

Claim 2. The population of dendritic cells of claim 1, wherein the cancer is from a tissue of one or more of endodermal origin, mesodermal origin, or ectodermal origin.

Claim 3. The population of dendritic cells of claim 1, wherein the cancer is from a tissue of neural crest origin, and wherein the neural crest is of ectodermal origin.

Claim 4. The population of dendritic cells of claim 1, wherein the cancer is from a tissue of one or more of endodermal origin, mesodermal origin, or ectodermal origin, and where the cancer is melanoma of neural crest origin, colon cancer of endoderm origin, renal cancer of mesoderm origin, glioblastoma of ectoderm origin, or ovarian cancer of mixed mesoderm plus extra-embryonic origin.

Claim 5. The population of dendritic cells of claim 1, wherein greater than 80% of said cancer cells that are not treated in vitro with IFN-gamma or IFN-gamma mimetic, are autophagic and non-apoptotic.

Claim 6. A vaccine for the subject of claim 1 comprising the population of dendritic cells of claim 1.

Claim 7. The population of dendritic cells of claim 1, wherein essentially all of the cancer cells that are not treated with IFN-gamma or IFN-gamma mimetic are incapable of cell division.

Claim 8. The population of dendritic cells of claim 1, wherein essentially all of the cancer cells that are not treated with IFN-gamma or IFN-gamma mimetic are irradiated and incapable of cell division.

Claim 9. The population of dendritic cells of claim 1, wherein at least 80% of the cancer cells that are not treated with IFN-gamma or IFN-gamma mimetic are irradiated and incapable of cell division.

Claim 10. The population of dendritic cells of claim 1, wherein at least 80% of the cancer cells that are not treated with IFN-gamma or IFN-gamma mimetic are treated with a nucleic acid cross-linker and are incapable of cell division.

Claim 11. The population of dendritic cells of claim 1 that comprise one or more peptides derived from a melanoma-specific antigen that is S-100, HMB-45, Mel-2, Melan-A, Mel-5, MAGE-1, MART-1, or Tyrosinase, and wherein the cancer is melanoma.

Claim 12. The population of dendritic cells of claim 1, wherein the given subject is a human subject.

Claim 13. The population of dendritic cells of claim 1, wherein the given subject is a mammal that is not human.

Claim 14. A melanoma vaccine comprising:

at least one mature dendritic cell from a subject that has melanoma;

wherein the at least one mature dendritic cell had been contacted with at least one cancer tumor cell from the same subject,

wherein the at least one cancer tumor cell that is contacted with the at least one mature dendritic cell is non-dividing, autophagic, and non-apoptotic.

Claim 15. A method for stimulating immune response against a cancer-specific antigen comprising administering an immune-stimulatory amount of the dendritic cells of claim 1 to the subject.

Claim 16. The method of claim 15, wherein the immune response that is stimulated comprises one or more of $CD4^+$ T cell response, $CD8^+$ T cell response, and B cell response.

Claim 17. The method of claim 16, wherein the $CD4^+$ T cell response, $CD8^+$ T cell response, or B cell response, can be measured by ELISPOT assays, by intracellular cytokine staining assays, by tetramer assays, or by detecting antigen-specific antibody production.

Claim 18. The method of claim 15, wherein the immune response comprises a survival time that comprises 2-year overall survival (OS), and where the 2-year overall survival is at least about 60%.

Claim 19. The method of claim 15, wherein the administration comprises subcutaneous injections of the vaccine.

Claim 20. The method of claim 15, wherein the administration comprises injections of the vaccine given weekly for three months and then monthly for five months.

/ / /

Claim 21. A method for preparing a dendritic cell vaccine, involving cancer cells and dendritic cells from the same subject, the method comprising:

one or more cancer cells is treated with an agent that prevents cell division;

the one or more cancer cells are not treated in vitro with interferon-gamma (IFN-gamma) or with an IFN-gamma mimetic;

cancer cells that are autophagic and non-apoptotic are selected;

cancer cells that are non-autophagic and apoptotic are rejected; and, wherein the cancer cells that are autophagic and non-apoptotic are provided to one or more autologous dendritic cells, or, wherein peptides derived from the cancer cells that are autophagic and non-apoptotic are provided to one or more autologous dendritic cells.

Claim 22. A composition comprising:

at least one cancer cell that is not treated with interferon-gamma (IFN-gamma) from a first subject, and at least one antigen presenting cell (APC) from the same first subject, wherein the cancer cell is:

autophagic; and,

non-apoptotic.

Claim 23. The composition of claim 22, wherein the cancer cell is MHC class II-expressing.

Claim 24. The composition of claim 22, wherein the APC is a dendritic cell, a macrophage, or a B cell.

Claim 25. The composition of claim 22, wherein the at least one cancer cell comprises cancer-specific peptides, and wherein the cancer specific-peptides are substantially not contained in said APCs and are substantially not processed by said APCs.

Claim 26. The composition of claim 22, wherein the cancer cells comprise melanoma-specific peptides, and wherein the cancer specific-peptides are substantially contained in said APCs and are partially or substantially processed in said APCs.

/ / /

Claim 27. The composition of claim 22, wherein the cancer cell is loaded into the APC.

Claim 28. The composition of claim 22, wherein the cancer cell is not loaded into the APC.

Claim 29. The composition of claim 19, wherein autophagy is demonstrated by a test that assays microtubule-associated protein light chain 3 (LC3).

Claim 30. The composition of claim 22, wherein the cells are demonstrated to be non-apoptotic using at least one of the reagent, 7-aminoactinomycin D (7-ADD), or the reagent, annexin.

Claim 31. A method of stimulating immune response in a subject having a cancer and comprising cancer cells, wherein the subject is the same subject as the first subject, comprising administering an immunology effective amount of the composition of claim 22.

Claim 32. The composition of claim 22, wherein at least 90% of the cancer cells are not treated in vitro with IFN-gamma, and less than 10% of the cancer cells are treated in vitro with IFN-gamma.

Claim 33. A method for manufacturing the vaccine of claim 1 or the composition of claim 19, comprising contacting at least one cancer tumor cell to at least one antigen presenting cell (APC), wherein the at least one cancer tumor cell is from a first human subject, and wherein the at least one APC is from the same first human subject.

Claim 34. A method for preparing a dendritic cell vaccine, comprising: treating cancer cells acquired from a first subject with an agent that prevents cell division; wherein the cancer cells are not treated in vitro with IFN-gamma or an IFN-gamma mimetic; selecting cancer cells that are autophagic and non-apoptotic; and, contacting the selected cancer cells with autologous dendritic cells from the same first subject.

Claim 35. A composition that comprises a dendritic cell vaccine, as prepared by the method of claim 34.

Claim 36. A method for stimulating immune response against a cancer-specific antigen, comprising administering the composition of claim 34 to a subject that has cancer.

FIG.1

Pure tumor cell culture in flasks
Interferon-gamma treatment for 72 hours
Floating non-autophagic, apoptotic cells
Adherent autophagic, non-apoptotic cells
Tissue culture flask surface
Detached non-autophagic, apoptotic cell
Attached autophagic,non-apoptotic cell
Major histocompatibility complex II-over expressed in autophagic cells
Phosphatidylserine-apoptotic marker

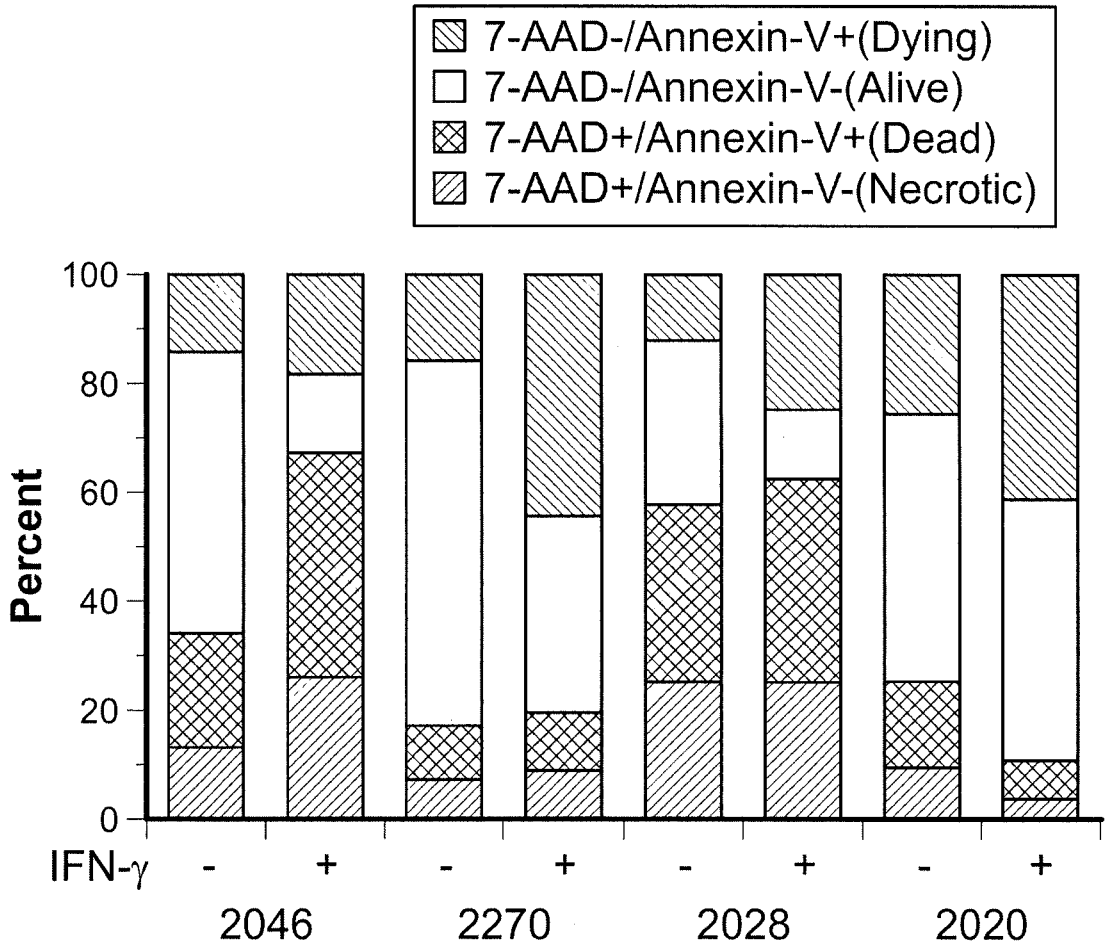

7-AAD-/Annexin-V+(Dying)
7-AAD-/Annexin-V-(Alive)
7-AAD+/Annexin-V+(Dead)
7-AAD+/Annexin-V-(Necrotic)
Percent
100
80
60
40
20
0
IFN-γ - + - + - + - +
2046
2270
2028
2020

FIG. 2A

FIG.2B

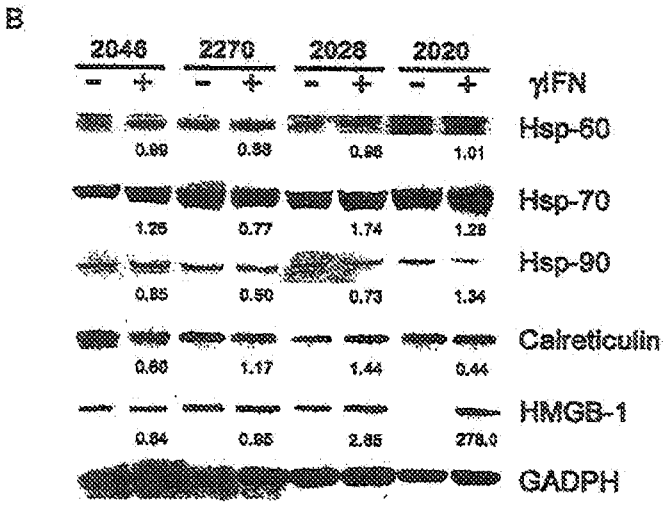

B
2046
2270
2028
2020
- + - + - + - +
γIFN
Hsp-60
0.89 0.93 0.98 1.01
Hsp-70
1.25 0.77 1.74 1.28
Hsp-90
0.85 0.50 0.73 1.34
Calreticulin
0.68 1.17 1.44 0.44
HMGB-1
0.84 0.85 2.85 278.0
GADPH

FIG.2C

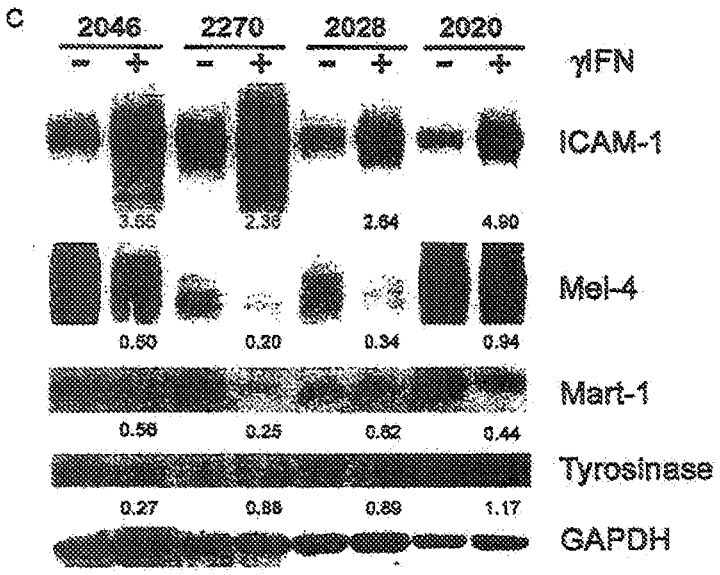

C
2046
2270
2028
2020
- + - + - + - +
γIFN
ICAM-1
3.66
2.36
2.64
4.90
Mel-4
0.50
0.20
0.34
0.94
Mart-1
0.56
0.25
0.62
0.44
Tyrosinase
0.27
0.88
0.89
1.17
GAPDH

FIG.2D

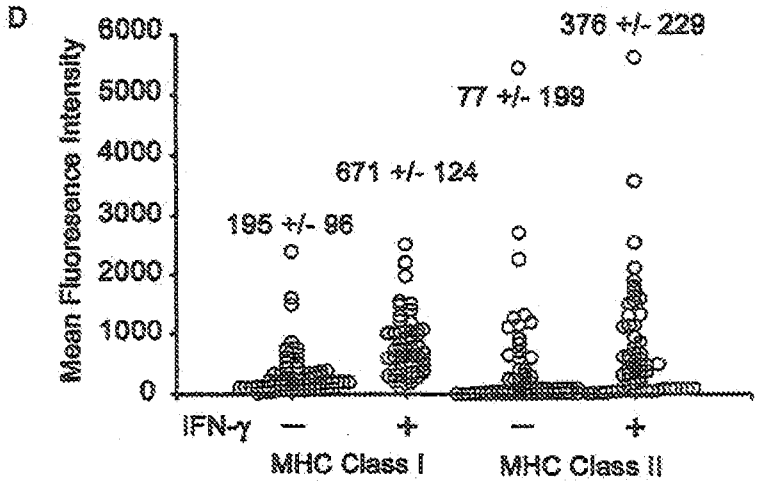
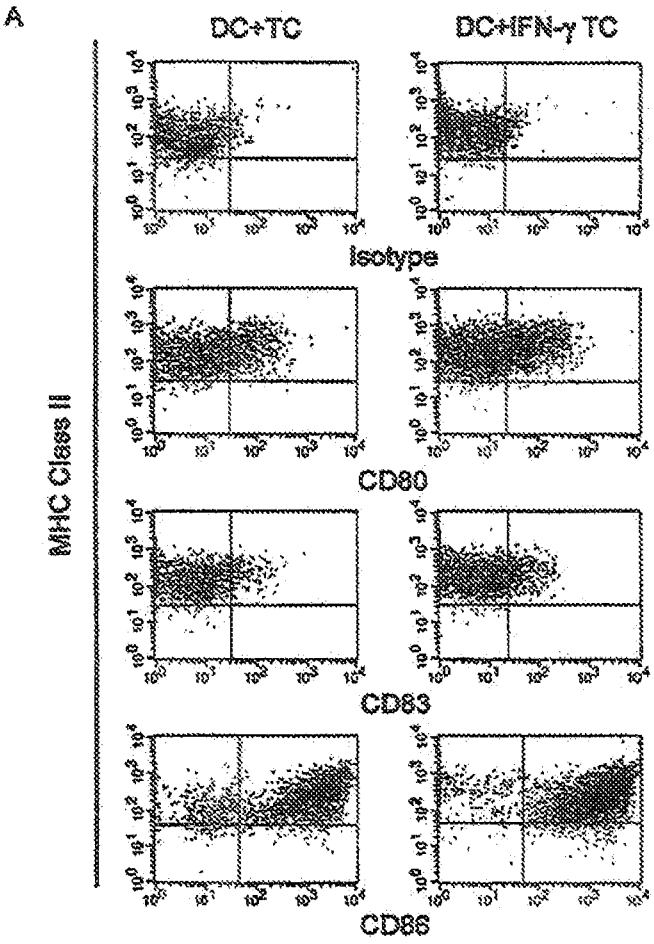

D
Mean Fluorescence Intensity
6000
5000
4000
3000
2000
1000
0
195 +/- 96
671 +/- 124
77 +/- 199
376 +/- 229
IFN-γ
—
+
—
+
MHC Class I
MHC Class II

FIG.3A



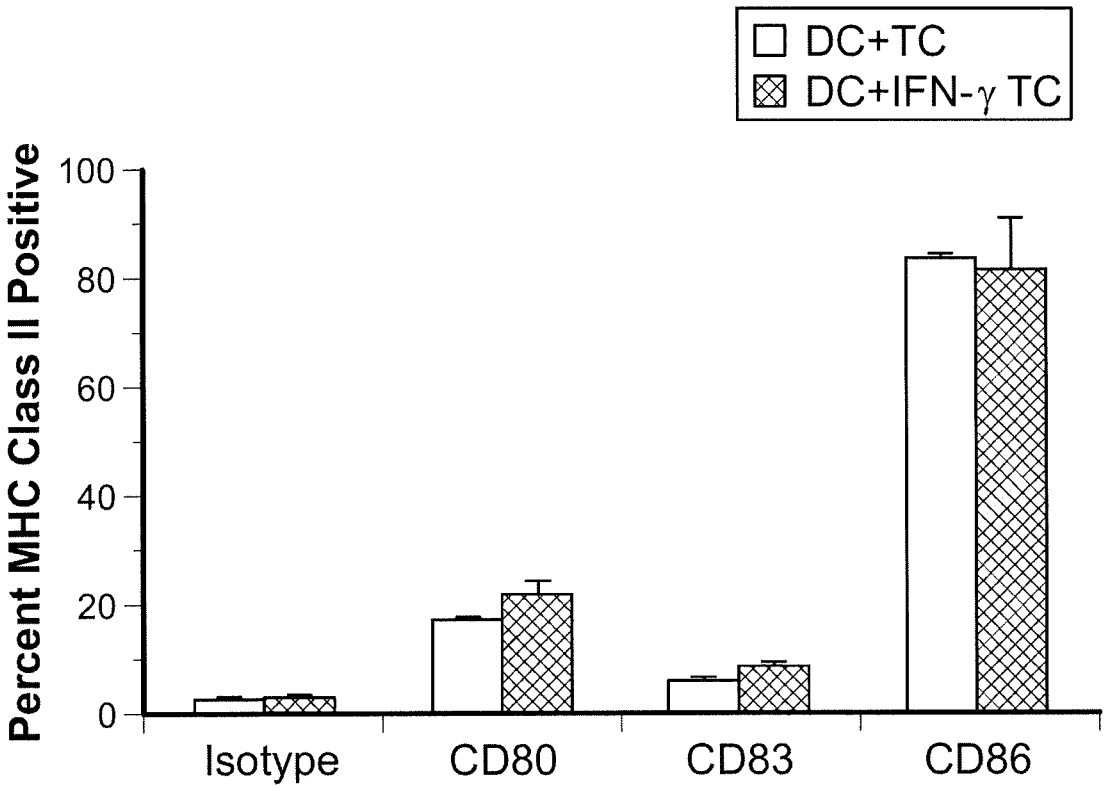
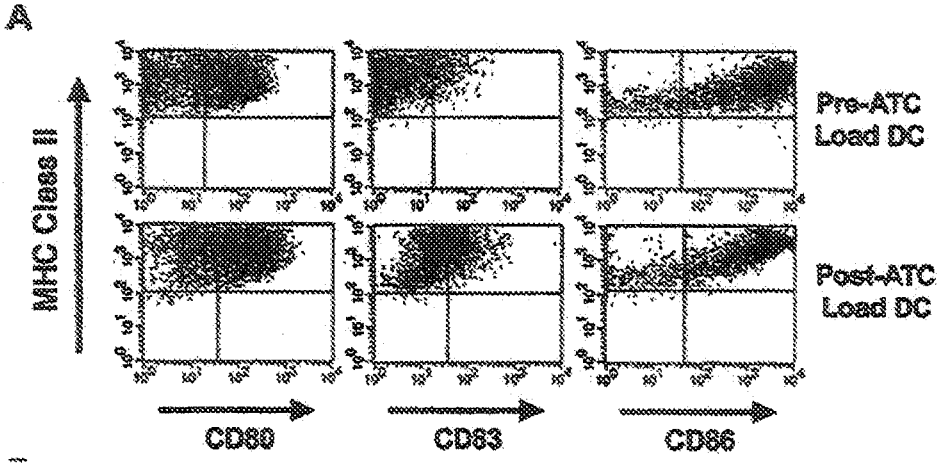

DC+TC
DC+IFN-γ TC
Percent MHC Class II Positive
100
80
60
40
20
0
Isotype
CD80
CD83
CD86

FIG. 3B

FIG.4A



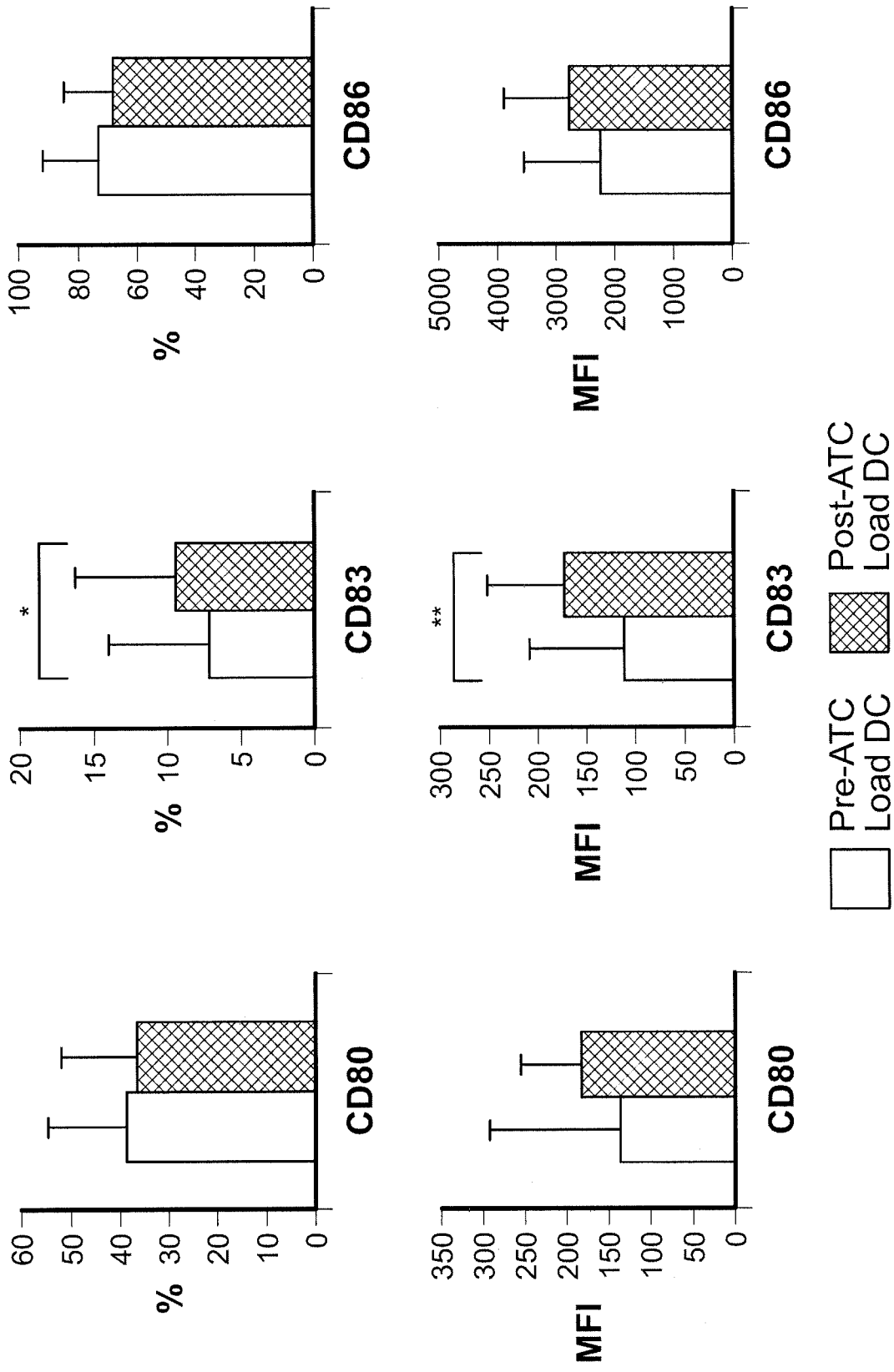
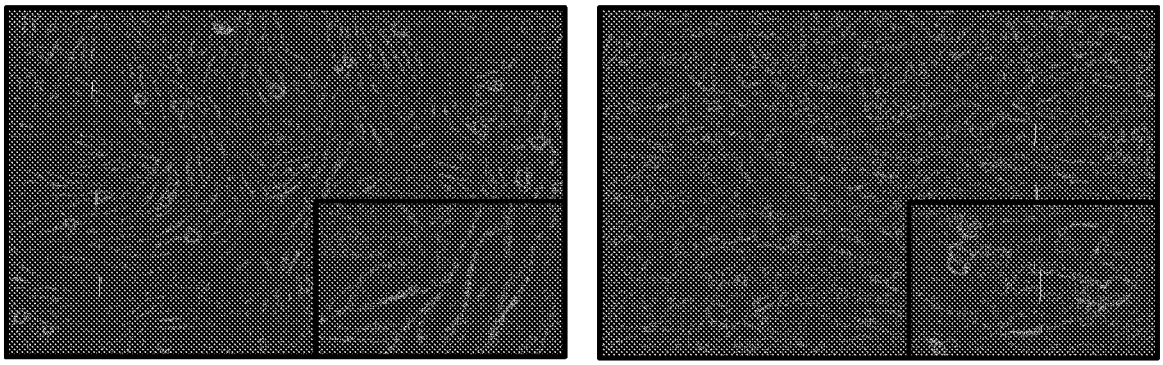
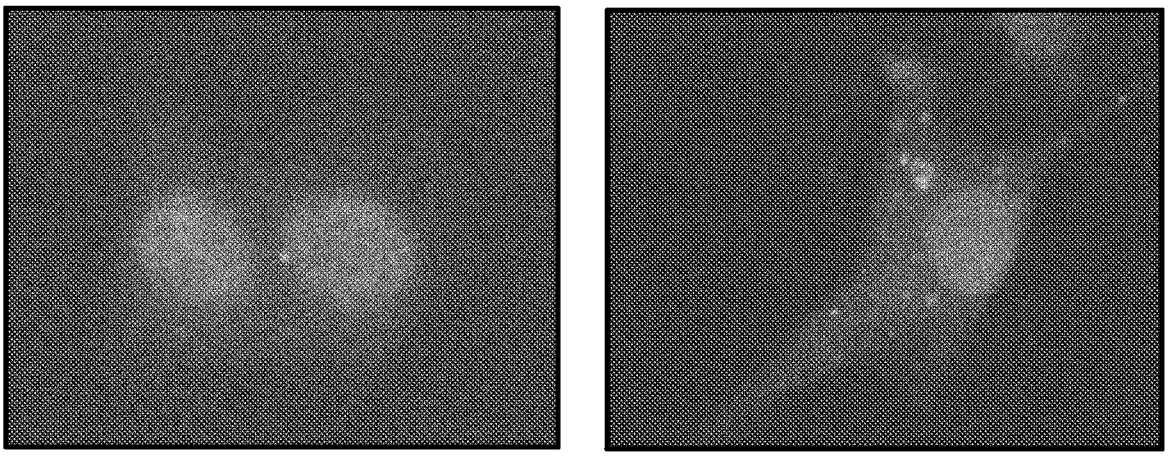


FIG. 4B



No IFN-γ IFN-γ

FIG. 5A



No IFN-γ IFN-γ

FIG. 5B

FIG.5C

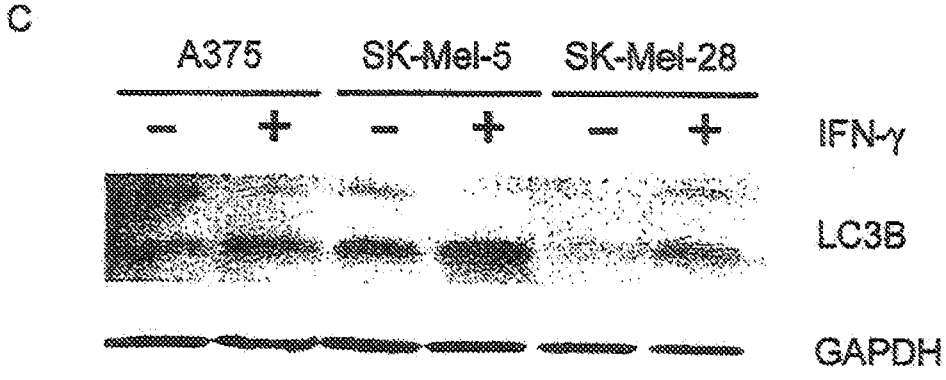


FIG.6A

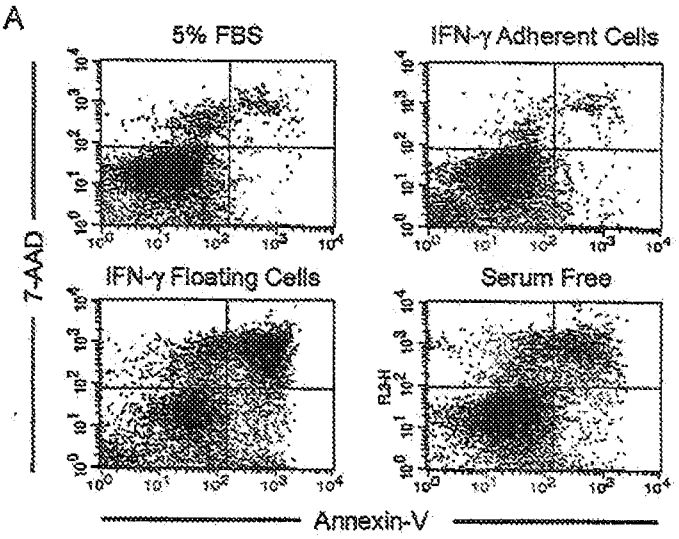
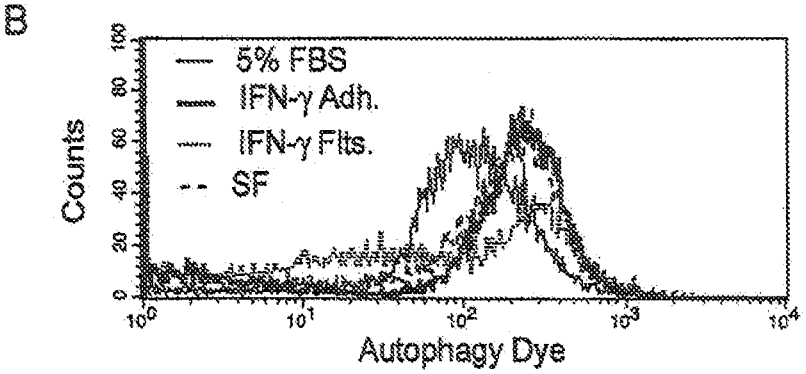

A
5% FBS
IFN-γ Adherent Cells
IFN-γ Floating Cells
Serum Free
7-AAD
Annexin-V
R2H

FIG.6B



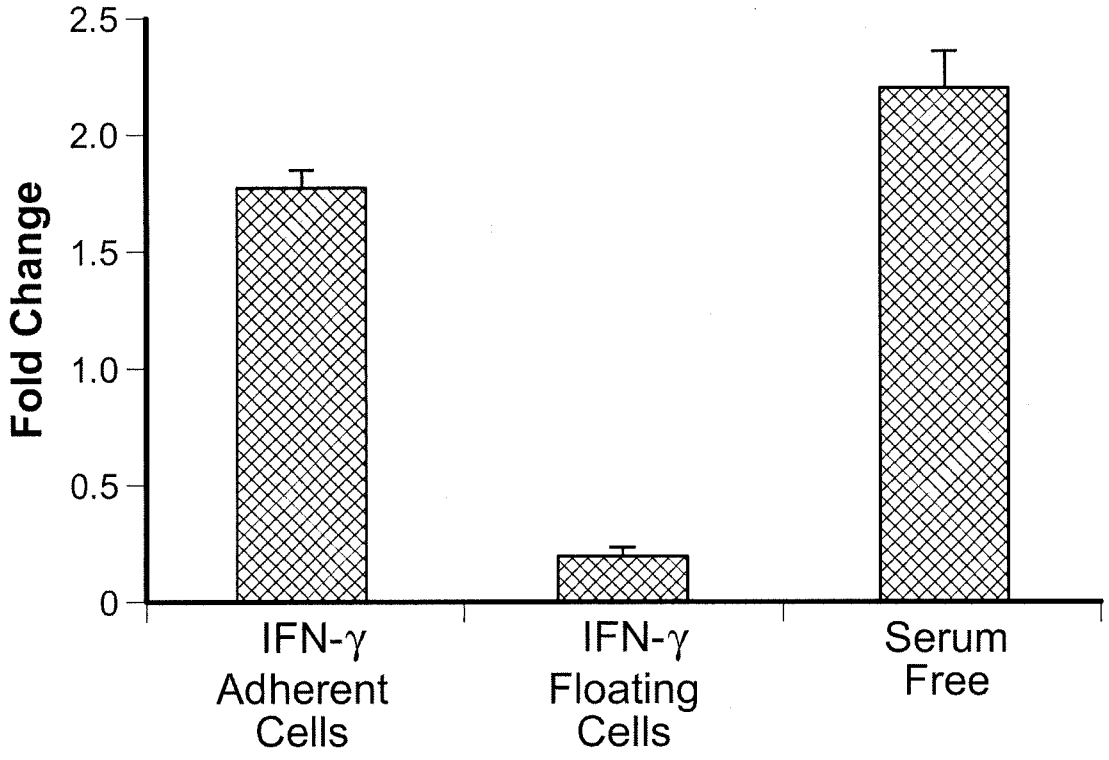

Fold Change
2.5
2.0
1.5
1.0
0.5
0
IFN-γ Adherent Cells
IFN-γ Floating Cells
Serum Free

