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(71) Applicant(s)
Alloplex Biotherapeutics

(72) Inventor(s)
Borriello, Frank

(74) Agent / Attorney
Spruson & Ferguson, GPO Box 3898, Sydney, NSW, 2001, AU

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(71) Applicant: **ALLOPLEX BIOTHERAPEUTICS** [US/US]; 3 Greeley Road, Winchester, MA 01890 (US).

(72) Inventor: **BORRIELLO, Frank**; 3 Greeley Road, Winchester, MA 01890 (US).

(74) Agent: **LUBIT, Beverly, W.**; McCarter & English LLP, Four Gateway Center, 100 Mulberry Street, Newark, NJ 07102 (US).

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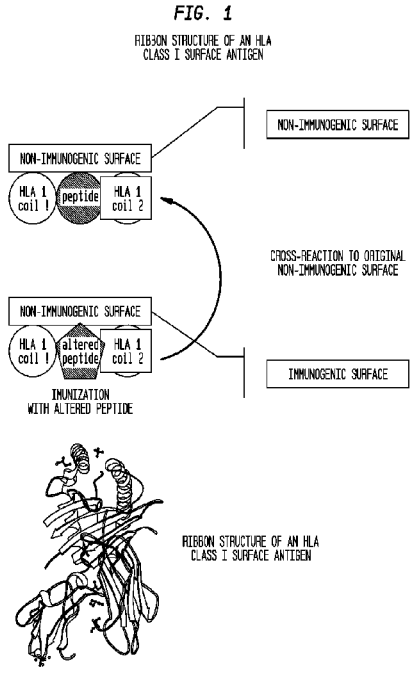
(54) Title: ALLOGENEIC TUMOR CELL VACCINE

FIG. 1



(57) Abstract: The described invention provides a tumor cell vaccine comprising genetically modified tumor cell line of a particular tumor type that stably expresses high levels of two or more immunomodulators. According to some embodiments, an immunogenic amount of the tumor cell line variants may be selected for concomitant expression of two or more of recombinant membrane expressed IgGl, CD40L, TNF-alpha, as well as membrane and soluble forms of GM- CSF, and Flt-3L peptides that are effective to elicit an anti-tumor immune response compared to the parent unmodified tumor cell line as measured in *vitro* by a one-way mixed lymphocyte tumor reaction assay using human peripheral blood mononuclear cells and the genetically modified allogeneic cell vaccine candidate. According to some embodiments, the tumor cell vaccine candidate will induce an immune response in the recipient cancer patient that cross reacts with the patient's own (autologous) tumor cells, the effects of which will be sufficient to result in enhanced anti-tumor immunity contributing to the increased survival of a vaccinated patient cohort compared to a matched unvaccinated patient cohort.

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ALLOGENEIC TUMOR CELL VACCINE

RELATED APPLICATIONS

[0001] This Application claims priority to United States Provisional Application No. 62/425,424, filed on November 22, 2016, the entire contents of which are incorporated by reference in their entirety herein.

FIELD OF THE INVENTION

[0002] The described invention relates generally to immunological approaches to the treatment of cancer, and more particularly to cancer vaccines comprising modified tumor cells.

BACKGROUND OF THE INVENTION

Immune response

[0003] Generally speaking, immune responses are initiated by an encounter between an individual and a foreign antigenic substance, e.g., an infectious microorganism. The infected individual rapidly responds with both a humoral immune response with the production of antibody molecules specific for the antigenic determinants/epitopes of the immunogen and a cell mediated immune response with the expansion and differentiation of antigen-specific regulatory and effector T-lymphocytes, including both cells that produce cytokines and killer T cells, capable of lysing infected cells. Primary immunization with a given microorganism evokes antibodies and T cells that are specific for the antigenic determinants/epitopes found on that microorganism, but that usually fail to recognize or recognize only poorly antigenic determinants expressed by unrelated microbes (Paul, W. E., "Chapter 1: The immune system: an

introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999), at p. 102).

[0004] As a consequence of this initial response, the immunized individual develops a state of immunologic memory. If the same or a closely related microorganism is encountered again, a secondary response ensues. This secondary response generally consists of an antibody response that is more rapid, greater in magnitude and composed of antibodies that bind to the antigen with greater affinity and are more effective in clearing the microbe from the body, and a similarly enhanced and often more effective T-cell response. However, immune responses against infectious agents do not always lead to elimination of the pathogen. (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999), at p. 102).

Immune Tolerance of Cancer

[0005] Cancer is characterized by genetic instability of particular cells but has also been described as a disorder of the immune system, based on the fact that the immune system fails, at least in certain segments of the afflicted human population, to respond optimally to cancerous cells that have taken on a distinctly non-self phenotype that should be recognized as foreign. Several reasons have been advanced to explain the basis of this observation. For example, first, cancer cells consist mainly of self-antigens, in striking contrast to the situation with infectious organisms. Some antigens that are classified as cancer antigens are actually normal antigens that are overexpressed, or normal antigens that have a mutation in only one or two amino acids in the polypeptide chain. Second, cancer cells down-regulate Major Histocompatibility Complex (MHC), and thus do not much present tumor cell-derived peptides by way of MHC. Third,

cancer cells, and associated tumor-associated macrophages, express cytokines that dampen the immune response (see, e.g., Yu et al (2007) Nature Rev. Immunol. 7:41 -51). This dampening is caused, for example, by the secretion of interleukin-10 (IL-10) by the cancer cells or by the associated macrophages. Fourth, unlike the situation with infections, cancer cells do not provide any immune adjuvant. Pathogens express a variety of naturally-occurring immune adjuvants, which take the form of toll-like receptor (TLR) agonists and NOD agonists (see, e.g., Kleinnijenhuis et al (2011) Clin. Dev. Immunol. 405310 (12 pages)). Generally, optimal activation of dendritic cells requires contact of an immune adjuvant with one or more toll-like receptors (TLRs) expressed by the dendritic cell. Without activation of the dendritic cell, contact between the dendritic cell and T cells (immune synapse) fails to result in optimal activation of the T cell.

Immune Surveillance and Immune Editing

[0006] Tumor immune editing is divided into three phases: an elimination phase, an equilibrium phase, and an escape phase. The elimination phase, also known as immune surveillance, is the process by which the immune system identifies cancerous or pre-cancerous cells and eliminates them before they grow out of control. This phase can be complete when all cancerous or precancerous cells are eliminated. If some tumor cells are not eliminated, a temporary state of equilibrium may be achieved between the immune system and tumor cell growth. In this equilibrium phase, tumors cells can either remain dormant or continue to evolve by accumulating further changes to genomic DNA that can modulate the antigens they present. During this process, the immune system exerts a selective pressure on evolving cells, whereby the tumor cells that are less able to be recognized have a survival advantage. Eventually the

immune response is unable to recognize cells of the tumor, resulting in the transition to the escape phase wherein tumor cells progressively grow out of control.

Tumor Microenvironment

[0007] The tumor microenvironment provides a consistently effective barrier to immune cell function because tumors actively downregulate all phases of anti-tumor immune responses using a spectrum of different strategies and mechanisms. Many molecular mechanisms that cause dysfunction of immune cells in the tumor microenvironment have been identified, including those directly mediated by factors produced by tumors, and others resulting from alterations of normal tissue homeostasis in the presence of cancer. Most human tumors appear to be able to interfere with one or more stages of immune cell development, differentiation, migration, cytotoxicity and other effector functions (T L Whiteside, The tumor microenvironment and its role in promoting tumor growth, Oncogene (2008) 27, 5904–5912).

[0008] One such mechanism involves accumulation in tumors of T_{reg} ($CD4^+CD25^{bright}$ $Foxp3^+$ T cells) and myeloid-derived cells ($CD34^+CD33^+CD13^+CD11b^+CD15^-$), which are common features of human tumors, and have been linked to poor prognosis in patients with cancer (T L Whiteside, The tumor microenvironment and its role in promoting tumor growth, Oncogene (2008) 27, 5904–5912). Under normal conditions, T_{reg} cells are involved in the important role of preventing autoimmunity, but in cancer, they expand, migrate to tumors, downregulate autologous effector T-cell proliferation and suppress anti-tumor responses of both $CD4^+CD25^-$ and $CD8^+CD25^-$ T cells using distinct molecular pathways. The T_{reg} cells in the tumor are a heterogeneous population of regulatory $CD3^+CD4^+$ T cells, comprising natural T_{reg}, antigen-specific Tr1 cells, and other less well defined subsets of suppressor cells. Tr1 cells are

induced in the tumor microenvironment, which is rich in IL-10, TGF-β, and prostaglandin E_2 (PGE_2), all of which have been shown to promote Tr1 generation (T L Whiteside, The tumor microenvironment and its role in promoting tumor growth, Oncogene (2008) 27, 5904–5912).

[0009] Myeloid suppressor cells (MSCs) also suppress T-cell responses in the tumor microenvironment, where they secrete TGF-β or induce TGF-β secretion. Immunosuppressive $CD34^+$ cell-derived myeloid cells have been identified in the peripheral blood of cancer patients. In tumor-bearing mice, MSCs accumulate in the spleen and peripheral circulation in very high amounts, exerting potent immunosuppression and favoring tumor growth. MSCs also control the availability of essential amino acids such as L-arginine and produce high levels of reactive oxygen species. The MSCs found in tumors also constitutively express iNOS and arginase 1, an enzyme involved in metabolism of L-arginine, which also synergizes with iNOS to increase superoxide and NO production, which have been found to interfere with lymphocyte responses. GM-CSF, which is also often secreted by tumor cells, recruits MSCs and induces dose-dependent *in vivo* immune suppression and tumor promotion, while at the same time, GM-CSF has been used as immune adjuvant in antitumor vaccines. GM-CSF was observed to increase a subset of TGF-β-producing MSCs in the circulation of patients with metastatic melanoma. The concurrent stimulatory and suppressive roles suggest that GM-CSF and MSCs are involved in maintaining immune homeostasis in normal tissue, but in the tumor microenvironment promote tumor cell escape (T L Whiteside, The tumor microenvironment and its role in promoting tumor growth, Oncogene (2008) 27, 5904–5912).