FIG. 6C

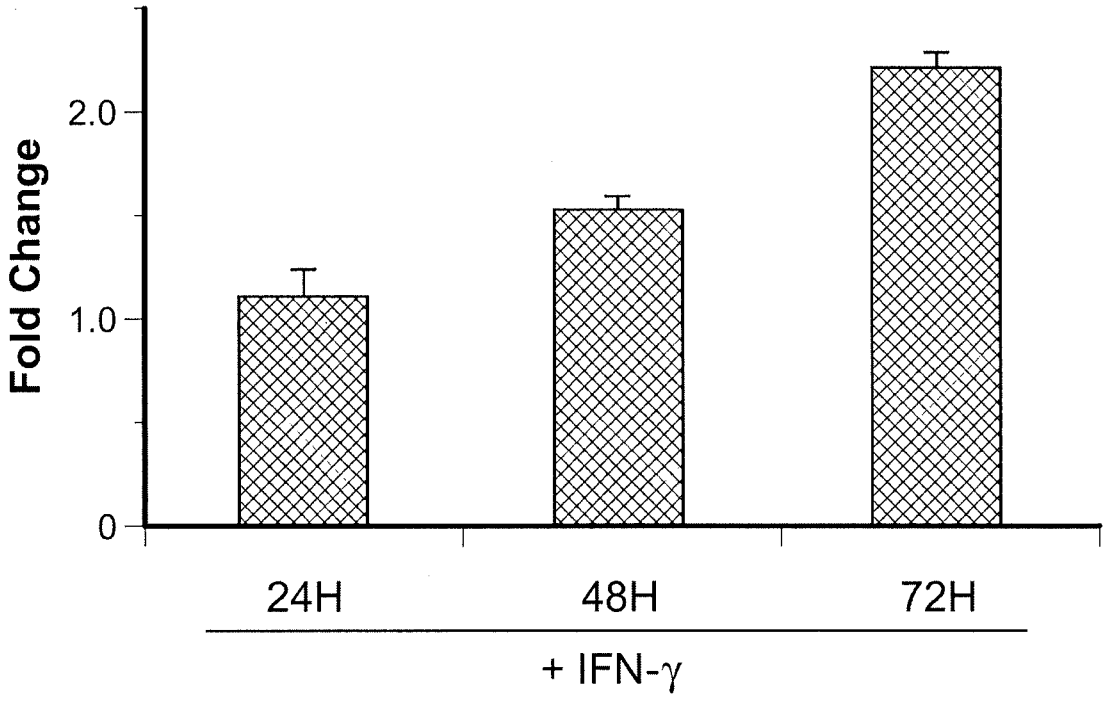


FIG. 7A

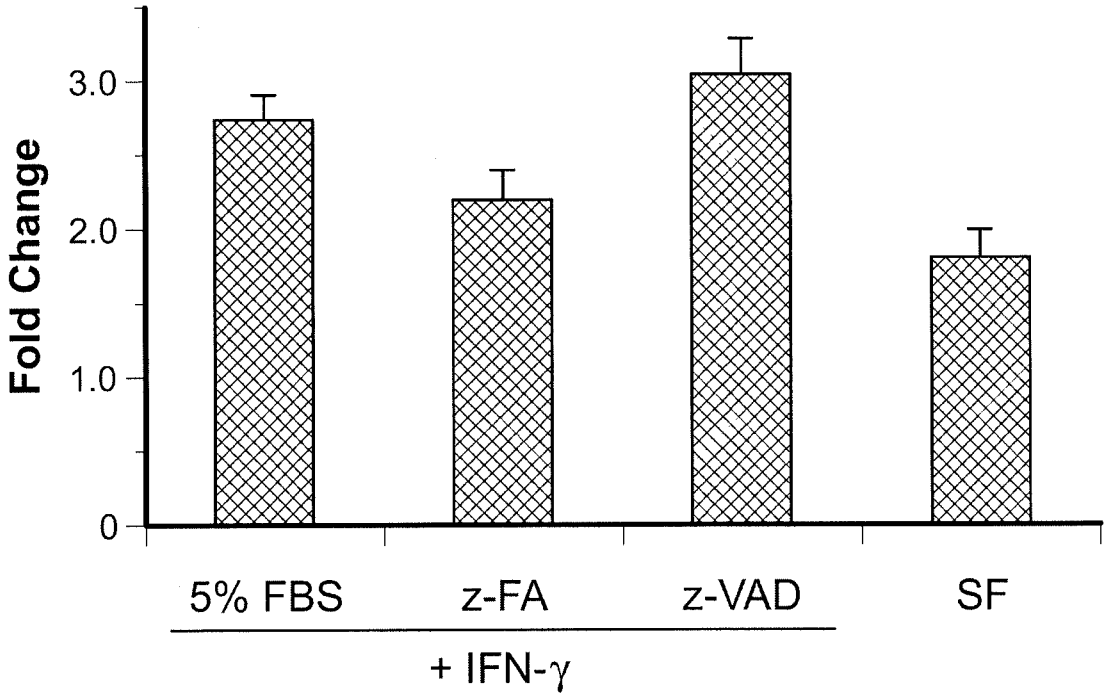
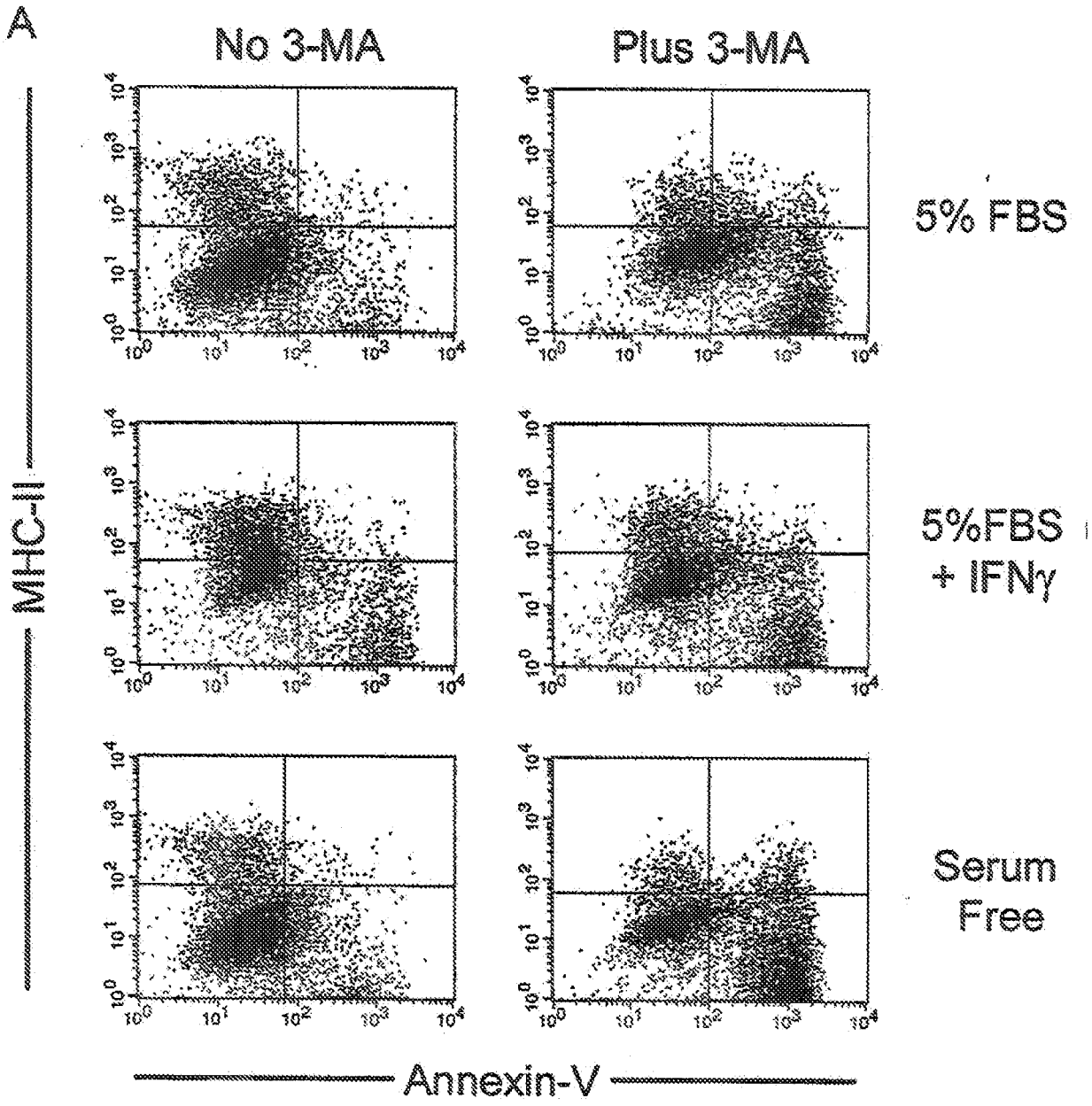


FIG. 7B

FIG.8



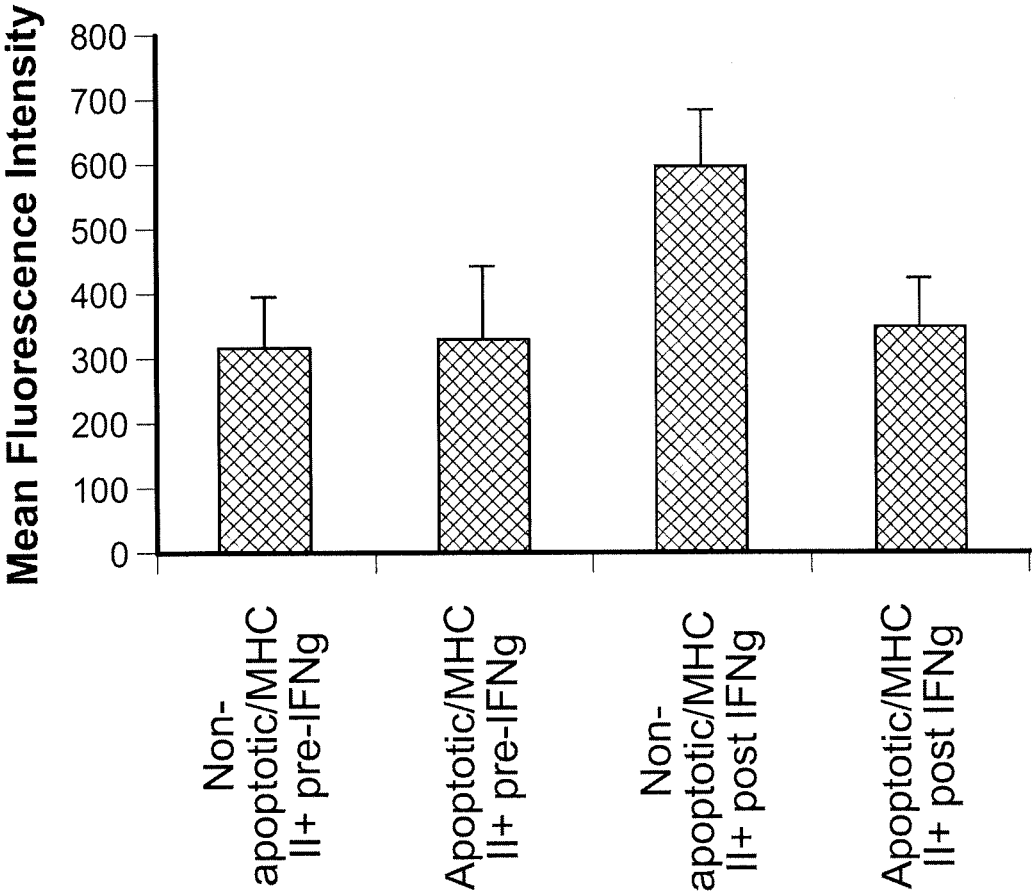

Mean Fluorescence Intensity
800
700
600
500
400
300
200
100
0
Non-apoptotic/MHC II+ pre-IFNg
Apoptotic/MHC II+ pre-IFNg
Non-apoptotic/MHC II+ post IFNg
Apoptotic/MHC II+ post IFNg

FIG. 9

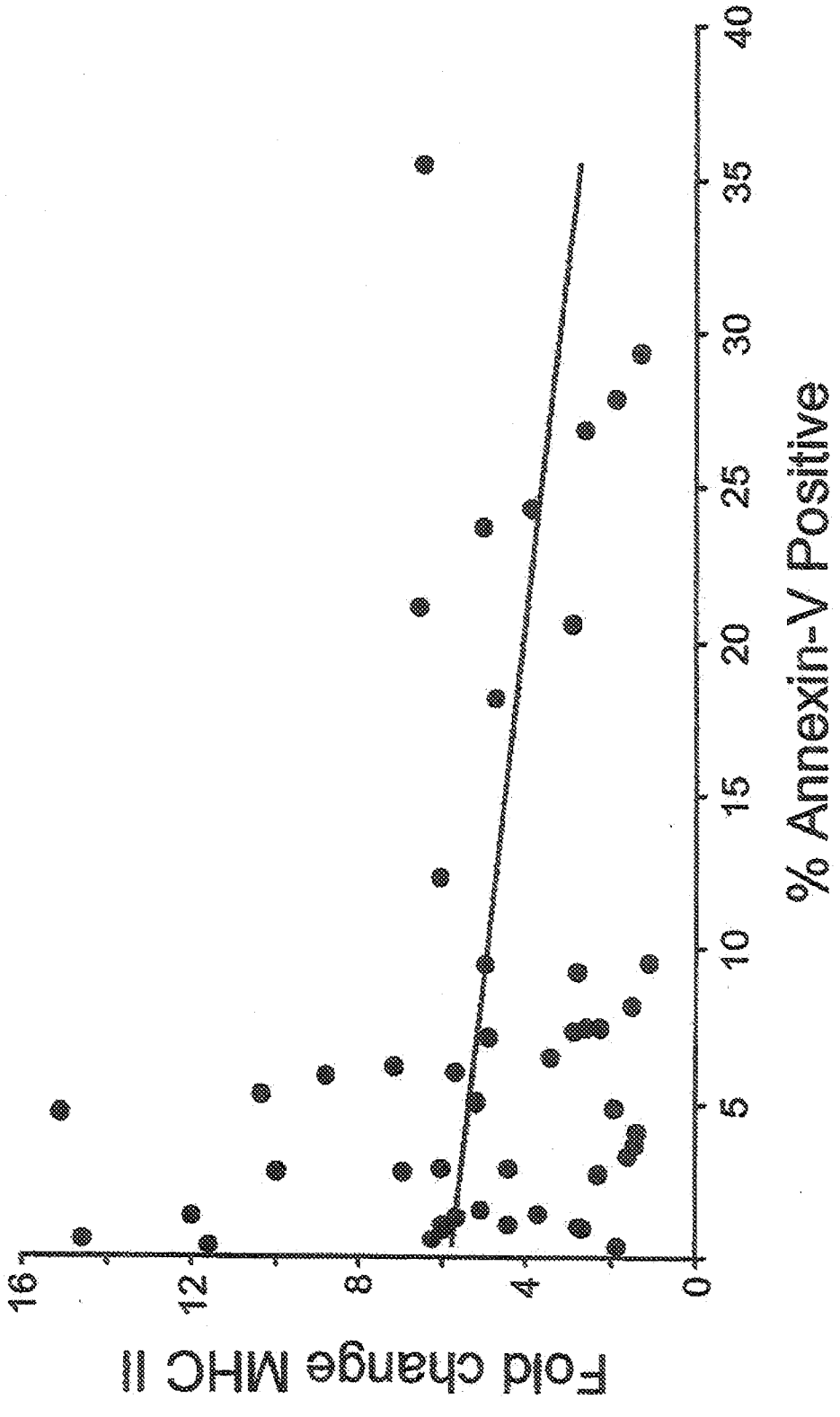

Fold change MHC II
16
12
8
4
0
% Annexin-V Positive
5
10
15
20
25
30
35
40

FIG.10

FIG.11

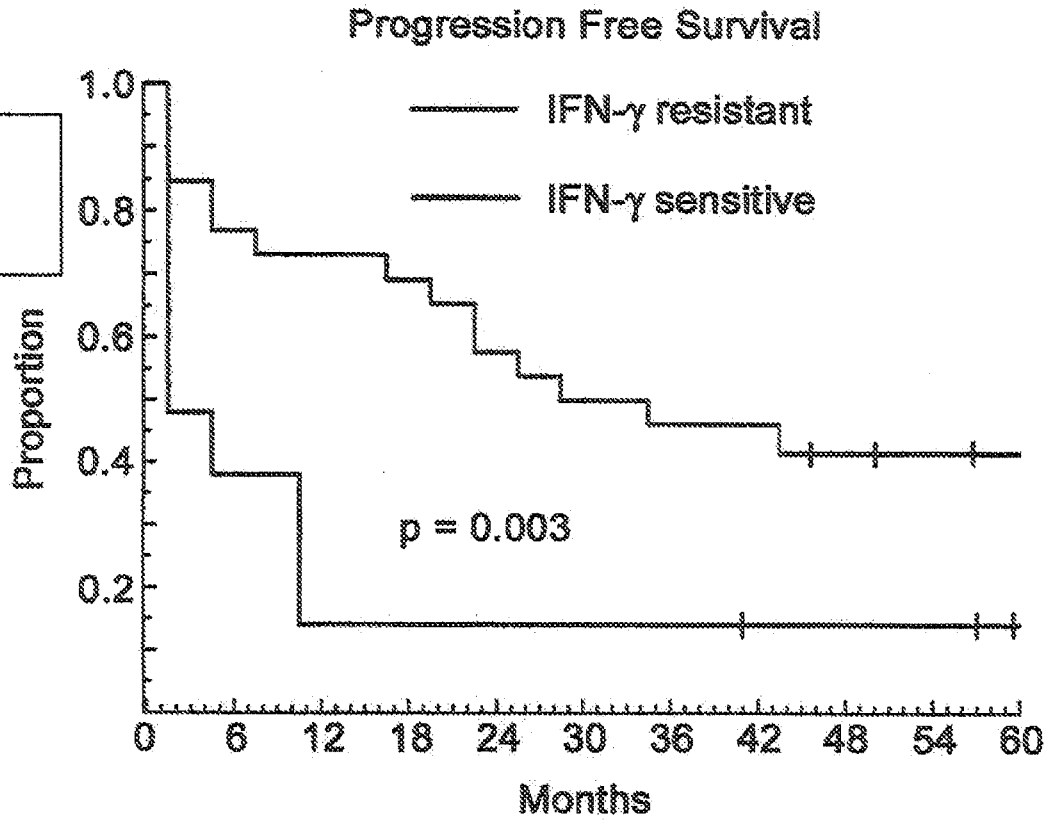
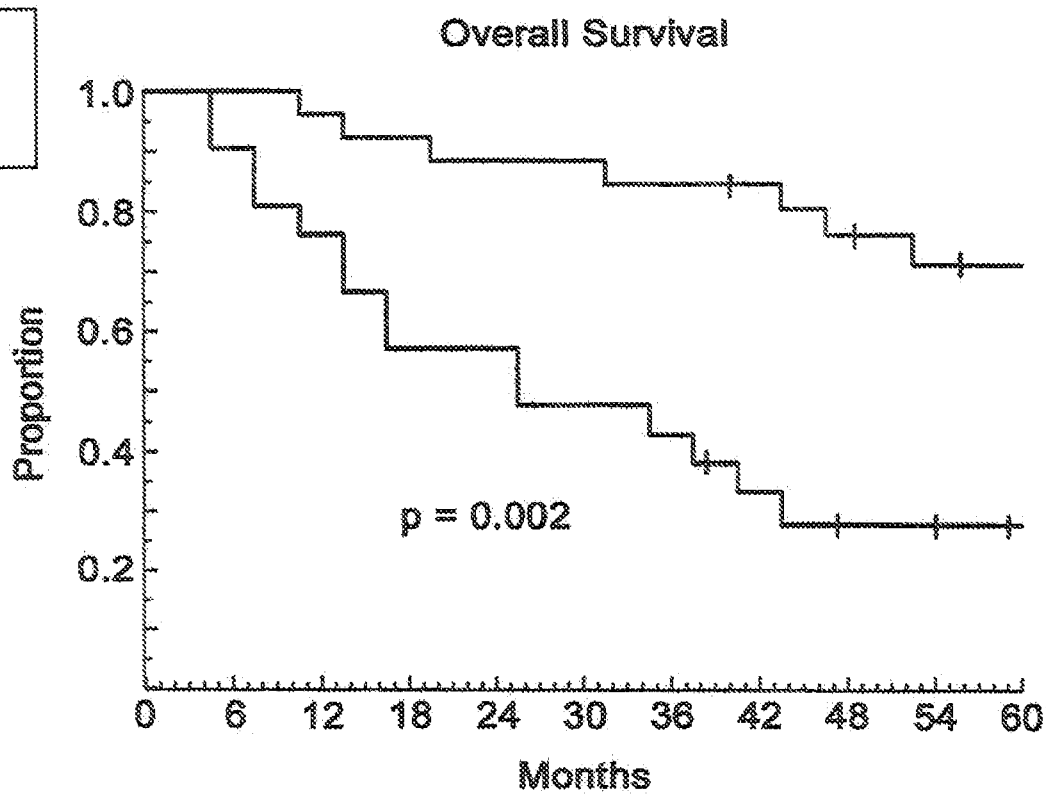

Progression Free Survival
IFN-γ resistant
IFN-γ sensitive
1.0
0.8
0.6
0.4
0.2
Proportion
p = 0.003
0
6
12
18
24
30
36
42
48
54
60
Months

FIG.12


Overall Survival
Proportion
1.0
0.8
0.6
0.4
0.2
p = 0.002
0
6
12
18
24
30
36
42
48
54
60
Months

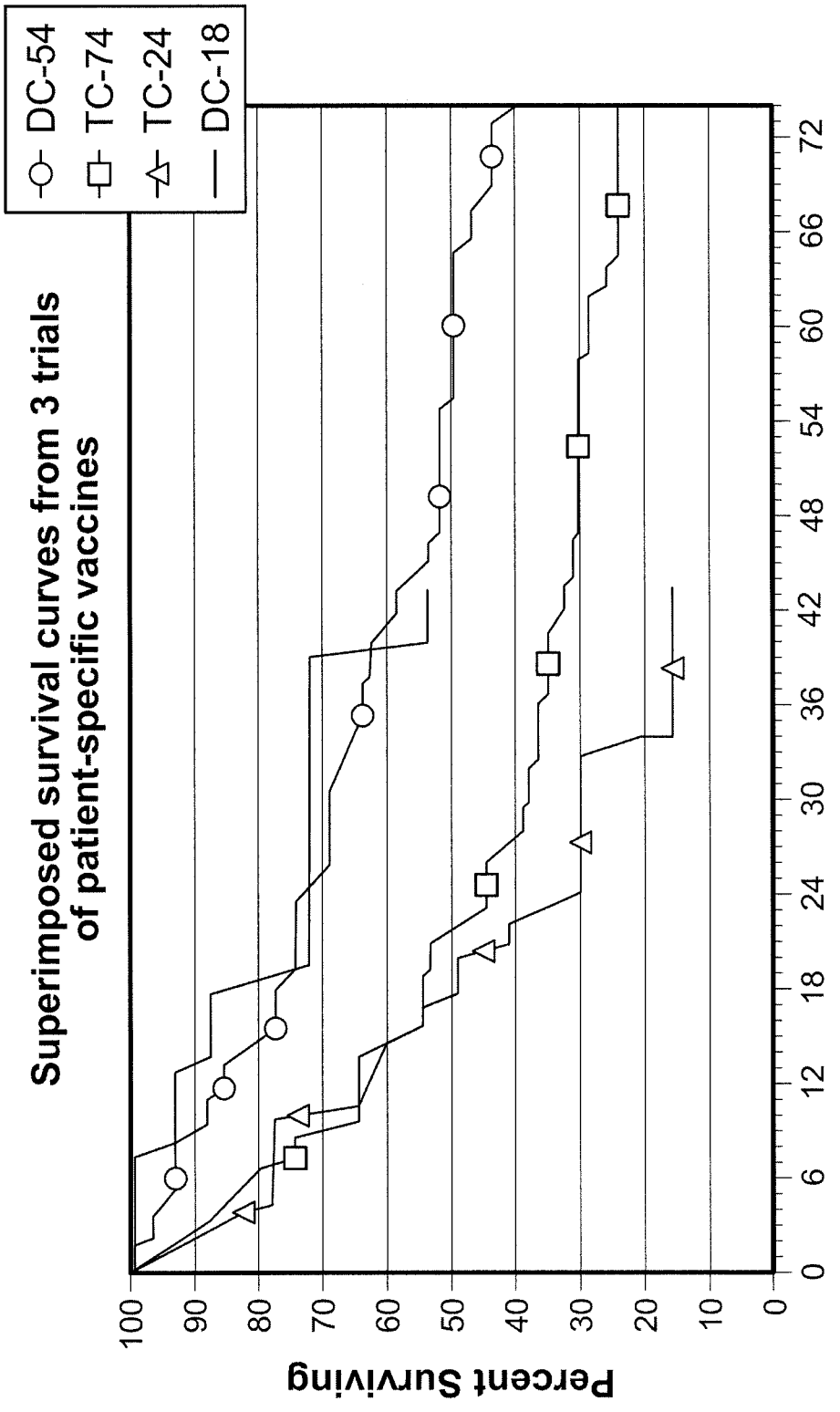

Superimposed survival curves from 3 trials of patient-specific vaccines
DC-54
TC-74
TC-24
DC-18
Percent Surviving
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
36
42
48
54
60
66
72
Months

FIG. 13

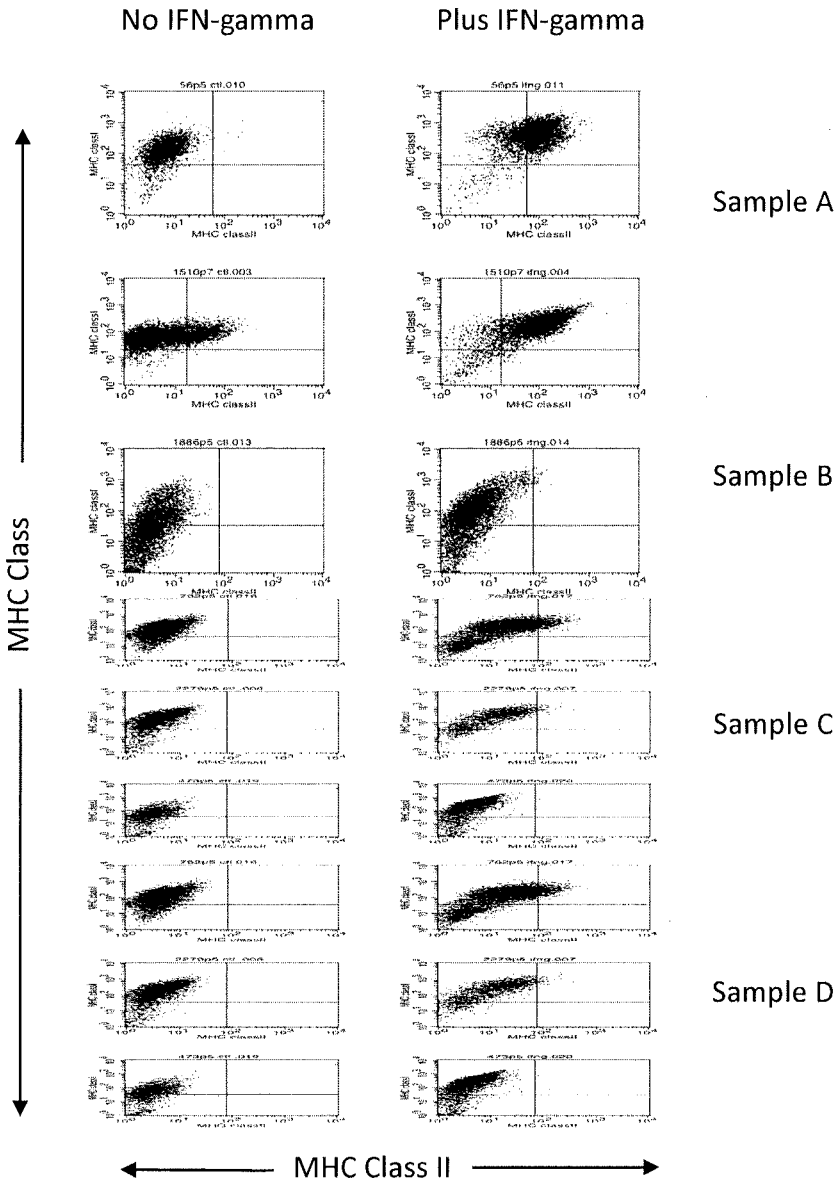

No IFN-gamma
Plus IFN-gamma
Sample A
Sample B
Sample C
Sample D
MHC Class
MHC Class II

Figure 14

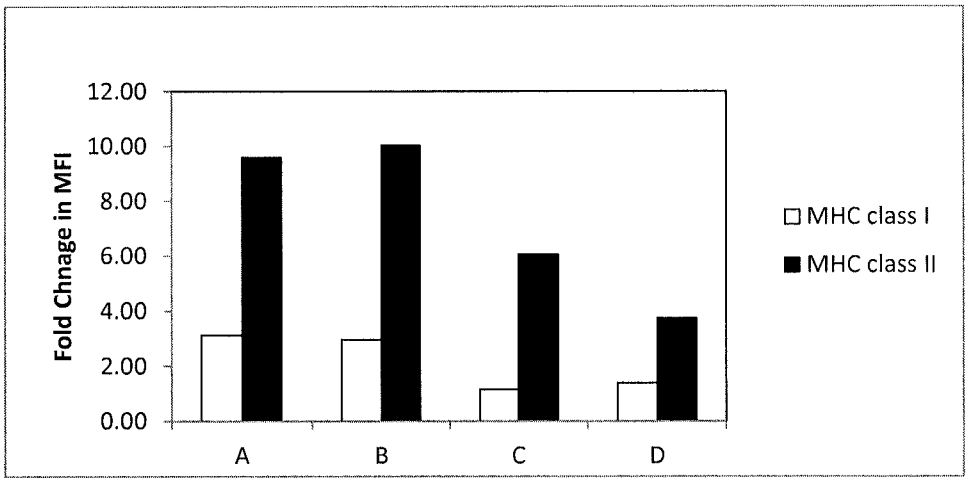

12.00
10.00
8.00
6.00
4.00
2.00
0.00
Fold Chnage in MFI
A
B
C
D
MHC class I
MHC class II

Figure 15

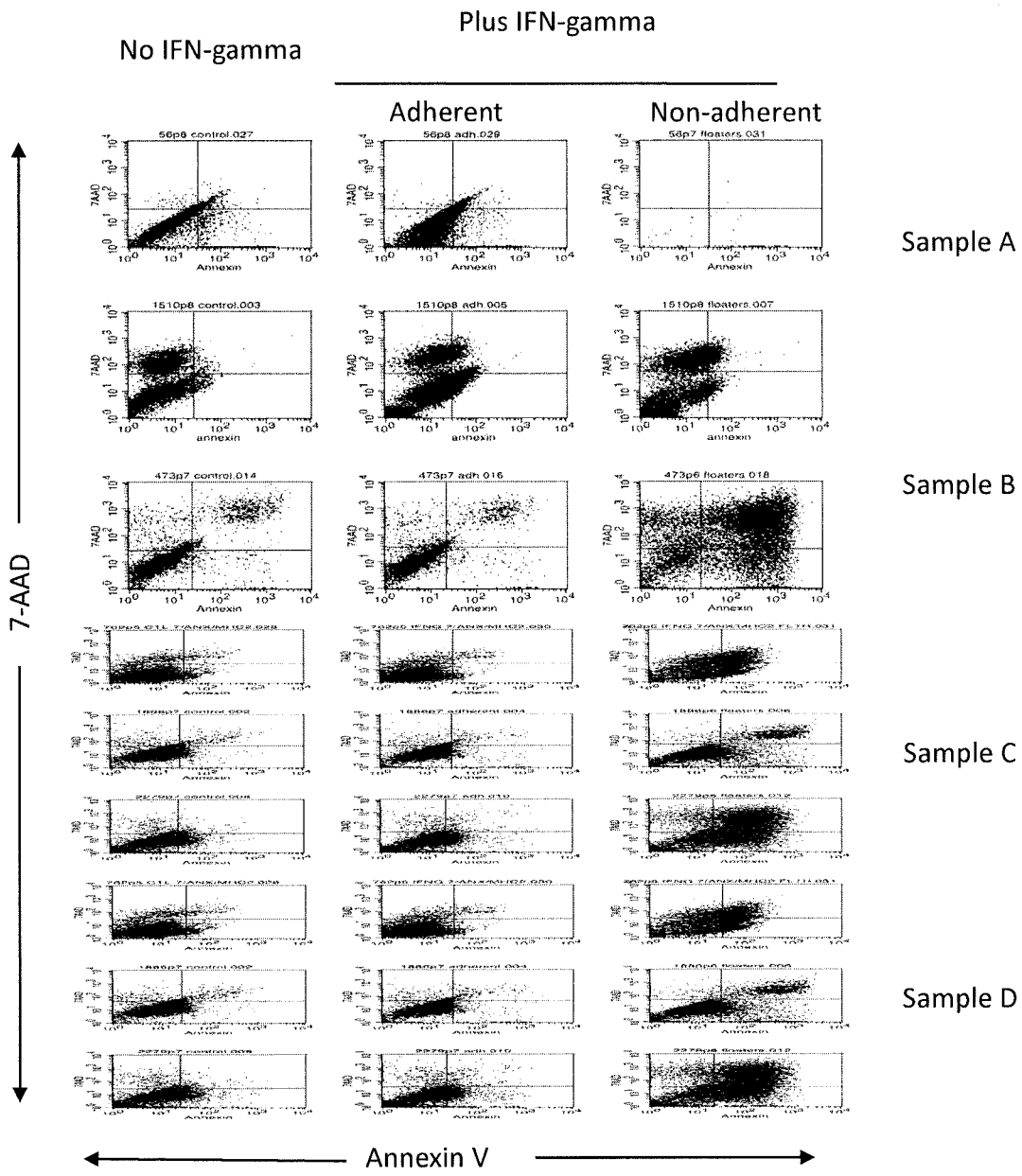

No IFN-gamma
Plus IFN-gamma
Adherent
Non-adherent
Sample A
Sample B
Sample C
Sample D
7-AAD
Annexin V

Figure 16

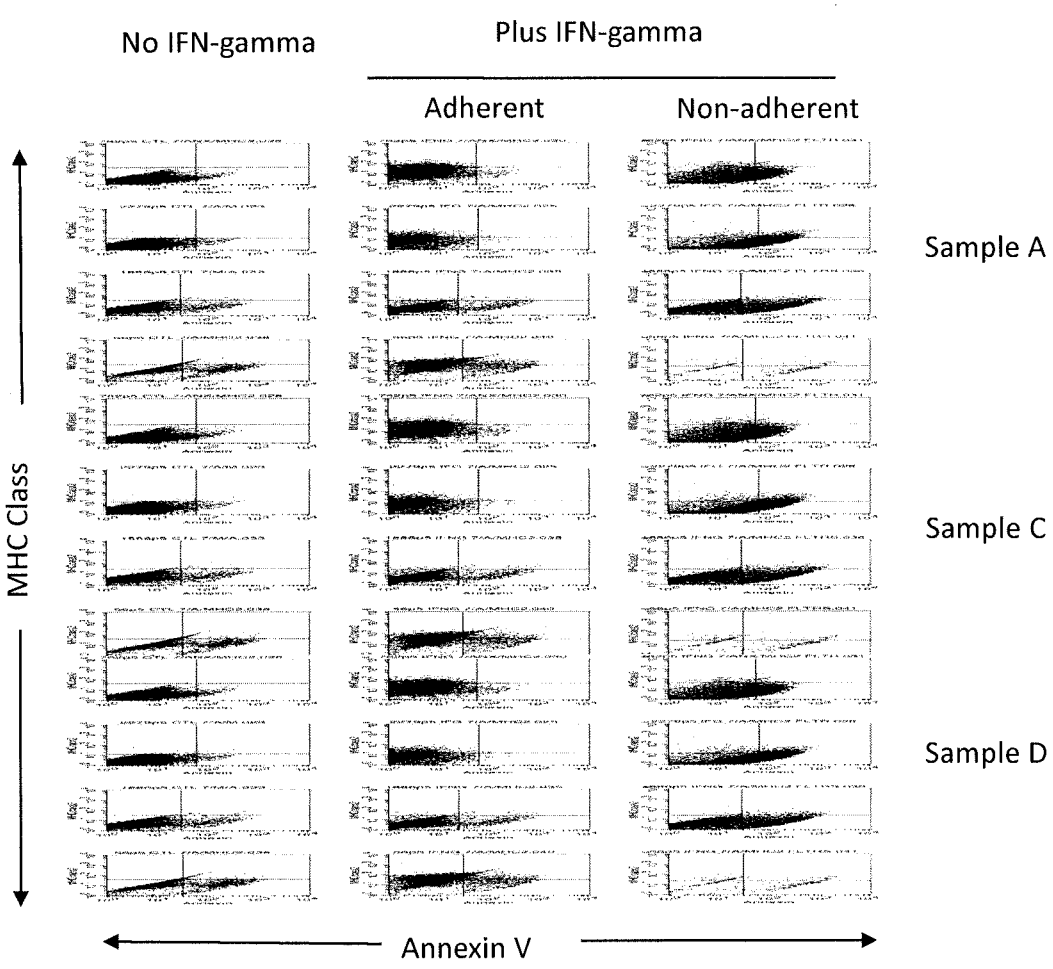

No IFN-gamma
Plus IFN-gamma
Adherent
Non-adherent
Sample A
Sample C
Sample D
MHC Class
Annexin V

Figure 17

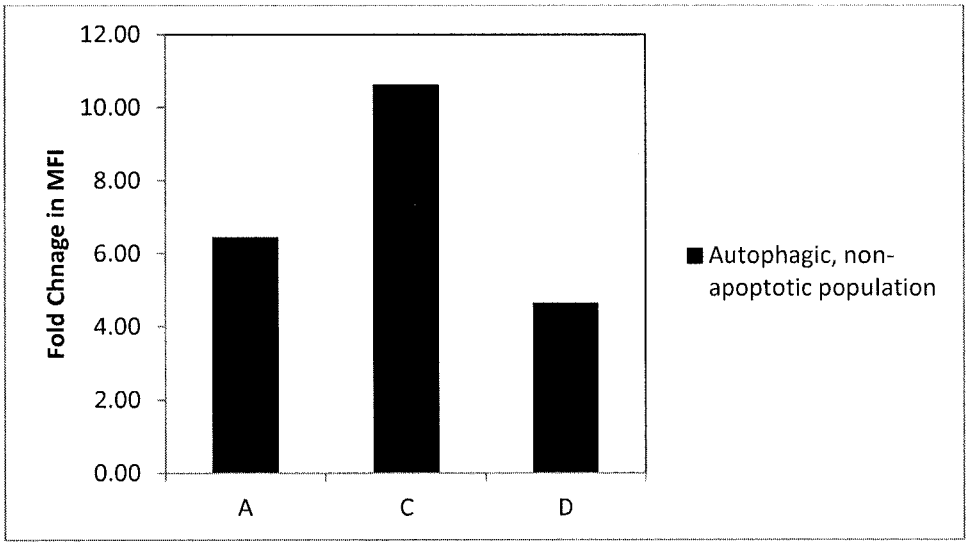

12.00
10.00
8.00
6.00
4.00
2.00
0.00
Fold Chnage in MFI
A
C
D
Autophagic, non-apoptotic population

Figure 18

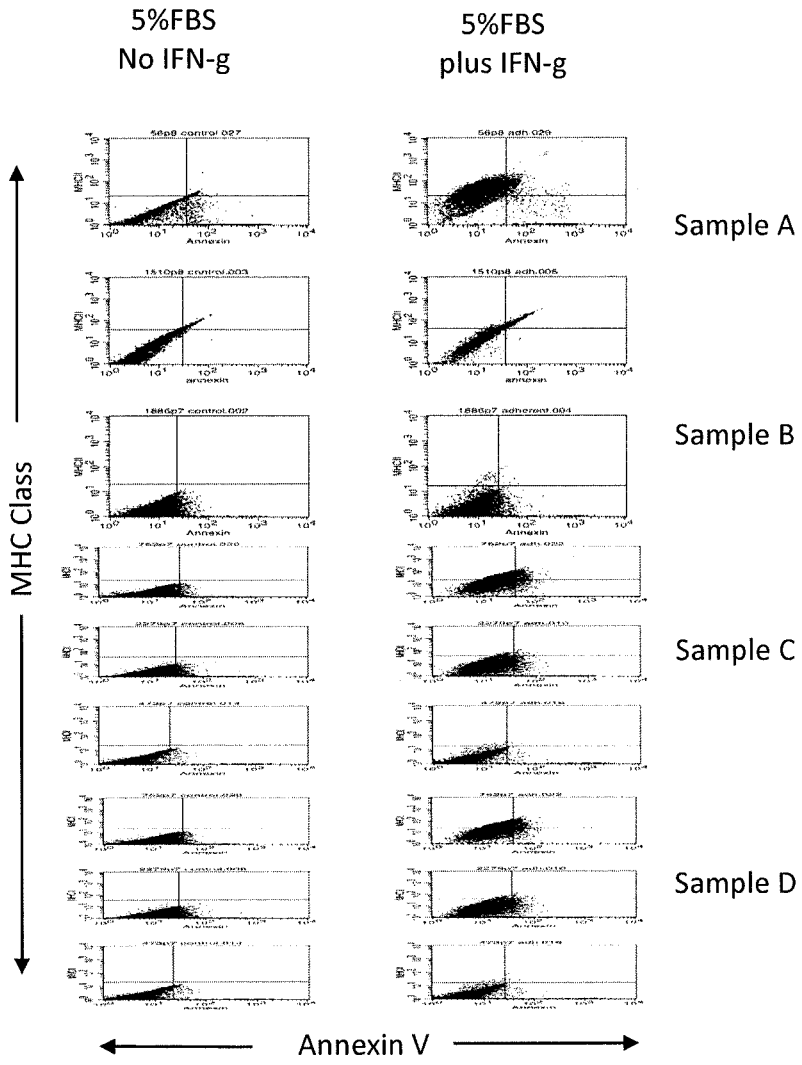

5%FBS
No IFN-g
5%FBS
plus IFN-g
Sample A
Sample B
Sample C
Sample D
MHC Class
Annexin V

Figure 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/024123

A. CLASSIFICATION OF SUBJECT MATTER

C12N 5/071(2010.01)i, C12N 5/09(2010.01)i, A61K 35/12(2006.01)i, A61P 35/00(2006.01)i

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)
C12N 5/071; A61K 39/00; A61P 31/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: cancer, dendritic cell, autophagic, interferon gamma

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LI, YUHUAN et al., `Efficient cross-presentation depends on autophagy in tumor cells`, Cancer Research, 01 September 2008, Vol.68, No.17, pp.6889-6895. See abstract; pages 6889, 6894 and 6895.	14
A		1-13,21-30,32-35
A	MITO, KAI et al., `IFNγ markedly cooperates with intratumoral dendritic cellvaccine in dog tumor models`, Cancer Research, 07 September 2010, Vol.70, No.18, pp.7093-7101. See abstract; pages 7093, 7094, 7096 and 7100; fig 1.	1-14,21-30,32-35
A	YAP, GEORGE S. et al., `Autophagic elimination of intracellular parasites`, Autophagy, 05 March 2007, Vol.3, No.2, pp.163-165. See abstract; pages 163 and 164; fig 1.	1-14,21-30,32-35
A	JAGANNATH, CHINNASWAMY et al., `Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells`, Nature Medicine, 01 March 2009, Vol.15, No.3, pp.267-276. See abstract; pages 267, 268 and 273.	1-14,21-30,32-35

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of 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	Date of mailing of the international search report
27 May 2013 (27.05.2013)	**30 May 2013 (30.05.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. 82-42-472-7140

Authorized officer
HEO, Joo Hyung
Telephone No. 82-42-481-8150

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/024123

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

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

1. ☒ Claims Nos.: 15-20,31,36
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 15-20, 31, and 36 pertain to methods for treatment of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.