Tumor Immunotherapy

[0010] Cancer therapy is evolving rapidly as new molecular targets are being discovered. Despite the advent of biologics targeting specific pathways (e.g., Herceptin®, Erbitux®) and small molecules designed against specific targets (tamoxifen, GLEEVEC™), nonspecific modalities such as chemotherapy and radiation remain a standard of care.

[0011] Anti-cancer immunotherapy has been a goal for many years with a variety of approaches being tested. One difficulty of developing this immunotherapy is that target antigens are often tissue specific molecules found on both cancer cells and normal cells, and either do not elicit immunity or show non-specificity regarding cell killing (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). Furthermore, tumor cells have features that make immune recognition difficult, such as loss of expression of antigens that elicit immune response, lack of major histocompatibility (MHC) class II, and downregulation of MHC class I expression. These features can lead to non-recognition of tumor cells by both CD4+ and CD8+ T cells (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). Tumors may also evade detection through active mechanisms, such as the production of immunosuppressive cytokines (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[0012] DCs generated ex vivo by culturing hematopoietic progenitor cells or monocytes with cytokine combinations have been tested as therapeutic vaccines in cancer patients for more than a decade (Ueno H, et al., Immunol. Rev. (2010) 234: 199-212). For example, treatment of metastatic prostate cancer with sipuleucel-T (also known as APC 8015), which is a cellular product based on enriched blood APCs that are briefly cultured with a fusion protein of prostatic acid phosphatase (PAP) and granulocyte macrophage colony-stimulating factor (GM-CSF),

resulted in an approximately 4-month-prolonged median survival in Phase III trials (Higano C S, et al., Cancer (2009) 115: 3670-3679; Kantoff P W, et al., N. Engl. J. Med. (2010) 363: 411-422). This study concluded that DC-based vaccines are safe and can induce the expansion of circulating CD4+ T-cells and CD8+ T-cells specific for tumor antigens. As a result of this and similar studies, sipuleucel-T has been approved by the US Food and Drug Administration (FDA) for the treatment of metastatic prostate cancer, thereby paving the clinical development and regulatory path for the next generation of cellular immunotherapy products (Palucka K and Banchereau J, Nature Reviews Cancer (April 2012) 12: 265-276).

[0013] Vaccination strategies involving DCs to induce tumor-specific effector T cells that can reduce the tumor mass specifically and that can induce immunological memory to control tumor relapse have been developed. For example, DCs can be provided with tumor-specific antigens by culturing DCs ex vivo with an adjuvant and a tumor-specific antigen, and then injecting these cells back into the patient. Tumor cells obtained from an excised tumor, needle biopsy, core biopsy, vacuum-assisted biopsy or peritoneal lavage have been used to generate immunogenic compositions comprising tumor-specific-antigen presenting dendritic cells.

Cancer Treatment Strategies

[0014] Antibody therapies such as Herceptin™ and Erbitux™ are passive immunotherapies, but have yielded considerable improvement in clinical outcome, as measured by, e.g. the recurrence rate, progression free survival and overall survival. More recently, PD-1 and CTLA4 inhibitors have been reported to block discrete checkpoints in an active host immune response allowing an endogenous anti-cancer immune response to be sustained. The term "immune checkpoints" refers to the array of inhibitory pathways that are necessary for maintaining self-

tolerance and modulating the duration and extent of immune responses to minimize damage to normal tissue. Immune checkpoint molecules such as PD-1, PD-L1, CTLA-4 are cell surface signaling receptors that play an important role in modulating the T-cell response in the tumor microenvironment. Tumor cells have been shown to utilize these checkpoints to their benefit by up regulating their expression and activity. With the tumor cell's ability to commandeer some immune checkpoint pathways as a mechanism of immune resistance, it has been hypothesized that checkpoint inhibitors that bind to molecules of immune cells to activate or inactivate them may relieve the inhibition of an immune response. Recent discoveries have identified immune checkpoints or targets, like PD-1, PD-L1, PD-L2, CTLA4, TIM3, LAG3, CCR4, OX40, OX40L, IDO, and A2AR, as proteins responsible for immune evasion. Specific immune checkpoint inhibitors, including antibodies against CTLA-4, PD-1 receptor or its ligand PD-L1 have produced impressive results in the clinic in a range of cancers, leading to FDA approvals for YERVOY™ (Ipilimumab; CTLA-4 antagonist), OPDIVO™ (Nivolumab; PD-1 antagonist) and KEYTRUDA™ (Pembrolizumab; PD-1 antagonist) in multiple tumor indications and with ongoing registration trials in many more. This method of therapy, however, can only be successful if a pre-existing antitumor immune response is present within a patient (Pardoll, D., The blockade of immune checkpoints in cancer immunotherapy, Nature Reviews: Cancer, Vol. 12, April 2012, 253). Recent cellular therapies, such as chimeric antigen receptor T-cell therapy (CAR-T), attempt to use synthetic biology to redirect T-cells to specific cell surface tumor antigens. Genetic modification of T-cells is used to confer tumor antigen recognition by transgenic expression of chimeric antigen receptor (CAR). CARs are engineered molecules that can be introduced into T cells to enable them to target tumor antigens (Frey, N.V., Porter, D.L., The Promise of Chimeric Antigen Receptor T-Cell Therapy, Oncology (2016); 30(1)) pii

219281). CAR T cells have been shown to have some efficacy against hematologic malignancies and to a lesser extent solid tumors. CAR T therapy, however, has been shown to cause several types of toxicities, including cytokine release syndrome, neurological toxicity, non-tumor recognition, and anaphylaxis (Bonifant CL, et al., Toxicity and management in CAR T-cell therapy, Molecular Therapy — Oncolytics (2016) 3, 16011).

[0015] Cellular vaccines have also been used as a cancer treatment. GVAX™, a prototypical example, is a GM-CSF gene transduced tumor vaccine within either an autologous or allogeneic population of tumor cells. It is believed that GM-CSF secretion of genetically modified tumor cells stimulates cytokine release at the vaccine site to activate antigen presenting cells to induce a tumor specific cellular immune response (Eager, R. & Nemunaitis, J., GM-CSF Gene-Transduced Tumor Vaccines, Molecular Therapy, Vol. 12, No. 1, 18 (July 2005)). However, GVAX™ yielded only limited clinical responses.

[0016] Dendritic cell (DC)-tumor cell fusions have been developed to generate hybrid cells that express the relevant tumor associated antigens derived from the parent tumor cells, and also have the ability to process and present such antigens to appropriate cells of the immune system. The DC-tumor cell fusions provide a greater variety of tumor antigens, but have been met with limited success in human trials, likely due to the autologous components required, the heterogeneity of the product caused by maturation of DC cells, and variations in antigen loading (Browning, M., Antigen presenting cell/tumor cell fusion vaccines for cancer, Human Vaccines & Immunotherapeutics 9:7, 1545–1548; July 2013; Butterfield, L., Dendritic Cells in Cancer Immunotherapy Clinical Trials: Are We Making Progress?, Frontiers of Immunology, 2013 4: 454).

Cells of the Immune System

[0017] There are a large number of cellular interactions that comprise the immune system. These interactions occur through specific receptor-ligand pairs that signal in both directions so that each cell receives instructions based on the temporal and spatial distribution of those signals.

[0018] Murine models have been highly useful in discovering immunomodulatory pathways, but clinical utility of these pathways does not always translate from an inbred mouse strain to an outbred human population, since an outbred human population may have individuals that rely to varying extents on individual immunomodulatory pathways.

[0019] Cells of the immune system include lymphocytes, monocytes/macrophages, dendritic cells, the closely related Langerhans cells, natural killer (NK) cells, mast cells, basophils, and other members of the myeloid lineage of cells. In addition, a series of specialized epithelial and stromal cells provide the anatomic environment in which immunity occurs, often by secreting critical factors that regulate growth and/or gene activation in cells of the immune system, which also play direct roles in the induction and effector phases of the response. (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999), at p. 102).

[0020] The cells of the immune system are found in peripheral organized tissues, such as the spleen, lymph nodes, Peyer's patches of the intestine and tonsils. Lymphocytes also are found in the central lymphoid organs, the thymus, and bone marrow where they undergo developmental steps that equip them to mediate the myriad responses of the mature immune system. A substantial portion of lymphocytes and macrophages comprise a recirculating pool of cells found in the blood and lymph, providing the means to deliver immunocompetent cells to sites where

they are needed and to allow immunity that is generated locally to become generalized. (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999), at p. 102).

[0021] The term "lymphocyte" refers to a small white blood cell formed in lymphatic tissue throughout the body and in normal adults making up about 22-28% of the total number of leukocytes in the circulating blood that plays a large role in defending the body against disease. Individual lymphocytes are specialized in that they are committed to respond to a limited set of structurally related antigens through recombination of their genetic material (e.g. to create a T cell receptor and a B cell receptor). This commitment, which exists before the first contact of the immune system with a given antigen, is expressed by the presence of receptors specific for determinants (epitopes) on the antigen on the lymphocyte's surface membrane. Each lymphocyte possesses a unique population of receptors, all of which have identical combining sites. One set, or clone, of lymphocytes differs from another clone in the structure of the combining region of its receptors and thus differs in the epitopes that it can recognize. Lymphocytes differ from each other not only in the specificity of their receptors, but also in their functions. (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999), at p. 102).

[0022] Two broad classes of lymphocytes are recognized: the B-lymphocytes (B-cells), which are precursors of antibody-secreting cells, and T-lymphocytes (T-cells).