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

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

Remark on Protest

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/024123

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02-053176 A2 (HADASIT MEDICAL RESEARCH SERVICES AND DEVELOPMENT LTD.) 11 July 2002 See abstract; claims 1, 5 and 6.	1-14,21-30,32-35
A	US 2008-0311142 A1 (YU, JOHN S. et al) 18 December 2008 See abstract; claims 1, 2 and 13.	1-14,21-30,32-35

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/024123

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 02-053176 A2	11.07.2002	AU 2002-219485 A1	16.07.2002
		IL 140796 D0	10.02.2002
		WO 02-053176 A3	19.12.2002
US 2008-0311142 A1	18.12.2008	CA 2700436 A1	03.04.2008
		CA 2700579 A1	03.04.2008
		US 2008-311141 A1	18.12.2008
		US 8097256 B2	17.01.2012
		WO 2008-039969 A2	03.04.2008
		WO 2008-039969 A3	20.11.2008
		WO 2008-039974 A2	03.04.2008
		WO 2008-039974 A3	17.07.2008

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 104540937 A
(43)申请公布日 2015.04.22

(21)申请号 201380017689.2
(22)申请日 2013.01.31
(30)优先权数据
61/594304 2012.02.02 US
(85)PCT国际申请进入国家阶段日
2014.09.29
(86)PCT国际申请的申请数据
PCT/US2013/024123 2013.01.31
(87)PCT国际申请的公布数据
WO2013/116505 EN 2013.08.08
(71)申请人 新干细胞肿瘤学有限责任公司
地址 美国加利福尼亚州
(72)发明人 A. 科恩富思 R. 迪尔曼
(74)专利代理机构 中国专利代理(香港)有限公司 72001
代理人 初明明 吕彩霞
(51)Int.Cl.
C12N 5/0784(2010.01)
C12N 5/09(2010.01)
A61K 39/00(2006.01)
A61P 35/00(2006.01)

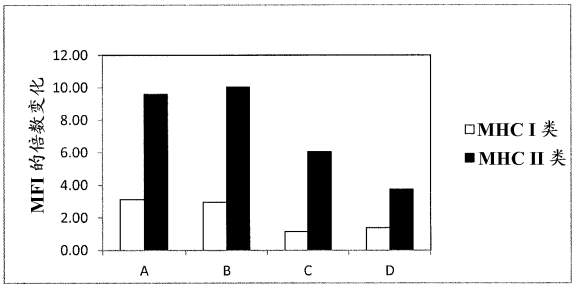
权利要求书3页 说明书30页 附图25页

(54)发明名称

多能胚层起源抗原呈递癌症疫苗

(57)摘要

本公开内容提供用于治疗或预防起源于各胚层(内胚层、中胚层、外胚层、神经嵴)的癌症的试剂、方法和药盒。所述试剂包括干扰素-γ(IFN-γ)反应性癌细胞,其中所述细胞是自噬的和非凋亡的癌细胞,且其中所述细胞表达MHC II类。



CN 104540937 A

1.一种哺乳动物树突细胞群,其包含:

取自患有癌症的受试者的癌细胞的癌特异性肽;

其中所述癌特异性肽自所述癌细胞由树突细胞在体外获得;

其中所述癌细胞不用干扰素-γ(IFN-γ)或IFN-γ模拟物体外处理;

其中大于约60百分比(%)的不用IFN-γ或IFN-γ模拟物体外处理的所述癌细胞是自噬的和非凋亡的,和,

其中所述树突细胞和癌细胞来自于同一受试者。

2.权利要求1的树突细胞群,其中所述癌症来自于内胚层起源、中胚层起源或外胚层起源中的一种或多种的组织。

3.权利要求1的树突细胞群,其中所述癌症来自于神经嵴起源的组织,且其中所述神经嵴是外胚层起源的。

4.权利要求1的树突细胞群,其中所述癌症来自于内胚层起源、中胚层起源或外胚层起源中的一种或多种的组织,且其中所述癌症是神经嵴起源的黑素瘤、内胚层起源的结肠癌、中胚层起源的肾癌、外胚层起源的成胶质细胞瘤或混合型中胚层加胚外起源的卵巢癌。

5.权利要求1的树突细胞群,其中大于80%的不用IFN-γ或IFN-γ模拟物体外处理的所述癌细胞是自噬的和非凋亡的。

6.一种用于权利要求1的受试者的疫苗,其包含权利要求1的树突细胞群。

7.权利要求1的树突细胞群,其中基本所有的不用IFN-γ或IFN-γ模拟物处理的癌细胞不能够细胞分裂。

8.权利要求1的树突细胞群,其中基本所有的不用IFN-γ或IFN-γ模拟物处理的癌细胞被照射并且不能够细胞分裂。

9.权利要求1的树突细胞群,其中至少80%的不用IFN-γ或IFN-γ模拟物处理的癌细胞被照射并且不能够细胞分裂。

10.权利要求1的树突细胞群,其中至少80%的不用IFN-γ或IFN-γ模拟物处理的癌细胞用核酸交联剂处理并且不能够细胞分裂。

11.权利要求1的树突细胞群,其包含一种或多种来源于黑素瘤特异性抗原的肽,所述抗原是S-100、HMB-45、Mel-2、Melan-A、Mel-5、MAGE-1、MART-1或酪氨酸酶,且其中所述癌症为黑素瘤。

12.权利要求1的树突细胞群,其中所述特定的受试者为人受试者。

13.权利要求1的树突细胞群,其中所述特定的受试者为非人哺乳动物。

14.一种黑素瘤疫苗,其包含:

来自患有黑素瘤的受试者的至少一种成熟树突细胞;

其中所述至少一种成熟树突细胞与来自同一受试者的至少一种癌症肿瘤细胞接触,

其中与至少一种成熟树突细胞接触的至少一种癌症肿瘤细胞是不分裂的、自噬的和非凋亡的。

15.一种用于刺激针对癌特异性抗原的免疫应答的方法,所述方法包括将免疫刺激量的权利要求1的树突细胞给予受试者。

16.权利要求15的方法,其中所述被刺激的免疫应答包括$CD4^+$ T细胞应答、$CD8^+$ T细胞应答和B细胞应答中的一种或多种。

17.权利要求 16 的方法，其中 $CD4^+$ T 细胞应答、$CD8^+$ T 细胞应答或 B 细胞应答可通过 ELISPOT 测定法、通过胞内细胞因子染色测定法、通过四聚体测定法或通过检测抗原特异性抗体产生来测定。

18.权利要求 15 的方法，其中所述免疫应答包括含 2 年总体生存率 (OS) 的生存时间，且其中 2 年总体生存率为至少约 60%。

19.权利要求 15 的方法，其中所述给药包括皮下注射疫苗。

20.权利要求 15 的方法，其中所述给药包括每周一次给予疫苗注射达 3 个月，然后每月一次达 5 个月。

21.一种用于制备包含来自同一受试者的癌细胞和树突细胞的树突细胞疫苗的方法，所述方法包括：

一种或多种癌细胞用阻止细胞分裂的作用剂处理；

所述一种或多种癌细胞不用干扰素 -γ (IFN-γ) 或 IFN-γ 模拟物体外处理；

选择是自噬的和非凋亡的癌细胞；

排除是非自噬的和凋亡的癌细胞；且其中将是自噬的和非凋亡的癌细胞提供给一种或多种自体树突细胞，或者，其中将来源于是自噬的和非凋亡的癌细胞的肽提供给一种或多种自体树突细胞。

22.一种组合物，其包含：

来自第一受试者的不用干扰素 -γ (IFN-γ) 处理的至少一种癌细胞和来自同一第一受试者的至少一种抗原呈递细胞 (APC)，其中所述癌细胞是：

自噬的；和，

非凋亡的。

23.权利要求 22 的组合物，其中所述癌细胞是表达 MHC II 类的。

24.权利要求 22 的组合物，其中所述 APC 是树突细胞、巨噬细胞或 B 细胞。

25.权利要求 22 的组合物，其中所述至少一种癌细胞包含癌特异性肽，且其中所述癌特异性肽基本上不包含在所述 APC 内，并且基本上不被所述 APC 加工。

26.权利要求 22 的组合物，其中所述癌细胞包含黑素瘤特异性肽，且其中所述癌特异性肽基本上包含在所述 APC 内，且在所述 APC 中被部分加工或被充分加工。

27.权利要求 22 的组合物，其中将所述癌细胞加载至 APC 中。

28.权利要求 22 的组合物，其中不将所述癌细胞加载至 APC 中。

29.权利要求 19 的组合物，其中自噬通过测定微管相关蛋白轻链 3 (LC3) 的试验证实。

30.权利要求 22 的组合物，其中使用试剂 7- 氨基放线菌素 D (7-ADD) 或试剂膜联蛋白的至少一种证实细胞是非凋亡的。

31.一种在患有癌症和包含癌细胞的受试者中刺激免疫应答的方法，其中所述受试者与第一受试者是同一受试者，所述方法包括给予免疫有效量的权利要求 22 的组合物。

32.权利要求 22 的组合物，其中至少 90% 的癌细胞不用 IFN-γ 体外处理，且小于 10% 的癌细胞用 IFN-γ 体外处理。

33.一种用于制备权利要求 1 的疫苗或权利要求 19 的组合物的方法，所述方法包括使至少一种癌症肿瘤细胞与至少一种抗原呈递细胞 (APC) 接触，其中所述至少一种癌症肿瘤细胞来自于第一人受试者，且其中所述至少一种 APC 来自于同一第一人受试者。

34. 一种用于制备树突细胞疫苗的方法，所述方法包括：将获自第一受试者的癌细胞用阻止细胞分裂的作用剂处理；其中所述癌细胞不用 IFN-γ 或 IFN-γ 模拟物体外处理；选择是自噬的和非凋亡的癌细胞；且使所选择的癌细胞与来自同一第一受试者的自体树突细胞接触。

35. 一种组合物，其包含按权利要求 34 的方法制备的树突细胞疫苗。

36. 一种用于刺激针对癌特异性抗原的免疫应答的方法，所述方法包括将权利要求 34 的组合物给予患有癌症的受试者。

多能胚层起源抗原呈递癌症疫苗

[0001] 相关申请

本申请要求2012年2月2日提交的美国临时申请号61/594,304的完全巴黎公约优先权和权益,所述临时申请的内容通过该引用予以结合,就像以其整体在本文中完整提供。

[0002] 领域

本公开内容涉及治疗来源于各胚胎胚层的癌症、筛选适于治疗的受试者、组合物、方法和药盒。

[0003] 背景

癌症的特征在于缺乏针对癌症的有效免疫应答。免疫应答的缺乏可产生于例如这样的事实:出于缺乏肿瘤细胞表达的MHC而必然缺乏肿瘤细胞对肿瘤抗原的呈递、出于巨噬细胞与肿瘤结合(在肿瘤处巨噬细胞表达降低免疫应答的细胞因子)以及出于T调节性细胞(Treg)的免疫抑制活性,因此许多肿瘤抗原是"自身抗原"。缺乏针对肿瘤的免疫应答还由以下事实引起:肿瘤细胞趋于不表达刺激先天免疫应答的分子,即刺激toll样受体(TLR)或核苷酸结合寡聚结构域(NOD)样受体的分子。癌症包括实体瘤以及血液癌症,例如白血病和骨髓增生异常综合征。

[0004] 胚胎胚层为内胚层、中胚层、外胚层和神经嵴。神经嵴来源于外胚层,但有时被视为第四胚层。肿瘤细胞(neoplastic cell)可鉴定为来源于这些胚层之一。例如,乳腺癌、肉瘤和结肠癌分别产生于来源于胚胎外胚层、中胚层和内胚层的组织(Singer等(1997) J. Clin. Endocrinol. Metabol. 82:1917-1922)。本公开内容提供刺激免疫应答的试剂,所述免疫应答针对来源于产生自各胚胎胚层的组织的癌症以及针对混合型胚胎起源的肿瘤。

[0005] 免疫系统包括细胞免疫、体液免疫和补体应答。细胞免疫包括涉及树突细胞、$CD8^+$ T细胞(细胞毒性T细胞;细胞毒性淋巴细胞)和$CD4^+$ T细胞(辅助T细胞)的细胞和事件的网络。树突细胞(DC)捕获多肽抗原,其中这些抗原可获自DC以外,或由感染性生物在DC内生物合成。DC加工该多肽,产生长度约10个氨基酸的肽,将该肽转移至MHC I类或MHC II类形成复合体,并使复合体穿梭到DC的表面。当携带MHC I类/肽复合体的DC接触$CD8^+$ T细胞时,结果是$CD8^+$ T细胞的活化和增殖。至于MHC II类的作用,当携带MHC II类/肽复合体的DC接触$CD4^+$ T细胞时,结果是$CD4^+$ T细胞的活化和增殖(Munz等(2010) Curr. Opin. Immunol. 22:89-93;Monaco (1995) J. Leukocyte Biol. 57:543-547;Robinson等(2002) Immunology 105:252-262)。虽然树突细胞将抗原呈递给T细胞可"激活"该T细胞,但是激活的T细胞可能不能够发起有效的免疫应答。由$CD8^+$ T细胞所致的有效免疫应答常常需要DC被许多相互作用中的一个或多个在先刺激。这些相互作用包括$CD4^+$ T细胞与DC直接接触(通过$CD4^+$ T细胞的CD40配体与DC的CD40受体接触),或TLR激动剂与树突细胞的toll样受体(TLR)之一直接接触。

[0006] 体液免疫是指B细胞和抗体。B细胞转化成浆细胞,而浆细胞表达和分泌抗体。幼稚B细胞的特征在于它们不表达标志物CD27,而抗原特异性B细胞却表达CD27(Perez-Andres等(2010) Cytometry Part B 78B (Suppl. 1) S47-S60)。所分泌的抗体随后可与驻留在肿瘤细胞表面的肿瘤抗原结合。结果是被感染的细胞或肿瘤细胞被抗体加

标签。随着抗体与受感染的细胞或肿瘤细胞结合，结合的抗体介导杀死受感染的细胞或肿瘤细胞，其中杀死通过 NK 细胞进行。虽然 NK 细胞未以 T 细胞配置成识别特异性靶抗原的方式配置成识别特异性靶抗原，但 NK 细胞与抗体恒定区结合的能力，使得 NK 细胞能够特异性地杀死被抗体加标签的细胞。NK 细胞的抗体识别受与抗体的 Fc 部分结合的 Fc 受体 (NK 细胞的 Fc 受体）介导。这种杀死类型被称为抗体依赖性细胞毒性 (ADCC)。NK 细胞也可杀死不依赖于 ADCC 机制的细胞，而这种杀死需要靶细胞中 MHC I 类的表达丧失或缺乏（参见例如 Caligiuri (2008) Blood 112:461-469)。

[0007] “迟发型超敏反应应答”技术可用来分辨主要涉及细胞免疫或主要涉及体液免疫的免疫应答。迟发型超敏反应应答的阳性信号表明细胞应答（参见例如 Roychowdhury 等 (2005) AAPS J. E834-E846)。

[0008] 自噬是一个稳态过程，籍此将胞质组分和细胞器递送到溶酶体用于降解。自噬还与对胞内病原体的先天性和适应性免疫应答有关，胞质抗原籍此被加载到主要组织相容性复合体 (MHC) II 类分子上用于 $CD4^+$ T 细胞识别。

[0009] 描述

如本文包括随附权利要求书中所用，单词例如“a”、“an”和“the”的单数形式包括其相应的复数对象，除非文中另有明确说明。本文引用的所有参考文献通过引用结合到本文中，其程度就像每个单独的出版物、专利、公开的专利申请和序列表以及所述出版物和专利文献中的图表和附图，具体而单独说明通过引用予以结合一样。

[0010] 公开内容

本公开内容提供包含取自患有癌症的受试者的癌细胞的癌特异性肽的哺乳动物树突细胞群；其中癌特异性肽自所述癌细胞由树突细胞在体外获得；其中癌细胞不用干扰素 -γ (IFN-γ）或 IFN-γ 模拟物体外处理；其中大多数的癌细胞不用干扰素 -γ (IFN-γ）或 IFN-γ 模拟物体外处理；其中小于约 40% 的癌细胞用干扰素 -γ (IFN-γ）或 IFN-γ 模拟物体外处理；其中小于约 30% 的癌细胞用干扰素 -γ (IFN-γ）或 IFN-γ 模拟物体外处理；其中小于约 20% 的癌细胞用干扰素 -γ (IFN-γ）或 IFN-γ 模拟物体外处理；其中小于约 10% 的癌细胞用干扰素 -γ (IFN-γ）或 IFN-γ 模拟物体外处理；其中小于约 5% 的癌细胞用干扰素 -γ (IFN-γ）或 IFN-γ 模拟物体外处理；其中小于约 1% 的癌细胞用干扰素 -γ (IFN-γ）或 IFN-γ 模拟物体外处理；其中大于 60 百分比 (%) 的不用 IFN-γ 或 IFN-γ 模拟物体外处理的所述癌细胞是自噬的和非凋亡的，且其中树突细胞和癌细胞来自同一受试者；其中大于 70 百分比 (%) 的不用 IFN-γ 或 IFN-γ 模拟物体外处理的所述癌细胞是自噬的和非凋亡的，且其中树突细胞和癌细胞来自同一受试者；其中大于 80 百分比 (%) 的不用 IFN-γ 或 IFN-γ 模拟物体外处理的所述癌细胞是自噬的和非凋亡的，且其中树突细胞和癌细胞来自同一受试者；其中大于 90 百分比 (%) 的不用 IFN-γ 或 IFN-γ 模拟物体外处理的所述癌细胞是自噬的和非凋亡的，且其中树突细胞和癌细胞来自同一受试者。

[0011] 在其中要求不加入 IFN-γ 的一些实例中，本公开内容涉及显著的或相当大的量。换种方式说，要求是至少 60% 的癌细胞不用 IFN-γ 处理（或暴露于 IFN-γ)。在一些实例中，至少 70% 的癌细胞不用 IFN-γ 处理（或暴露于 IFN-γ)。在一些实例中，至少 80% 的癌细胞不用 IFN-γ 处理（或暴露于 IFN-γ)。在一些实例中，至少 90% 的癌细胞不用 IFN-γ

处理（或暴露于 IFN-γ）。在一些实例中，至少 95% 的癌细胞不用 IFN-γ 处理（或暴露于 IFN-γ）。在一些实例中，至少 98% 的癌细胞不用 IFN-γ 处理（或暴露于 IFN-γ）。在一些实例中，至少 99% 的癌细胞不用 IFN-γ 处理（或暴露于 IFN-γ）。在一些实例中，至少 100% 的癌细胞不用 IFN-γ 处理（或暴露于 IFN-γ）。在上述情况下，“处理”和“暴露”是指外源加入的 IFN-γ 或 IFN-γ 模拟物。因此，如果使用说明或方案要求至少 60% 的癌细胞是未处理的，则接受该方案并在 IFN-γ 中只处理 30% 的细胞的使用者，将成功遵从方案的要求。然而，如果接受方案的使用者犯错且用 IFN-γ 处理 62% 细胞，则使用者将不能符合该方案的要求。本公开内容未设置所添加的 IFN-γ 或 IFN-γ 模拟物的任何具体浓度或经处理或未处理的癌细胞的量。在所添加的 IFN-γ 或 IFN-γ 模拟物接触癌细胞时，本公开内容未设置暴露的任何具体时限。本公开内容未设置对所添加的 IFN-γ 或 IFN-γ 模拟物的功效的任何要求。

[0012] 本公开内容提供包含取自患有癌症的受试者的癌细胞的癌特异性肽的哺乳动物树突细胞群；其中癌特异性肽自所述癌细胞由树突细胞在体外获得；其中癌细胞不用干扰素 -γ （IFN-γ）或 IFN-γ 模拟物体外处理；其中大于 60%、或大于 70%、或大于 80%、或大于 90%、或大于 95%、或大于 98%、或大于 99% 的不用 IFN-γ 或 IFN-γ 模拟物体外处理的所述癌细胞是自噬的和非凋亡的，且其中树突细胞和癌细胞来自同一受试者。

[0013] 在要求加入 IFN-γ 的那些实例中，例如来自受试者的树突细胞群包含取自患有癌症的受试者的癌细胞的癌特异性肽，其中所述癌特异性肽自用 IFN-γ 或 IFN-γ 模拟物体外处理的所述癌细胞由树突细胞在体外获得，其中大于 60 百分比 (%) 的用 IFN-γ 或 IFN-γ 模拟物体外处理的所述癌细胞是自噬的和非凋亡的，且其中树突细胞和癌细胞来自同一受试者。在其它实施方案中，所要求的是大于 65%、大于 70%、大于 75%、大于 80%、大于 90%、大于 95%、大于 98%、大于 99% 或 100% 的癌细胞是自噬的和非凋亡的。但是上述示例性方案（其中要求加入 IFN-γ）没有对用 IFN-γ 或 IFN-γ 模拟物处理的总癌细胞的百分比明确提出要求，没有对 IFN-γ 或 IFN-γ 模拟物的浓度提出任何要求，没有对所添加的 IFN-γ 或 IFN-γ 模拟物对癌细胞具有任何特定水平的功效提出任何要求。然而，在备选的示例性实施方案（其中要求加入 IFN-γ）中，所要求的是至少 20%、至少 30%、至少 40%、至少 50%、至少 60%、至少 70%、至少 80%、至少 90%、至少 95%、至少 98%、至少 99% 或 100% 的癌细胞暴露于外源加入的 IFN-γ 或 IFN-γ 模拟物（或与之接触）。“群”可根据以下方面定义：停留在培养瓶、烧杯、生物反应器、滴定板孔、给定滴定板的所有孔、瓶、批号、癌细胞来源于其中的特定肿瘤活检样品、特定批次的癌细胞首次进行维持培养或生长培养的日期和时间等。在排斥性实施方案中，本公开内容可排除非来自于所需培养瓶、烧杯、生物反应器、特定肿瘤活检样品、维持培养批次、生长培养批次等的细胞、癌细胞、树突细胞或用肿瘤抗原处理树突细胞。

[0014] 还提供上述树突细胞群，其中癌症来自于内胚层起源、中胚层起源或外胚层起源的一种或多种的组织。还提供上述树突细胞群，其中癌症来自于神经嵴起源的组织，且其中神经嵴是外胚层起源的。还提供上述树突细胞群，其中癌症来自于内胚层起源、中胚层起源或外胚层起源的一种或多种的组织，且其中癌症是神经嵴起源的黑素瘤、内胚层起源的结肠癌、中胚层起源的肾癌、外胚层起源的成胶质细胞瘤或混合型中胚层加胚外起源的卵巢癌。

[0015] 还提供上述树突细胞群，其中大于 80% 的不用 IFN-γ 或 IFN-γ 模拟物体外处理的所述癌细胞是自噬的和非凋亡的。还提供用于上述受试者的包含上述树突细胞群的疫苗。还提供上述树突细胞群，其中基本所有的不用 IFN-γ 或 IFN-γ 模拟物处理的癌细胞不能细胞分裂。还提供上述树突细胞群，其中基本所有的不用 IFN-γ 或 IFN-γ 模拟物处理的癌细胞被照射并且不能够细胞分裂。还提供上述树突细胞群，其中至少 80% 的不用 IFN-γ 或 IFN-γ 模拟物处理的癌细胞被照射并且不能够细胞分裂。还提供上述树突细胞群，其中至少 80% 的不用 IFN-γ 或 IFN-γ 模拟物处理的癌细胞用核酸交联剂处理并且不能够细胞分裂。

[0016] 还提供权利要求 1 的上述树突细胞群，其包含一种或多种来源于黑素瘤特异性抗原的肽，所述黑素瘤特异性抗原为 S-100、HMB-45、Mel-2、Melan-A、Mel-5、MAGE-1、MART-1 或酪氨酸酶，且其中癌症为黑素瘤。还提供上述树突细胞群，其中指定受试者为人受试者。还提供上述树突细胞群，其中指定受试者为非人哺乳动物。在疫苗实施方案中，提供包含以下的癌症疫苗 ：来自患有癌症的受试者的至少一种成熟树突细胞 ；其中至少一种成熟树突细胞已与来自同一受试者的至少一种癌症肿瘤细胞接触，其中与至少一种成熟树突细胞接触的至少一种癌症肿瘤细胞是不分裂的、自噬的和非凋亡的。还提供用于刺激针对癌特异性抗原的免疫应答的方法，所述方法包括将免疫刺激量的上述树突细胞群给予受试者。

[0017] 还提供上述方法，其中被刺激的免疫应答包括 CD4+ T 细胞应答、CD8+ T 细胞应答和 B 细胞应答的一种或多种。还提供上述方法，其中 CD4+ T 细胞应答、CD8+ T 细胞应答或 B 细胞应答可通过 ELISPOT 测定法、通过胞内细胞因子染色测定法、通过四聚体测定法或通过检测抗原特异性抗体产生来测量。还提供上述方法，其中免疫应答包括含 2 年总体生存率 (OS) 的生存时间，且其中 2 年总体生存率为至少 60%。还提供上述方法，其中给药包括皮下注射疫苗。还提供上述方法，其中给药包括每周一次给予疫苗注射达 3 个月，然后每月一次达 5 个月。还提供用于制备包括来自同一受试者的癌细胞和树突细胞的树突细胞疫苗的方法，所述方法包括 ：一种或多种癌细胞用阻止细胞分裂的作用剂处理 ；所述一种或多种癌细胞不用干扰素 -γ （IFN-γ）或 IFN-γ 模拟物体外处理 ；选择是自噬的和非凋亡的癌细胞 ；排除是非自噬的和凋亡的癌细胞 ；且其中将是自噬的和非凋亡的癌细胞提供给一种或多种自体树突细胞，或者，其中将来源于是自噬的和非凋亡的癌细胞的肽提供给一种或多种自体树突细胞。

[0018] 下面提供组合物实施方案。提供包括以下的组合物 ：来自第一受试者的不用干扰素 -γ （IFN-γ）处理的至少一种癌细胞，和来自同一第一受试者的至少一种抗原呈递细胞 (APC)，其中癌细胞是 ：自噬的 ；和非凋亡的。还提供上述组合物，其中癌细胞是表达 MHC II 类的。还提供上述组合物，其中 APC 是树突细胞、巨噬细胞或 B 细胞。还提供上述组合物，其中至少一种癌细胞包含癌特异性肽，且其中癌特异性肽基本上不包含在所述 APC 内，并且基本上不被所述 APC 加工。还提供上述组合物，其中癌细胞包含癌特异性肽，且其中癌特异性肽基本上包含在所述 APC 内，且在所述 APC 中被部分加工或被充分加工。

[0019] 还提供上述组合物，其中将癌细胞加载至 APC 中。还提供上述组合物，其中不将癌细胞加载至 APC 中。还提供上述组合物，其中自噬通过测定微管相关蛋白轻链 3 (LC3) 的试验证实。还提供上述组合物，其中使用试剂 7- 氨基放线菌素 D (7-ADD) 或试剂膜联蛋白的至少一种证实细胞是非凋亡的。在方法实施方案中，提供在患有癌症和包含癌细胞的受

试者中刺激免疫应答的方法，其中受试者与第一受试者是同一受试者，所述方法包括给予免疫有效量的上述组合物。还提供上述组合物，其中至少 90% 的癌细胞不用 IFN-γ 体外处理，且小于 10% 的癌细胞用 IFN-γ 体外处理。

[0020] 下面提供制备方法的实施方案。提供用于制备上述疫苗或上述组合物的方法，所述方法包括使至少一种癌症肿瘤细胞与至少一种抗原呈递细胞 (APC) 接触，其中至少一种癌症肿瘤细胞来自于第一人受试者，且其中至少一种 APC 来自于同一第一人受试者。还提供用于制备树突细胞疫苗的方法，其包括 ：将获自第一受试者的癌细胞用阻止细胞分裂的作用剂处理 ；其中癌细胞不用 IFN-γ 或 IFN-γ 模拟物体外处理 ；选择是自噬的和非凋亡的癌细胞 ；且使所选择的癌细胞与来自同一第一受试者的自体树突细胞接触。还提供包含通过上述方法制备的树突细胞疫苗的组合物。此外，提供用于刺激针对癌特异性抗原的免疫应答的方法，所述方法包括将上述组合物给予患有癌症的受试者。

[0021] 公开了包含来自患有黑素瘤和包含黑素瘤细胞的指定受试者的黑素瘤特异性肽的哺乳动物树突细胞群 ；其中所述黑素瘤特异性肽自不用 IFN-γ 或 IFN-γ 模拟物体外处理的所述黑素瘤细胞由树突细胞在体外获得，其中大于 60 百分比 (%) 的不用 IFN-γ 或 IFN-γ 模拟物体外处理的所述黑素瘤细胞是自噬的和非凋亡的，且其中树突细胞和黑素瘤细胞来自于同一受试者。

[0022] 还公开了上述哺乳动物树突细胞群，其中 ：大于 80% 的所述黑素瘤细胞是自噬的和非凋亡的。

[0023] 公开了上述树突细胞，其中基本上所有的不用 IFN-γ 或 IFN-γ 模拟物处理的黑素瘤细胞不能够细胞分裂 ；以及上述树突细胞，其中基本上所有的不用 IFN-γ 或 IFN-γ 模拟物处理的黑素瘤细胞被照射并且不能够细胞分裂 ；以及上述树突细胞，其中至少 80% 的不用 IFN-γ 或 IFN-γ 模拟物处理的黑素瘤细胞被照射并且不能够细胞分裂 ；以及上述树突细胞，其中至少 80% 的不用 IFN-γ 或 IFN-γ 模拟物处理的黑素瘤细胞用核酸交联剂处理并且不能够细胞分裂。

[0024] 还公开了包含上述哺乳动物树突细胞的上述群体的疫苗。

[0025] 还公开了上述树突细胞，其中基本上所有的黑素瘤特异性肽来自于不能够细胞分裂的黑素瘤细胞。

[0026] 此外，公开了上述树突细胞，其中基本上所有的黑素瘤特异性肽来自于因为黑素瘤细胞被照射过而不能够细胞分裂的黑素瘤细胞。

[0027] 公开了上述树突细胞，其中基本上所有的黑素瘤特异性肽来自于因为黑素瘤细胞的染色体被核酸交联剂交联而不能够细胞分裂的黑素瘤细胞。

[0028] 还公开了上述树突细胞，其包含来自于用放射处理的黑素瘤细胞的黑素瘤特异性肽。

[0029] 公开了上述树突细胞，其包含黑素瘤特异性肽，其中全部所述肽来自于用放射处理的黑素瘤细胞。

[0030] 还公开了上述树突细胞，其包含来源于以下黑素瘤特异性抗原的一种或多种肽 ：S-100、HMB-45、Mel-2、Melan-A、Mel-5、MAGE-1、MART-1 或酪氨酸酶。

[0031] 公开了上述树突细胞，其中基本上所有的黑素瘤特异性肽来自于经体外处理不能够细胞分裂的黑素瘤细胞。

[0032] 还公开了上述树突细胞，其中指定受试者为人受试者。

[0033] 公开了上述树突细胞，其中指定受试者为非人哺乳动物。

[0034] 公开了黑素瘤疫苗，其包含来自患有黑素瘤的受试者的至少一种成熟树突细胞，其中使至少一种成熟树突细胞与来自同一受试者的至少一种黑素瘤肿瘤细胞接触，其中与至少一种成熟树突细胞接触的至少一种黑素瘤肿瘤细胞是不分裂的、自噬的和非凋亡的。

[0035] 还公开了用于刺激针对黑素瘤特异性抗原的免疫应答的方法，所述方法包括将免疫刺激量的权利要求 1 的树突细胞给予受试者。

[0036] 公开了其中受试者患有黑素瘤且确实包含黑素瘤细胞。

[0037] 公开了上述方法，其中被刺激的免疫应答包括 CD4+ T 细胞应答、CD8+ T 细胞应答和 B 细胞应答的一种或多种。

[0038] 公开了上述方法，其中 CD4+ T 细胞应答、CD8+ T 细胞应答或 B 细胞应答可通过 ELISPOT 测定法、通过胞内细胞因子染色测定法、通过四聚体测定法或通过检测抗原特异性抗体产生来测定。

[0039] 还公开了上述方法，其中免疫应答包括含 2 年总体生存率 (OS) 的生存时间，且其中 2 年总体生存率为至少 60%。

[0040] 公开了上述方法，其中给药包括皮下注射疫苗。

[0041] 公开了上述方法，其中给药包括每周一次给予疫苗注射达 3 个月，然后每月一次达 5 个月。

[0042] 还公开了用于制备包括来自同一受试者的黑素瘤细胞和树突细胞的树突细胞疫苗的上述方法，所述方法包括 :将一种或多种黑素瘤细胞用阻止细胞分裂的作用剂处理 ;所述一种或多种黑素瘤细胞不用干扰素 -γ (IFN-γ) 或 IFN-γ 模拟物体外处理 ;选择是自噬的和非凋亡的黑素瘤细胞 ;排除是非自噬的和凋亡的黑素瘤细胞 ;且其中将是自噬的和非凋亡的黑素瘤细胞提供给一种或多种自体树突细胞，或者，其中将来源于是自噬的和非凋亡的黑素瘤细胞的肽提供给一种或多种自体树突细胞。