B-Lymphocytes

[0023] B-lymphocytes are derived from hematopoietic cells of the bone marrow. A mature B-cell can be activated with an antigen that expresses epitopes that are recognized by its cell surface. The activation process may be direct, dependent on cross-linkage of membrane Ig molecules by the antigen (cross-linkage-dependent B-cell activation), or indirect, via interaction with a helper T-cell, in a process referred to as cognate help. In many physiological situations, receptor cross-linkage stimuli and cognate help synergize to yield more vigorous B-cell responses (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999)).

[0024] Cross-linkage dependent B-cell activation requires that the antigen express multiple copies of the epitope complementary to the binding site of the cell surface receptors, because each B-cell expresses Ig molecules with identical variable regions. Such a requirement is fulfilled by other antigens with repetitive epitopes, such as capsular polysaccharides of microorganisms or viral envelope proteins. Cross-linkage-dependent B-cell activation is a major protective immune response mounted against these microbes (Paul, W. E., "Chapter 1: The immune system: an introduction", Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999)).

[0025] Cognate help allows B-cells to mount responses against antigens that cannot cross-link receptors and, at the same time, provides costimulatory signals that rescue B cells from inactivation when they are stimulated by weak cross-linkage events. Cognate help is dependent on the binding of antigen by the B-cell's membrane immunoglobulin (Ig), the endocytosis of the antigen, and its fragmentation into peptides within the endosomal/lysosomal compartment of the cell. Some of the resultant peptides are loaded into a groove in a specialized set of cell surface

proteins known as class II major histocompatibility complex (MHC) molecules. The resultant class II/peptide complexes are expressed on the cell surface and act as ligands for the antigen-specific receptors of a set of T-cells designated as $CD4^+$ T-cells. The $CD4^+$ T-cells bear receptors on their surface specific for the B-cell's class II/peptide complex. B-cell activation depends not only on the binding of the T cell through its T cell receptor (TCR), but this interaction also allows an activation ligand on the T-cell (CD40 ligand) to bind to its receptor on the B-cell (CD40) signaling B-cell activation. In addition, T helper cells secrete several cytokines that regulate the growth and differentiation of the stimulated B-cell by binding to cytokine receptors on the B cell (Paul, W. E., "Chapter 1: The immune system: an introduction, "Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999)).

[0026] During cognate help for antibody production, the CD40 ligand is transiently expressed on activated $CD4^+$ T helper cells, and it binds to CD40 on the antigen-specific B cells, thereby transducing a second costimulatory signal. The latter signal is essential for B cell growth and differentiation and for the generation of memory B cells by preventing apoptosis of germinal center B cells that have encountered antigen. Hyperexpression of the CD40 ligand in both B and T cells is implicated in pathogenic autoantibody production in human SLE patients (Desai-Mehta, A. et al., "Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production," J. Clin. Invest. Vol. 97(9), 2063-2073, (1996)).

T-Lymphocytes

[0027] T-lymphocytes derived from precursors in hematopoietic tissue, undergo differentiation in the thymus, and are then seeded to peripheral lymphoid tissue and to the

recirculating pool of lymphocytes. T-lymphocytes or T cells mediate a wide range of immunologic functions. These include the capacity to help B cells develop into antibody-producing cells, the capacity to increase the microbicidal action of monocytes/macrophages, the inhibition of certain types of immune responses, direct killing of target cells, and mobilization of the inflammatory response. These effects depend on T cell expression of specific cell surface molecules and the secretion of cytokines (Paul, W. E., "Chapter 1: The immune system: an introduction", Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999)).

[0028] T cells differ from B cells in their mechanism of antigen recognition. Immunoglobulin, the B cell's receptor, binds to individual epitopes on soluble molecules or on particulate surfaces. B-cell receptors see epitopes expressed on the surface of native molecules. While antibody and B-cell receptors evolved to bind to and to protect against microorganisms in extracellular fluids, T cells recognize antigens on the surface of other cells and mediate their functions by interacting with, and altering, the behavior of these antigen-presenting cells (APCs). There are three main types of APCs in peripheral lymphoid organs that can activate T cells: dendritic cells, macrophages and B cells. The most potent of these are the dendritic cells, whose only function is to present foreign antigens to T cells. Immature dendritic cells are located in tissues throughout the body, including the skin, gut, and respiratory tract. When they encounter invading microbes at these sites, they endocytose the pathogens and their products, and carry them via the lymph to local lymph nodes or gut associated lymphoid organs. The encounter with a pathogen induces the dendritic cell to mature from an antigen-capturing cell to an APC that can activate T cells. APCs display three types of protein molecules on their surface that have a role in activating a T cell to become an effector cell: (1) MHC proteins, which present foreign antigen

to the T cell receptor; (2) costimulatory proteins which bind to complementary receptors on the T cell surface; and (3) cell-cell adhesion molecules, which enable a T cell to bind to the APC for long enough to become activated ("Chapter 24: The adaptive immune system," Molecular Biology of the Cell, Alberts, B. et al., Garland Science, NY, (2002)).

[0029] T-cells are subdivided into two distinct classes based on the cell surface receptors they express. The majority of T cells express T cell receptors (TCR) consisting of α and β-chains. A small group of T cells express receptors made of γ and δ chains. Among the α/β T cells are two sub-lineages: those that express the coreceptor molecule CD4 ($CD4^+$ T cells); and those that express CD8 ($CD8^+$ T cells). These cells differ in how they recognize antigen and in their effector and regulatory functions.

[0030] $CD4^+$ T cells are the major regulatory cells of the immune system. Their regulatory function depends both on the expression of their cell-surface molecules, such as CD40 ligand whose expression is induced when the T cells are activated, and the wide array of cytokines they secrete when activated.

[0031] T cells also mediate important effector functions, some of which are determined by the patterns of cytokines they secrete. The cytokines can be directly toxic to target cells and can mobilize potent inflammatory mechanisms.

[0032] In addition, T cells, particularly $CD8^+$ T cells, can develop into cytotoxic T-lymphocytes (CTLs) capable of efficiently lysing target cells that express antigens recognized by the CTLs (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999)).

[0033] T cell receptors (TCRs) recognize a complex consisting of a peptide derived by proteolysis of the antigen bound to a specialized groove of a class II or class I MHC protein. $CD4^+$ T cells recognize only peptide/class II complexes while $CD8^+$ T cells recognize peptide/class I complexes (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999)).

[0034] The TCR's ligand (i.e., the peptide/MHC protein complex) is created within APCs. In general, class II MHC molecules bind peptides derived from proteins that have been taken up by the APC through an endocytic process. These peptide-loaded class II molecules are then expressed on the surface of the cell, where they are available to be bound by $CD4^+$ T cells with TCRs capable of recognizing the expressed cell surface complex. Thus, $CD4^+$ T cells are specialized to react with antigens derived from extracellular sources (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999)).

[0035] In contrast, class I MHC molecules are mainly loaded with peptides derived from internally synthesized proteins, such as viral proteins. These peptides are produced from cytosolic proteins by proteolysis by the proteosome and are translocated into the rough endoplasmic reticulum. Such peptides, generally composed of nine amino acids in length, are bound into the class I MHC molecules and are brought to the cell surface, where they can be recognized by $CD8^+$ T cells expressing appropriate receptors. This gives the T cell system, particularly $CD8^+$ T cells, the ability to detect cells expressing proteins that are different from, or produced in much larger amounts than, those of cells of the remainder of the organism (e.g., viral

antigens) or mutant antigens (such as active oncogene products), even if these proteins in their intact form are neither expressed on the cell surface nor secreted (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999)).

[0036] T cells can also be classified based on their function as helper T cells; T cells involved in inducing cellular immunity; suppressor T cells; and cytotoxic T cells.

Helper T Cells

[0037] Helper T cells are T cells that stimulate B cells to make antibody responses to proteins and other T cell-dependent antigens. T cell-dependent antigens are immunogens in which individual epitopes appear only once or a limited number of times such that they are unable to cross-link the membrane immunoglobulin (Ig) of B cells or do so inefficiently. B cells bind the antigen through their membrane Ig, and the complex undergoes endocytosis. Within the endosomal and lysosomal compartments, the antigen is fragmented into peptides by proteolytic enzymes, and one or more of the generated peptides are loaded into class II MHC molecules, which traffic through this vesicular compartment. The resulting peptide/class II MHC complex is then exported to the B-cell surface membrane. T cells with receptors specific for the peptide/class II molecular complex recognize this complex on the B-cell surface. (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia (1999)).

[0038] B-cell activation depends both on the binding of the T cell through its TCR and on the interaction of the T-cell CD40 ligand (CD40L) with CD40 on the B cell. T cells do not constitutively express CD40L. Rather, CD40L expression is induced as a result of an interaction

with an APC that expresses both a cognate antigen recognized by the TCR of the T cell and CD80 or CD86. CD80/CD86 is generally expressed by activated, but not resting, B cells so that the helper interaction involving an activated B cell and a T cell can lead to efficient antibody production. In many cases, however, the initial induction of CD40L on T cells is dependent on their recognition of antigen on the surface of APCs that constitutively express CD80/86, such as dendritic cells. Such activated helper T cells can then efficiently interact with and help B cells. Cross-linkage of membrane Ig on the B cell, even if inefficient, may synergize with the CD40L/CD40 interaction to yield vigorous B-cell activation. The subsequent events in the B-cell response, including proliferation, Ig secretion, and class switching of the Ig class being expressed, either depend or are enhanced by the actions of T cell-derived cytokines (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999)).