[0043] 公开了包含以下的组合物 :来自第一受试者的不用干扰素 -γ (IFN-γ) 处理的至少一种黑素瘤细胞，和来自同一第一受试者的至少一种抗原呈递细胞 (APC)，其中黑素瘤细胞是 :自噬的和非凋亡的。

[0044] 此外，公开了上述组合物，其中黑素瘤细胞是表达 MHC II 类的。

[0045] 还公开了上述组合物，其中 APC 是树突细胞、巨噬细胞或 B 细胞。

[0046] 公开了上述组合物，其中至少一种黑素瘤细胞包含黑素瘤特异性肽，且其中黑素瘤特异性肽基本上不包含在所述 APC 内，并且基本上不被所述 APC 加工。

[0047] 还公开了上述组合物，其中黑素瘤细胞包含黑素瘤特异性肽，且其中黑素瘤特异性肽基本上包含在所述 APC 内，且在所述 APC 中被部分加工或被充分加工。还公开了上述组合物，其中将黑素瘤细胞加载到 APC 中。公开了上述组合物，其中黑素瘤细胞不加载到 APC 中。

[0048] 公开了上述组合物，其中自噬通过测定微管相关蛋白轻链 3 (LC3) 的试验证实。

[0049] 公开了上述组合物，其中使用试剂 7- 氨基放线菌素 D (7-ADD) 或试剂膜联蛋白的至少一种证实细胞是非凋亡的。

[0050] 公开了在患有黑素瘤和包含黑素瘤细胞的受试者中刺激免疫应答的方法，其中受

试者与第一受试者是同一受试者，所述方法包括给予免疫有效量的上述组合物。

[0051] 公开了上述组合物，其中至少90%的黑素瘤细胞不用IFN-γ体外处理，且小于10%的黑素瘤细胞用IFN-γ体外处理。

[0052] 公开了用于制备上述疫苗或上述组合物的方法，所述方法包括使至少一种黑素瘤肿瘤细胞与至少一种抗原呈递细胞(APC)接触，其中至少一种黑素瘤肿瘤细胞来自于第一人受试者，且其中至少一种APC来自于同一第一人受试者。

[0053] 公开了用于制备树突细胞疫苗的方法，其包括：将获自第一受试者的黑素瘤细胞用阻止细胞分裂的作用剂处理；其中黑素瘤细胞不用IFN-γ或IFN-γ模拟物体外处理；选择是自噬的和非凋亡的黑素瘤细胞；并使所选择的黑素瘤细胞与来自同一第一受试者的自体树突细胞接触。

[0054] 公开了包含通过上述方法制备的树突细胞疫苗的组合物。

[0055] 公开了用于刺激针对黑素瘤特异性抗原的免疫应答的方法，所述方法包括将上述组合物给予患有黑素瘤的受试者。

[0056] 公开了包含来自第一受试者的至少一种黑素瘤细胞和来自同一第一受试者的至少一种抗原呈递细胞(APC)的组合物，其中黑素瘤细胞是：自噬的、非凋亡的和表达MHC II类的。在本公开内容中，IFN-γ处理的黑素瘤细胞不加载到APC中，且其中IFN-γ处理的黑素瘤细胞不加载到APC中。另一方面，包括了上述组合物，其中黑素瘤细胞来自于I期、II期、III期或IV期黑素瘤受试者。另外，预期上述组合物、相关药盒和相关方法，其中APC包含至少一种树突细胞。

[0057] 一方面，本公开内容的药物组合物、试剂和相关方法使用是7-AAD阴性和膜联蛋白V阴性的癌细胞的制备物。该群体可以是例如约99% 7-AAD阴性和约99%膜联蛋白V阴性，或至少95% 7-AAD阴性和至少95%膜联蛋白V阴性，或至少90% 7-AAD阴性和至少90%膜联蛋白V阴性，以提供非限制性实例。

[0058] 此外，包括了上述组合物，其中自噬通过测定微管相关蛋白轻链3 (LC3)的试验证实；和上述组合物，其中使用试剂7-氨基放线菌素D (7-AAD)或试剂膜联蛋白的至少一种证实细胞是非凋亡的。

[0059] 在方法方面，提供制备上文公开的组合物的方法，所述方法包括从第一受试者中取出至少一种黑素瘤细胞，从第一受试者中取出至少一种APC，使黑素瘤细胞与APC接触；以及用于刺激针对受试者或患者的黑素瘤的免疫应答的方法，所述方法包括将上述组合物给予受试者。

[0060] 在药盒方面，本公开内容提供用于测试针对受试者的肿瘤抗原的免疫应答的药盒，其中受试者用上述方法的一种或多种治疗，且其中药盒包含检测体液免疫应答、细胞免疫应答或先天免疫应答的试剂。

[0061] 附图

图1. 显示从贴壁细胞分离漂浮细胞的方法的图示。

[0062] 图2. 直方图与标志物表达。

[0063] 图3. 显示MHC II类与标志物表达的流式细胞术。

[0064] 图4. 流式细胞术与标志物表达。

[0065] 图5. 细胞相衬显微照片。

[0066] 图 6. 流式细胞术。

[0067] 图 7. 直方图。

[0068] 图 8. 显示 MHC II 类与膜联蛋白 -V 的流式细胞术。

[0069] 图 9. 直方图。

[0070] 图 10. MHC II 类表达与膜联蛋白 -V 阳性百分比的示图。

[0071] 图 11. 生存曲线。

[0072] 图 12. 生存曲线。

[0073] 图 13. 生存曲线。

[0074] 图 14. 显示 MHC 表达的流式细胞术。

[0075] 图 15. MHC I 类和 MHC II 类的表达。

[0076] 图 16. 显示 7-AAD 与膜联蛋白 V 的流式细胞术。

[0077] 图 17. 显示 MHC 表达与膜联蛋白 V 的流式细胞术。

[0078] 图 18. MHC II 类的表达。

[0079] 图 19. 显示 MHC 表达与膜联蛋白 V 的流式细胞术。

[0080] 定义

在没有限制的情况下，免疫刺激量可以是这样的量：提高 ELISPOT 测定结果可测量的量、提高 ICS 测定结果可测量的量、提高四聚体测定结果可测量的量、提高血液抗原特异性 CD4+ T 细胞群可测量的量、提高血液抗原特异性 CD8+ T 细胞群可测量的量，或其中当与合适的对照相比时，提高至少 10%、20%、30%、40%、50%、60%、70%、80%、90%、100%、1.5 倍、2.0 倍、3.0 倍等。合适的对照可为对照疫苗，其中树突细胞不加载黑素瘤细胞，或不加载来源于黑素瘤细胞的肽。

[0081] 在具体的语句重复提及“癌症 / 癌”时，除非另有说明，否则它将假设癌症是相同类型的癌症。例如，在引述“获自患有癌症的受试者的癌细胞的癌特异性肽”时，它意指下述含义。如果患癌症的受试者患有黑素瘤，则肽是黑素瘤特异性肽，且癌细胞是黑素瘤细胞。

[0082] 为了提供黑素瘤的非限制性实例，术语“黑素瘤特异性抗原”包括通常与黑素瘤有关的抗原，其中与同其它癌症有关的不同，该抗原被认为是黑素瘤独特的，另外，术语“黑素瘤特异性抗原”包括常与黑素瘤有关的抗原，且其中该抗原还与其它类型的癌症（例如乳腺癌、结肠直肠癌等）有关。

[0083] 在照射本公开内容的黑素瘤细胞的情况下，“照射”优选通过 γ- 照射，但也包括通过 X 射线、电子、中子、质子、电磁照射、可见光、紫外光等照射。一方面，照射起阻止黑素瘤细胞细胞分裂的作用。另一方面，照射阻止细胞分裂，但还使细胞蛋白质变性。作为照射的备选方式，本公开内容通过物理破坏（例如超声处理、空化 (cavitation)、脱水、离子耗尽），或通过暴露于一种或多种盐产生的毒性，来阻止黑素瘤细胞细胞分裂。

[0084] 如“大于 60% 的黑素瘤特异性肽”中的术语“百分比”是指肽分子的数目，而不是指不同的抗原确定的肽 (different antigenically distinct peptide) 的数目。如“大于 80% 的黑素瘤特异性肽”中的术语“百分比”是指肽分子的数目，而不是指不同的抗原确定的肽的数目。如“小于 40% 的黑素瘤特异性肽”中的术语“百分比”是指肽分子的数目，而不是指抗原确定的肽的数目。如“小于 20% 的黑素瘤特异性肽”中的术语“百分比”是指肽分子的数目，而不是指抗原确定的肽的数目，等等。

[0085] 如“大于 60% 的黑素瘤特异性肽”中的术语“肽”是指肽分子、寡肽分子和多肽分子的数目的总和。如“大于 80% 的黑素瘤特异性肽”中的术语“肽”是指肽分子、寡肽分子和多肽分子的数目的总和。如“小于 40% 的黑素瘤特异性肽”中的术语“肽”是指肽分子、寡肽分子和多肽分子的数目的总和。如“小于 20% 的黑素瘤特异性肽”中的术语“肽”是指肽分子、寡肽分子和多肽分子的数目的总和，等等。

[0086] 在来源于一种或多种癌细胞的肽的情况下，“来源于”包括以下方面。例如，可通过匀浆器或通过渗透爆裂，使癌细胞破裂，产生粗提物。可将粗提物的肽、寡肽和多肽暴露于树突细胞中，接着肽被树突细胞加工。来源于还包括用完整癌细胞提供给树突细胞，其中癌细胞是活的，或其中癌细胞已用照射处理但仍有代谢活性，或其中癌细胞已用核酸交联剂处理但仍有代谢活性。“来源于”包括癌细胞碎片、游离癌细胞蛋白和照射过的癌细胞的混合物，其被树突细胞摄取，因此来源于癌细胞。

[0087] “给予”当应用于人、哺乳动物、哺乳动物受试者、动物、兽药受试者、安慰剂受试者、研究受试者、实验受试者、细胞、组织、器官或生物流体时，无限制地是指使外源配体、试剂、安慰剂、小分子、药剂、治疗剂、诊断剂或组合物与受试者、细胞、组织、器官或生物流体等接触。“给予”可涉及例如治疗、药代动力学、诊断、研究、安慰剂和实验方法。细胞的处理包括使试剂与细胞接触，以及使试剂与流体接触，其中所述流体与细胞接触。“给予”还包括例如细胞用试剂、诊断、结合组合物或用另一种细胞体外和离体处理。

[0088] “激动剂”在涉及配体和受体时，包括刺激受体的分子、分子的组合、复合物或试剂的组合。例如，粒细胞 - 巨噬细胞集落刺激因子 (GM-CSF) 的激动剂可包括 GM-CSF、GM-CSF 的突变蛋白或衍生物、GM-CSF 的肽模拟物、模拟 GM-CSF 的生物功能的小分子或刺激 GM-CSF 受体的抗体。拮抗剂，在涉及配体和受体时，包括抑制、对抗、减量调节受体和 / 或使受体脱敏的分子、分子的组合或复合物。“拮抗剂”包括抑制受体的组成型活性的任何试剂。组成型活性是在缺乏配体 / 受体相互作用时显现的活性。“拮抗剂”还包括抑制或阻止受体受刺激的 (或受调节的) 活性的任何试剂。举例来说，GM-CSF 受体的拮抗剂包括而绝不限于与配体 (GM-CSF) 结合并阻止配体与受体结合的抗体，或与受体结合并阻止配体与受体结合的抗体，或其中抗体锁定受体呈无活性构象。

[0089] 除非另有明确说明，或上下文另有规定，否则术语“表达”包括下列方面。表达包括 mRNA 生物合成、多肽生物合成、例如通过翻译后修饰的多肽活化或通过改变亚细胞位置或通过募集至染色质所致表达的活化。换句话说，“增加的表达”包括增加的生物合成，或通过磷酸化引起的增加的活性，或通过从细胞溶胶迁至核引起的增加的活性。

[0090] 抗原呈递细胞 (APC) 是用于向 T 细胞呈递抗原的免疫系统的细胞。APC 包括树突细胞、单核细胞、巨噬细胞、边缘区 Kupffer 细胞、小胶质细胞、朗格汉斯细胞、T 细胞和 B 细胞 (参见例如 Rodriguez-Pinto 和 Moreno (2005) Eur. J. Immunol. 35:1097- 1105)。树突细胞出现于至少两种谱系。第一谱系包括 pre-DC1、骨髓样 DC1 和成熟 DC1。第二谱系包括 $CD34^{++}CD45RA^{-}$ 早期祖代多能细胞、$CD34^{++}CD45RA^{+}$ 细胞、$CD34^{++}CD45RA^{++}$ $CD4^{+}$ $IL\text{-}3Ralpha^{++}$ pro-DC2 细胞、$CD4^{+}CD11c^{-}$ 浆细胞样 pre-DC2 细胞、淋巴样人 DC2 浆细胞样衍生的 DC2 和成熟 DC2 (参见例如 Gilliet 和 Liu (2002) J. Exp. Med. 195:695-704 ;Bauer 等 (2001) J. Immunol. 166:5000-5007 ;Arpinati 等 (2000) Blood 95:2484-2490 ;Kadowaki 等 (2001) J. Exp. Med. 194:863-869 ;Liu (2002) Human Immunology 63:1067-1071 ;

McKenna 等(2005) J. Virol. 79:17-27 ;O'Neill 等(2004) Blood 104:2235-2246 ;Rossi 和 Young (2005) J. Immunol. 175:1373-1381 ;Banchereau 和 Palucka (2005) Nat. Rev. Immunol. 5:296-306)。

[0091] "有效量"无限制地包括可以改善、逆转、减轻、预防或诊断医学病况或病症的症状或体征的量。除非另有明确说明或通过上下文说明,否则"有效量"不限于足以改善病况的最低量。可毫无任何限制地通过生物标志物或通过临床参数测量疾病或病症的严重程度以及预防、治疗或减轻疾病或病症的治疗的能力。生物标志物包括血细胞计数、血清、尿液或脑脊液中的代谢物水平、肿瘤细胞计数、癌症干细胞计数、肿瘤水平。肿瘤水平可通过 RECIST 标准 (Eisenhauer 等 (2009) Eur. J. Cancer. 45:228-247) 测定。表达标志物包括 mRNA 的遗传表达或基因扩增、抗原的表达和多肽的表达。临床参数毫无任何限制地包括无进展生存率 (PFS)、6 个月 PFS、无病生存率 (DFS)、至进展的时间 (TTP)、至远端转移的时间 (TDM) 和总体生存率。

[0092] 被"标记的"的组合物是可通过以下方法直接或间接检测的 :分光镜方法、光化学方法、生物化学方法、免疫化学方法、同位素方法或化学方法。例如,有用的标记包括 ^{32}P、^{33}P、^{35}S、^{14}C、^{3}H、^{125}I、稳定同位素、表位标签荧光染料、电子致密试剂、底物或酶,例如用于酶联免疫测定法或 fluorettes (参见例如 Rozinov 和 Nolan (1998) Chem. Biol. 5:713-728)。

[0093] 免疫系统调节剂

下面总体上涉及 toll 样受体 (TLR) 激动剂、消耗 T 调节性细胞 (Treg) 的作用剂、可提高 $CD8^+$ T 细胞或 $CD4^+$ T 细胞活性的作用剂和可调节免疫系统的其它作用剂。本公开内容提供试剂、给药方法、诊断方法和使用下列试剂的一种或两种及其组合刺激针对肿瘤抗原的免疫应答的方法。

[0094] 本公开内容提供与树突细胞疫苗一起使用的免疫佐剂和其它免疫系统调节剂。提供 toll 样受体 (TLR) 激动剂,例如以下激动剂。咪唑并喹啉类 (例如咪喹莫德和瑞喹莫德) 是 TLR7 或 TLR8 激动剂。刺激浆细胞样树突细胞的 TLR7 引起 IFN-α 表达。刺激骨髓样树突细胞的 TLR7 引起白介素 -12 表达和随后的 Th1 型免疫应答。CpG 寡核苷酸 (CpG ODN) 是 TLR9 激动剂。CpG ODN 以 3 个类别 CpG-A、CpG-B 和 CpG-C 存在。因为其对浆细胞样 DC 的 IFN-α 产生作用,因此 CpG-A 刺激 NK 细胞。CpG-B 诱导 IFN-α,并增量调节 pDC 和 B 细胞上的共刺激标志物。TLR3 激动剂包括多聚肌苷酸 - 多聚胞苷酸 (poly I:C),其是病毒双链 RNA (dsRNA) 的类似物。TLR4 激动剂包括单磷酰脂质 A (MPL),其是明尼苏达沙门氏菌 (*Salmonella minnesota*) 脂多糖的衍生物,并且用作针对人乳头瘤病毒的疫苗的一部分。减毒细菌用于抗癌疗法。牛分枝杆菌 (*Mycobacterium bovis*) 刺激 TLR2 和 TLR4 和 TLR9。单核细胞增多性李斯特氏菌 (*Listeria monocytogenes*) 刺激各种 TLR (Dubensky 等人的 US 2007/0207171,其通过引用以其整体结合到本文中)。另参见 Galluzzi 等 (2012) Oncolmmunology. 1:699-716 ;Adams (2009) Immunotherapy. 1:949-964。

[0095] 还提供 α- 半乳糖基神经酰胺 (α-GalCer) (Schwaab 和 Ernstoff (2011) Therapy. 4:369-377)。α-GalCer 激发表达某些 T 细胞受体的 NKT 细胞活化 (Lopez-Sagaseta 等 (2012) PLoS Biol. 10:e1001412 (11 页))。还提供 NOD 受体的激动剂。NOD 受体包括 NOD1 和 NOD2。NOD 激动剂包括 N- 乙酰基胞壁酰基 -L- 丙氨酰基 -D- 异谷

酰胺(胞壁酰二肽(MDP)),其与NOD 2结合。NOD激动剂包括γ-D-谷氨酰基-内消旋-二氨基庚二酸(iE-DAP),其与NOD1结合。NOD激动剂包括脱胞壁酰肽(DMP),其与NOD1结合。参见例如Uehara等(2006) J. Immunol. 177:1796-1804)。NOD激动剂来源于肽聚糖的片段。

[0096] 还提供抑制T调节性细胞(Treg)的作用剂。这些作用剂包括环磷酰胺、吉西他滨和消耗Treg的抗体(参见例如Le和Jaffee (2012) Cancer Res. 72:3439-3444;Klages等(2010) Cancer Res. 70:7788-7799)。吉西他滨降低骨髓衍生的抑制性细胞(MDSC)的免疫抑制活性(Le等(2009) Int. Immunopharmacol. 9:900-909)。抗CD25抗体可消耗Treg,并且已用于癌症治疗(Klages等(2010) Cancer Res. 70:7788-7799)。达克珠单抗是一种抗CD25抗体,其阻断白介素-2 (IL-2)与CD25结合,因此阻断Treg维持所需要的信号。某些Treg表达高水平的CTLA4 (CD45RA$^+$ Treg)。抗CTLA4抗体(例如伊匹木单抗)靶向CTLA4。伊匹木单抗用于治疗黑素瘤(Rech等(2012) Immunotherapy. 4:1103-1105)。

[0097] 淋巴细胞活化基因3 (LAG-3)阻滞剂(例如抗LAG-3抗体或可溶性LAG-3 (例如LAG-3 Ig)),可提高对癌症或感染的免疫应答。抗LAG-3抗体降低Treg的活性(参见例如Huang等(2004) Immunity 21:503-513;Triebel (2003) Trends Immunol. 24:619-622)。

[0098] 可获得直接靶向CD8$^+$ T细胞或CD4$^+$ T细胞的抗体。这些抗体包括靶向共刺激受体(4-1 BB、OX40和GITR)或阻断共抑制受体(CTLA-4、PD-1和PD-L1)的那些抗体。GITR是糖皮质激素诱导的TNFR相关蛋白。参见例如Schaer等(2010) Curr. Opin. Investig. Drugs. 11:1378-1386)。抗GITR可终止Treg的抑制功能,因此解释了抗GITR在抗癌疗法中的功效(Coe等(2010) Cancer Immunol. Immunother. 59:1367-1377)。DTA-1是一种抗GITR抗体。

[0099] 评价免疫应答的方法

本公开内容还提供ELISPOT测定法、胞内细胞因子染色(ICS)和四聚体测定法,用于表征免疫应答(参见例如Pardoll的US 2007/0190029;Chattopadhyay (2008) Cytometry A. 2008 73:1001-1009;Vollers (2008) Immunology. 123:305-313;Lalvani等(1997) J. Exp. Med. 186:859-865;Waldrop (1997) J. Clin. Invest. 99:1739-1750;Hudgens (2004) J. Immunol. Methods 288:19-34;Goulder (2001) J. Virol. 75:1339-1347;Goulder (2000) J. Exp. Med. 192:1819-1831;Anthony (2003) Methods 29:260-269;Badovinac和Harty (2000) J. Immunol. Methods 238:107-117)。可通过用于肿瘤学临床试验的终点,包括客观反应(RECIST标准)、总体生存率、无进展生存率(PFS)、无病生存率、至远端转移的时间、6个月PFS、12个月PFS等,来评价患者的免疫应答。

[0100] 抗原

成胶质细胞瘤特异性抗原包括PTPRZ1、EGFR、SEC61G、TNC、HER2、TRP-2、gp100、MAGE-1、IL13Ralpha2、AIM-2 (Phuphanich等(2013) Cancer Immunol. Immunother. 62:125-135;Neidert等(2012) J. Neurooncol.)。结肠直肠癌特异性抗原包括SPARC、CEA、Cep55/c10orf3 (Inoue等(2010) Int. J. Cancer. 127:1393-1403;Parkhurst等(2011) Mol. Ther. 19:620-626;Inoda等(2011) Exp. Mol. Pathol. 90:55-60)。肾癌特异性抗原包括碳酸酐酶IX (CA-IX)、MUC-1和NYESO-1和5T4 (Tykodi等(2012) J. Immunother. 35:523-533)。卵巢癌特异性抗原包括WT1、间皮素、NY-ESO-1、p53、携带特异性突变的

p53、HER-2/neu、叶酸受体-α、IGFBP、MUC1、MUC4、MUC16、EpCAM、CTA (Dohi 等 (2011) Anticancer Res. 31:2441-2445 ;Vermeij等(2012) Curr. Pharm. Des. 18:3804- 3811 ; Preston 等 (2011) Immunother. 3:539-556)。特异性可产生自抗原的氨基酸序列、肿瘤细胞表达该抗原的程度、抗原的翻译后修饰等。某些类型的癌细胞的特异性也可产生自多种肿瘤抗原的特定指纹。特异性还可产生自这样的事实,即具体的抗原虽然由各种各样的肿瘤细胞表达,但只在针对较少数的肿瘤类型的免疫疗法中具有特定用途。特异性还可产生自这样的事实,即特定集合的MHC I类可呈递和MHC II类可呈递表位存在于特定的多肽或多肽片段上。此外,可通过删除可激发免疫耐受性的一种或多种肽,在所给予的抗原中产生特异性。技术人员可容易地查到相关的核酸和多肽序列,例如美国政府网址ncbi.nlm.nih。

[0101] 疫苗

本公开内容的疫苗可以是纯预防性的,其可用于治疗现有的癌症或肿瘤前病症,或可用于预防之前治疗的癌症的复发。本公开内容的树突细胞疫苗可通过真皮内、结内、粘膜或皮下途径、或上述的任何组合给予。每个剂量可包含约10 x 10^3个树突细胞、20 x 10^3个细胞、50 x 10^3个细胞、100 x 10^3个细胞、200 x 10^3个细胞、500 x 10^3个细胞、1 x 10^6个细胞、2 x 10^6个细胞、20 x 10^6个细胞、50 x 10^6个细胞、100 x 10^6个细胞、200 x 10^6、500 x 10^6、1 x 10^9个细胞、2 x 10^9个细胞、5 x 10^9个细胞、10 x 10^9个细胞等。给药频率可为例如每周一次、每周两次、每两周一次、每三周一次、每四周一次、每月一次、每两月一次、每三月一次、每四月一次、每五月一次、每六月一次等。其中发生给药的总天数可为1天、2天或3、4、5、6、7、8、9、10、11、12、13、14、15、16、17、18、19或20天等。要了解,任何规定的给药可包括在同一日注射两次或更多次。一方面,本公开内容包括树突细胞用完整肿瘤细胞加载,其中至少10%、其中至少20%、至少30%、至少40%、至少50%、至少60%、至少70%、至少80%、至少90%、至少95%或至少99%的加载到树突细胞的黑素瘤细胞来源的蛋白质停留在完整肿瘤细胞中。在非限制性实施方案中,将树突细胞疫苗装在培养瓶、小瓶、瓶、注射器、导管、套管等中。对于给药,至少20%、至少30%、至少40%、至少50%、至少60%、至少70%、至少80%、至少90%、至少95%、至少99%的给予的树突细胞为成熟树突细胞。

[0102] 疫苗均质性

在实施方案中,本公开内容提供包含树突细胞的疫苗,所述树突细胞含有来源于体外加载的黑素瘤肽,其中疫苗包含树突细胞/黑素瘤细胞的比率为至少5/95、10/90、20/80、30/70、40/60、50/50、60/40、70/30、80/20、90/10、95/5、98/2、99/1等的树突细胞(含有黑素瘤肽的DC和不含黑素瘤肽的DC的总和)。还提供包含树突细胞的疫苗,所述树突细胞含有来源于体外加载的黑素瘤肽,其中疫苗包含[树突细胞]/[既非DC也非黑素瘤的细胞]的比率为至少5/95、10/90、20/80、30/70、40/60、50/50、60/40、70/30、80/20、90/10、95/5、98/2、99/1等的树突细胞(含有黑素瘤肽的DC和不含黑素瘤肽的DC的总和)。本公开内容提供分区容器,其中第一区室含有黑素瘤细胞,第二区室含有树突细胞。两个区室可被防止黑素瘤细胞与树突细胞接触的膜、滤器、阀、管道、联接器分开,但是在手工转移时,或在除去膜或打开阀时允许黑素瘤细胞与树突细胞接触,使黑素瘤细胞、黑素瘤细胞片段和/或黑素瘤肽加载到树突细胞中。

[0103] 干扰素-γ模拟物

本公开内容包括模拟物,例如干扰素-γ模拟物,例如模拟肽95-132 (Ahmed (2007)

J. Immunol. 178:4576-4583;Fulcher (2008) FEBS Lett. 582:1569-1574)。IFN模拟物包括例如具有相同的干扰素-γ激动剂活性的抗体。

[0104] 使黑素瘤细胞灭活

本公开内容提供组合物和方法,其中例如通过辐射或通过核酸交联剂使癌细胞灭活。示例性的交联剂具有交联DNA但留下未被修饰的蛋白质的能力。核酸烷基化剂(alkylator)可以是β-丙氨酸、N-(吖啶-9-基)、2-[双(2-氯乙基)氨基]乙酯。在一些实施方案中,靶向核酸的化合物是被UVA照射激活的补骨脂素化合物。例如,核酸靶向化合物可以是4'-(4-氨基-2-氧杂)丁基-4,5',8-三甲基补骨脂素(本文亦称为"S-59")。细胞可用150微摩尔补骨脂素S-59和3 J/cm^2 UVA光(FX 1019照射装置,Baxter Fenwal,Round Lake,IL)灭活。用S-59灭活称为光化学处理,并导致细胞彻底失活。可测试不同浓度的核酸交联剂在使细胞灭活中的功效,例如阻止细胞分裂的功效。S-59的特征是其交联核酸、但留下未被修饰的完整蛋白质的能力。可将细胞悬浮于5 mL含有0、1、10、100和1000 nM的补骨脂素S-59的盐水中。如下照射每个样品。可加入100 nM浓度的S-59。可按约2 J/cm^2 (FX1019照射装置,Baxter Fenwal,Round Lake,III.)的剂量对样品进行UVA照射。然后将各样品转移到15 mL管中,离心,除去上清液,然后用5 mL盐水洗涤,离心,除去上清液,并将最后的沉淀悬浮于0.5 mL盐水中(Dubensky的美国专利号7,833,775和Dubensky的7,691,393)。

[0105] 非凋亡癌细胞的富集

可通过例如采用从是非自噬的和凋亡的细胞中分离是非凋亡的和自噬的细胞的技术,在是非凋亡的黑素瘤细胞中富集黑素瘤细胞群,其中分离通过使自噬的和非凋亡的细胞粘附在表面上进行,其中其它细胞是漂浮的。还可通过借助于对磷脂酰丝氨酸有特异性的抗体除去凋亡的细胞,来获得在非凋亡的黑素瘤细胞中富集的群体。可获得通过固定化抗体除去细胞的技术(Onodera (1998) Ther. Apher. 2:37-42)。可获得对磷脂酰丝氨酸有特异性的抗体(例如,EMD Millipore,Billerca,MA)。此外,大部分黑素瘤细胞群可用荧光抗磷脂酰丝氨酸抗体标记,其中加标签的凋亡的黑素瘤细胞通过流式细胞术、亲和色谱法、免疫磁性分离除去(参见例如Hoeppener (2012) Recent Results Cancer Res. 195:43-58;Dainiak (2007) Adv. Biochem. Eng. Biotechnol. 106:1-18)。该技术一般适用于在是黑素瘤细胞的癌细胞中富集,也适用于不是黑素瘤细胞的癌细胞。

[0106] 凋亡抑制剂

凋亡抑制剂Z-VAD (Z-VAD-fmk)可获自例如Enzo Life Sciences (Exeter,UK)、R & D Systems (Minneapolis,MN)、Tocris Biosciences (Bristol,UK)、BioMol (Plymouth Meeting,PA)和EMD Chemicals (Gibbstown,NJ)。Z-VAD-fmk是一种合成肽Z-Val-Ala-Asp(OMe)-CH_2F。胱天蛋白酶是蛋白酶家族的半胱氨酸-天冬氨酸特异性成员。通过死亡受体连接例如TRAIL、FAS;通过DNA损伤、应激、血清饥饿和在一些细胞类型中通过干扰素,来激活胱天蛋白酶。胱天蛋白酶在高度调节的细胞凋亡的过程(包括核断裂、染色质浓缩和胞质完整性丢失)中起关键作用。全胱天蛋白酶(pan-capase)抑制剂z-VAD-fmk (苄氧羰基-缬氨酰基-丙氨酰基-天冬氨酰基[O-甲基]-氟甲基酮)不可逆地结合胱天蛋白酶的催化部位并抑制其在诱导细胞凋亡中的功能。抑制细胞在响应IFN-γ时进行细胞凋亡的能力可以是籍此产生是非凋亡但是自噬的细胞而无需通过洗涤除去漂

浮的凋亡细胞的选择步骤的手段。

[0107] 本公开内容提供药物、试剂、药盒（包括诊断药盒），其中药物、试剂和药盒包含树突细胞、抗体或抗原。还提供用于给予包含至少一种树状细胞和至少一种抗原的组合物的方法、用于刺激抗体形成的方法、用于刺激 ADCC 的方法、用于刺激补体依赖性细胞毒性的方法及用于测定患者适合性、用于在临床试验或普通医学治疗的情况下测定患者纳入 / 排斥标准和用于预测对药物或试剂的反应的方法和药盒。描述了补体依赖性细胞毒性（参见例如Goodman等(1990) J. Clin. Oncol. 8:1083-1092 ;Cheson (2010) J. Clin. Oncol. 28:3525-3530)。本公开内容的药物组合物、试剂和相关方法包括 CD83 阳性树突细胞，其中 CD83 通过加载 IFN-γ 处理的癌细胞来诱导。在本公开内容的 CD83 方面，CD83 被诱导至少 2%、至少 3%、至少 4%、6%、7%、8%、9%、10% 等。

[0108] 图 1显示在用 IFN-γ 处理前（左）和在用 IFN-γ 处理 72 小时后（右）培养的肿瘤细胞的示图。在处理后，培养的肿瘤细胞是漂浮的、非自噬的和凋亡的，或是贴壁的、自噬的和非凋亡的。漂浮细胞显示表达凋亡标志物磷脂酰丝氨酸。漂浮细胞显示相对少地表达 MHC II 类，而贴壁细胞显示过量表达 MHC II 类。

[0109] 图 2A-D显示用于加载树突细胞的 IFN-γ 处理的自体肿瘤细胞的特征。在含 15% FBS/ECS 的 RPMI 中，将自体黑素瘤肿瘤细胞用或不用 1000 IU/mL IFN-γ 处理 72 小时，收获，用 100Gy 照射，并冷藏保存。然后将细胞在 AIMV 中融化，取样品用于流式细胞术和用于制备进行免疫印迹法的细胞裂解物，然后进行 DC 的抗原加载。显示了 4 个不同的自体黑素瘤细胞系的实例（图 2A、图 2B 和图 2C)。通过 IFN-γ 处理自体肿瘤细胞诱导主要组织相容性复合体（图 2D)。在用或不用 1000 IU/mL IFN-γ 处理 72 小时后收获肿瘤细胞，然后测定 MHC I 类和 II 类。使用对照同种型抗体鉴定阳性群体。深色数据点表示中值平均荧光 +/-95% 置信区间。N = 65。在照射后，通过测定检查黑素瘤细胞，确保没有任何有丝分裂。

[0110] 一方面，本公开内容不包括用于加载树突细胞的非自体肿瘤细胞，并且不包括使用用于加载树突细胞的非自体肿瘤细胞的方法。

[0111] 图 3A和 3B描述了用或不用干扰素 -γ 处理的自体黑素瘤细胞系加载的树突细胞的表型。将一组 4 个自体黑素瘤细胞系用或不用 1000 IU/mL 的 IFN-γ 处理 72 小时，照射并冷藏保存。然后将细胞在 AIMV 中融化，与自体树突细胞混合约 24 小时，随后收获并通过流式细胞术测定 CD80、CD83、CD86 和 MHC II 类的表达（图 3A)。数据概括于图 3B。显示了平均值 ± SD，n = 4。

[0112] 图 4A和 4B显示用于剂量制剂的树突细胞的表型。通过流式细胞术，针对 CD80、CD83、CD86 和 MHC II 类的表达，对在用 IFN-γ 处理的、经照射的自体肿瘤细胞加载之前 (ATC 加载前 DC，N = 53) 和加载之后 (ATC 加载后 DC，N = 65) 的 DC 样品进行了评价。使用 FACS Caliber® 珠粒设定初始流式细胞仪仪器设置，其随后在数据采集期间保持恒定（图 4A)。图 4B 中，比较了 ATC 加载前和 ATC 加载后的百分比表达和平均荧光强度 (MFI) 的值 ±SD。*p = 0.019 和 **p = 0.0009。

[0113] 图 5A-5C显示干扰素 -γ 处理的黑素瘤细胞进行自噬。在 5%FBS/RMPI 中，将选择的市售得到的黑素瘤细胞系与 1000 IU/mL IFN-γ 一起温育 72 小时。在温育期结束时拍摄 SK-5-Mel 细胞培养物的相衬显微照片（图 5A)，显示具有表明自噬体的空泡的放大细

胞。通过在用 IFN-γ 处理前用 GFP-LC3B 构建体转染证实了自噬体形成（图 5B)。通过使用 LC3B 抗体的蛋白质印迹法证实了在 IFN-γ 处理后的自噬诱导（图 5C)，其鉴定出已表明与自噬管 (autophagic vessel) 形成有关的 LC3 的快速迁移形式。

[0114] 图 6A和 6B揭示响应干扰素 -γ 时所诱导的细胞凋亡和自噬。使 SK-5-Mel 细胞与 1000 IU/mL 的 IFN-γ 一起温育 72 小时，之后收集非贴壁群和贴壁群，使用 7-AAD 和膜联蛋白 -V，通过流式细胞术测定细胞凋亡和自噬（图 6A)。通过流式细胞术，经测量生产商提供的染料的平均峰移强度（图 6B)，使用 Enzo Cyto-ID 自噬检测染料测量自噬。与 5% FBS/RPMI 相比的峰移倍数变化见图 6C，其中无血清用作自噬诱导的阳性对照。

[0115] 图 7揭示了在黑素瘤细胞中在阻断胱天蛋白酶活性后的自噬诱导不影响响应 IFN-γ 的自噬诱导。在 20uM 全胱天蛋白酶抑制剂 z-VAD 或其对照化合物 z-FA 存在下，将 SK-5-Mel 细胞用 1000 IU/mL 的 IFN-γ 处理 72 小时。收获细胞，并通过流式细胞术测定自噬，如图 6C 所示。

[0116] 图 8显示在 10 uM 自噬抑制剂 3- 甲基腺嘌呤 (3-MA) 存在下，与 1000 IU/mL 的 IFN-γ 一起温育 72 小时的 SK-5-Mel 细胞。然后收获细胞，并通过流式细胞术，测定细胞凋亡和 MHC II 类 (HLA-DR) 表达。

[0117] 图 9显示针对 MHC II 类或细胞凋亡的变化，对自患者肿瘤样本 (N = 36) 产生的肿瘤细胞系的 IFN-γ 处理的细胞进行测定。所示数据是平均荧光强度 (MFI) 的平均数 ± SE。

[0118] 图 10显示通过流式细胞术，自用于加载树突细胞以用于患者特异性疫苗免疫疗法的样品，测定 IFN-γ 处理的细胞 (N = 54) 的 MHC II 类或细胞凋亡。显示了 MHC II 类平均荧光强度 (MFI) 的倍数变化和百分比凋亡细胞（膜联蛋白 -V 阳性）。

[0119] 图 11和图 12显示在接受了加载自噬的、非凋亡的干扰素 -γ 处理的肿瘤细胞的树突细胞的患者中，MHC II 类诱导和细胞凋亡缺乏（干扰素 -γ 抵抗）间的关系与更好的无进展生存率（图 11) 和总体生存率（图 12) 有关。

[0120] 图 13显示 3 个试验的生存曲线。示图 (Kaplan-Meier 图）是一种阶梯曲线，显示在临床试验期间研究受试者生存的百分比。各组命名为 DC-54（实心圆）；TC-74（实心正方形）；TC-24（实心三角形）；和 DC-18（直线）。用 TC-24 出现最差生存率。次最差生存率是用 TC-74。TC-24 是指在包括 24 名受试者的研究中肿瘤细胞的疫苗。

[0121] 图 14显示 3 个试验的生存曲线。试验是与图 13 所公开的那些相同的临床试验，但有较后时间点获得的其它数据。

[0122] 其它公开内容

自体树突细胞产生

在补充 IL-4 (CellGenix，Freisberg，Germany) 和 GM-CSF (Berlex，Seattle，WA) 各 1,000 IU/mL 的无抗生素 AIM-V 培养基 (Invitrogen，Grand Island，NY) (DC 培养基）中，通过 ficoled 单采血液成分术产品的塑料粘附方法 (Choi 等 (1998) Clin. Cancer Res. 4:2709-2716；Luft 等 (1998) Exp. Hematol. 26:489-500；Cornforth 等 (2011) Cancer Immunol. Immunother. 60:123-131) 产生树突细胞。然后培养瓶经培养 6 天后，加载 IFN-γ 处理的、经照射的自体肿瘤细胞。

[0123] IFN-γ 自体肿瘤细胞系产生和药物的制备

按 照 Cornforth 等 人 (Cornforth 等 (2011) Cancer Immunol. Immunother. 60:123-131 ;Dillman 等 (1993) J. Immunother. Emphasis Tumor Immunol. 14:65-69 ; Dillman 等 (2000) Cancer Biother. Radiopharm. 15:161-168),产生纯的肿瘤细胞。然后使肿瘤细胞与 1,000U/mL 的干扰素 -γ (InterMune,Brisbane,CA) 一起温育 72 小时,用来自铯源的 100Gy 照射,并冷藏保存 (Selvan 等 (2007) Int. J. Cancer 122:1374-1383 ; Selvan 等 (2010) Melanoma Res. 20:280-292)。从冷藏保存中回收 IFN-γ 处理和照射的肿瘤细胞,用磷酸盐缓冲盐水 (PBS) 洗涤,然后加入到培养的树突细胞 (DC) 中,然后温育约 24 小时。通过用橡胶刮棒轻轻刮擦,收获加载抗原的 DC,并冷藏保存。获得等分的 IFN-γ 处理或未处理的肿瘤细胞和加载的 DC 用于流式细胞术评价和锥虫蓝排斥测定。

[0124] 皮肤黑素瘤的分期

可将本公开内容的药物或试剂给予黑素瘤患者,其中黑素瘤诊断为 I 期、II 期、III 期或 IV 期 (Mohr 等 (2009) Ann. Oncology (Suppl. 6) vi14-vi21)。例如,I 期是指患有原发性黑素瘤、没有局部或远端转移证据的患者。II 期包括没有淋巴病或远端转移证据的患者,其中患者通过以下进一步表征 :例如病变大于 1mm,且覆盖在上皮上面的溃疡厚小于或等于 2mm ;或病变大于 2mm,且上皮溃疡厚小于或等于 4mm。III 期黑素瘤包括具有病理学记载的涉及局部淋巴结或移动中 (in-transit) 或随体 (satellite) 转移的病变,其中患者可具有例如 1、2、3 或 4 个或更多个受累淋巴结。IV 期黑素瘤定义为存在远端转移,其中转移仅位于远端皮肤、皮下组织或淋巴结 ;其中转移包括肺转移 ;或其中转移包括所有其它的内脏部位。

[0125] 本公开内容包括用于是预防性给药 (即用于尚未或从未诊断为黑素瘤的受试者) 的方法。包括这样的给药方法,其中受试者早期被诊断为黑素瘤、早期已成功接受治疗以根治黑素瘤 (或经历过自发性完全缓解) 和其中在根治后预防性地使用给药。

[0126] 肿瘤抗原

不意指任何限制,本公开内容的黑素瘤细胞表达 Mage、Mart-1、Mel-5、HMB45、S100 或酪氨酸酶的一种或多种 (Dillman 等 (2011) Cancer Biotherapy Radiopharmaceuticals 26:407-415)。一方面,肿瘤抗原的检测使用未暴露于 IFN-γ 的细胞,而另一方面,肿瘤抗原的检测在用 IFN-γ 处理的细胞中进行 (参见例如 Cornforth 等 (2011) Cancer Biotherapy Radiopharmaceuticals 26:345-351)。包括了表达一种或多种黑素瘤抗原的黑素瘤细胞,或包含一种或多种分离的黑素瘤抗原的组合物,如 Dubensky 等人的 US2007/0207171 所公开,其通过引用以其整体结合到本文中。

[0127] 测量细胞凋亡

可用许多试剂 (例如荧光染料标记的膜联蛋白)、通过用染料 (例如碘化丙锭和 7- 氨基放线菌素 D (7-AAD)) 染色、通过测定线粒体内膜电位的丧失、通过测量胱天蛋白酶的活化或切割,来检测或测量细胞凋亡。参见例如 George 等 (2004) Cytometry Part A. 59A:237-245。细胞凋亡的早期事件是磷脂酰丝氨酸暴露于质膜的外表面,这可通过荧光染料标记的膜联蛋白检测。可用得方法可区分活细胞、坏死细胞、早期凋亡细胞和晚期凋亡细胞。本公开内容使用在 IFN-γ 处理后,通过 7-ADD 测定不是凋亡的、通过膜联蛋白 V 测定不是凋亡的、通过细胞凋亡测定不是凋亡的 (Dillman 等 (2011) Cancer Biotherapy Radiopharmaceuticals 26:407-415) 或通过生物标志物 Bcl-2、胱天蛋白酶 -3、P53 或存

活蛋白的一种或多种而不是凋亡的 (Karam 等 (2007) Lancet Oncol. 8:128-136) 黑素瘤细胞。本公开内容的药物组合物、试剂和相关方法不包括 IFN-γ 处理的是凋亡的黑素瘤细胞，其中细胞凋亡按照例如被授予专利权的 Herlyn 等人的美国专利号 7,544,465、被授予专利权的 Thorpe 等人的美国专利号 7,714,109 测定，所述专利通过引用结合到本文中。

[0128] 测量自噬

自噬是用于许多蛋白质和一些细胞器降解的一个天然存在的过程。自噬介导蛋白质和细胞器更新、饥饿反应、细胞分化、细胞死亡等。微管相关蛋白轻链 3 (LC3) 将用于监测自噬。在一种方法中，自噬可通过测量 LC3 转化 (其包括 LC3-I 转化成 LC3-II) 来检测。LC3-II 的量与自噬体的数目相关。详细地讲，LC3 是胞质的和可溶的，而 LC3-II 存在于膜上。LC3-II 有较大的分子量，因为它与脂质缀合。LC3 加工可通过例如蛋白质印迹测量，而自噬、自噬泡和自噬体可通过显微镜测量。可例如通过检测已加工的 LC3-II、通过早期与晚期自噬区室间的比例或通过自噬的容积对自噬进行定量测定。参见 (Mizushima 和 Yoshimori (2007) Autophagy 3:542-546:634-641 ;Tanida 等 (2008) Methods Mol. Biol. 445:77-88 ;Eng 等 (2010) Autophagy 6:634-641)。一方面，本公开内容利用自噬作为筛选工具，用于选择合适的自噬癌细胞，其中可根据一个或多个特定阶段中自噬的出现来选择细胞。这些自噬阶段包括 :(1) 通过自噬体分隔出胞质区室，(2) 自噬体与溶酶体融合形成自体溶酶体，和 (3) 自体溶酶体内容物被溶酶体内的蛋白酶降解。另一方面，本公开内容包括大部分显示第一阶段的细胞、大部分为第二阶段、大部分为第三阶段、大部分为第一和第二阶段、大部分为第二和第三阶段或大部分显示所有三个阶段的细胞。又一方面，本公开内容包含显示第一阶段、第二阶段、第三阶段、第一和第二、第二和第三阶段的细胞或显示所有三个阶段的细胞。

[0129] 干扰素 -γ (IFN-γ)信号转导

IFN-γ (II 型干扰素) 信号转导取决于许多基因的表达，例如 IFN-γ 受体、STAT1、STAT2、STAT1 同二聚体、STAT1/STAT2 异二聚体、IRF-1、GAS 和 IRF-E。研究表明，IFN-γ 信号转导取决于 IFN-γ 受体 (IFNGR1 链 ;IFNGR2 链)。细胞表面上 IFNGR 的低表达可阻断 IFN-γ 信号转导的某些方面 (Schroder 等 (2004) J. Leukocyte boil. 75:163-189)。一方面，本公开内容不包括使用显示低的 IFNGR 表面表达的癌细胞。另一方面，本公开内容针对表达 STAT1 同二聚体的细胞筛选癌细胞，使用这些细胞，且基本上不包括不表达 STAT1 同二聚体的细胞。又一方面，本公开内容预期针对具有 STAT1 磷酸化 (丝氨酸 -727) 的细胞筛选细胞。还预期不包括来自具有 STAT1 基因中功能突变丧失的患者的癌细胞 (参见例如 Dupuis 等 (2001) Science 293:300-303 ;Schroder 等 (2004) J. Leukoc. Biol. 75:163-189)。下面涉及 IRF 基因家族。IRF-1、IRF-2 和 IRF-9 都参与 IFN-γ 信号转导。本公开内容包括使用表达这些 IRF 基因家族基因的一种或多种的癌细胞，或不包括不表达这些基因的一种或多种的癌细胞。

[0130] IFN-γ 响应基因

本公开内容包括需要使用响应 IFN-γ 的黑素瘤细胞或肿瘤发生前黑素瘤细胞的生物材料、组合物、试剂和方法。可通过测定一种或多种 IFN-γ 响应基因的表达，来鉴定、区分和选择黑素瘤细胞。已鉴定出许多 IFN-γ 响应基因 (参见例如 Halonen 等 (2006) J. Neuroimmunol. 175:19-30 ;MacMicking (2004) 11:601-609 ;Boehm 等 (1997)

15:749-795)。所述测定可包括从患者中取出一个或多个黑素瘤细胞，在添加的 IFN-γ 存在和不存在下培养该细胞，并测定对 IFN-γ 的响应性。在该测定中，可通过测定对转录因子与 IFN-γ 诱导的基因的启动子结合、对自 IFN-γ 诱导的基因的 mRNA 的表达、对表达的多肽等的敏感性，来检测 IFN-γ 诱导的基因表达。IFN-γ 响应基因可包括例如用于免疫应答的基因、编码转录因子、转运蛋白的基因、细胞凋亡基因、用于细胞生长或维持的基因、用于脂质代谢的基因、介导胞吞或胞吐的基因、胞内信号转导基因、葡萄糖代谢基因、细胞粘附基因以及没有确定功能的基因。

[0131] 一方面，本公开内容不包括这样的黑素瘤细胞，即在 IFN-γ 处理的情况下显示 MHC II 类表达降低、显示 MHC II 类表达无可检出的变化、显示 MHC II 类表达增加 10% 或更少、显示 MHC II 类表达增加 15% 或更少、显示 MHC II 类表达增加 20% 或更少、25% 或更少、30% 或更少、40% 或更少、50% 或更少等。一方面，百分比值是指存在于得自指定受试者或患者的活检样品或部分活检样品中黑素瘤细胞群的平均表达值。

[0132] 用于 IFN-γ 响应癌细胞筛选的 IFN-γ 可诱导基因的非限制性清单

ab000677，JAB/SOCS1 ;m63961，IFN-γ 可诱导蛋白 (mag-1) ;m35590，巨噬细胞炎性蛋白 1-β ;m19681，MCP-1 (JE) ;y07711，斑联蛋白 ;M34815，IFN-γ 诱导的单核因子 (MIG) ;m33266，干扰素可诱导蛋白 10 (IP-10) ;U44731，嘌呤核苷酸结合蛋白 ;U88328，细胞因子信号转导 -3 超家族 (SOCS-3) ;M21065，干扰素调节因子 1 ;M63630，GTP 结合蛋白 (IRG-47) ;U19119，G- 蛋白样 LRG-47 ;L27990，Ro 蛋白 ;M31419，204 干扰素可激活蛋白 ;af022371，干扰素可诱导蛋白 203 ;U28404，MIP-1 α 受体 ;U43085，糖皮质激素减毒反应 39 ;x56123，踝蛋白 ;m31419，204 干扰素可激活蛋白 ;U53219，GTPase IGTP ;l38444，T 细胞特异性蛋白质 ;M31418，202 干扰素可激活蛋白 ;d38417，芳基烃受体 ;m26071，组织因子 (mtf) ;D13759，Cot 原癌基因 ;M18194，纤连蛋白 ;u59463，ICH-3 ;M13945，pim-1 原癌基因 ;L20450，DNA 结合蛋白 (参见 Gil 等 (2001) Proc. Natl. Acad. Sci 98:6680-6685)。本公开内容包括使用 IFN-γ 诱导的基因 CIITA (参见例如 Chan 等 (2010) J. Leukocyte Biol. 88:303-311 ; Kwon 等 (2007) Mol. Immunol. 44:2841-2849)。

[0133] 本公开内容包括测量下列 IFN-γ 可诱导基因的一种或多种的表达，作为用于限定或选择给予药物的患者的筛选程序。所述基因包括 (基因 1) FCGR1A、(基因 2) IL6R、(基因 3) CXCL9、(基因 4) CLCSF14、(基因 5) UBD、(基因 6) C/EBPα 和 (基因 7) MHC2TA (CIITA) (参见 Waddell 等 (2010) PLoS ONE 5:e9753)。在限定程序中还包括使用这些基因的特定群，例如基因 1 和 2、2 和 3、3 和 4、4 和 5、5 和 6、6 和 7、1 和 3、1 和 4、1 和 5、1 和 6、1 和 7、2 和 4、2 和 5、2 和 6、2 和 7、3 和 5、3 和 6、3 和 7、4 和 6、4 和 7、5 和 7，以及 3 种基因的组合，例如 1、2、3 ;或 3、4、5 ;或 4、5、6 ;或 5、6、7 ;或 1、3、4 ;或 1、3、5 ;或 1、3、6 ;或 1、3、7 ;或 1、2、4 ;或 1、2、5 ;或 1、2、6 ;或 1、2、7 等。(这些基因编号是任意的)。

[0134] 不包括小于 90% 是自噬的、小于 80% 是自噬的、小于 70% 是自噬的、小于 60% 是自噬的、小于 50% 是自噬的、小于 40% 是自噬的等黑素瘤细胞群。

[0135] 不包括小于 90% 是非凋亡的、小于 80% 是非凋亡的、小于 70% 是非凋亡的、小于 60% 是非凋亡的、小于 50% 是非凋亡的、小于 40% 是非凋亡的等黑素瘤细胞群。

[0136] 不包括小于 90% 是非贴壁的、小于 80% 是非贴壁的、小于 70% 是非贴壁的、小于 60% 是非贴壁的、小于 50% 是非贴壁的、小于 40% 是非贴壁的等黑素瘤细胞群。

[0137] 测量 MHC II类的表达

例如,可使用对 MHC II 类基因产物有特异性的抗体或核酸探针,测量 MHC II 类的表达。这些 MHC II 类基因产物包括 HLA-DPA1、HLA-DPB1、HLA-DQA1、HLA-DQB1、HLA-DRA、HLA-DRB1 以及 HLA-DM 和 HLA-DO (参见例如 Apostolopoulos 等 (2008) Human Vaccines 4:400-409)。

[0138] 例如,本公开内容包括需要表达 STAT1 和 STAT2、具有活性 STAT1 信号转导途径、具有活性 STAT2 信号转导途径或具有活性 STAT1 和 STAT2 信号转导途径的黑素瘤细胞的试剂、治疗方法和诊断方法。

[0139] 本公开内容提供产生具有危险比 (hazard ratio,HR) 小于 1.0、HR 小于 0.9、HR 小于 0.8、HR 小于 0.7、HR 小于 0.6、HR 小于 0.5、HR 小于 0.4、HR 小于 0.3 等的生存率数据的药物组合物或药用试剂、相关给药方法和治疗方法。本公开内容产生总体生存数据、无进展生存数据、至进展的时间数据等。还提供至少 40%、至少 50%、至少 60%、至少 70%、至少 75%、至少 80%、至少 85%、至少 90%、至少 95% 等的 6 个月 PFS。此外,提供至少 40%、至少 50%、至少 60%、至少 70%、至少 75%、至少 80%、至少 85%、至少 90%、至少 95% 等的 6 个月总体生存率。另外提供至少 40%、至少 50%、至少 60%、至少 70%、至少 75%、至少 80%、至少 85%、至少 90%、至少 95% 等的 1 年 (或 2 年) PFS。此外,提供至少 40%、至少 50%、至少 60%、至少 70%、至少 75%、至少 80%、至少 85%、至少 90%、至少 95% 等的 1 年 (或 2 年) 总体生存率 (参见例如 U.S. Dept. of Health and Human Services. Food and Drug Administration. Guidance for Industry. Clinical trial endpoints for the approval of cancer drugs and biologics (2005 年 4 月))。

[0140] IFN-γ 和自噬的诱导

在 IFN-γ 处理后自噬的诱导,如通过主要组织相容性 II 类复合体表达的增加所测量,可用来测定对系统 IFN-γ 处理的响应。如果活检黑素瘤肿瘤细胞在培养时暴露于 IFN-γ 中,进行自噬但无细胞凋亡,则这就表明这些患者可有利地响应系统 IFN-γ 处理。另外,如果从活检样品建立成功的细胞系,则患者还可获利于细胞疗法产品,所述产品由 IFN-γ 处理的纯化的肿瘤细胞系制备,所述细胞系来自于自噬但非凋亡的贴壁细胞群。

[0141] 本公开内容包括分离和表征主要组织相容性复合体,其分离自从用干扰素 -γ 处理的肿瘤细胞系中收集的自噬非凋亡细胞。主要组织相容性复合体含有对 $CD4^+$ T 细胞有特异性的抗原,并且与抗体介导的免疫应答有关。该复合体可表示大的抗原库,所述抗原因在自噬细胞中诱导的溶酶体介导的抗原加工的作用所致可能不存在于非自噬细胞中。

[0142] 因自噬肿瘤细胞表面的高水平的主要组织相容性复合体所致,自 IFN-γ 处理的细胞系产生的非凋亡自噬肿瘤细胞可与树突细胞融合以提高抗原呈递。该过程将产生因两种细胞类型融合产生的新的细胞产物。

[0143] 自噬响应 IFN-γ 的诱导过程可以不引起细胞凋亡的方式诱导。通过肿瘤细胞用胱天蛋白酶抑制剂和干扰素 γ 处理的组合,可阻断细胞死亡过程 (和最终 tolergeneic 凋亡细胞的形成) 而不抑制自噬的诱导或主要组织相容性 II 类复合体的增加。

[0144] 清除凋亡细胞同时保留有活力的自噬细胞的方法

在临床试验中,黑素瘤研究表明存在凋亡细胞与生存率差之间的相关性 (Cornforth 等 (2011) Cancer Immunol. Immunother. 60:123-131 ;Dillman 等 (2011) Cancer

Biother. Radiopharmaceuticals 26:407-415)。下面的研究调查了在体外 IFN-γ 处理黑素瘤肿瘤细胞后自噬、细胞凋亡和 MHC II 类分子的诱导。

[0145] 研究方法如下。将自体和模式黑素瘤肿瘤细胞系与 1000 IU/mL 的 IFN-γ 一起温育 72 小时后，测定自噬、细胞凋亡和 MHC II 类表达。通过用抗 LC3 II 的抗体的免疫印迹和通过带有 Enzo's CytoID® 自噬检测试剂盒的流式细胞术检测自噬。分别使用 7-AAD 和膜联蛋白 -V 染色和抗 MHC II 类的抗体，通过流式细胞术测定细胞凋亡和 MHC II 类诱导。7-AAD 嵌入 dsDNA 中。7-AAD 被有活力的细胞排斥，因此可用来检测死细胞。

[0146] 研究结果表明，IFN-γ 在黑素瘤细胞系中诱导自噬和凋亡细胞群两者。凋亡群主要存在于非贴壁群中，而自噬细胞保持附着在培养瓶上。用抑制剂 3- 甲基腺嘌呤 (3-MA) 阻断自噬抑制响应 IFN-γ 时 MHC II 类阳性细胞的诱导 (39.4% IFN-γ 相对于 10.0% IFN-γ + 3-MA)。用全胱天蛋白酶抑制剂 Z-VAD 抑制胱天蛋白酶活性阻止细胞凋亡但不干扰 IFN-γ 处理的细胞的自噬 (2.75 ± 0.15 IFN-γ 相对于 3.04 ± 0.27 IFN-γ + Z-VAD，倍数变化)。最后，细胞凋亡的诱导与自噬和 MHC II 类诱导的水平降低有关。本公开内容提供清除凋亡细胞的方法或程序，同时在 IFN-γ 处理后保留有活力的自噬细胞可提高这种基于细胞的免疫疗法类型的有效性。

[0147] IFN-γ 与抑制针对肿瘤的免疫应答有关 (参见例如 Hallermalm (2008) J. Immunol. 180:3766-3774 ;Romieu-Mourez (2010) Cancer Res. 70:7742-7747 ;Lee (2005) Clinical Cancer Res. 11:107-112)。

[0148] 肿瘤可以是不同程度的分化细胞的异质群。肿瘤的黑素瘤细胞的 IFN-γ 处理可作用于某些更易于细胞凋亡的更加分化的细胞。通过从抗原来源清除这些细胞，结果可以是在接种后损失对肿瘤块的某些作用，这通过肿瘤大小消退慢或无明显消退来解释。研究表明，凋亡细胞不激活树突细胞 (Sauter (2000) J. Exp. Med. 191:423-434)。

[0149] IFN-γ 可起使单核细胞分化从 DC 偏向巨噬细胞的作用。制备物中 IFN-γ 的量可通过使表型偏向较少特异化的巨噬细胞，影响 DC 的不完全分化。

[0150] IFN-γ 可用来增加 MHC II 类分子，并具有向 T 细胞的直接呈递。然而，Ii 蛋白 (钙防卫蛋白) 与 MHC II 类分子的共诱导阻止内源肿瘤抗原自 MHC II 类分子的呈递。

[0151] 第一项研究的材料与方法

自体树突细胞产生

树突细胞通过之前描述的塑料粘附方法产生 (Choi (1998) Clin. Cancer Res. 4:2709-2716 ;Luft (1998) Exp. Hematol. 26:489-500)。简单地说，对自体单采血液成分术产物进行聚蔗糖 - 泛影葡胺 (ficoll-hypaque) (GE Healthcare，Buckinghamshire，United Kingdom) 密度梯度分离。在细胞培养瓶 (Corning-Costar，Corning，NY) 中，将所得外周血单核细胞按 15 x 10^6 个细胞 /mL 接种于补充了 IL-4 (CellGenix，Freisberg，Germany) 和 GM-CSF (Berlex，Seattle，WA) 各 1,000 IU/mL 的无抗生素 AIM-V 培养基 (Invitrogen，Grand Island，NY) (DC 培养基) 中。在 1 小时温育后，弃去非贴壁群，将新的 DC 培养基加入培养瓶中。次日上午，弃去非贴壁细胞，培养瓶用环境温度 PBS 洗涤一次，加入新的 DC 培养基。然后将培养瓶培养 6 天，届时进行流式细胞术评价，以测定通过该方法产生的 DC 的百分比和表型 (加载前 DC)。

[0152] 自体肿瘤细胞系产生

将按之前报告产生和表征的纯的肿瘤细胞增殖到 2 亿个细胞，然后如前所述与 1000 IU/mL 的 IFN-γ (InterMune，Brisbane，CA) 一起在含 15%FBS/ECS 的 RPMI (完全培养基) 中温育 72 小时，用来自铯源的 100 Gy 照射，并冷藏保存 (Choi (1998) Clin. Cancer Res. 4:2709-2716 ；Luft (1998) Exp. Hematol. 26:489-500 ；Dillman (1993) J. Immunother. Emphasis Tumor Immunol. 14:65-69)。从冷藏保存回收 IFN-γ 处理和照射的肿瘤细胞，用 PBS 洗涤 3x，然后加至体外培养的 DC 中，并温育约 24 小时。通过用橡胶刮棒轻刮，收获加载抗原的 DC，并以 9-11 等分样品的等分量冷藏保存。获取等分细胞用于流式细胞术评价，其表示加载后的 DC 细胞。

[0153] 流式细胞术

树突细胞群的表型表征使用获自 BD Pharmingen San Diego，CA 的以下抗表面标志物单克隆抗体进行 ：与 PerCp 缀合的抗 MHC II 类、与 APC 缀合的抗 CD11c、与 PE 缀合的抗 CD86、抗 CD80、抗 CD83。使用同种型对照测定百分比阳性细胞。使用来自 BD Pharmingen 的与 FITC 缀合的针对 MHC I 类和 II 的抗体、膜联蛋白 -V-PE 和 7- 氨基 - 放线菌素 D (7-AAD)，进行肿瘤细胞的流式细胞术。在每次运行前使用 CaliBRITE 流式细胞术校准 (BD Pharmingen)，流式细胞术数据采集期间使用相同的仪器设置。

[0154] 免疫印迹测定法

用哺乳动物蛋白质提取试剂 (Mammalian Protein Extraction Reagent) (Thermo Scientific，Rockford，IL) 加蛋白酶抑制剂混合物 (Roche，Indianapolis，IN) 在冰上按 10,000 个细胞 /uL 制备胞质细胞裂解物。将约 25 uL/ 泳道的细胞裂解物在 12.5% tris- 甘氨酸凝胶上分离，转移到 PVDF 膜上，用针对以下的抗体探查 ：钙网蛋白 (MBL，Woburn，MA)、Hsp-60、Hsp-70、Hsp-90 (R&D Systems，Minneapolis，MN)、HMBG-1 (Cell Signaling，Danvers，MA)、ICAM-1 (Santa Cruz Biotech，Santa Cruz，CA)、Mel-4、Mart-1 (Signet，Emeryville，CA)、酪氨酸酶 (Upstate，Lake Placid，NY) 和 GADPH (Calbiochem，Darmstadt，Germany)。

[0155] 免疫组织化学

采用免疫细胞化学程序，测定由黑素瘤系表达的一组抗原。在 1000 IU/mL IFN-γ 存在或不存在下，将细胞培养在 8 室培养载玻片 (Thermo Fisher，Rochester，NY) 上。72 小时后，细胞用 1X 磷酸盐缓冲盐水 (PBS) 洗涤 3 次，并在冷丙酮中固定。在阻断内源过氧化物酶后，将细胞与针对所列出的抗原的合适的第一抗体一起温育。使用生物素化抗小鼠或兔免疫球蛋白、超灵敏酶 (Super Sensitive enzyme) 缀合的链霉抗生物素标记和辣根过氧化物酶色原、和底物试剂盒 (Biogenex，San Ramon，CA)，进行免疫组织化学。用同种型匹配对照抗体，对下列抗人多克隆或单克隆抗体的反应性进行了研究 ：S-100 和 HMB-45 (Biogenex，San Ramon，CA)、Mel-2、Mel-5、Mart-1 (Signet，Dedham，MA)、酪氨酸酶和 Mage-1 (Thermo Scientific，Fremont，CA)、Melan-A、HLA-I 类和 HLA-II 类 (Dako，Denmark)。

[0156] 统计分析

等方差的双尾、两样品的 Student t 检验。通过 p 值≤ 0.05，确定显著差异。

[0157] 第一项研究的结果

在自体黑素瘤肿瘤细胞系中，在响应在完全培养基中与 IFN-γ 温育 72 小时时差异性地诱导细胞死亡。在用与不用 IFN-γ 处理的细胞中进行的锥虫蓝染料排斥测定，揭示了在

IFN-γ 处理的细胞中趋于较低存活力的明显趋势 (89.1 ± 6.8% 相对于 84.9 ± 9.3%，p = 0.014，N = 47)。通过流式细胞术，针对细胞凋亡诱导对 4 个自体黑素瘤细胞系样品的分析 (图 1A) 揭示了黑素瘤细胞差异性地对 IFN-γ 诱导的细胞凋亡的作用敏感，其中一些细胞显示更多晚期细胞凋亡或“死亡”群 (7-AAD+/ 膜联蛋白 -V+)，而其它细胞显示早期细胞凋亡或“濒死”群 (7-AAD-/ 膜联蛋白 -V+) 的迹象。在 IFN-γ 处理后得到的凋亡细胞的存在与无进展生存率和总体生存率显著下降有关 (Cornforth (2010) Cancer Immunol. Immunother。对干扰素 -γ 对用于患者特异性树突细胞免疫疗法的黑素瘤细胞的促凋亡作用的抵抗与改进的总体生存率有关)。对数秩 (log-rank) 检验显示与在黑素瘤肿瘤细胞的 IFN-γ 处理时较低的存活力和研究中的患者的总体生存率显著相关。

[0158] 针对可能是重要的免疫介质的各种分子，对来自在 IFN-γ 存在或不存在时温育的细胞的裂解物进行了免疫印迹法 (图 1B)。在用 IFN-γ 处理的黑素瘤细胞的背景下，热激蛋白显得被差异性调节，但依然很大程度存在于细胞制备物中，尤其在 hsp-70 的情况下。内质网蛋白质钙网蛋白和高迁移率族 box-1 蛋白 (HMGB-1)，在用 IFN-γ 处理时在某些情况下显得增量调节 (图 1B)。相比之下，普通黑素瘤抗原 (mel-4、Mart-1 和酪氨酸酶) 一般显得被 IFN-γ 减量调节，而 ICAM-1，一种与对淋巴细胞介导的细胞毒性的敏感性有关的淋巴细胞粘附分子 (Hamai (2008) Cancer Res. 68:9854-9864)，被显著地增量调节 (图 1C)。实际上，发现 IFN-γ 处理的黑素瘤肿瘤细胞对细胞毒性 T 淋巴细胞 (CTL) 活性更敏感。另外，一组黑素瘤相关抗原的免疫组织化学揭示在所检查的许多抗原中，IFN-γ 导致抗原表达减量调节 (表 1)。

[0159] 使用 IFN-γ 导致主要组织相容性复合体 I 类和 II 类增量调节 (Bohn (1998) J. Immunol. 161:897-908)。如图 1D 所示，用 IFN-γ 处理自体黑素瘤细胞导致 MHC I 类几乎普遍和显著地上调 ($p = 2.8 \times 10^{-8}$)，其倍数诱导中值为 2.91 ± 1.13 (95% C.I.)。另外，MHC II 类的平均荧光强度 (MFI) 也显著较高，但只是少些如此 (p = 0.039)，其诱导中值为 4.23 ± 2.66 (95% C.I.)。自体黑素瘤细胞表面上 MHC II 类分子的水平一般低于 MHC I 类分子的水平，但在 70% 的情况下，MHC II 类分子由于 MHC II 类表达初始水平低，因此在响应 IFN-γ 处理时诱导超过两倍。在将抗原加载到树突细胞期间肿瘤细胞中这些分子的存在可为将 MHC 复合体“交叉装饰 (cross dressing)”到抗原呈递细胞上提供机会 (Dolan (2006) J. Immunol. 277:6018-6024，Dolan (2006) J. Immunol. 176:1447-1455)。

[0160] 将一组 4 个代表性自体黑素瘤细胞系与 IFN-γ 一起温育，并按等量加载到树突细胞中，然后通过流式细胞术，测定树突细胞的 CD80、CD83、CD86 和 MHC-II 类表达。结果表明，在加载 IFN-γ 处理的黑素瘤细胞时观察到表达 CD83 的树突细胞的百分比阳性群小但可观的增加 (图 2)。另外，在 CD86 点图 (左上象限) 中注意到更多未加工的肿瘤细胞，这导致百分比 CD86 阳性群中可识别的降低，表明 IFN-γ 未处理肿瘤细胞仍存在。这种作用更可能是因为 IFN-γ 诱导细胞凋亡所致，因为如之前的报告，凋亡细胞更可能被树突细胞吞噬。

[0161] 如图 3 所示，DC 加载前的样品显示，它们表达 CD80 (39.0 ± 16.2%)、CD83 (7.1 ± 6.9%)、CD86 (73.6 ± 19.5%)，并且是 MHC II 类阳性的，其存活力为 96.2 ± 5.0%。加载的 DC 具有显著较高的 CD83 百分比 (9.4 ± 7.1%，p = 0.019)，其显著较高的平均荧光强度 (MFI) (172.9 ± 79.0，p = 0.0009) 表明用经照射的 IFN-γ 处理的肿瘤细胞加载 DC

诱导某些树突细胞成熟（图 3B）。

[0162] 第一项研究的讨论

技术人员实施了在抗肿瘤免疫情况下用于抗原加载、成熟和给药的方案以及对基于树突细胞 (DC) 的免疫疗法的指导。这种治疗类型包括使用纯化的自体肿瘤细胞作为抗原来源，并含有患者特异性肿瘤相关抗原库 (Selvan (2010) Melanoma Res. 20:280-292；Dillman (2007) Cancer Biother. Radiopharm. 22:309-321)。一些临床试验正使用未纯化的自体团块肿瘤。这种抗原来源可能具有污染性成纤维细胞和坏死组织 (O'Rourke (2007) Melanoma Res. 17:316-322)。肿瘤干细胞相关抗原可存在于纯化的细胞系中 (Dillman (2006) New Engl. J. Med. 355:1179-1181)。IFN-γ 处理增加 MHC II 类分子的表达。MHC II 类分子对响应基于树状细胞的疗法是重要的。存在于被吞噬的材料中的分子，例如钙网蛋白、HMGB-1 和热激蛋白，可对成熟信号作出贡献，其中这种贡献可附加到细胞因子混合物的贡献中。本发明的 DC 制备物显示趋于成熟的趋势，这可能与晚期凋亡细胞的吞噬相关 (Ip (2004) J. Immunol. 173:189-196)。使用凋亡细胞与树突细胞的产生相互关联，所述树突细胞相对于加载肿瘤细胞裂解物或坏死细胞的树突细胞，对刺激淋巴细胞 IFN-γ 分泌更有效，表明了加载凋亡细胞的树突细胞可能在体内更有效。对 IFN-γ 的促凋亡作用的抵抗可能与更好的临床结果有关 (Cornforth (2010) Cancer Immunol. Immunother. 60:123-131)。通过成熟 DC 的白介素 -12 (IL-12) 分泌可导致强大的细胞毒性淋巴细胞 (CTL；$CD8^+$ T 细胞）活性。解决了离体成熟是否导致持久肿瘤免疫的问题。另已表明，因未成熟 DC 所致调节性 T 细胞的诱导风险（其可抑制抗原特异性 CTL)，随细胞因子成熟的 DC 而发生。正在对导致成熟的信号转导事件顺序的重新评价进行研究，以改进 DC 成熟方案。因此，在该研究中，使用经照射的完整肿瘤细胞作为抗原来源，无需离体细胞因子成熟，可能是 DC 免疫疗法的一种更优选的方法，因为本文呈现的证据表明，DC 已开始成熟过程。在注射时，这些“成熟中的” DC 可通过分泌趋化因子（其将吸引经许可的抗原特异性 CD40L 表达 $CD4^+$ T 细胞），来完成成熟过程。血清趋化因子，比如在响应佐剂 GM-CSF 时由树突细胞产生的 CCL17/TARC，与更好的无进展生存率相关。在某些情况下，通过树突细胞激活淋巴细胞可能需要共刺激分子（比如 CD80 和 CD86) 的表达。作为成熟的标志物，CD83 在成熟树突细胞上表达，可能与会诱导更有效的免疫应答的树突细胞相对应 (Prazma (2008) Immunol. Lett. 115:1-8)。这代表了在药物制备中所有细胞的一部分。在任一个药物方案中，仅成熟 DC 的数目，可能与或可能不与更好的患者反应关联。

[0163] 第一项研究的表格

表 1：在基于患者特异性细胞的树突细胞疗法所使用的黑素瘤细胞系中常见肿瘤相关抗原在响应干扰素 -γ 时表达水平的改变。

表1

抗原	无基础表达	基础表达	IFN-γ处理后的变化	
			无	减量
S-100	74.1%	25.9%	42.9%	57.1%
HMB-45	18.5	81.5	54.5	45/5
Mel-2	3.7	96.3	46.2	53.8
Melan-A	11.1	88.9	29.2	70.8
Mel-5	18.5	81.5	72.7	27.3
MAGE-1	51.9	48.1	38.5	61.5
MART-1	11.1	88.9	14.8	85.2
酪氨酸酶	25.9	74.1	40.0	60.0