[0039] $CD4^+$ T cells tend to differentiate into cells that principally secrete the cytokines IL-4, IL-5, IL-6, and IL-10 (T_H2 cells) or into cells that mainly produce IL-2, IFN-γ, and lymphotoxin (T_H1 cells). The T_H2 cells are very effective in helping B-cells develop into antibody-producing cells, whereas the T_H1 cells are effective inducers of cellular immune responses, involving enhancement of microbicidal activity of monocytes and macrophages, and consequent increased efficiency in lysing microorganisms in intracellular vesicular compartments. Although $CD4^+$ T cells with the phenotype of T_H2 cells (i.e., IL-4, IL-5, IL-6 and IL-10) are efficient helper cells, T_H1 cells also have the capacity to be helpers (Paul, W. E., "Chapter 1: The immune system: an introduction, "Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999)).

T cell Involvement in Cellular Immunity Induction

[0040] T cells also may act to enhance the capacity of monocytes and macrophages to destroy intracellular microorganisms. In particular, interferon-gamma (IFN-γ) produced by helper T cells enhances several mechanisms through which mononuclear phagocytes destroy intracellular bacteria and parasitism including the generation of nitric oxide and induction of tumor necrosis factor (TNF) production. T_{H1} cells are effective in enhancing the microbicidal action, because they produce IFN-γ. In contrast, two of the major cytokines produced by T_{H2} cells, IL-4 and IL-10, block these activities (Paul, W. E., "Chapter 1: The immune system: an introduction," Fundamental Immunology, 4th Edition, Ed. Paul, W. E., Lippicott-Raven Publishers, Philadelphia, (1999)).

Regulatory T (Treg) Cells

[0041] Immune homeostasis is maintained by a controlled balance between initiation and downregulation of the immune response. The mechanisms of both apoptosis and T cell anergy (a tolerance mechanism in which the T cells are intrinsically functionally inactivated following an antigen encounter (Scwartz, R. H., "T cell anergy", Annu. Rev. Immunol., Vol. 21: 305-334 (2003)) contribute to the downregulation of the immune response. A third mechanism is provided by active suppression of activated T cells by suppressor or regulatory $CD4^+$ T (Treg) cells (Reviewed in Kronenberg, M. et al., "Regulation of immunity by self-reactive T cells", Nature, Vol. 435: 598-604 (2005)). $CD4^+$ Tregs that constitutively express the IL-2 receptor alpha (IL-2Rα) chain ($CD4^+$ $CD25^+$) are a naturally occurring T cell subset that are anergic and suppressive (Taams, L. S. et al., "Human anergic/suppressive $CD4^+CD25^+$ T cells: a highly differentiated and apoptosis-prone population", Eur. J. Immunol. Vol. 31: 1122-1131 (2001)).

Depletion of $CD4^+CD25^+$ Tregs results in systemic autoimmune disease in mice. Furthermore, transfer of these Tregs prevents development of autoimmune disease. Human $CD4^+CD25^+$ Tregs, similar to their murine counterpart, are generated in the thymus and are characterized by the ability to suppress proliferation of responder T cells through a cell-cell contact-dependent mechanism, the inability to produce IL-2, and the anergic phenotype *in vitro*. Human $CD4^+CD25^+$ T cells can be split into suppressive ($CD25^{high}$) and nonsuppressive ($CD25^{low}$) cells, according to the level of CD25 expression. A member of the forkhead family of transcription factors, FOXP3, has been shown to be expressed in murine and human $CD4^+CD25^+$ Tregs and appears to be a master gene controlling $CD4^+CD25^+$ Treg development (Battaglia, M. et al., "Rapamycin promotes expansion of functional $CD4^+CD25^+Foxp3^+$ regulator T cells of both healthy subjects and type 1 diabetic patients", J. Immunol., Vol. 177: 8338-8347, (2006)).

Cytotoxic T Lymphocytes

[0042] $CD8^+$ T cells that recognize peptides from proteins produced within the target cell have cytotoxic properties in that they lead to lysis of the target cells. The mechanism of CTL-induced lysis involves the production by the CTL of perforin, a molecule that can insert into the membrane of target cells and promote the lysis of that cell. Perforin-mediated lysis is enhanced by granzymes, a series of enzymes produced by activated CTLs. Many active CTLs also express large amounts of fas ligand on their surface. The interaction of fas ligand on the surface of CTL with fas on the surface of the target cell initiates apoptosis in the target cell, leading to the death of these cells. CTL-mediated lysis appears to be a major mechanism for the destruction of virally infected cells.

Priming

[0043] The term "unprimed cells" (also referred to as virgin, naïve, or inexperienced cells) as used herein refers to T cells and B cells that have generated an antigen receptor (TCR for T cells, BCR for B cells) of a particular specificity, but have never encountered the antigen. The term "priming" as used herein refers to the process whereby T cells and B cell precursors encounter the antigen for which they are specific.

[0044] For example, before helper T cells and B cells can interact to produce specific antibody, the antigen-specific T cell precursors must be primed. Priming involves several steps: antigen uptake, processing, and cell surface expression bound to class II MHC molecules by an antigen presenting cell, recirculation and antigen-specific trapping of helper T cell precursors in lymphoid tissue, and T cell proliferation and differentiation (Janeway, CA, Jr., "The priming of helper T cells", Semin. Immunol., Vol. 1(1): 13-20 (1989)). Helper T cells express CD4, but not all CD4 T cells are helper cells. Id. The signals required for clonal expansion of helper T cells differ from those required by other CD4 T cells. The critical antigen-presenting cell for helper T cell priming appears to be a macrophage; and the critical second signal for helper T cell growth is the macrophage product interleukin 1 (IL-1). Id. If the primed T cells and/or B cells receive a second, co-stimulatory signal, they become activated T cells or B cells.

Lymphocyte Activation

[0045] The term "activation" or "lymphocyte activation" refers to stimulation of lymphocytes by specific antigens, nonspecific mitogens, or allogeneic cells resulting in synthesis of RNA, protein and DNA and production of lymphokines; it is followed by proliferation and differentiation of various effector and memory cells. For example, a mature B cell can be activated by an encounter with an antigen that expresses epitopes that are recognized by its cell

surface immunoglobulin Ig. The activation process may be a direct one, dependent on cross-linkage of membrane Ig molecules by the antigen (cross-linkage-dependent B cell activation) or an indirect one, occurring most efficiently in the context of an intimate interaction with a helper T cell ("cognate help process"). T-cell activation is dependent on the interaction of the TCR/CD3 complex with its cognate ligand, a peptide bound in the groove of a class I or class II MHC molecule. The molecular events set in motion by receptor engagement are complex. Among the earliest steps appears to be the activation of tyrosine kinases leading to the tyrosine phosphorylation of a set of substrates that control several signaling pathways. These include a set of adapter proteins that link the TCR to the ras pathway, phospholipase Cγ1, the tyrosine phosphorylation of which increases its catalytic activity and engages the inositol phospholipid metabolic pathway, leading to elevation of intracellular free calcium concentration and activation of protein kinase C, and a series of other enzymes that control cellular growth and differentiation. Full responsiveness of a T cell requires, in addition to receptor engagement, an accessory cell-delivered costimulatory activity, e.g., engagement of CD28 on the T cell by CD80 and/or CD86 on the APC. The soluble product of an activated B lymphocyte is immmunoglobulins (antibodies). The soluble product of an activated T lymphocyte is lymphokines.

[0046] Chemokines are chemotactic cytokines, which constitute a family of low molecular mass (8-11 kDa) structurally-related proteins with diverse immune and neural functions (Mackay C.R., "Chemokines: immunology's high impact factors", Nat Immunol., Vol. 2: 95-101, (2001)); (Youn B. et al., "Chemokines, chemokine receptors and hematopoiesis", Immunol Rev, Vol. 177: 150-174, (2000)) that can be categorized into four subfamilies (C, CC, CXC and CX3C) based on the relative positions of conserved cysteine residues (Rossi D. et al., "The biology of

chemokines and their receptors", Annu Rev Immunol,, Vol. 18: 217-242, (2000)). Chemokines are essential molecules in directing leucocyte migration between blood, lymph nodes and tissues. They constitute a complex signaling network because they are not always restricted to one type of receptor (Loetscher P. et al., "The ligands of CXC chemokine receptor 3, I-TAC, Mig, and IP10, are natural antagonists for CCR3", J. Biol. Chem., Vol. 276: 2986–2991, (2001)). Chemokines affect cells by activating surface receptors that are seven-transmembrane-domain G-protein-coupled receptors. Leukocyte responses to particular chemokines are determined by their expression of chemokine receptors. The binding of the chemokine to the receptor activates various signaling cascades, similar to the action of cytokines that culminate in the activation of a biological response. Secretion of the ligands for the CCR5 receptor, regulated upon activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α/ and MIP-1β (Schrum S. et al., "Synthesis of the CC-chemokines MIP-1alpha, MIP-1beta, and RANTES is associated with a type 1 immune response", J Immunol, Vol. 157: 3598–3604, (1996)) and the ligand for CXC chemokine receptor 3 (CXCR3), induced protein (IP)-10 (Taub D.D. et al., "Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells", J Exp Med., Vol. 177:1809–1814, (1993)) have been associated with unwanted heightened T_{H1} responses. Additionally, elevated damaging pro-inflammatory cytokine levels of IL-2 and IFN-γ correlate with type 1 diabetes (T1D) (Rabinovitch A. et al., "Roles of cytokines in the pathogenesis and therapy of type 1 diabetes", Cell Biochem Biophys, Vol. 48(2-3): 159-63, (2007)). Chemokines have been observed in T_{H1} pancreatic infiltrates and other inflammatory lesions characterized by T cell infiltration (Bradley L.M. et al., "Islet-specific Th1, but not Th2, cells secrete multiple

chemokines and promote rapid induction of autoimmune diabetes", J Immunol, Vol. 162:2511–2520, (1999)).