[0164] N = 27 个样品。

[0165] 第二项研究的材料与方法

黑素瘤细胞系

市售获得的黑素瘤细胞系 A375、SK-Mel-5 和 SK-Mel-28 购自美国典型培养物保藏中心 (American Type Culture Collection) (目录号 :CRL-1619、HTB-70 和 HTB-72)。将 A375、SK-Mel-5 和 SK-Mel-28 保持在含 5% 胎牛血清的 RPMI-1640 (Invitrogen, 目录号 11875-085) 中。全胱天蛋白酶抑制剂 z-VAD-fmk 及其对照化合物 z-FA-fmk 购自 BD Pharmingen (目录号 :550377 和 550411)。按照生产商说明书进行 GFP-LC3 的转染 (InvivoGen, 目录号 psetz-gfplc3 和 lyec-12), 并使用 DP72 数码相机, 在 Olympus BX-51 显微镜上拍摄显微照片。测定前, 将肿瘤细胞系与 1000 U/mL 的 IFN-γ (InterMune, Cat#) 一起温育 72 小时。患者特异性细胞系如所述如下产生 (Hamai (2008) Cancer Res. 68:9854-9864 ;Tyring (1984) J. Natl. Cancer Inst. 73:1067-1073) :对手术肿瘤样品进行酶消化, 在补充胎牛血清和富含牛血清 (Omega Scientific, San Diego, CA) 加 1 mM 丙酮酸钠、1 mM 谷氨酰胺和 HEPES 缓冲液的 RPMI-1640 组织培养基中培养。使用 Nikon DS-L1 数码显微镜相机, 在 Olympus CK-2 显微镜上拍摄相衬显微照片。

[0166] 自体树突细胞产生

在补充 IL-4 (CellGenix, Cat#) 和 GM-CSF (Berlex, Seattle, WA) 各 1,000 IU/mL 的无抗生素 AIM-V 培养基 (Invitrogen, Cat#) (DC 培养基) 中, 通过 ficoled 单采血液成分术产品的塑料粘附方法产生树突细胞 (Selvan (2007) Int. J. Cancer. 122:1374-1383 ; Cornforth (2010) Cancer Immunol. 60:123-131)。然后在用 IFN-γ 处理的、经照射的自体肿瘤细胞加载前, 将培养瓶培养 6 天。

[0167] 流式细胞术

使用针对 MHC II 类的抗体、膜联蛋白 -V 和 7- 氨基 - 放线菌素 D (7-AAD), 对在响应 IFN-γ 时的肿瘤细胞死亡和主要组织相容性 II 类表达的变化进行了分析, 并在 Beckton-Dickenson FACS Calibur® 流式细胞仪中获取。

[0168] 蛋白质印迹法

黑素瘤肿瘤细胞裂解物经 10-12.5% SDS-PAGE 分离, 转移到硝化纤维上, 用第一抗体探测过夜, 然后第二抗体缀合并通过 Novex AP 显色底物 (Invitrogen, Carlsbad, CA) 显影

以显现条带。分别按生产商推荐的 1:100 和 1:10,000 稀释度,使用针对 LC3-B 抗体 (Cell Signaling Technologies,Boston,MA) 和 GADPH (EMD biosciences,Germany) 的抗体。

[0169] 第二项研究的描述

研究在 IFN-γ 处理后,体外黑素瘤肿瘤细胞的自噬、细胞凋亡和 MHC II 类分子的诱导。将自体和模式黑素瘤肿瘤细胞系与 1000 IU/mL 的 IFN-γ 一起温育 72 小时后,测定自噬、细胞凋亡和 MHC II 类表达。通过用抗 LC3 II 的抗体的免疫印迹和通过带有 Enzo's CytoID 自噬检测试剂盒的流式细胞术,对自噬进行检测。分别使用 7-AAD 和膜联蛋白 -V 染色和抗 MHC II 类的抗体,通过流式细胞术测定了细胞凋亡和 MHC II 类诱导。

[0170] 第二项研究的结果

结果表明,IFN-γ 诱导黑素瘤细胞系的自噬和凋亡细胞群两者。凋亡群主要存在于非贴壁群中,而自噬细胞保持附着在培养瓶上。用抑制剂 3- 甲基腺嘌呤 (3-MA) 阻断自噬抑制在响应 IFN-γ 时 MHC II 类阳性细胞的诱导 (39.4% IFN-γ 相对于 10.0% IFN-γ + 3-MA)。用全胱天蛋白酶抑制剂 Z-VAD 抑制胱天蛋白酶活性阻止细胞凋亡但不干扰 IFN-γ 处理的细胞的自噬 (2.75 ± 0.15 IFN-γ 相对于 3.04 ± 0.27 IFN-γ + Z-VAD,倍数变化)。细胞凋亡的诱导与自噬和 MHC II 类表达水平降低相关。接受是加载自体肿瘤细胞的树突细胞 (其是来源于干扰素 -γ 处理的纯化肿瘤细胞系的非凋亡自噬细胞) 的患者,具有改善的无进展和总体生存率 (分别为 p 0.003 和 p 0.002)。在 IFN-γ 处理后清除凋亡细胞同时保留有活力的自噬细胞的方法,可提高这种类型的基于细胞的免疫疗法的有效性。

[0171] 研究的汇总分析

自体、增殖性、自我更新肿的瘤细胞 (公认的肿瘤干细胞和 / 或早期祖细胞),对于建立转移癌的新贮库 (depot) 是重要的,并且可能是用于疫苗的优异抗原来源。这些研究解决了用来自所述细胞的抗原进行免疫对存活率的影响。

[0172] 方法

从 3 个连续的 II 期试验中汇总数据,全部数据包括患有被证实为转移性黑素瘤的患者,在使用来自自体肿瘤细胞的细胞培养物的抗原的方案中对患者进行治疗。给予 S.C. 注射每周一次达 3 周,然后每月一次达 5 个月 :74 名患者注射经照射的肿瘤细胞 (TC) :54 名患者注射与经照射的自体肿瘤细胞 (NCI-V01-1646) 共培养的自体树突细胞 (DC) :在随机 II 期试验中,24 名患者注射 TC,18 名注射 DC。

[0173] 结果

表 2 概括了各试验的总体生存率 (OS)。在汇总分析中,有 98 名 TC 和 72 名 DC 患者。就年龄 (51,52)、男性 (62%,62%)、治疗时无疾病证据 (46%,47%) 和在治疗时存在 M1c 内脏疾病 (13%,14%) 而言,特征相似。用 DC 治疗的患者中 OS 较长 (中位值 63.1 月相对于 20.2 月,5 年 OS 51% 相对于 26%,$p^{=}$0.0002 Mantle-Cox 对数秩检验)。随机试验中 OS 的差异亦是显著的 (p=0.007)。

[0174] 用来自自体增殖、自我更新的肿瘤细胞的抗原引发的患者特异性 DC 疫苗与令人鼓舞的长期生存率有关,并且在被诊断为转移性黑素瘤的患者群中优于患者特异性 TC 疫苗。

表2

疫苗	患者数	死亡数	OS中位值	2年OS	5年OS
TC	74	60	20.3月	45%	28%
DC (使用IFN-γ处理的黑素瘤细胞)	54	31	58.4月	72%	50%
TC	24	16	15.9月	31%	---
DC (无IFN-γ处理的黑素瘤细胞)	18	5	未达到	72%	---

[0175] 来自患者特异性疫苗的3个试验的生存曲线见图13。连续的I期和II期临床试验使用自体肿瘤细胞,与自体树突细胞或不与树突细胞组合进行。给予皮下注射一周一次达三(3)个周,然后一月一次达五(5)个月,给74名患者注射未用IFN-γ预处理的经照射的肿瘤细胞(TC):给54名患者注射与用IFN-γ预处理的经照射的自体肿瘤细胞共培养的自体树突细胞(DC):在随机II期试验中,给24名患者注射未用IFN-γ预处理的TC,给18人注射DC加未用IFN-γ预处理的TC。

[0176] 图14显示3个试验的生存曲线,其中试验与图13公开的试验是同一临床试验,但是具有从较后时间点获取的其它数据,从对两个图中的步阶图的比较来看是明显的。临床试验中的黑素瘤细胞TC-24和TC-74不接受IFN-γ。临床试验中的黑素瘤细胞DC-TC-18不接受IFN-γ。临床试验中的黑素瘤细胞DC-TC-54的确得到IFN-γ。

[0177] 用于制备树突细胞疫苗的非限制性标准操作程序包括以下方面(表3)。在扩增后收获肿瘤细胞时,对各患者的肿瘤细胞批次,进行以下操作。需要约2.2亿个细胞制备肿瘤细胞疫苗批次。将任何额外的细胞冷藏保存作为备份细胞。按22 ml培养基中220 x 10^6个细胞制备原液细胞悬液,按下列方法进行分配(表3)。

表3. 操作程序

使用	所需细胞总数	第一次操作	第二次操作	最终安排
TC疫苗剂量或DC加载细胞	1.50亿	原液15 ml加到50 ml圆锥管中，加入25 ml AIM-V，并照射	在10个小的冷冻管中冷藏保存照射后的细胞	保存以备用于患者治疗

[0178] 试验 #2 :DC 2000-2006 (NCI-V01-1646)。加载来自经照射的自体肿瘤细胞的抗原的自体树突细胞作为患者特异性疫苗的 II 期试验 (BB-IND 8554) :树突细胞 (DC) 疫苗。在用于该试验的疫苗的生产中,将自体增殖性肿瘤细胞与 IFN-γ 一起共温育,冷藏保存,然后接着与自体树突细胞一起共温育。将各等分的细胞悬浮于 500 微克的 GM-CSF 用于注射。

[0179] 试验 #3 :DC vs. TC 2007-2011 (NCT00436930) :转移性黑素瘤患者中由佐剂 GM-CSF 加增殖性肿瘤细胞组成的自体疫苗 vs. GM-CSF 加加载增殖性肿瘤细胞的树突细胞组成自体疫苗的随机 II 期试验 (BB-IND 8554 和 BB-IND 5838) :'MAC VAC'。第 3 个试验是测定在上述两种方法中是否有差异的随机试验。在肿瘤细胞生产中不使用 IFN-γ 。正如上述 DC 试验,所有患者随机接受与 500 微克 GM-CSF 一起 s.c. 注射的 TC 或 DC 一周一次达 3 个周,然后每月一次达 5 个月。DC 组中患者的预计 72% 的 2 年生存率与之前 54 名患者 DC 试验中观察的 71% 的 2 年生存率 (其中中位生存率为 5 年) 相当。

[0180] 有关结肠癌、卵巢癌、成胶质细胞瘤和肾癌细胞的信息

下面提供得自结肠癌细胞 (内胚层)、卵巢癌细胞 (混合型中胚层加胚外层)、成胶质细胞瘤细胞 (外胚层) 和肾癌细胞 (中胚层) 研究的流式细胞术数据。这些细胞类型各自的胚胎学起源点用圆括号标注。黑素瘤细胞具有神经嵴起源。表 3 概括了肿瘤细胞类型,而表 4 概括了这些细胞实验的结果,其中细胞用干扰素 -γ (IFN-γ) 处理。

[0181] 肿瘤细胞与成体干细胞具有多处相似性,例如在更成熟的细胞类型中自我增殖和分化的潜力。下列数据表明自噬是可被诱导成来源于所有胚胎层的肿瘤细胞的现象。除起源于来自神经嵴的细胞的黑素瘤以外,本研究涉及在来源于内胚层 (结肠)、中胚层 (肾)、外胚层 (成胶质细胞瘤) 和混合型中胚层加胚外层 (卵巢) 的组织中产生的肿瘤。

表3. 肿瘤类型的图例	
样品	肿瘤类型
A	结肠
B	卵巢
C	成胶质细胞瘤
D	肾细胞癌

[0182] 表 4 公开了以下结果。在干扰素 -γ 处理后的贴壁细胞群中，在不存在细胞凋亡（磷脂酰丝氨酸暴露）时，在基于 MHC II 类复合体诱导所检查的肿瘤类型中显示自噬被诱导。

表4		对干扰素-γ的响应			
样品	肿瘤类型	MHC I类的诱导	MHC II类的诱导	细胞凋亡的诱导	自噬的诱导
A	结肠	是	是	否	是
B	卵巢	是	是	否	是
C	成胶质细胞瘤	否	是	是	是
D	肾细胞癌	否	是	是	是

[0183] 材料与方法

按照 Dillman 和 Comforth 等人的文献，从结肠、卵巢、成胶质细胞瘤和肾细胞癌中生产纯的肿瘤细胞 (Cornforth 等 (2011) Cancer Immunol. Immunother. 60:123-131 ;Dillman 等 (1993) J. Immunother. Emphasis Tumor Immunol. 14:65-69 ;Dillman 等 (2000) Cancer Biother. Radiopharm. 15:161-168 ;Dillman 等 (1999) 14:443-449 ;Dillman 等 (2000) 15:161-168)。

[0184] 简单地说，将来自手术样品的肿瘤活检样品酶促加工成单细胞悬液，并通过差异附着和血清饥饿产生纯化的细胞系。将所产生的细胞系在液氮中冷藏保存，融化，并用或不用含干扰素 -γ 的 5% 胎牛血清 /RPMI 培养基处理 72 小时。在暴露于干扰素 -γ 后，通过流式细胞术，对自贴壁细胞分离的非贴壁细胞进行分析。所研究的标志物为 MHC I 类、MHC II 类、膜联蛋白 V（细胞凋亡）和 7-ADD（存活力）。

[0185] 图 14 公开了以下方面。数据来自于没有任何分离成贴壁细胞和非贴壁细胞的原液细胞。干扰素 -γ 处理增加主要组织相容性复合体的表达 :结肠 (A)、卵巢 (B)、成胶质细胞瘤 (C) 和肾细胞癌 (D)。在用或不用 1000 IU/mL IFN-γ 处理 72 小时后，收获肿瘤细胞系，然后通过流式细胞术测定 MHC I 类和 II 类（图 1A)。图 15 显示以下方面。使用对照同种型抗体鉴定阳性群体。主要组织相容性复合体 I 类和 II 类的平均荧光强度 (MFI) 的倍

数变化概括于图 15 中。

[0186] 图 16 披露了以下方面。通过 IFN-γ 处理来自各种不同来源的肿瘤细胞诱导细胞凋亡。结肠 (A)、卵巢 (B)、成胶质细胞瘤 (C) 和肾细胞癌 (D)。在用或不用 1000 IU/mL IFN-γ 处理 72 小时后，收获肿瘤细胞系，然后通过膜联蛋白 -V 测定磷脂酰丝氨酸暴露，通过 7-AAD 测定细胞存活力。

[0187] 图 17 公开了以下方面。在贴壁细胞群中（但不在非贴壁群中），通过 IFN-γ 处理来自各种不同来源的肿瘤细胞诱导非凋亡的自噬 MHC II 类表达细胞。结肠癌 (A)、成胶质细胞瘤 (C) 和肾细胞癌 (D)。通过用 HBSS 洗涤除去非贴壁肿瘤细胞群，在用或不用 1000 IU/mL IFN-γ 处理 72 小时后收获贴壁细胞，然后通过膜联蛋白 -V 测定磷脂酰丝氨酸暴露，并在 7-AAD 阴性流分中测定主要组织相容性复合体 II 类（图 17）。

[0188] 图 18 公开了以下方面。使用对照同种型抗体鉴定阳性群体。在非贴壁细胞群中，主要组织相容性复合体 II 的平均荧光强度 (MFI) 的倍数变化概括于图 18。因为 MHC II 类表达是自噬的代表，因此该图的平均荧光强度 (MFI) 数据表示倍数变化，在图中表示为结肠癌细胞 (A)、成胶质细胞瘤 (C) 和肾细胞癌细胞 (D) 的自噬的增加的变化。所用细胞来自非凋亡群。MFI 的变化是响应 IFN-γ 处理的。

[0189] 图 19 公开了以下方面。该图公开了来自所检查的 4 种肿瘤类型的贴壁非凋亡细胞（膜联蛋白 -V）中自噬的诱导。在用或不用 1000 IU/mL IFN-γ 处理 72 小时后，通过用 HBSS 洗涤除去非贴壁群，收获贴壁细胞，通过膜联蛋白 -V 测定磷脂酰丝氨酸暴露，并测定 7-AAD 阴性流分中的主要组织相容性复合体 II 类。结肠 (A)、卵巢 (B)、成胶质细胞瘤 (C) 和肾细胞癌 (D)。

[0190] 结果

MHC II 类分子通常专门在特化细胞例如树突细胞、单核吞噬细胞、B 淋巴细胞、一些内皮细胞和胸腺中表达。如表 4 所示，发现由于上述体外操作，通过所研究的所有样品明确表示出 MHC II 类的表达。一些样品对 IFN-γ 更敏感，并导致细胞凋亡的诱导，这可能是与凋亡途径蛋白质（例如胱天蛋白酶和 bcl 家族蛋白）表达有关的一个特征。

[0191] 在所述体外操作后肿瘤细胞上的 MHC II 类分子表达是溶酶体加工的结果，因此该结果表明自噬的诱导发生在来源于本文以结肠、卵巢、成胶质细胞瘤和肾细胞癌为例的所有胚胎胚层（外胚层、中胚层和内胚层）的肿瘤细胞系的贴壁非凋亡群中。这种对干扰素 -γ 的响应与用黑素瘤细胞观察到的响应相同，表明这个特征可用作生产非凋亡自噬细胞群的方式，我们表明该细胞群是用于癌症的基于细胞的免疫疗法的关键组分。由于自噬所致的 MHC II 类呈递性肿瘤细胞可与辅助 T 淋巴细胞 ($CD4^+$ T 细胞）直接相互作用，并诱导免疫应答。与只基于树突细胞、未经操作的肿瘤细胞群或来自肿瘤的细胞裂解物的其它免疫疗法相比，树突细胞与自噬肿瘤细胞的复合体在患者中诱导更快和更强的应答，正如我们的临床数据所表明的一样。

[0192] 使用 MHC II 类表达作为响应干扰素 -γ 处理的自噬的替代标志物得到本文和所引用的著作（表明自噬被描述为参与内源蛋白质迁移到 MHC II 类加载区室）提供的证据的充分支持。通过自噬细胞表面 MHC II 类复合体表达的增加，揭示了这种作用（参见例如 Crotzer 和 Blum (2009) J. Immunol. 182:3335-3341）。本公开内容或引用的优先权文献，表明尽管用干扰素 -γ 处理，但使用自噬抑制剂 3- 甲基腺嘌呤导致 MHC II 类表达丧

失。因此，MHC II 类诱导的测量可用作自噬的替代标志物。

[0193] 因此，虽然显示、描述和指出本公开内容适于其示例性实施和 / 或方面的新的基本特征，但应了解本领域技术人员可在其示例性实施、公开内容和方面的形式和细节上进行各种省略、再组合和替换和变化，而不偏离本公开内容和 / 或权利要求书的精神。例如，明确指出以基本相同的方式执行基本相同的功能以达到相同结果的要素和 / 或方法步骤的所有组合都在本公开内容的范围内。而且，应认识到，连同任何公开的形式或实施所显示和 / 或描述的结构和 / 或要素和 / 或方法步骤可并入任何其它公开或描述或建议的形式或实施中作为设计选择的普通内容。因此，无意限制本公开内容的范围。所有这类修改都预期在其所随附的权利要求书的范围内。

[0194] 本说明书所引用的所有出版物、专利、专利申请、参考文献和序列表均通过引用结合到本文中就像全部阐述于本文中一样。

[0195] 提供符合 37 CFR §1.72(b) 的摘要以允许读者快速查明该技术内容的性质和要点。按摘要并非用来解释或限制权利要求书的范围或含义的认识，来提交摘要。

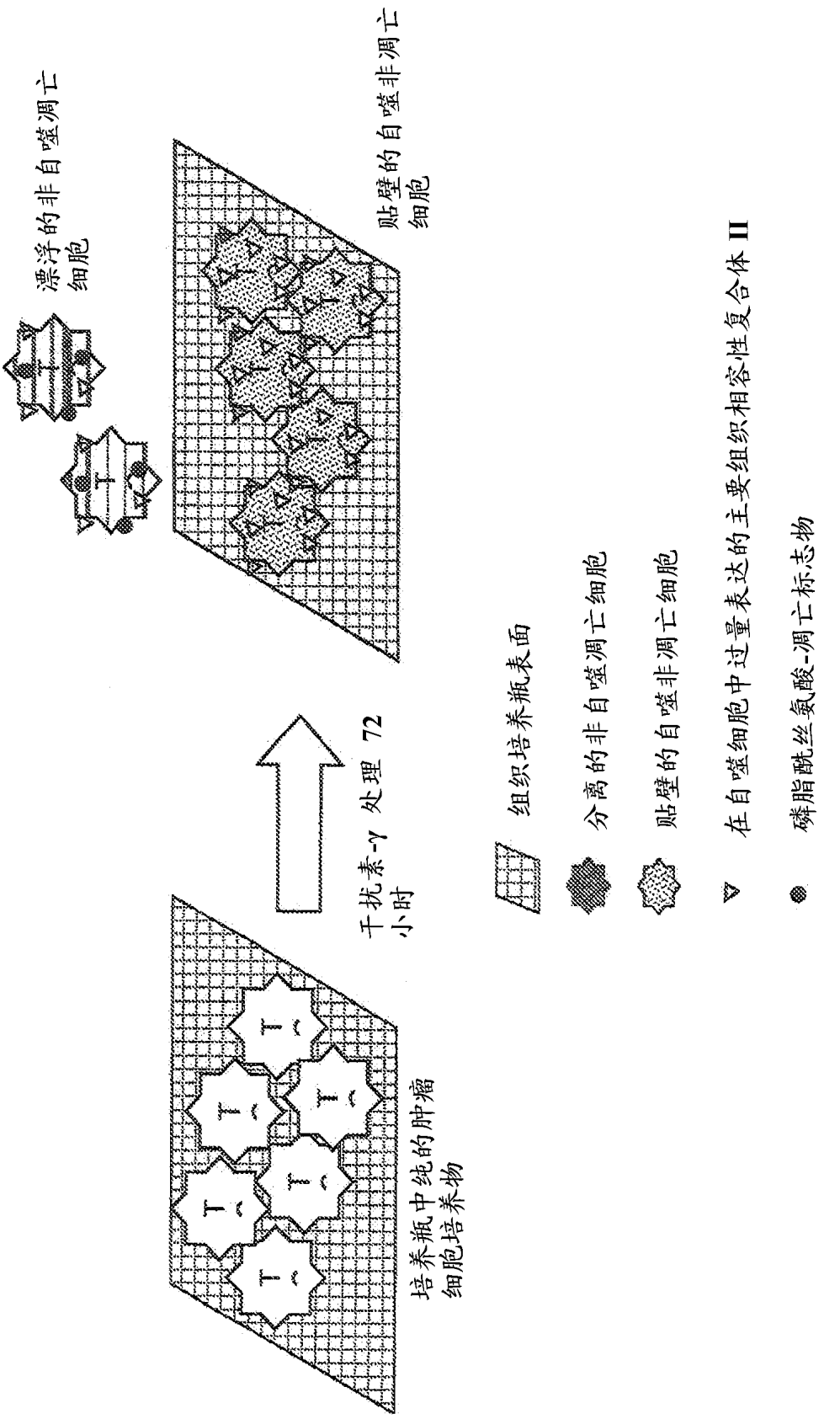

漂浮的非自噬凋亡细胞
贴壁的自噬非凋亡细胞
培养瓶中纯的肿瘤细胞培养物
千扰素-γ 处理 72 小时
组织培养瓶表面
分离的非自噬凋亡细胞
贴壁的自噬非凋亡细胞
在自噬细胞中过量表达的主要组织相容性复合体 II
磷脂酰丝氨酸-凋亡标志物

图 1

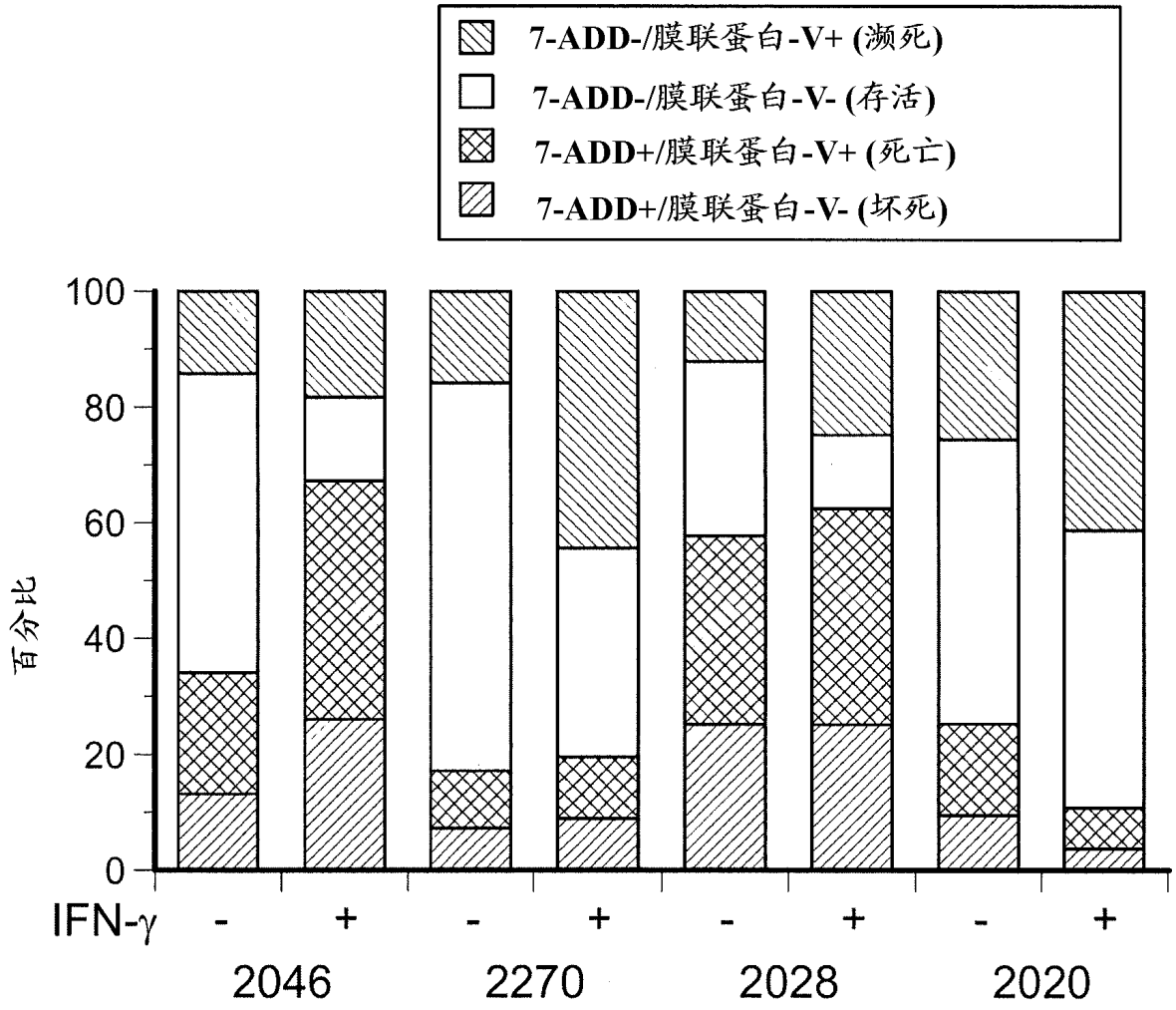

7-ADD-/膜联蛋白-V+ (濒死)
7-ADD-/膜联蛋白-V- (存活)
7-ADD+/膜联蛋白-V+ (死亡)
7-ADD+/膜联蛋白-V- (坏死)
百分比
100
80
60
40
20
0
IFN-γ
-
+
-
+
-
+
-
+
2046
2270
2028
2020

图 2A

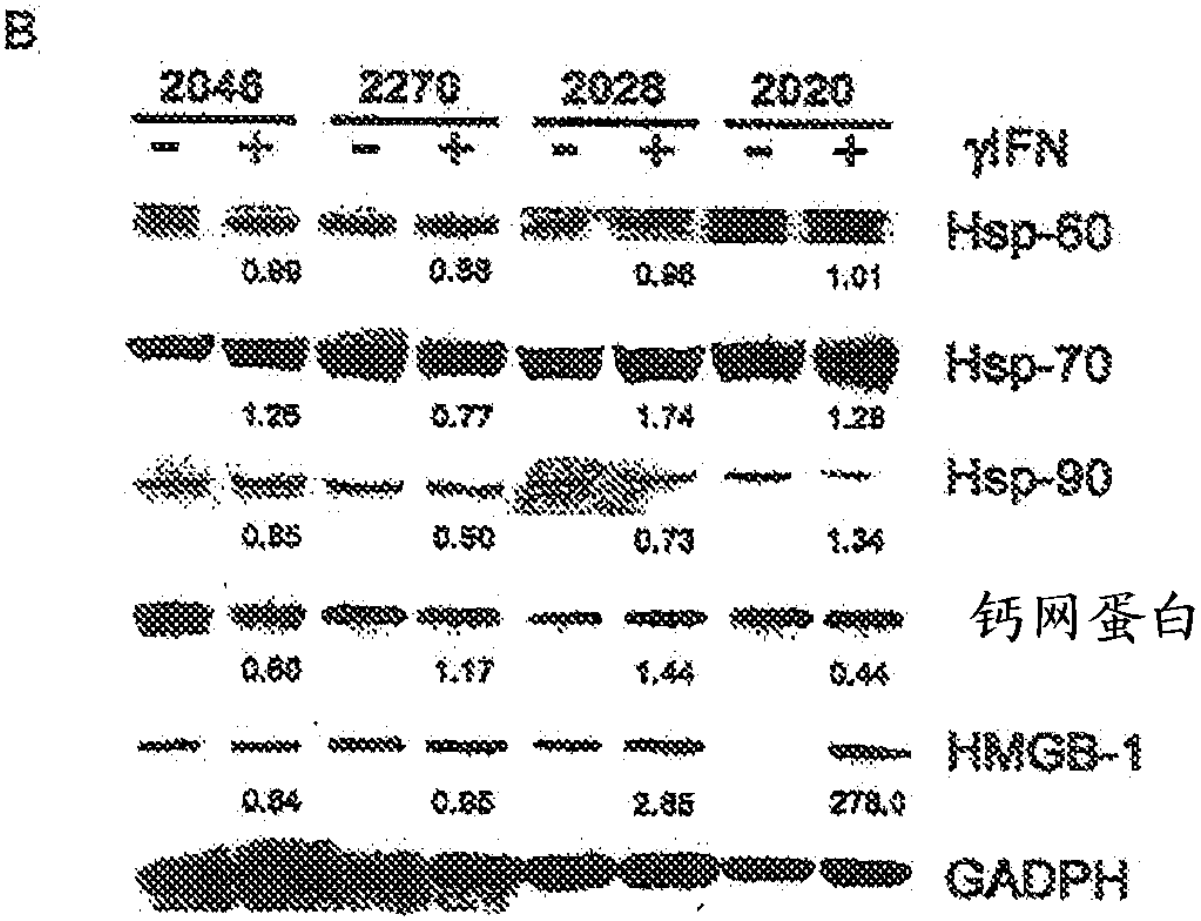

B
2048
2270
2028
2020
- + - + - + - +
γIFN
Hsp-60
0.89 0.33 0.96 1.01
Hsp-70
1.25 0.77 1.74 1.28
Hsp-90
0.85 0.50 0.73 1.34
钙网蛋白
0.60 1.17 1.44 0.44
HMGB-1
0.84 0.85 2.85 278.0
GADPH

图 2B

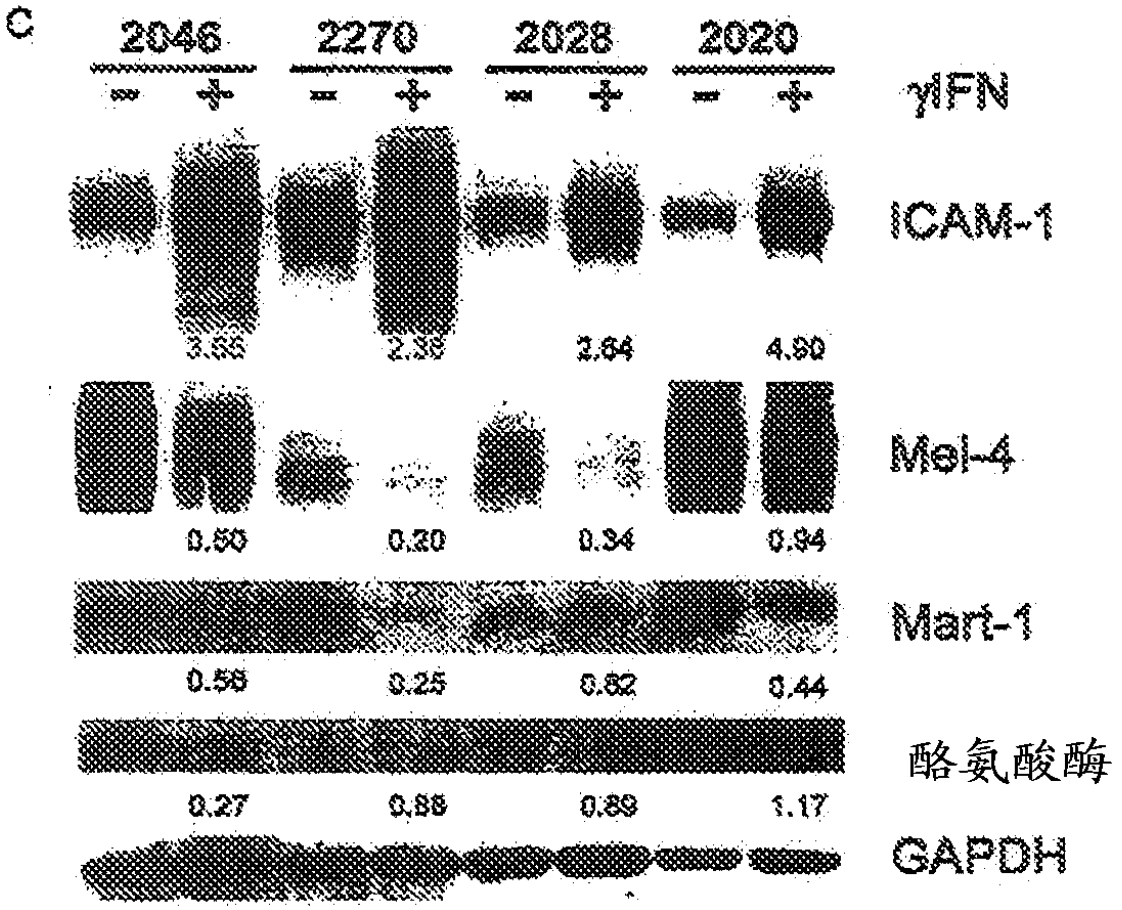

C
2046
2270
2028
2020
- + - + - + - +
γIFN
ICAM-1
3.56
2.38
2.64
4.90
Mel-4
0.50
0.20
0.34
0.94
Mart-1
0.56
0.25
0.62
0.44
酪氨酸酶
0.27
0.88
0.89
1.17
GAPDH

图 2C

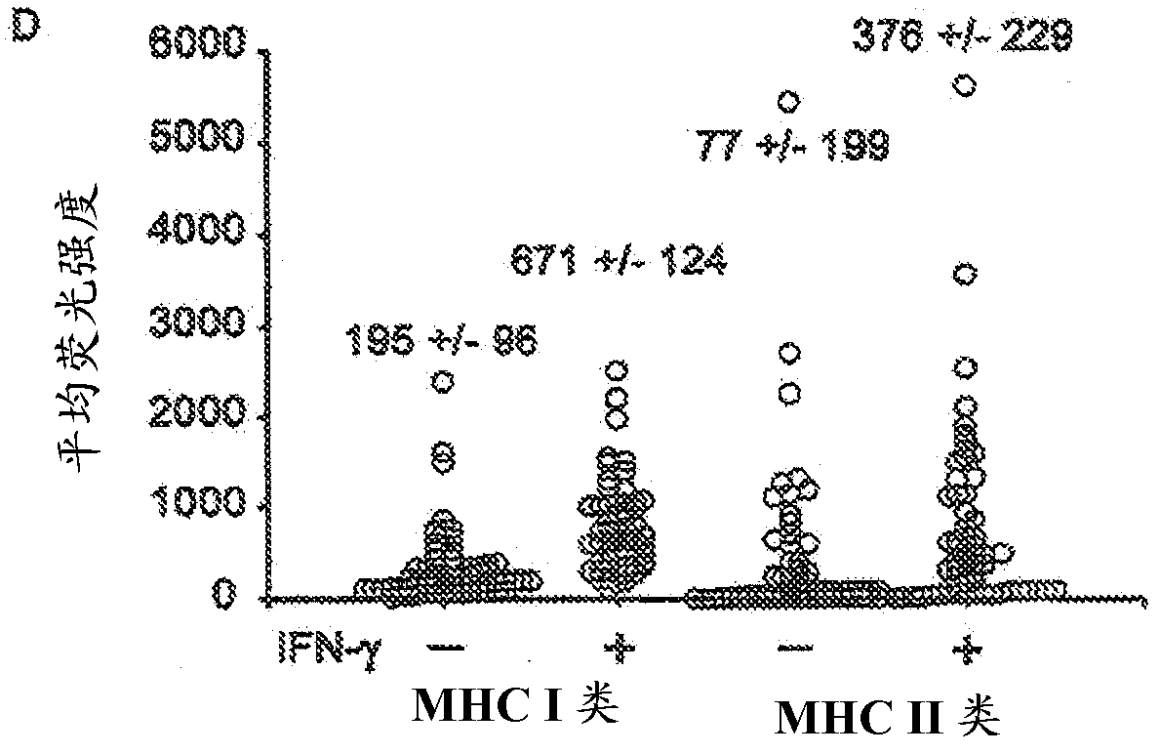

D
6000
5000
4000
3000
2000
1000
0
平均荧光强度
376 +/- 229
77 +/- 199
671 +/- 124
195 +/- 86
IFN-γ
—
+
—
+
MHC I 类
MHC II 类