[0047] Pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α in the plasma have been primarily detected and involved in the insulin resistance and development of T2D which are kept in check and modulated by the anti-inflammatory and immune suppressive cytokines TGF-β1 and IL-10 (Alexandraki K. et al., "Inflammatory process in type 2 diabetes: The role of cytokines", Annals of the New York Academy of Sciences, 1084: 89-117, (2006); Kumar N.P. et al. 2015. Eur J Immunol. doi: 10.1002/eji.201545973 ahead of print). IL-17A is a well-known pro-inflammatory cytokine involved in several autoimmune diseases.

Immune Tolerance

[0048] The immune system is tolerant of self-antigens, i.e., it can discriminate between antigenic determinants expressed on foreign substances, and antigenic determinants expressed by tissues of the host. The capacity of the system to ignore host antigens, referred to as immune tolerance or immunological tolerance, is an active process involving the elimination or inactivation of cells that could recognize self-antigens through immunologic tolerance (Fundamental immunology, 4th Edn, William E. Paul, Ed. Lippincott-Raven Publishers, Philadelphia, (1999), at p. 2).

[0049] Immune tolerance is classified into 1) central tolerance or 2) peripheral tolerance depending on where the state is originally induced, i.e., whether it is in the thymus and bone marrow (central) or in other tissues and lymph nodes (peripheral). The biological mechanisms whereby these forms of tolerance are established are distinct, but the resulting effect is similar

(Raker V. K. et al., "Tolerogenic Dendritic Cells for Regulatory T Cell Induction in Man", Front Immunol, Vol., 6(569): 1-11, (2015)).

[0050] Central tolerance, the principal way in which the immune system is educated to discriminate self-molecules from non-self-molecules, is established by deleting autoreactive lymphocyte clones at a point before they mature into fully immunocompetent cells. It occurs during lymphocyte development in the thymus and bone marrow for T and B lymphocytes, respectively (Sprent J. et al., "The thymus and central tolerance", Philos Trans R Soc Lond B Biol Sci, Vol. 356(1409): 609–616, (2001)). In these tissues, maturing lymphocytes are exposed to self-antigens presented by thymic epithelial cells and thymic dendritic cells, or bone marrow cells. Self-antigens are present due to endogenous expression, importation of antigen from peripheral sites via circulating blood, and in the case of thymic stromal cells, expression of proteins of other non-thymic tissues by the action of the transcription factor AIRE (Murphy, Kenneth. Janeway's Immunobiology: 8th ed. Chapter 15: Garland Science. (2012), pp. 611–668; Klein L., "Aire gets company for immune tolerance", Cell, Vol. 163(4):794-795, (2015)). Those lymphocytes that have receptors that bind strongly to self-antigens are removed by means of apoptosis of the autoreactive cells, or by induction of anergy (Id. at pp. 275–334). Weakly autoreactive B cells may also remain in a state of immunological inactivity where they do not respond to stimulation of their B cell receptor. Some weakly self-recognizing T cells are alternatively differentiated into natural regulatory T cells (nTreg cells), which act as sentinels in the periphery to lower potential instances of T cell autoreactivity (Id. at pp. 611–668).

[0051] The deletion threshold is more stringent for T cells than for B cells since T cells are the main populations of cells that can cause direct tissue damage. Furthermore, it is more

advantageous for the organism to let its B cells recognize a wider variety of antigens, so that they can elicit antibodies against a greater diversity of pathogens. Since B cells can only be fully activated after confirmation by more self-restricted T cells that recognize the same antigen, autoreactivity is held in great check (Murphy, Kenneth. Janeway's Immunobiology: 8th ed. Chapter 8: Garland Sciences. pp. 275–334).

[0052] This process of negative selection ensures that T and B cells that potentially may initiate a potent immune response to the individual's own tissues are destroyed while preserving the ability to recognize foreign antigens. This step in lymphocyte education is detrimental to preventing autoimmunity. Lymphocyte development and education is most active in fetal development, but continues throughout life as immature lymphocytes are generated, slowing as the thymus degenerates and the bone marrow shrinks in the adult life (Murphy, Kenneth. Janeway's Immunobiology: 8th ed. Chapter 8: Garland Sciences. (2012), pp. 275–334; Jiang T.T., "Regulatory T cells: new keys for further unlocking the enigma of fetal tolerance and pregnancy complications", J Immunol., Vol. 192(11): 4949-4956, (2014)).

[0053] Peripheral tolerance develops after T and B cells mature and enter the peripheral tissues and lymph nodes (Murphy, Kenneth. Janeway's Immunobiology: 8th ed. Chapter 8: Garland Sciences. pp. 275–334). It is set forth by a number of overlapping mechanisms that predominantly involve control at the level of T cells, especially $CD4^+$ helper T cells, which orchestrate immune responses and give B cells the confirmatory signals that the B cells need in order to progress to produce antibodies. Inappropriate reactivity toward a normal self-antigen that was not eliminated in the thymus can occur, since the T cells that leave the thymus are relatively, but not completely, safe. Some will have receptors (TCRs) that can respond to self-

antigens that the T cell did not encounter in the thymus (Murphy, Kenneth. Janeway's Immunobiology: 8th ed. Chapter 8: Garland Sciences. (2012), pp. 275–334). Those self-reactive T cells that escape intra-thymic negative selection in the thymus can inflict cell injury unless they are deleted in the peripheral tissue chiefly by nTreg cells.

[0054] Autoimmune regulator (Aire), usually expressed in thymic medullary epithelial cells, plays a role in immune tolerance by mediating ectopic expression of peripheral self-antigens and mediating the deletion of auto-reactive T cells. (Metzger T.C. et al., "Control of central and peripheral tolerance by Aire", Immunol. Rev. 2011, Vol. 241: 89-103, (2011)).

[0055] Appropriate reactivity towards certain antigens can also be suppressed by induction of tolerance after repeated exposure. Naïve $CD4^+$ helper T cells differentiate into induced Treg cells (iTreg cells) in the peripheral tissue, or accordingly, in nearby lymphoid tissue (lymph nodes, mucosal-associated lymphoid tissue, etc.). This differentiation is mediated by IL-2 produced upon T cell-activation, and TGF-β from any of a variety of sources, including tolerizing dendritic cells (DCs) or other antigen presenting cells (Curotto de Lafaille et al., "Effective recruitment and retention of older adults in physical activity research: PALS study", Immunity, Vol. 30(6): 626–635, (2009)).

T-memory Cells

[0056] Following the recognition and eradication of pathogens through adaptive immune responses, the vast majority (90–95%) of T cells undergo apoptosis with the remaining cells forming a pool of memory T cells, designated central memory T cells (TCM), effector memory T cells (TEM), and resident memory T cells (TRM) (Clark, R.A., "Resident memory T cells in human health and disease", Sci. Transl. Med., 7, 269rv1, (2015)).

[0057] Compared to standard T cells, these memory T cells are long-lived with distinct phenotypes such as expression of specific surface markers, rapid production of different cytokine profiles, capability of direct effector cell function, and unique homing distribution patterns. Memory T cells exhibit quick reactions upon re-exposure to their respective antigens in order to eliminate the reinfection of the offender and thereby restore balance of the immune system rapidly. Increasing evidence substantiates that autoimmune memory T cells hinder most attempts to treat or cure autoimmune diseases (Clark, R.A., "Resident memory T cells in human health and disease", Sci. Transl. Med., Vol. 7, 269rv1, (2015)).

The Complement System

[0058] The complement system comprises over 30 different proteins that circulate in blood plasma. In the absence of an infection, the complement proteins circulate in an inactive form. In the presence of a pathogen, the complement proteins become activated to kill the pathogen either directly or by facilitating phagocytosis. There are three ways in which the complement system is activated.

[0059] Antibody-dependent cell mediated cytotoxicity (ADCC) is a mechanism by which effector cells of the immune system (e.g. natural killer cells) actively lyse target cells that have been bound by antibodies. The ADCC killing mechanism of an antibody-coated target cell by a cytotoxic effector cell is through a nonphagocytic process. This process involves the release of the content of cytotoxic granules or by the expression of cell death-inducing molecules. ADCC is triggered through interaction of target-bound antibodies (belonging to IgG or IgA or IgE classes) with certain Fc receptor glycoproteins present on the effector cell surface that bind the Fc region of immunoglobulins (Ig). Effector cells that mediate ADCC include natural killer (NK) cells,

monocytes, macrophages, neutrophils, eosinophils and dendritic cells. ADCC is dependent on a number of parameters such as density and stability of the antigen on the surface of the target cell, antibody affinity, and FcR-binding affinity.

[0060] In contrast with ADCC, complement dependent cell cytotoxicity (CDCC) is a process of the immune system that kills pathogens by damaging target cell membrane without the involvement of antibodies. This alternative pathway is initiated by spontaneous hydrolysis and activation of the complement component C3, which binds directly to microbial surfaces. Alternatively the lectin pathway is initiated by soluble carbohydrate binding proteins that bind to specific carbohydrate molecules on microbial surfaces.

[0061] Each of the ADCC and CDCC mechanisms generates a C3 convertase that cleaves C3, leaving behind C3b bound to the pathogen's surface and releasing C3a. This results in recruitment of phagocytic cells to the site of an infection, phagocytosis of pathogens by immune cells, and/or formation of a membrane attack complex (MAC) that disrupts pathogen cell membrane and causes cell lysis.

Co-stimulatory Molecules

[0062] Co-stimulatory molecules are the highly active immunomodulatory proteins that play a critical role in the development and maintenance of an immune response (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). The two signal hypothesis of T cell response involves the interaction between an antigen bound to an MHC molecule and the T cell receptor (TCR), and an interaction of a co-stimulatory molecule and its ligand. Specialized APCs, which are carriers of a co-stimulatory second signal, are able to activate T cell responses following binding of the MHC molecule with TCR. By contrast,

somatic tissues do not express the second signal and thereby induce T cell unresponsiveness (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). The two signal model explains the peripheral tolerance to self-antigens and why cancer cells can evade immune detection: tumor cells rarely express co-stimulatory molecules, and thereby lack the second signal critical to activating T cells.

[0063] Furthermore, many of the co-stimulatory molecules involved in the two-signal model can be blocked by co-inhibitory molecules that are expresses by normal tissue and by cancer cells (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). In fact, many types of interacting immunomodulatory molecules expressed on a wide variety of tissues may exert both stimulatory and inhibitory functions depending on the immunologic context (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[0064] DNA motifs consisting of an unmethylated CpG dinucleotide flanked by two 5' purines (e.g. GpA) and two 3' pyrimidines (e.g. TpC or TpT) are capable of stimulating an innate immune response by mimicking bacterial DNA. CpG oligodeoxynucleotides can be used as immune adjuvants to improve the function of professional antigen-presenting cells and increase generation of humoral and cellular vaccine-specific immune responses. CpG DNA is able to directly activate dendritic cells and B cells, resulting in the induction of both innate and adaptive immune responses (Bode, C., CpG DNA as a vaccine adjuvant, Expert Rev Vaccines. 2011 Apr; 10(4): 499–511).

[0065] Cell-surface immunomodulatory molecules can be grouped according to structure into two large families of receptors/ligands: the B7/CD28 immunoglobulin family and the Tumor

Necrosis Factor (TNF)-related family (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). Many members of these families have been characterized and evaluated for cancer immunotherapy.

CD28/CTLA-4/B7-1/B7-2 Family

[0066] B7-1(CD80) and B7-2 (CD86) are expressed on activated APCs and bind to CD28 on T cells, providing the necessary co-stimulation for naïve T-cell activation, inducing IL-2 production, cell division, and the inhibition of activation induced cell death (AICD) (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). A homologue to CD28, Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4, CD152), binds both B7-1 and B7-2 molecules and, in contrast to CD28, inhibits T-cell proliferation. B7 molecules therefore have two ligands, CD28 and CTLA-4, with opposing effects on T cells (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[0067] Ligation of CTLA-4 in isolation may cause apoptosis of T cells, whereas CTLA-4 ligation in conjunction with signaling via the TCR and CD28 inhibits T-cell activation. Accordingly, CTLA-4 -/- mice develop a fatal lymphoproliferative disorder (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[0068] The differential expression of CD28 and CTLA-4 in time and location at the cell surface has implications for their respective roles in the generation of immune responses. While CD28 is uniformly distributed throughout the membrane and aggregates rapidly to the immunologic synapse with T-cell activation, CTLA-4 is present in intracellular vesicles and is mobilized to the cell surface later (Kaufman and Wolchok eds., General Principles of Tumor

Immunotherapy, Chpt 5, 67-121 (2007)). Mobilization of CTLA-4 is tightly regulated by B7.1 expression on the APC, and by the strength of TCR stimulation. As a result, CTLA-4 may act to attenuate the T cell response, limiting the activity of high affinity T-cell clones (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[0069] CTLA-4 has been implicated in many aspects of immune regulation. For example, it may be involved in causing T cell anergy, modulating memory T cell responses, shaping diversity of a polyclonal T cell response, and raising levels of inhibitory cytokines TGF-beta and IL10 (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). CTLA-4 may also "back-signal" via B7 to down-regulate dendritic cell activation markers (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). CTLA-4 may also play a role in regulatory T-cell (Treg) function, as it is expressed on Tregs and on cutaneous T cell lymphoma, which may arise from Tregs (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[0070] The CD28/B7/CTLA-4 co-stimulatory mechanism has been investigated for cancer immunotherapy, such as by transfecting tumors with B7 molecules and use of anti-CTLA-4 antibodies.

[0071] In initial experiments involving transfection of B7.1 into poorly immunogenic melanoma cell lines, tumors grew but then regressed in a CD8+ T-cell-dependent process. Furthermore, animals treated with B7.1 melanoma cells became immune to further tumor challenge, demonstrating induction of immunologic memory, and inoculation with B7-expressing tumor cells caused regression of small pre-existing B7-negative tumors. Generally, larger tumors (greater than 2-3mm) were not affected, and similar results were seen with B7.2-

expressing tumors. Similar results have been shown in other tumor models including lymphoma and prostate cancer. The B7 surface molecule appears to be directly contacting and activating T cells, and B7-transfected tumor cells appear to function as APCs. Despite these promising results, human clinical trials of B7-containing vaccines have demonstrated increased immune response, but with only limited clinical benefit. (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[0072] Anti-CTLA antibodies have been shown to be effective in murine colon carcinoma and fibrosarcoma, as well as in murine models of prostate cancer, breast cancer, and melanoma, but not in some models of poorly immunogenic tumors. Anti-CTLA-4 antibodies have also been combined with other modes of immunotherapy and conventional therapies (e.g. surgery, chemotherapy) in mouse models. The results of mouse models and human studies suggest that mechanisms by which CTLA-4 blockade enhances anti-tumor immunity are not due to regulatory T cell–mediated suppression but instead to enhanced proliferation of effector T cells through down-regulation of CTLA-4-mediated inhibition (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). Human clinical trials of anti-CTLA-4 antibodies have demonstrated potential as an immune therapy, but because CTLA-4 plays a role in controlling T-cell responses, blocking its activity has the potential to lead to autoimmunity.

PD-1/PD-L1 (B7-H1), PD-L2 (B7-DC)

[0073] Programmed Death-1 (PD-1) is expressed by activated T cells, and is thought to be primarily an inhibitory modulator. Evidence from murine models suggests that expression of PD-L1 may protect tumors from the immune system. PD-L1 on tumors causes apoptosis in tumor-

reactive T cells, and a myeloma cell line expressing PD-L1 fails to grow in PD-1 knock-out mice. In one model, PD-L1 blocking antibodies cured mice of squamous cell carcinoma. In another model, PD-L1 blocking antibodies restored responsiveness to immunologic therapy with a 4-1BB (CD137) agonist. Furthermore, PD-1 -/- T cells have been shown to have enhanced anti-tumor characteristics. PD-L1 may also play an important role in the function of "suppressor" myeloid cells. It was reported that culturing dendritic cells in the presence of blocking antibody enhanced the development of T-cell responses against ovarian cancer. The mechanism through which PD-L1 may mediate immune suppression is through Interleukin-10 (IL-10) production. In contrast, the other PD-1 ligand, PD-L2, stimulated immunity in mice to the poorly immunogenic B16 melanoma (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[0074] Many human cancers have been found to express PD-L1 including tumors of the breast, cervix, lung, ovary, colon, as well as melanoma, glioblastoma and primary T cell lymphomas, which is consistent with the role of the PD-L1 pathway in tumor immune evasion. Furthermore, a poor prognosis in esophageal cancer and renal cell cancer may be associated with expression of PD-L1. Similarly, PD-L2 is highly expressed in Hodgkin lymphoma cell lines and may also serve as a prognostic marker (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

CD27/CD70

[0075] Upon T cell activation, CD27 is transiently up-regulated and is also expressed on B cells and NK cells. The ligand of CD27, CD70, is expressed on activated lymphocytes and mature dendritic cells. A transition from central-memory to effector-memory phenotype is

associated with loss of CD27 expression on CD8+ T cells, and CD27 -/- mice show impaired memory T cell function along with decreased accumulation in peripheral tissues during viral infection. In contrast, mice with constitutive CD27 expression display accumulating increased T cell populations, and ultimately develop a paucity of B cells and eventually succumb to a lethal T-cell immunodeficiency, possibly due to an excessive shift in the T-cell population towards a terminally differentiated, non-reproducing memory phenotype (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

OX40/OX40L

[0076] OX40 (CD134) is expressed only on activated T-cells, and predominately CD4+ T cells. Its ligand, OX40L, is found on a wide variety of immune cells including activated B cells, T cells, dendritic cells, and vascular epithelial cells. Ligation of OX40 on T-cells promotes survival, expansion, and cytokine production, and studies in knock-out animals show that OX40 is critical for CD4, but not CD8 responses. OX40 is also important for the homeostasis and development of Tregs. In the context of immunotherapy, OX40 ligation may reverse T-cell anergy and render silent epitopes immunogenic (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[0077] Various strategies of augmenting OX40 signaling in anti-tumor T cells have shown promise in developing tumor immunotherapy in mouse models. OX40 ligation has been found to increase tumor-free survival and cure some mice in animal models of cancers such as melanoma, sarcoma, colon cancer, breast cancer, and glioma. Furthermore, treatment was effective in animal models of metastatic disease, where mice developed strong anti-tumor T-cell responses, in particular memory CD4+ T-cells, which protected them from further challenge with

the same tumor. Furthermore, vaccines comprising cells transfected with OX40L and GMCSF cure colon cancer in murine colon cancer models. OX40 ligation has also shown synergy with a combination of 4-1BB ligation and Interleukin 12 (IL-12). In total, evidence from murine studies suggests that ligation of OX40, combined with other immunotherapies, shows promise in the treatment of human cancers (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