图 2D

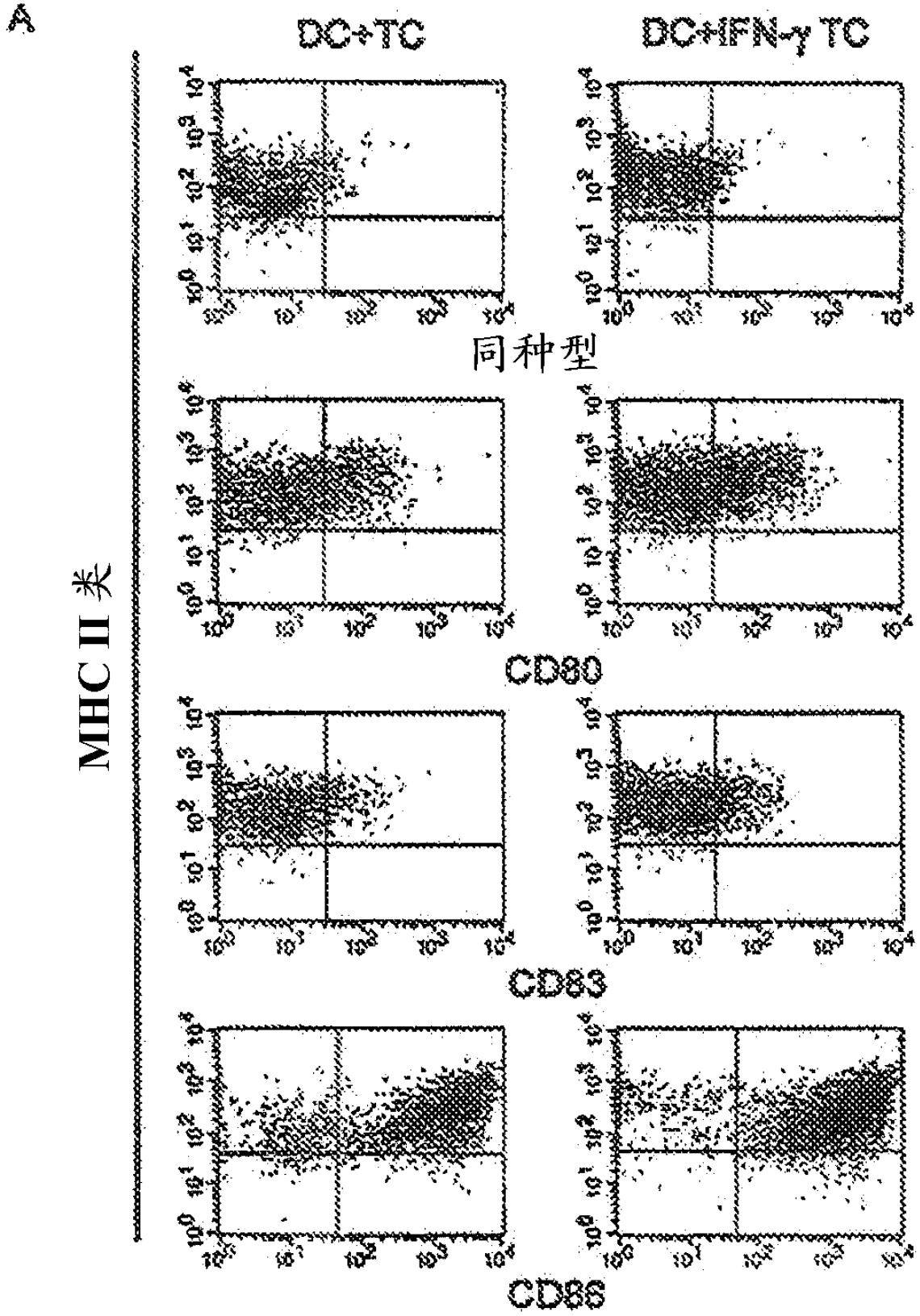


图 3A

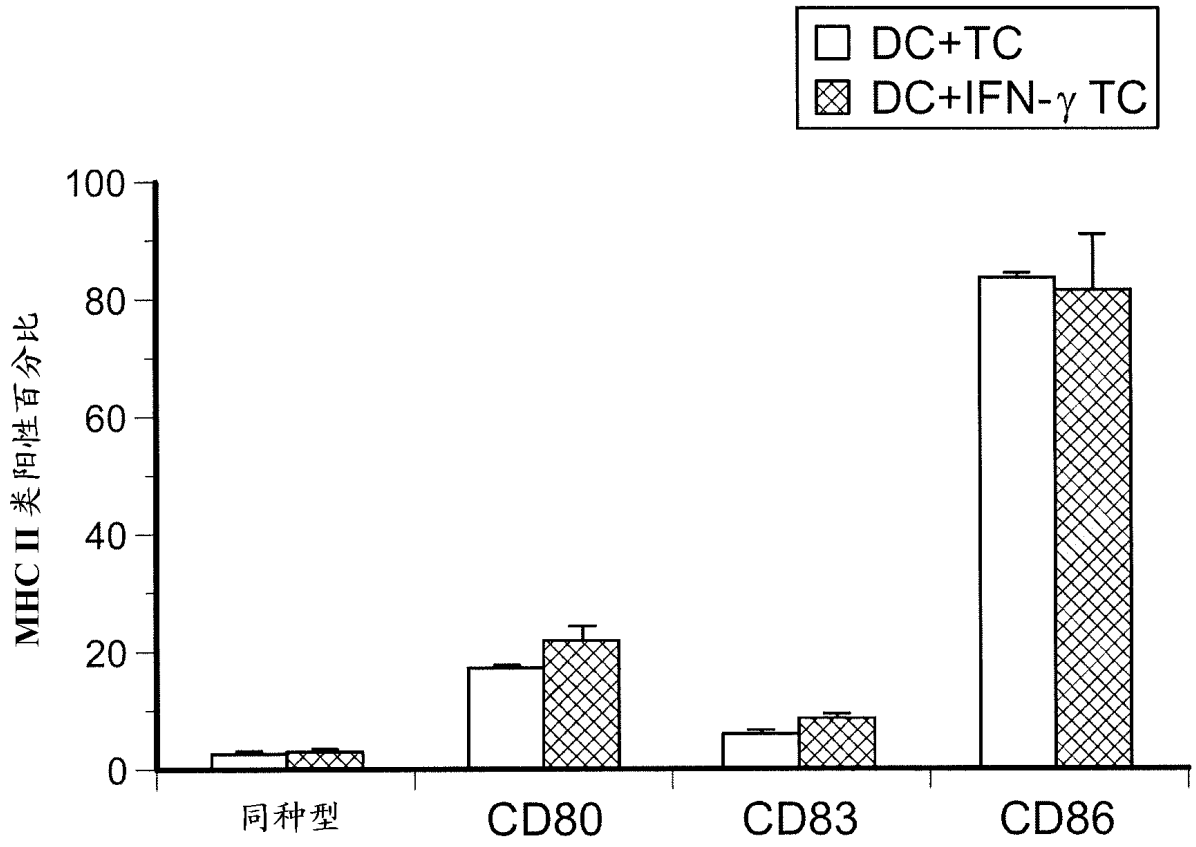

DC+TC
DC+IFN-γ TC
MHC II 类阳性百分比
100
80
60
40
20
0
同种型
CD80
CD83
CD86

图 3B

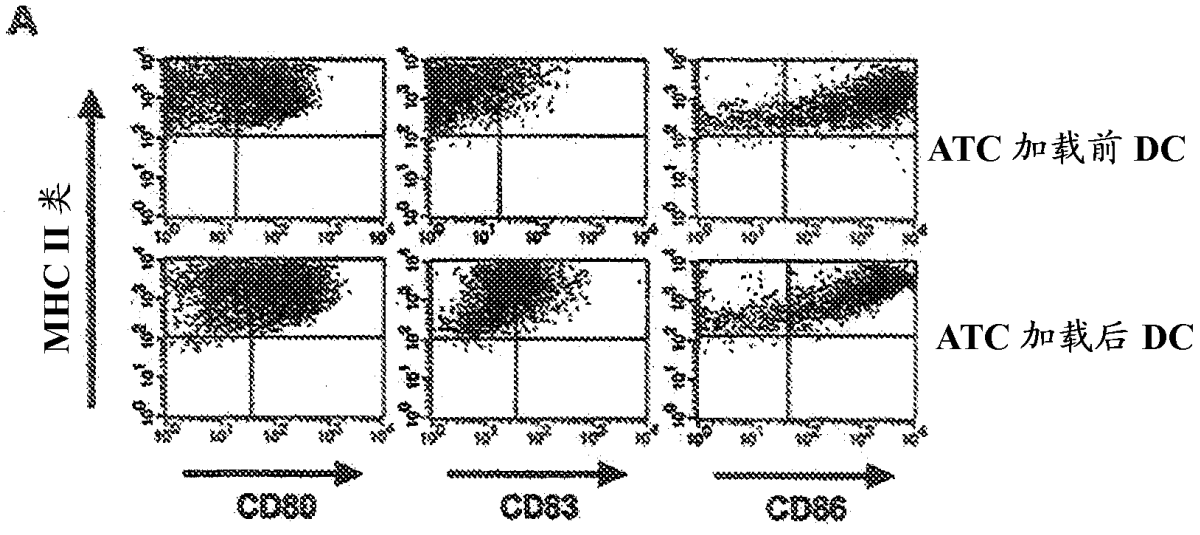

A
MHC II 类
ATC 加载前 DC
ATC 加载后 DC
CD80
CD83
CD86

图 4A

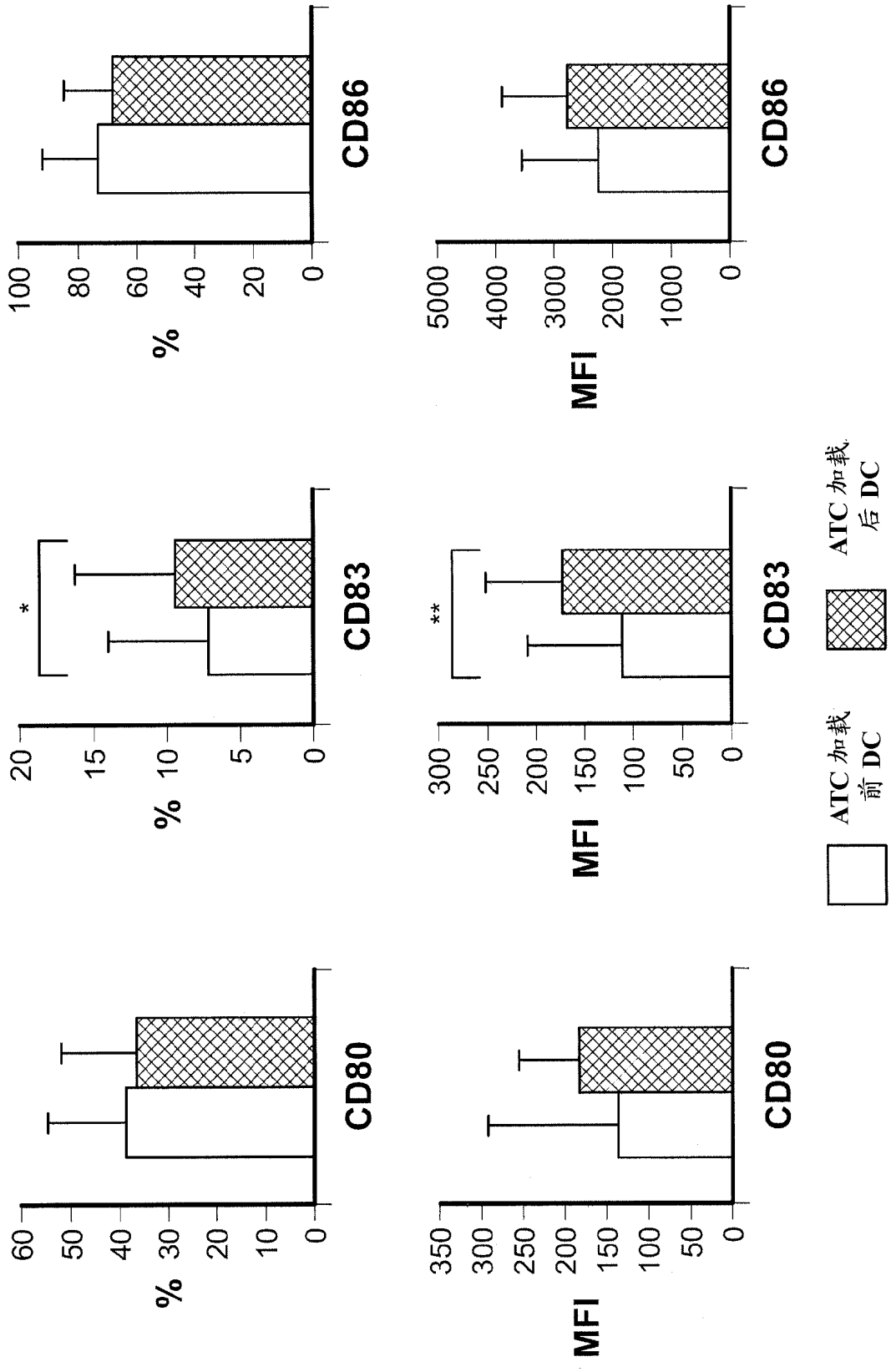

CD86
%
100
80
60
40
20
0
CD86
MFI
5000
4000
3000
2000
1000
0
CD83
%
*
20
15
10
5
0
CD83
MFI
**
300
250
200
150
100
50
0
ATC 加载后 DC
ATC 加载前 DC
CD80
%
60
50
40
30
20
10
0
CD80
MFI
350
300
250
200
150
100
50
0

图 4B

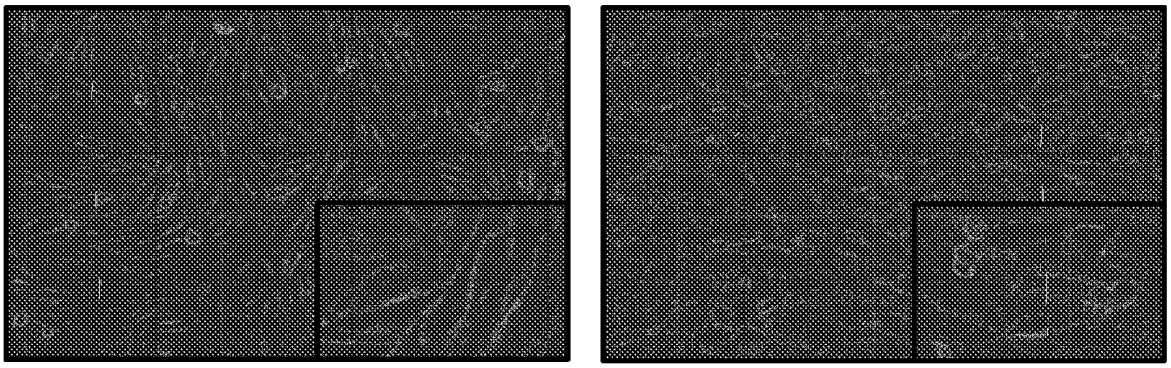


图 5A

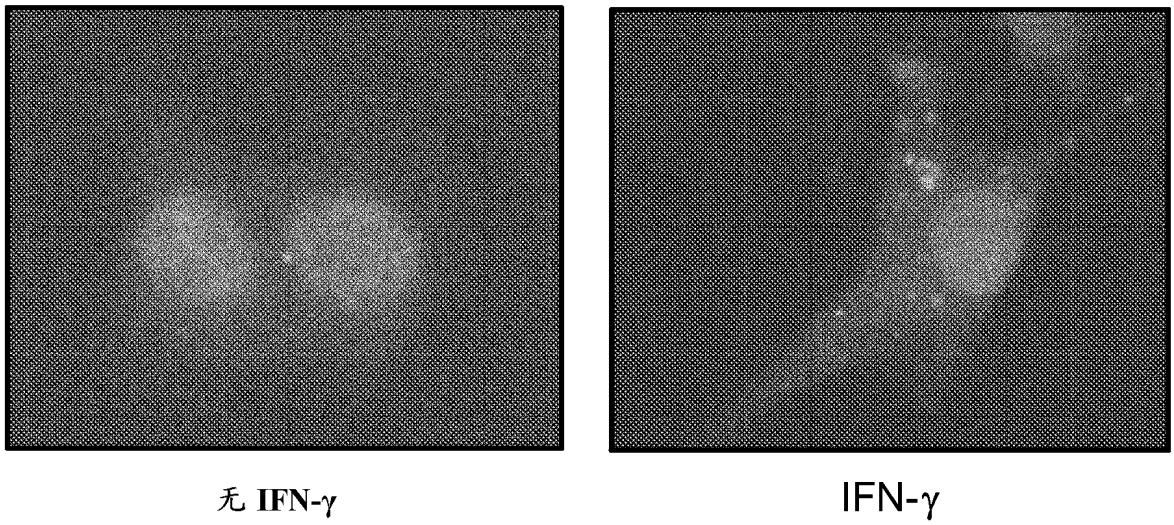


图 5B

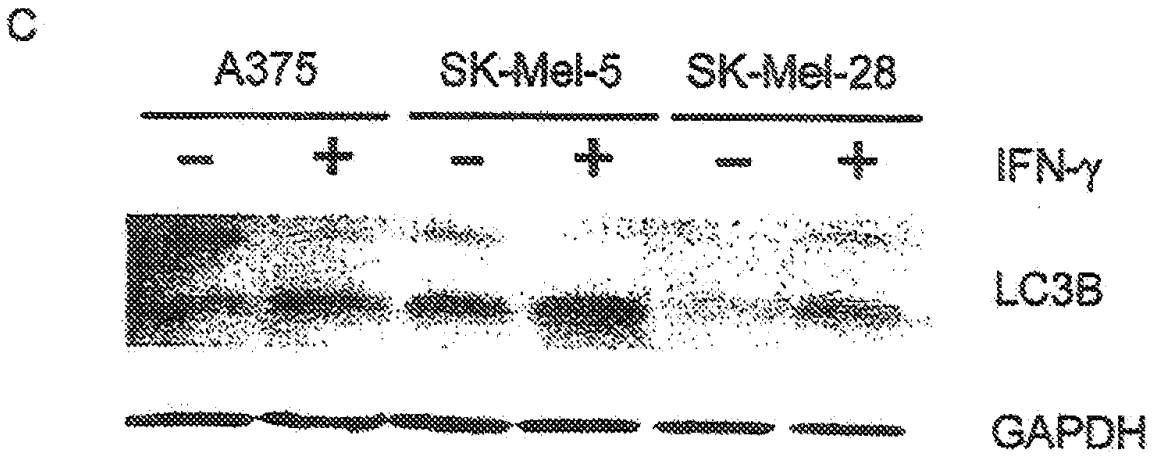

C
A375
SK-Mel-5
SK-Mel-28
-
+
-
+
-
+
IFN-γ
LC3B
GAPDH

图 5C

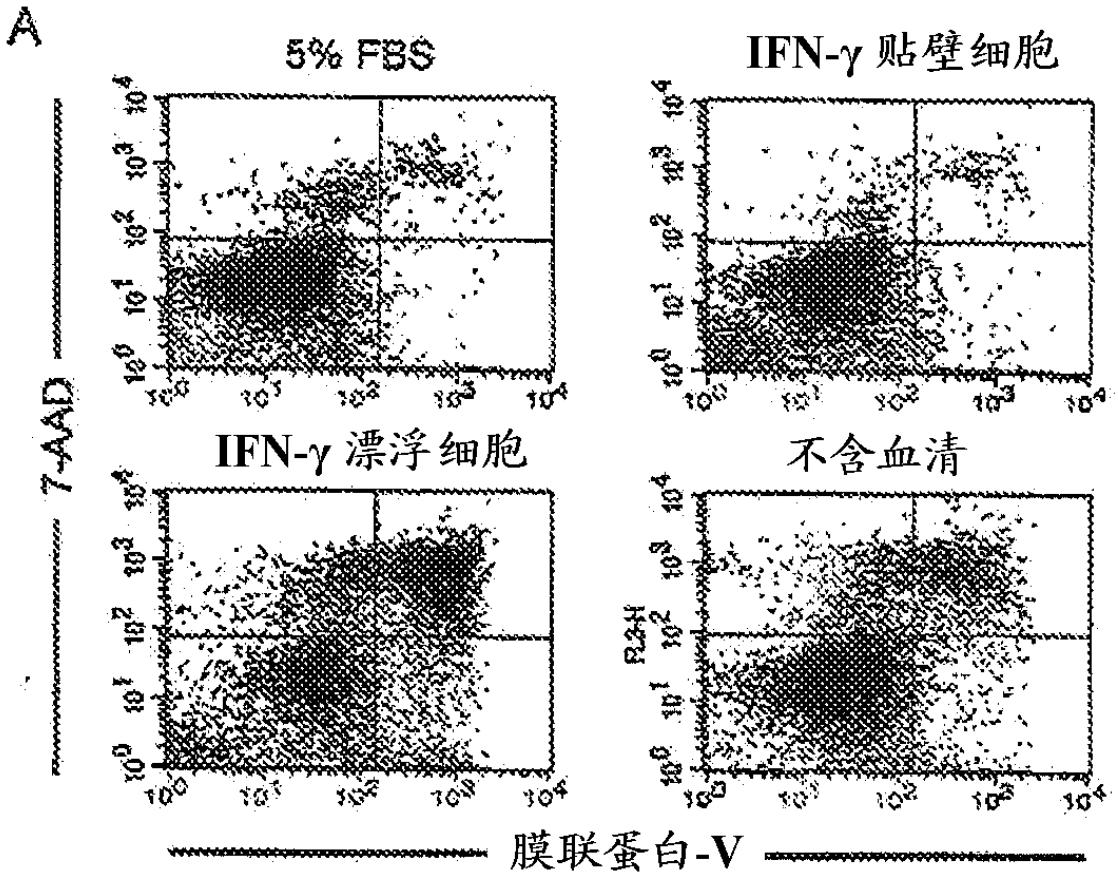

A
5% FBS
IFN-γ 贴壁细胞
IFN-γ 漂浮细胞
不含血清
7-AAD
膜联蛋白-V

图 6A

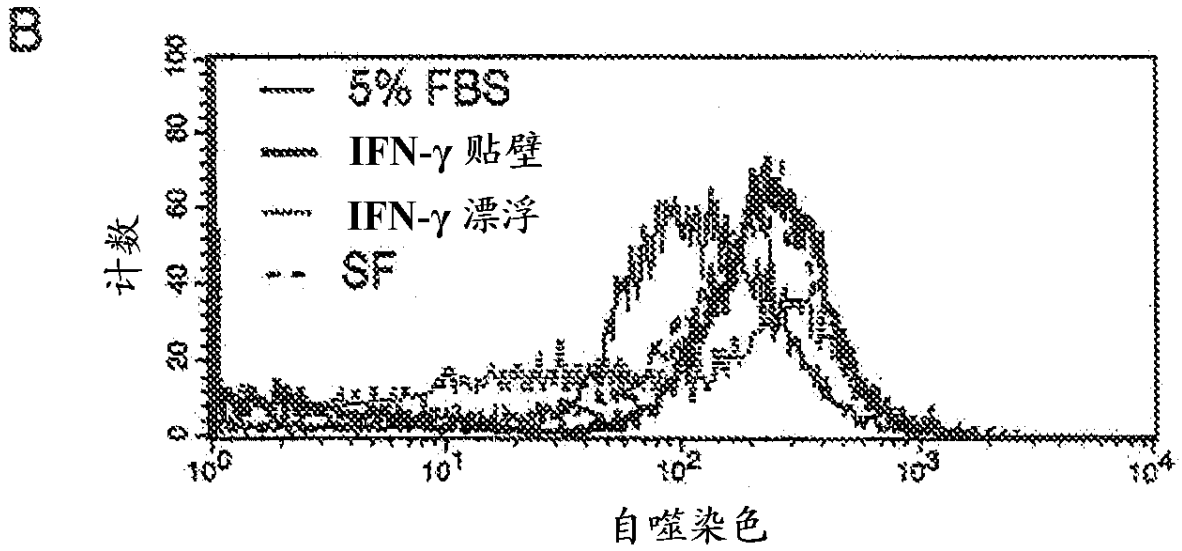

B
5% FBS
IFN-γ 贴壁
IFN-γ 漂浮
SF
计数
自噬染色

图 6B

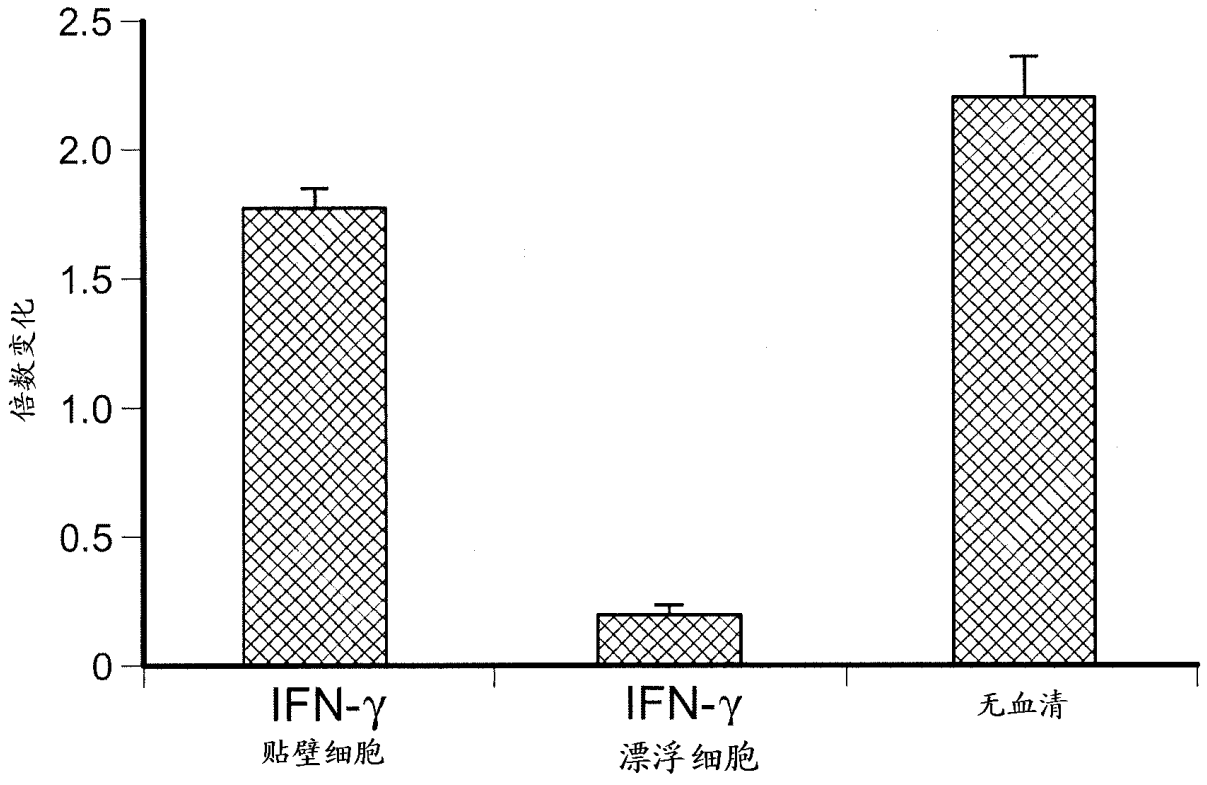

2.5
2.0
1.5
1.0
0.5
0
倍数变化
IFN-γ
贴壁细胞
IFN-γ
漂浮细胞
无血清

图 6C

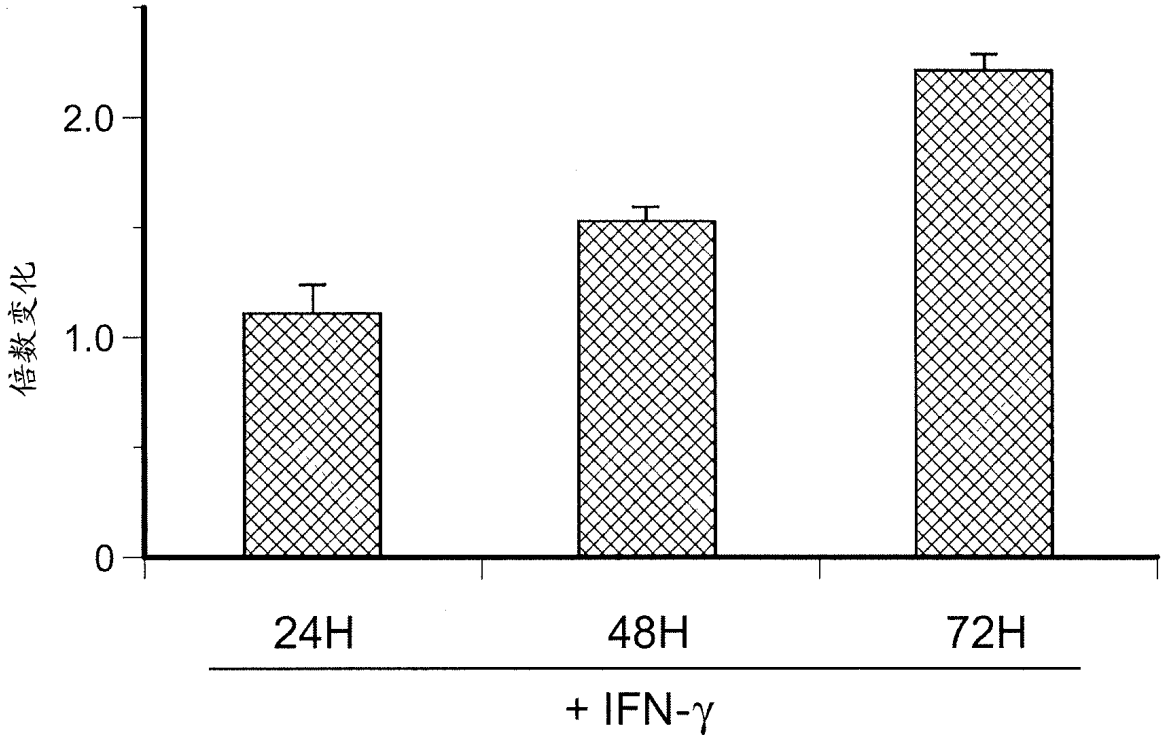


图 7A

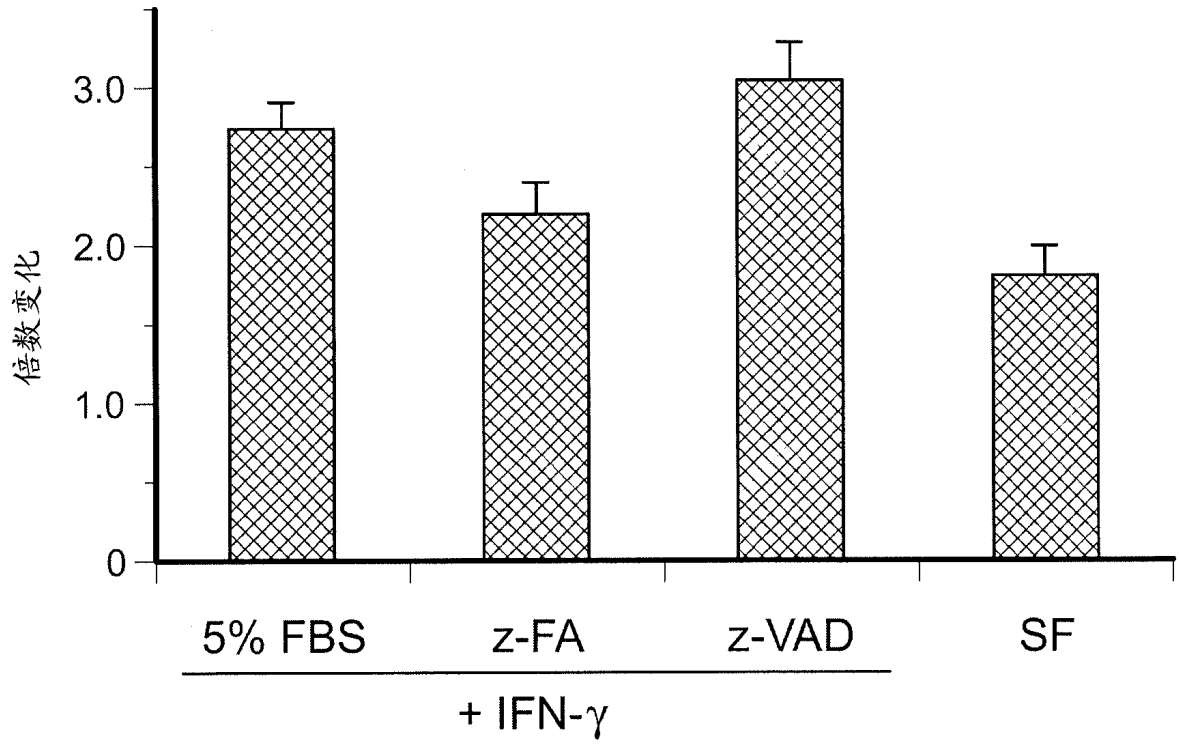


图 7B

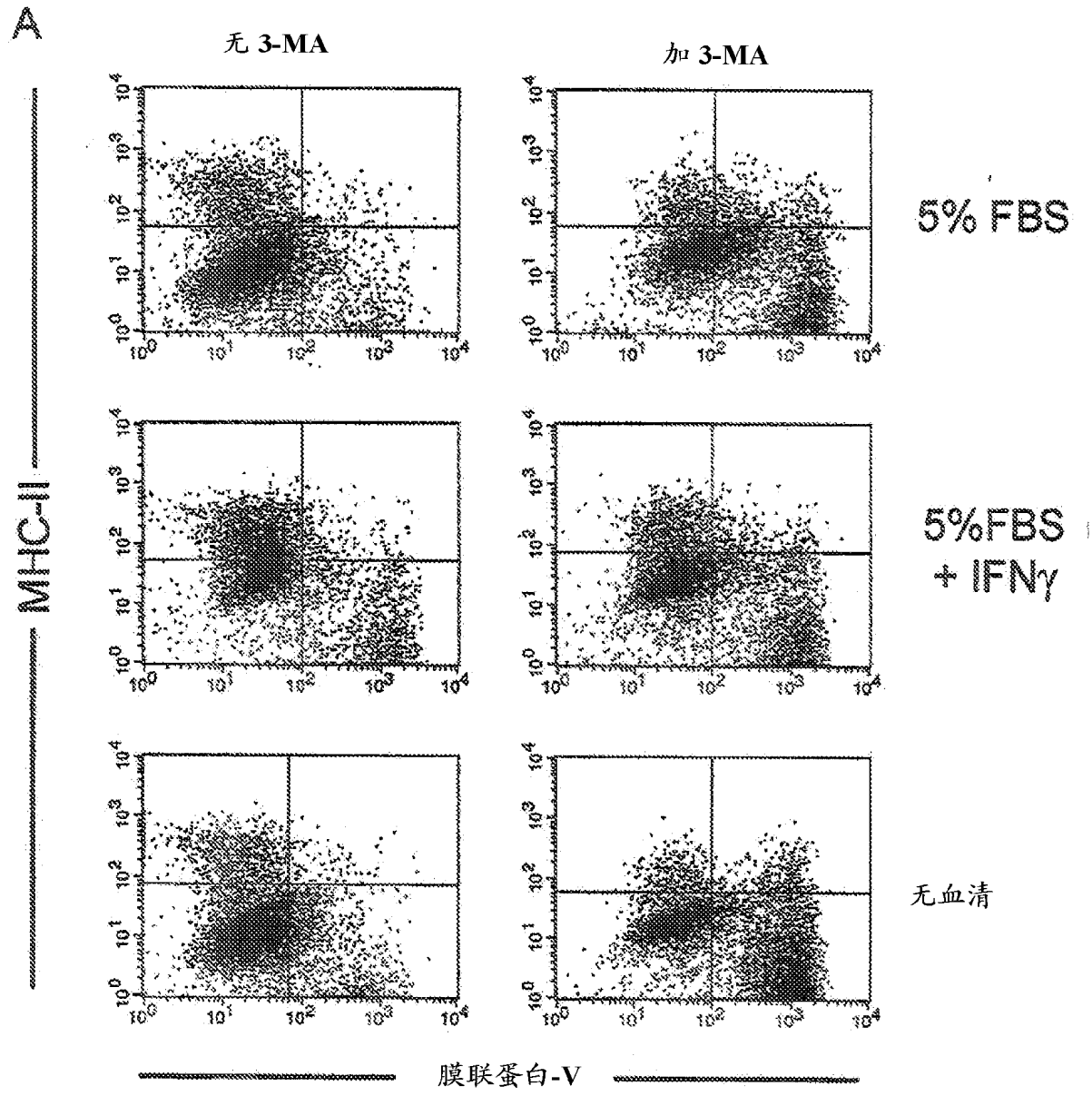


图 8

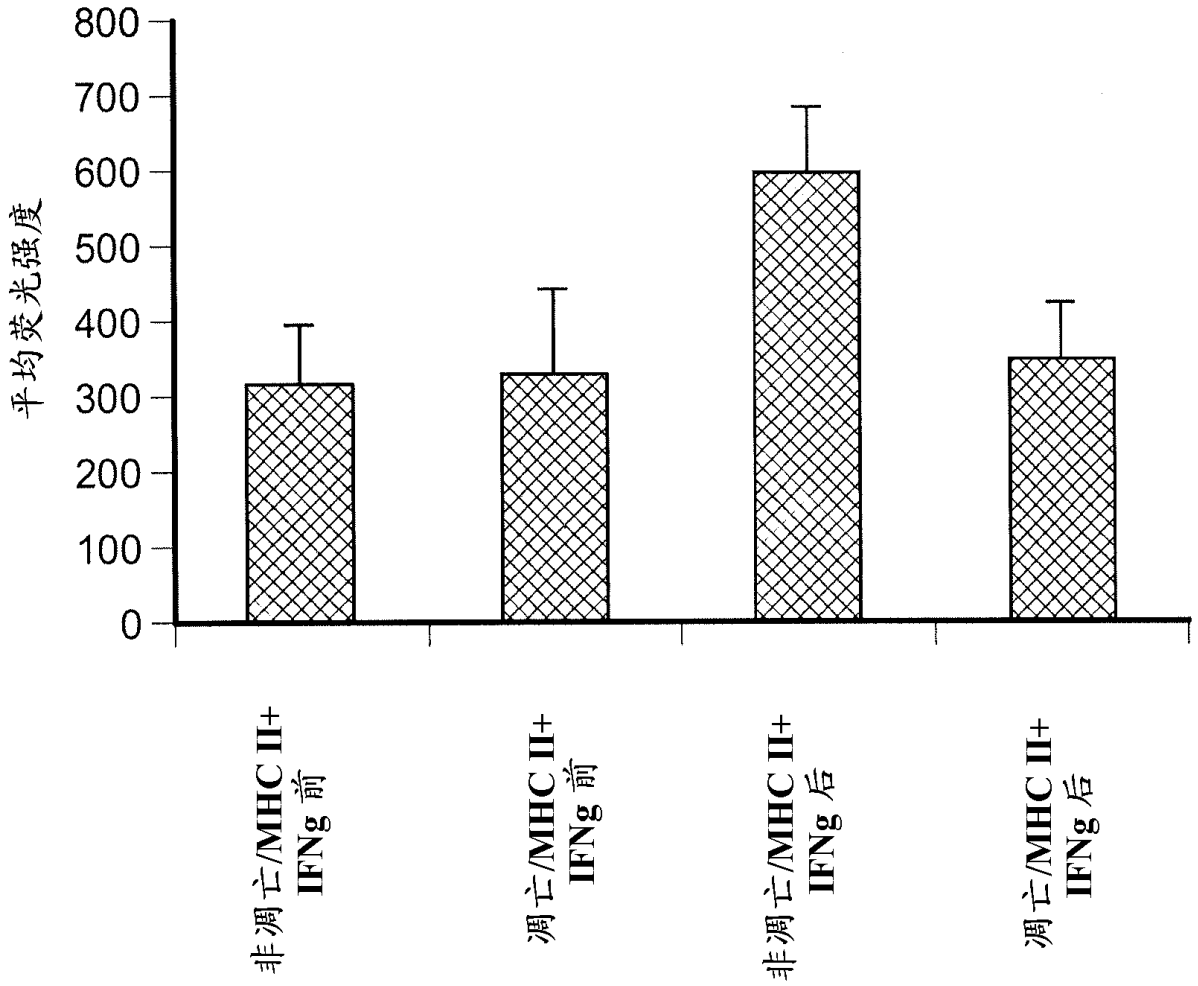

平均荧光强度
800
700
600
500
400
300
200
100
0
非凋亡/MHC II+ IFNg 前
凋亡/MHC II+ IFNg 前
非凋亡/MHC II+ IFNg 后
凋亡/MHC II+ IFNg 后

图 9

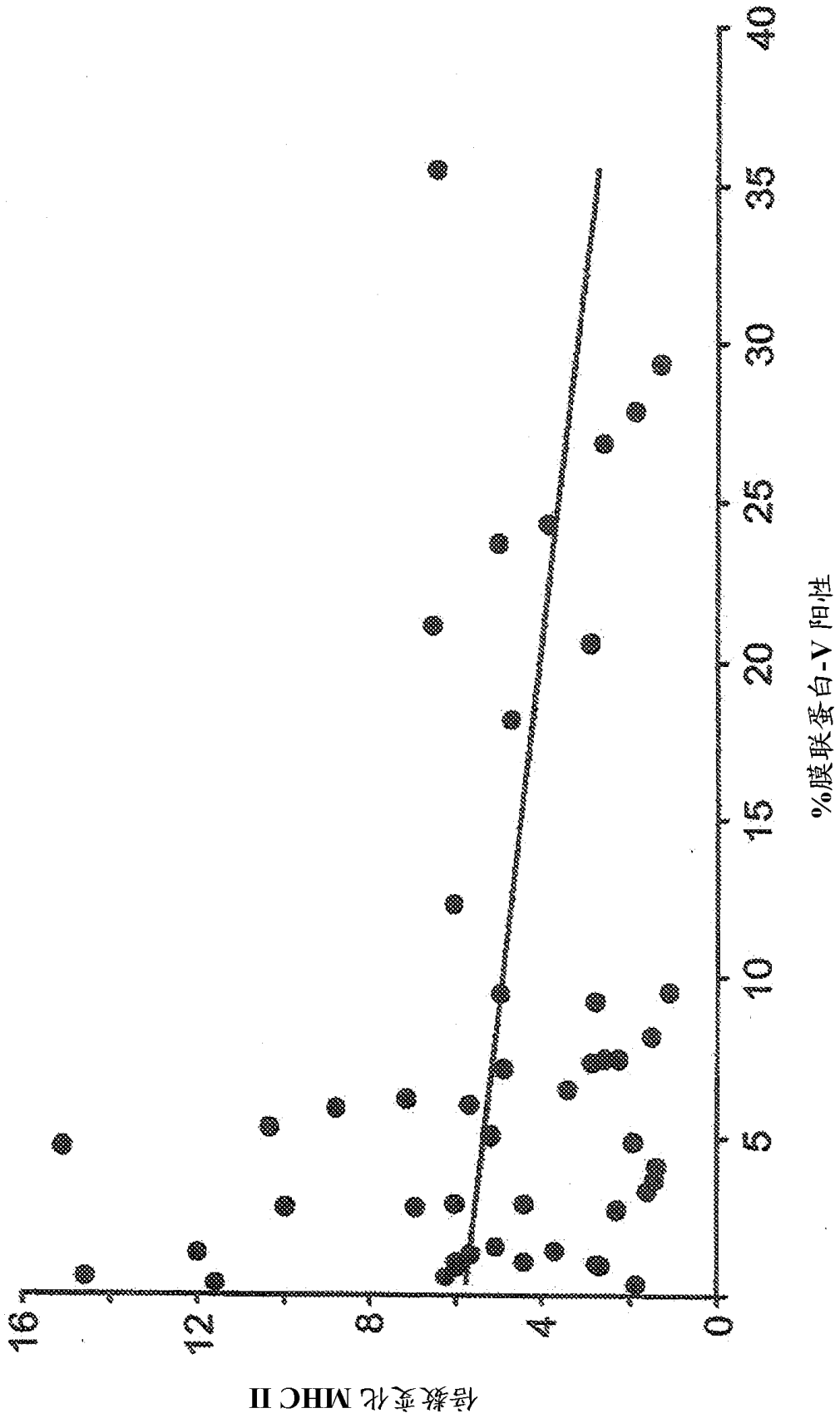


图 10

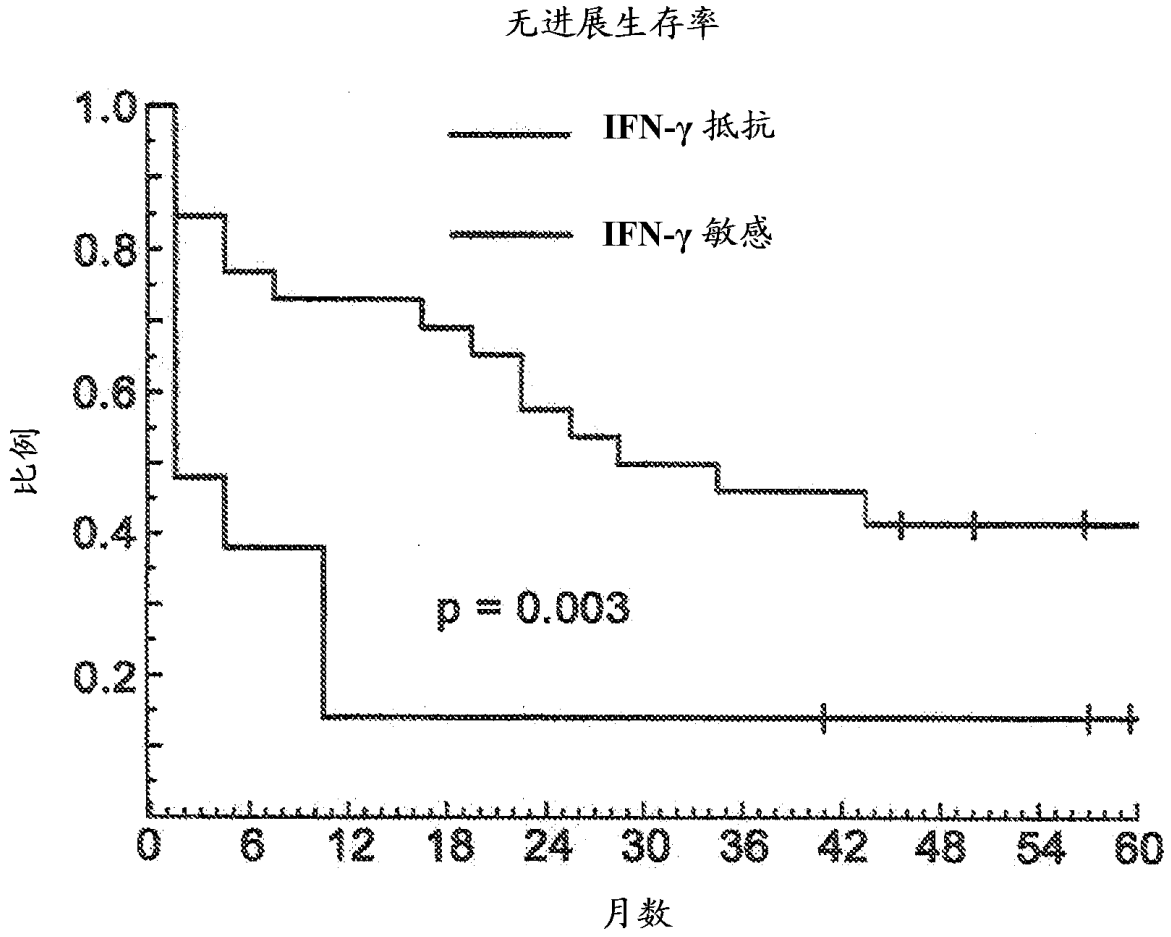

无进展生存率
IFN-γ 抵抗
IFN-γ 敏感
p = 0.003
比例
1.0
0.8
0.6
0.4
0.2
0
6
12
18
24
30
36
42
48
54
60
月数

图 11

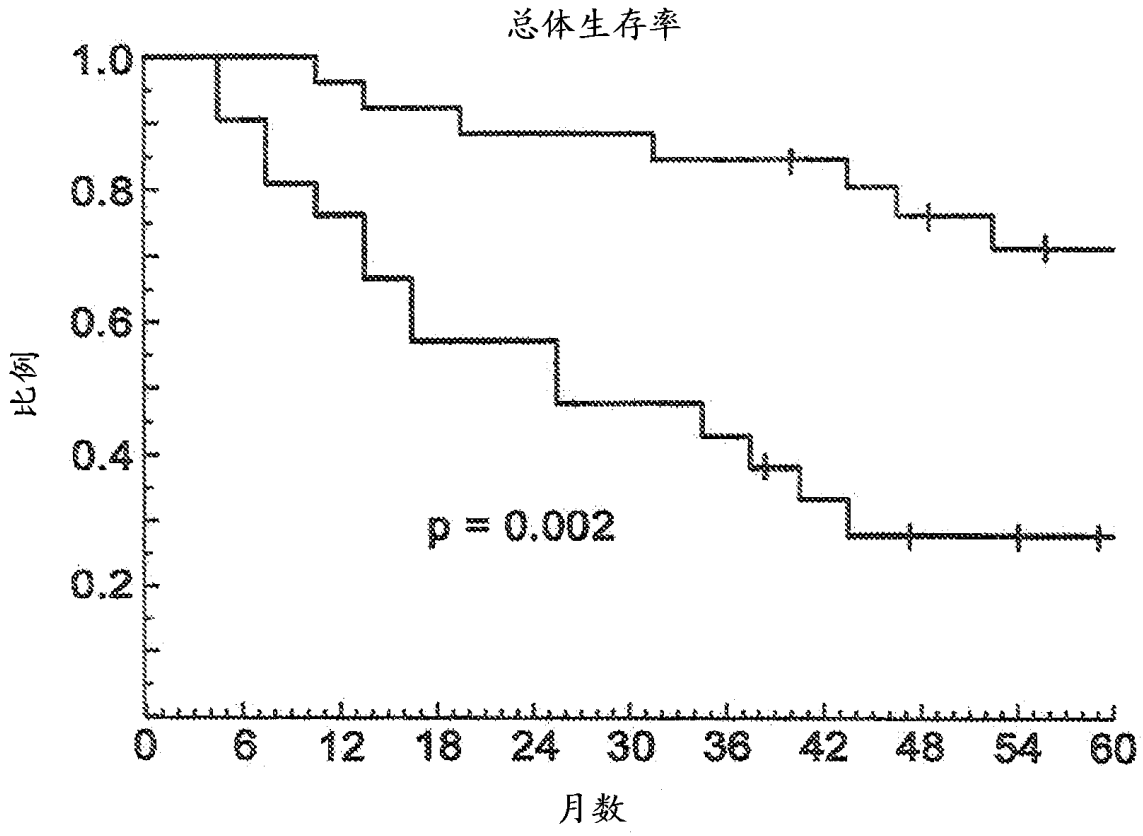


图 12

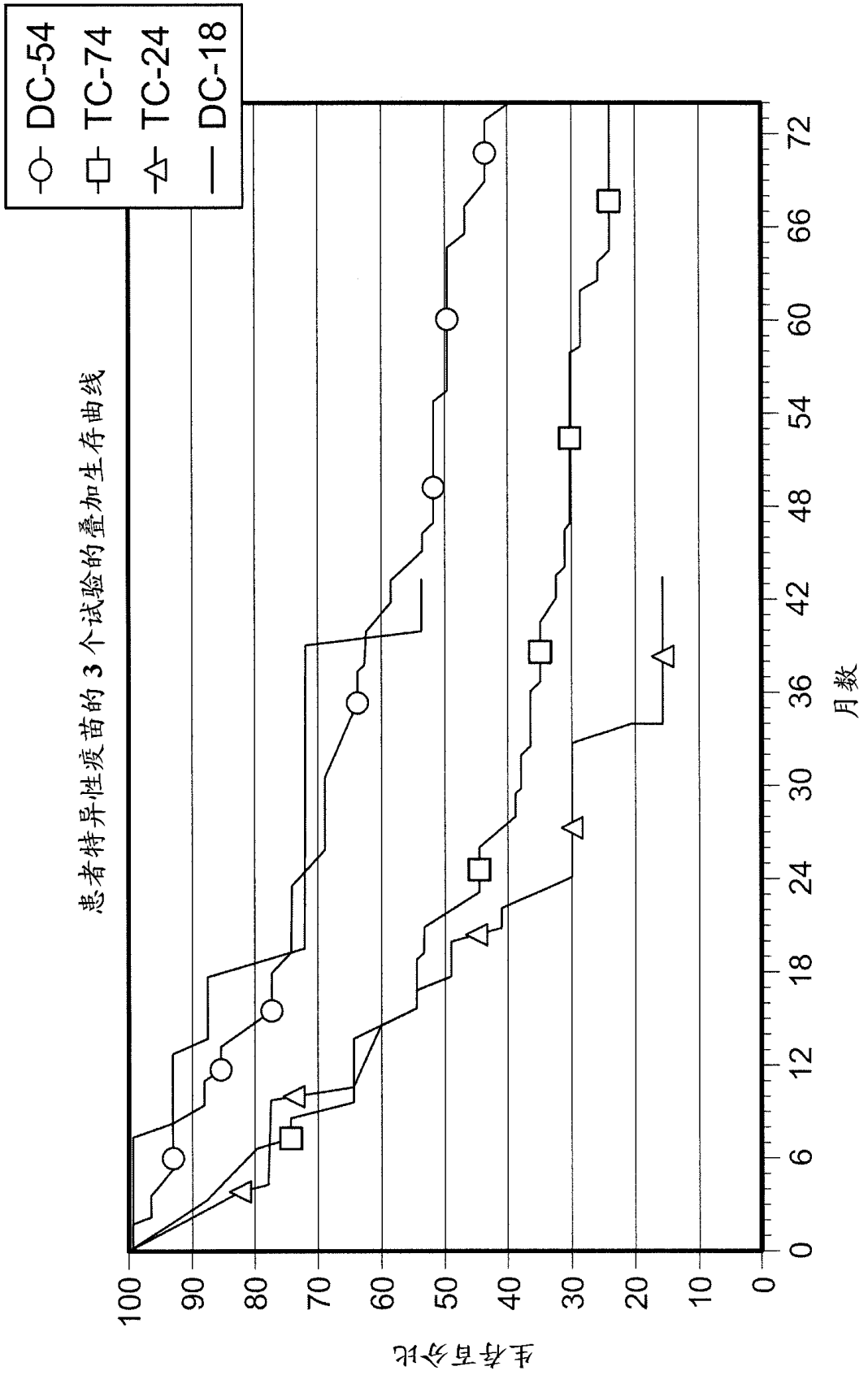

DC-54
TC-74
TC-24
DC-18
患者特异性疫苗的 3 个试验的叠加生存曲线
生存百分比
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
30
36
42
48
54
60
66
72
月数

图 13

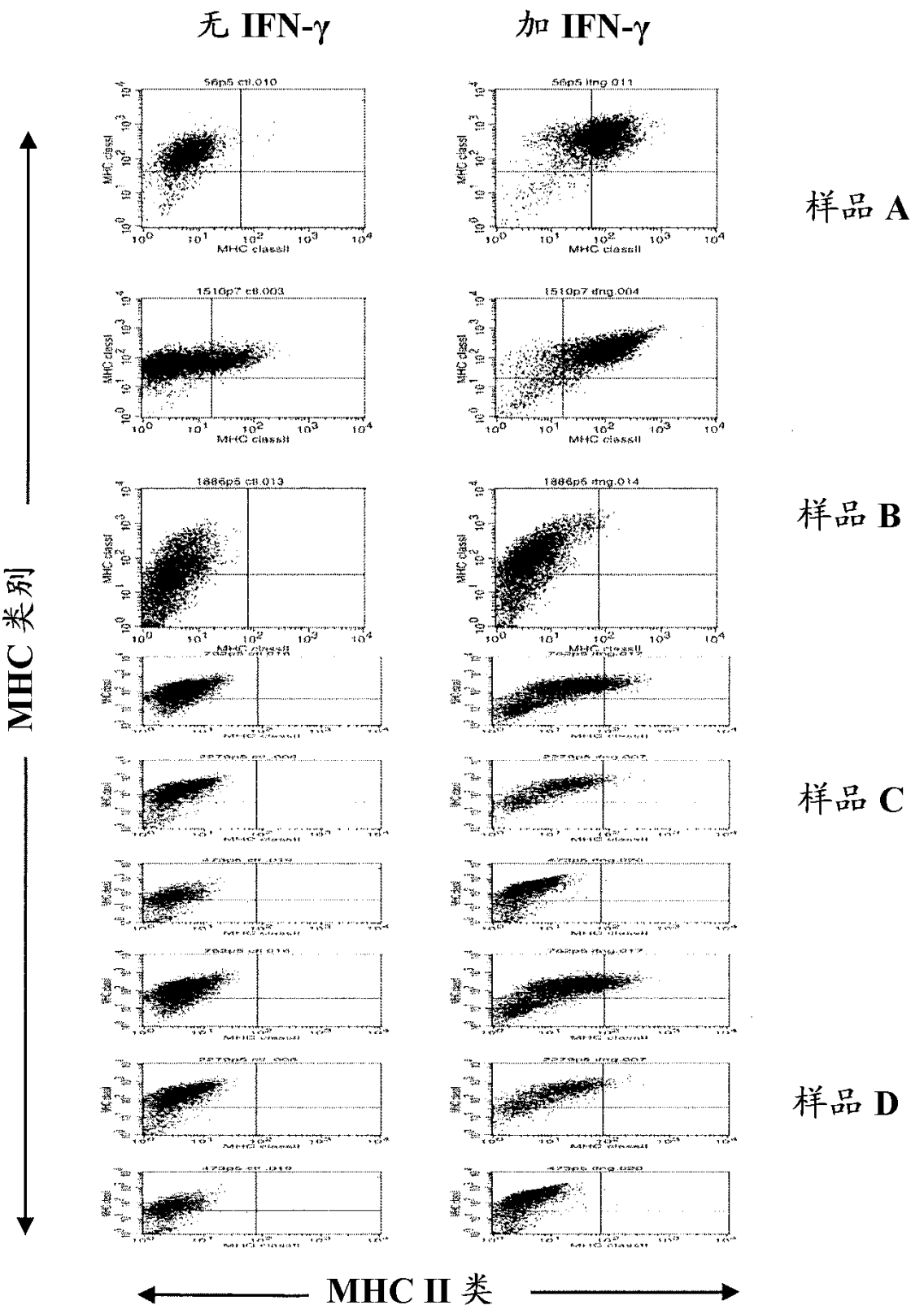


图 14

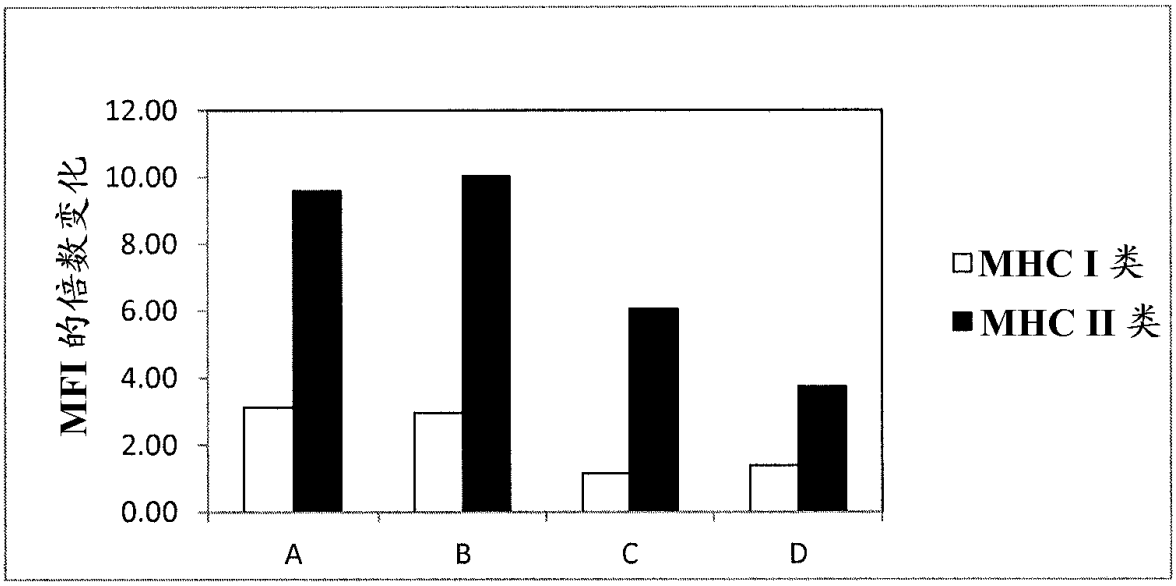


图 15

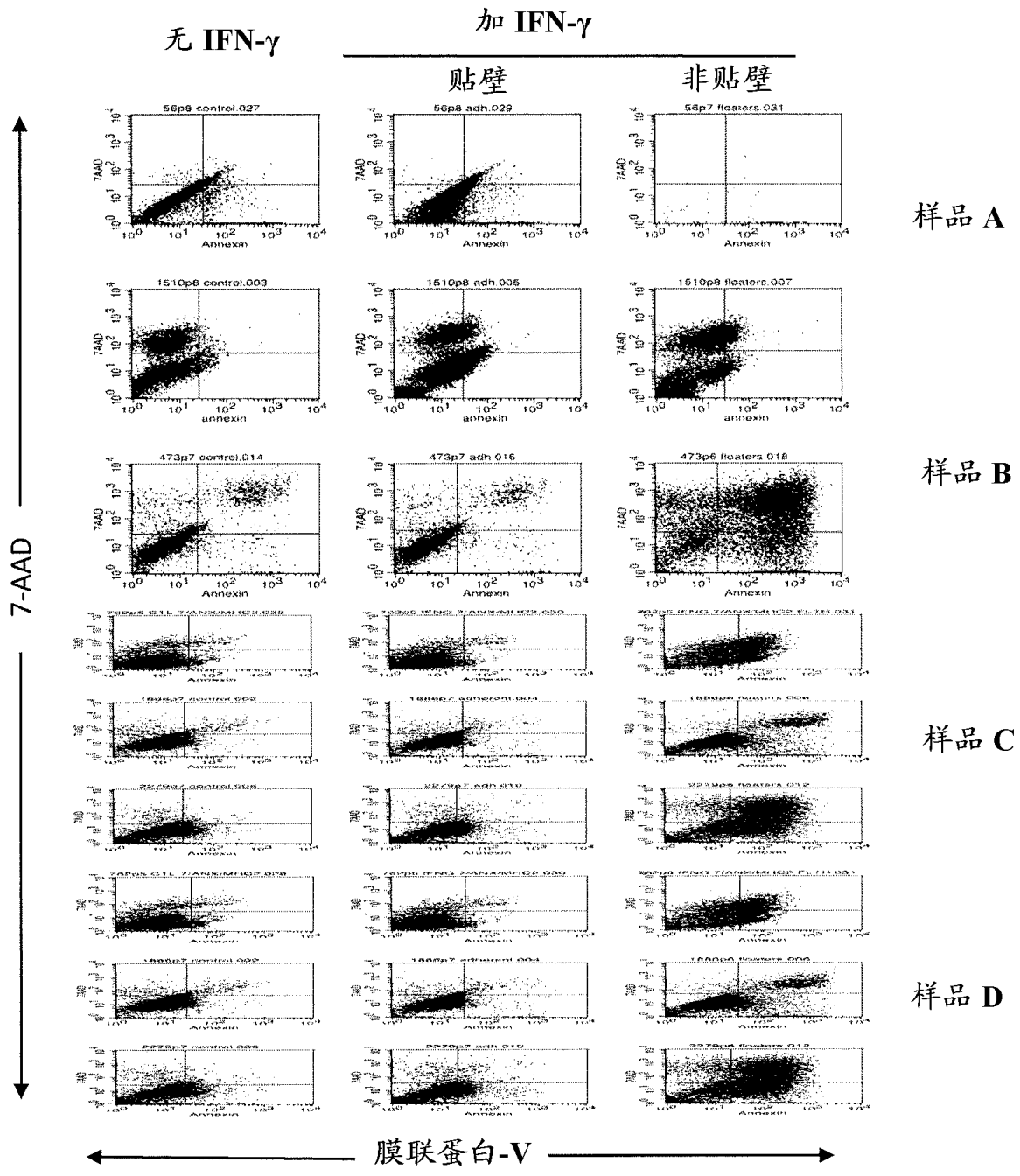


图 16

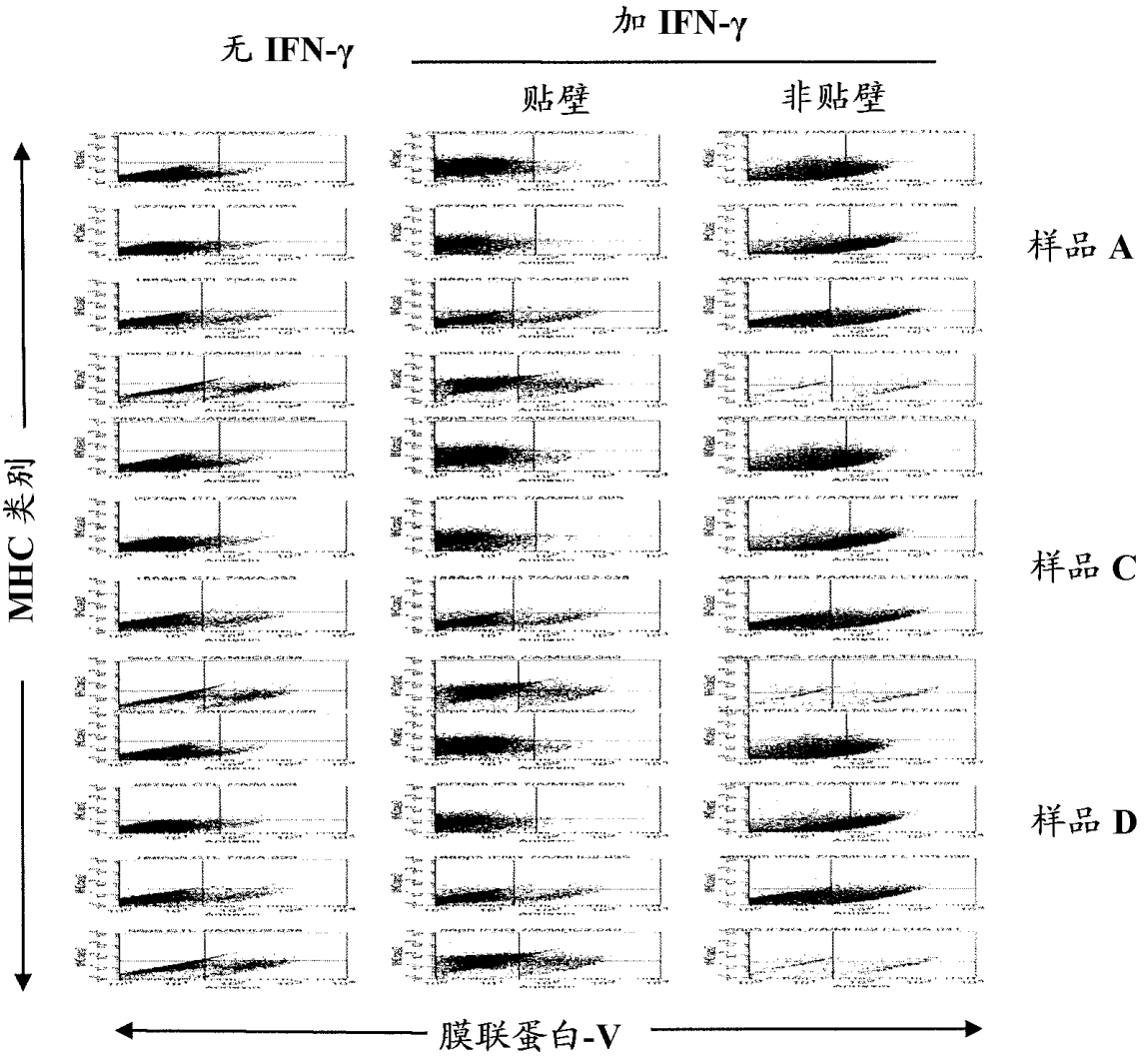


图 17

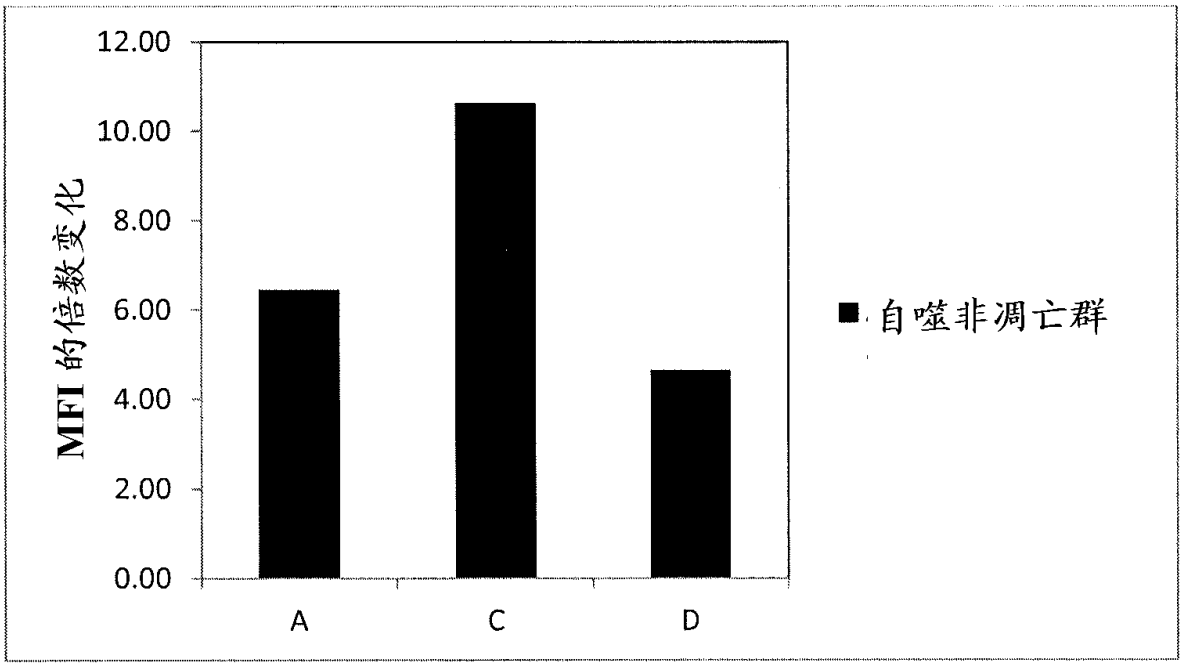


图 18

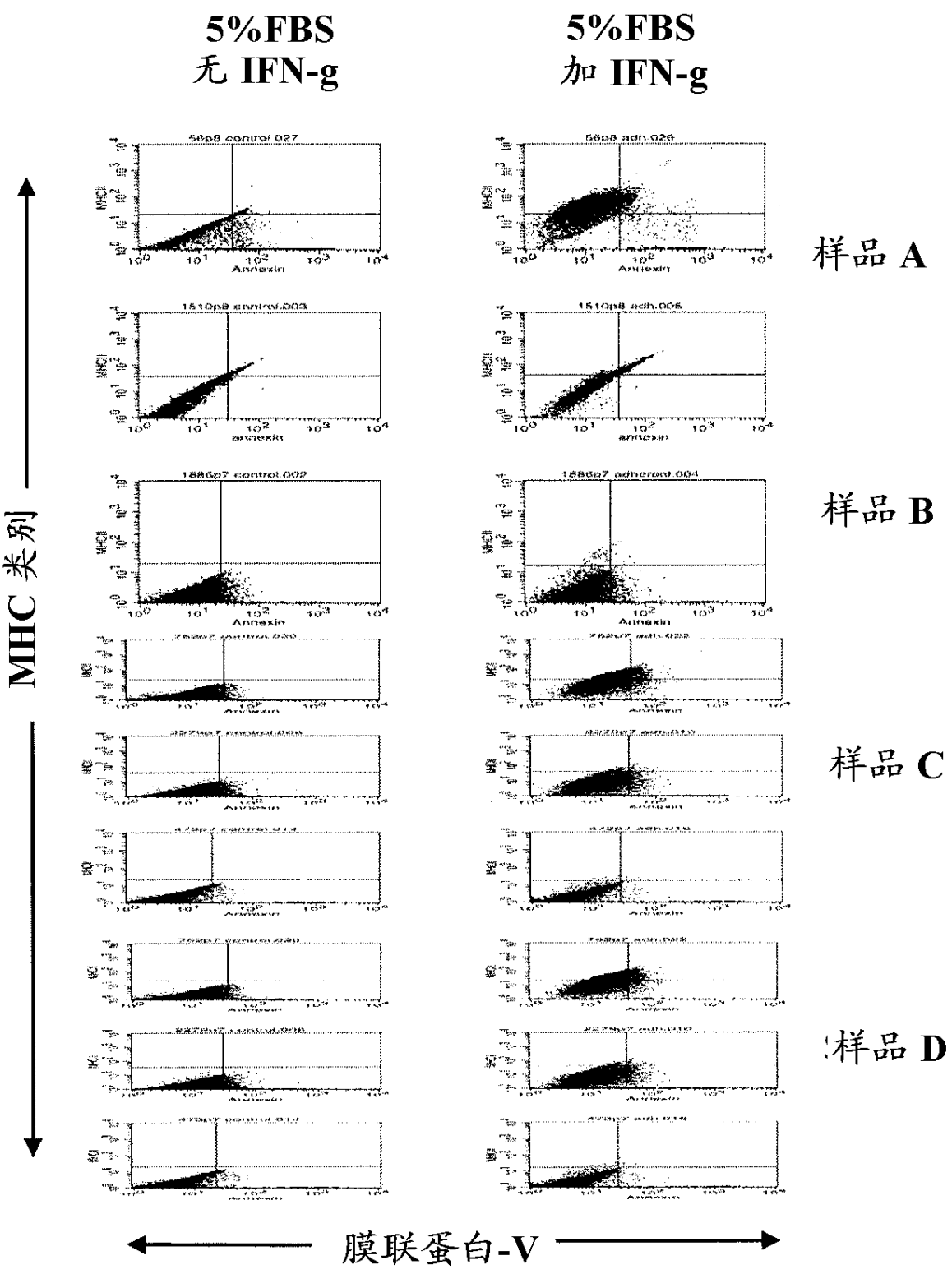


图 19