4-1BB/4-1BBL

[0078] 4-1BB (CD137) is expressed on activated T cells, NK cells, and dendritic cells, while 4-1BBL is expressed on activated antigen presenting cells (APCs). Studies have found that 4-1BB ligation particularly stimulates CD8+ T cells, and promotes their differentiation into effectors. It has been reported that 4-1BB signaling is able to reverse the anergy induced by soluble antigens and rescue CD28-/- CD8+ T cells. Accumulation of such T cells occurs in the elderly, during chronic inflammation, and cancer. In contrast, 4-1BB ligation has been shown to suppress CD4+ T cells and B cells. Agonist anti-4-1BB antibody has been identified as being able to reverse autoimmunity in mice (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[0079] Anti-4-1BB antibodies have been able to achieve eradication of established tumors in mouse models, and ligation of 4-1BB by systemically administered antibodies, as well as vaccination with 4-1-BBL expressing tumor cells, have been shown to cause rejection of tumors. Furthermore, tumor cells transfected with single-chain Fv fragments specific for 4-1BB have also been found to be effective anti-tumor agents. CD8+ T cells are believed to primarily be effectors in 4-1BB mouse models, but tumor rejection has also been identified as being

dependent on CD4+ T cells, NK cells, and on myeloid cells. Ligation of 4-1BB is ineffective, however, when CD28 is present and an immune response is already present. Thus, 4-1BB ligation has been used in combination with CD28 stimulation to target both pathways together (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

HVEM-LIGHT

[0080] Herpes Virus Entry Mediator (HVEM) is a biochemical switch regulating T cell activation in a costimulatory or co-inhibitory fashion. The stimulatory or inhibitory outcome depends on the specific ligand engaged (Cai, G., The CD160, BTLA, LIGHT/HVEM pathway: a bidirectional switch regulating T-cell activation, Immunol. Rev., May; 229(1):244-58 (2009)). HVEM binds to at least three ligands: lymphotoxin-like, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes (LIGHT), lymphotoxin alpha 3 (Ltα3), and B- and T-lymphocyte attenuator (BTLA). LIGHT, Ltα3, and BTLA are HVEM ligands. The binding of LIGHT or Ltα3 to HVEM delivers a costimulatory signal, while binding of BTLA to HVEM delivers a co-inhibitory signal. The LIGHT receptor binds two receptors in addition to HVEM: LTβR and CdR3/TR6. HVEM is found on resting T cells, monocytes, and immature dendritic cells. LIGHT can be found on activated T cells, monocytes, and NK cells, and also on immature dendritic cells. LIGHT signaling causes proliferation of T cells stimulated with CD3 or CD3/CD28, and can induce DC maturation, while over-expression of LIGHT can cause autoimmunity with increased T cell populations and inflammation of mucosal tissues. LIGHT deficiency causes CD8+ T-cell dysfunction. BTLA is expressed on activated T cells, B cells and dendritic cells, and its signals

can suppress T-cell responses (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[0081] LIGHT is believed to have an anti-tumor effect through apoptosis induction and immune activation, and it can kill tumors expressing HVEM via a death-domain pathway. Furthermore, transfection of tumor cells with LIGHT are capable of causing T-cell dependent tumor rejection, in some cases by inducing changes to tumor stromal cells facilitating entry of T cells into the tumor (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

CpG

[0082] DNA motifs consisting of an unmethylated CpG dinucleotide flanked by two 5' purines (e.g. GpA) and two 3' pyrimidines (e.g. TpC or TpT) are capable of stimulating an innate immune response by mimicking bacterial DNA. CpG oligodeoxynucleotides can be used as immune adjuvants to improve the function of professional antigen-presenting cells and increase generation of humoral and cellular vaccine-specific immune responses. CpG DNA is able to directly activate dendritic cells and B cells, resulting in the induction of both innate and adaptive immune responses (Bode, C., CpG DNA as a vaccine adjuvant, Expert Rev Vaccines. 2011 Apr; 10(4): 499–511). The efficacy of oligodeoxynucleotides containing unmethylated CpG motifs as an immune therapy adjuvant is dependent on the spatial and temporal proximity between the CpG and an antigen. Studies have shown that physically attaching a CpG oligonucleotide to an antigen can increase immunity to that antigen by more than 100-fold relative to a CpG oligonucleotide diffusely mixed with an antigen. Furthermore, CpG conjugated increases dendritic cell uptake of cell based vaccines, increases co-stimulatory molecule expression,

increases production of immunostimulatory cytokines, and causes expansion of cytotoxic T cells (Shirota, H., CpG-conjugated apoptotic tumor cells elicit potent tumor-specific immunity, Cancer Immunol Immunother (2011) 60:659–669 incorporated by reference herein in its entirety).

Immunogenic Potential of Vaccines

[0083] Vaccines against infectious agents are prime examples of specific receptor-ligand interactions being used to shape an immune response for the therapeutic goal of preventing or reducing infection (e.g. flu vaccine). Generally, an antigen is presented to the immune system in the context of an adjuvant (e.g., a synthetic small molecule immunomodulator).

[0084] The allogeneic tumor vaccines of the described invention are distinct from such vaccines in several key features. First, they are designed to be capable of treating existing tumors, although prevention of tumor formation is theoretically also possible. Second, their efficacy tends to be limited by the fact that while tumors express neoantigens (i.e. new, non-self elements) that are foreign and new to the individual, they are also undoubtedly human tumor cells and thus not always recognized as foreign (i.e. non-self) by the individual.

[0085] The aforementioned difficulties notwithstanding, evidence has now emerged that 1) endogenous antitumor responses exist, 2) that these immune responses can be modulated and 3) that this modulation can be measured in terms of overall survival in standard clinical trials.

[0086] According to aspects of the described invention, a series of immunomodulators that can be co-expressed either on a tumor cell line derived from a cancer patient, or on a multiply genetically modified allogeneic tumor cell line has been identified that, when used as a tumor vaccine, may serve 1) to efficiently load the broad array of tumor antigens into the endogenous

antigen presenting cells, 2) to efficiently stimulate several cell types by enhancing the normal signals received during an immune response, 3) to impede the mechanisms by which T regulatory cells suppress the immune response, 4) to impede the signals by which immune responses are generally resolved, and 5) to result in enhanced overall survival of cancer patients vaccinated with such a formulation. Although in certain embodiments, the modified tumor cell line can be derived from the patient who receives the vaccine, the allogeneic tumor cell line vaccine approach is distinct from a personalized therapy approach, because the modified tumor cells are not necessarily derived from the individual who ultimately receives the vaccine. Instead, an allogeneic tumor cell vaccine aims to focus an immune response on the many elements that individual tumors of the same tumor type have in common.

[0087] One strategy for exploiting the large number of potential tumor antigens for each individual type of cancer is to vaccinate with whole tumor cells to avoid accidentally excluding potentially relevant antigens. The invention described herein provides, among other things, a vaccine with whole tumor cells possessing an array of antigens and modified to express two or more immune modulators.

BRIEF SUMMARY OF THE INVENTION

[0087a] In a first aspect, the present invention provides a method of treating a melanoma cancer in a patient comprising the steps of: (a) preparing an allogeneic melanoma tumor cell line variant transfected to express two or more immunomodulator peptides, wherein the immunomodulator peptides are GM-CSF and Flt-3L, by: (1) providing an allogeneic parental tumor cell line; (2) transfecting or transducing recombinant DNA sequences coding for the two or more of immunomodulator peptides selected; (3) generating the melanoma tumor cell line

variants by selecting for tumor cell clones that stably express an immunogenic amount of the two or more immune modulator peptides, the clonally derived melanoma cell line variants comprise: (a) a soluble form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 13 and a membrane bound form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 14; (b) a membrane bound form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 5 and a soluble form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 44; or (c) a combination of (i) and (ii); (i) a combination of a soluble form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 13 and a membrane bound form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 14; and (ii) a combination of a membrane bound form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 5 and a soluble form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 44, (4) selecting in a mixed lymphocyte tumor cell reaction the clonally derived melanoma cell line variants from step (3) that expresses an immunostimulatory amount of the immunomodulators as measured by one or more of the following parameters of lymphocyte activation selected from cellular proliferation, cellular subset differentiation, cytokine release profile, and tumor cell lysis; wherein the selected clonally derived melanoma cell line variant is effective to stimulate activation of one or more of T cells, B cells, and dendritic cells; and (b) administering to the patient that has melanoma an immunostimulatory amount of the melanoma tumor cell line variant from step (4), wherein the immunostimulatory amount is effective to improve clinical outcome.

[0087b] In a second aspect, the present invention provides an allogeneic tumor cell vaccine comprising: (1) a tumor cell line variant comprising (a) two or more stably expressed recombinant membrane bound immunomodulatory molecules, wherein the immunomodulator peptides are GM-CSF and Flt-3L; and (b) stably expressed recombinant soluble GM-CSF peptides; and (2) a pharmaceutically acceptable carrier; wherein an immune stimulatory amount of the melanoma tumor cell line variant is effective to elicit an immune response that improves progression free survival, overall survival, or both relative to placebo controls.

[0087c] According to a third aspect, the present invention provides use of a melanoma tumor cell line variant in the manufacture of a medicament for treating a melanoma cancer in a patient, wherein said melanoma tumor cell line variant is an allogeneic melanoma tumor cell line variant transfected to express two or more immunomodulator peptides, wherein the immunomodulator peptides are GM-CSF and Flt-3L, prepared by: (1) providing an allogeneic melanoma parental tumor cell line; (2) transfecting or transducing recombinant DNA sequences coding for the two or more of immunomodulator peptides selected; (3) generating the melanoma tumor cell line variants by selecting for melanoma tumor cell clones that stably express an immunogenic amount of the two or more immune modulator peptides, the clonally derived melanoma cell line variants comprise: (a) a soluble form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 13 and a membrane bound form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 14; (b) a membrane bound form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 5 and a soluble form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 44; or (c) a combination of (i) and (ii); (i) a combination of a soluble

form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 13 and a membrane bound form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 14; and (ii) a combination of a membrane bound form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 5 and a soluble form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 44; (4) selecting in a mixed lymphocyte tumor cell reaction the clonally derived melanoma cell line variants from step (3) that expresses an immunostimulatory amount of the immunomodulators as measured by one or more of the following parameters of lymphocyte activation selected from cellular proliferation, cellular subset differentiation, cytokine release profile, and tumor cell lysis; wherein the selected clonally derived melanoma cell line variant is effective to stimulate activation of one or more of T cells, B cells, and dendritic cells; and wherein the medicament is to be administered to the patient in an immunostimulatory amount effective to improve clinical outcome.

[0088] According to some aspects, a method of treating a cancer in a patient comprises the steps of (a) preparing an allogeneic tumor cell line variant transfected to express two or more immunomodulator peptides by: (1) providing an allogeneic parental tumor cell line; (2) transfecting or transducing recombinant DNA sequences coding for two or more of immunomodulator peptides selected from IgG1, CD40L, TNF-alpha, GM-CSF, and Flt-3L; (3) generating the tumor cell line variants by selecting for tumor cell clones that stably express an

immunogenic amount of the two or more immune modulator peptides selected from IgG1, CD40L, TNF-alpha, GM-CSF, and Flt-3L; (4) selecting in a mixed lymphocyte tumor cell reaction clonally derived cell line variants by one or more of the following parameters selected from cellular proliferation, cellular subset differentiation, cytokine release profile, and tumor cell lysis; wherein the selected clonally derived cell line variant is effective to stimulate activation of one or more of T cells, B cells, and dendritic cells, and (b) administering to the patient that has cancer an immunostimulatory amount of the tumor cell line variant vaccine, wherein the immunostimulatory amount is effective to improve clinical outcome.

[0089] According to some embodiments, the immunomodulator peptides are selected from membrane expressed IgG1, CD40L, TNF-alpha, as well as membrane and soluble forms of GM-CSF, and Flt-3L.

[0090] According to some embodiments, the tumor cell line variant vaccine is effective to improve overall survival of cancer patients relative to placebo controls. According to some embodiments the parental tumor cell line is derived from a melanoma. According to some embodiments the parental tumor cell line is derived from a prostate cancer. According to some embodiments the parental tumor cell line is derived from a breast cancer.

[0091] According to some embodiments, the IgG1 immunomodulator peptide sequence is of at least 60% identity to SEQ ID NO: 45. According to some embodiments, the CD40L immune modulator peptides sequence is of at least 60% identity to SEQ ID NO: 7. According to some embodiments, the TNF-alpha immune modulator peptide sequence is of at least 60% identity to SEQ ID NO: 11. According to some embodiments, the GM-CSF immune modulator peptide sequence is of at least 60% identity to SEQ ID NO: 13 or SEQ ID NO: 5. According to some

embodiments, the Flt-3L immune modulator peptide sequence is of at least 60% identity to SEQ ID NO: 14 or SEQ ID NO: 44.

[0092] According to some aspects, an allogeneic tumor cell vaccine comprises (1) a tumor cell line variant comprising (a) two or more stably expressed recombinant membrane bound immunomodulatory molecules selected from IgG1, CD40L, TNF-alpha, and Flt-3L peptides; and (b) stably expressed recombinant soluble GM-CSF peptides; and (2) a pharmaceutically acceptable carrier; wherein an immune stimulatory amount of the tumor cell line variant is effective to elicit an immune response that improves progression free survival, overall survival, or both relative to placebo controls.

[0093] According to some embodiments, the tumor cell line variant expresses two or more of (a) a membrane bound IgG1 peptide with at least 60% identity to SEQ ID NO: 45; (b) a membrane bound CD40L peptide with at least 60% identity to SEQ ID NO: 7; (c) a membrane bound form of TNF-alpha peptide with at least 60% identity to SEQ ID NO: 11; (d) a membrane bound form of Flt-3L peptide with at least 60% identity to SEQ ID NO: 14; and (e) a soluble GM-CSF peptide with at least 60% identity to SEQ ID NO: 13.

[0094] According to some embodiments, the tumor cell line variant comprises a membrane bound fusion protein of CD40L peptide and TNF-alpha peptide. According to some embodiments, the CD40L peptide is of at least 60% identity to SEQ ID NO: 9, and the TNF-alpha peptide is of at least 60% identity to SEQ ID NO: 10. According to some embodiments, the TNF-alpha peptide is of at least 60% identity to SEQ ID NO: 11,. According to some embodiments, the tumor cell line variants comprise soluble GM-CSF and membrane bound IgG1, CD40L, TNF-alpha, and Flt-3L. According to some embodiments, the tumor cell line

variant comprises a fusion of CD40L and TNFa peptides. According to some embodiments, the tumor cell line variant comprises an immune modulator peptide sequence of at least 60% identity to SEQ ID NO: 31. According to some embodiments, the tumor cell line variant comprises membrane and soluble forms of GM-CSF and membrane and soluble forms of Flt-3L. According to some embodiments, the tumor cell line variant comprises membrane bound forms of IgG, CD40L, and TNF-alpha.

[0095] These and other advantages of the invention will be apparent to those of ordinary skill in the art by reference to the following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0096] FIG. 1 shows one embodiment of a heteroclitic cross reaction between a peptide native to a tumor cell line and a peptide native to a tumor cell of a patient receiving immunotherapy.

[0097] FIG. 2A shows a schematic of the core vectors.

[0098] FIG. 2B shows a schematic of the proteins encoded by Vectors 2-6.

[0099] FIG. 3A shows a schematic of the organization of the scFv-anti-biotin-G3hinge-mIgG1 vector.

[00100] FIG. 3B shows the nucleotide sequence of vector 1 (SEQ ID NO. 47).

[00101] FIG. 4A shows a schematic of the organization of the full anti-biotin – G3hinge-mIgG1 vector.

[00102] FIG. 4B shows the nucleotide sequence of vector 2 (SEQ ID NO. 48).

[00103] FIG. 5A shows a schematic of the organization of the sGM-CSF/ires/mFLT3L vector.

[00104] FIG. 5B shows the nucleotide sequence of vector 3 (SEQ ID NO. 49).

[00105] FIG. 6A shows a schematic of the organization of the sFLT3L/ires/(FLT3 signal-GM-CSF-Tm) vector.

[00106] FIG. 6B shows the nucleotide sequence of vector 4 (SEQ ID NO. 50).

[00107] FIG. 7A shows a schematic of the organization of the mCD40L vector.

[00108] FIG. 7B shows the nucleotide sequence of vector 5 (SEQ ID NO. 51).

[00109] FIG. 8A shows a schematic of the organization of the mTNFa vector.

[00110] FIG. 8B shows the nucleotide sequence of vector 6 (SEQ ID NO. 52).

[00111] FIG. 9A shows a schematic of the organization of the mRANKL /ires/FLT3 signal-V5- scFV anti-biotin-Tm vector.

[00112] FIG. 9B shows the nucleotide sequence of vector 7 (SEQ ID NO. 53).

[00113] FIG. 10 is a schematic that shows the general experimental format.

[00114] FIG. 11 is a panel of graphs that show the results of flow cytometry experiments. Forward (FSC) and side scatter (SSC) plots for size and granularity. SK lines are represented by a number code; SK, unmodified parent line; 3, secreted GM-CSF and membrane expressed FLT-3L; 4, secreted FLT3L and membrane expressed GM-CSF; 5, a non-cleavable form of CD40L; 6, a non-cleavable form of TNF-a; 3-4 is a combination of 3 and 4; 3-4-5 is a combination of 3,4 and 5; and 3-4-6 is a combination of 3,4 and 6. Cell lines 6, 3-4-5 and 3-4-6 display a larger and more granular phenotype likely owing to the presence of receptors for TNF-a and CD40L on cells of epithelial origin

[00115] FIG. 12 is a panel of graphs that show representative flow cytometry stains for CD4 cells in hPBMC in response to the indicated engineered cell lines with the indicated immunomodulators SK cell lines are represented by the following code; SK, unmodified parent line; 2, membrane expressed IgG1, 3, secreted GM-CSF and membrane expressed FLT-3L; 4, secreted FLT3L and membrane expressed GM-CSF; 5, a non-cleavable form of CD40L; and 6, a non-cleavable form of TNF.

[00116] FIG. 13 is a panel of graphs that show representative flow cytometry stains for the indicated engineered surface markers; GM-CSF, FLT3L, TNF-a and CD40L. SK lines are represented by a number code; SK, unmodified parent line; 3, secreted GM-CSF and membrane expressed FLT-3L; 4, secreted FLT3L and membrane expressed GM-CSF; 5, a non-cleavable form of CD40L; 6, a non-cleavable form of TNF-a; 3-4 is a combination of 3 and 4; 3-4-5 is a combination of 3,4 and 5; and 3-4-6 is a combination of 3,4 and 6.

[00117] FIG. 14A and FIG. 14B show the results of CyTOF mass cytometry single-cell phenotype analysis of hPBMC response to SK melanoma cells with modification by expression of immunomodulatory factors. FIG. 14A shows viSNE density contour plots of CyTOF staining data showing relative changes in immune cell subset abundance and phenotype. FIG. 14B shows single-cell phenotype analysis. SK lines are represented by a number code; SK, unmodified parent line; 3, secreted GM-CSF and membrane expressed FLT-3L; 4, secreted FLT3L and membrane expressed GM-CSF; 5, a non-cleavable form of CD40L; 6, a non-cleavable form of TNF-a; 3-4 is a combination of 3 and 4; 3-4-5 is a combination of 3,4 and 5; and 3-4-6 is a combination of 3,4 and 6.

[00118] FIG. 15A- FIG. 15D shows CyTOF monocyte cluster analysis of hPBMC indicating changes in the activation markers CD40 (FIG. 15A), CD86 (FIG. 15B), CD69 (FIG. 15C) and

CD25 (FIG. 15D) expression following 1 day stimulation with the indicated genetically modified SK lines at a 1:5 cell ratio. SK lines are represented by a number code; SK, unmodified parent line; 3, secreted GM-CSF and membrane expressed FLT-3L; 4, secreted FLT3L and membrane expressed GM-CSF; 5, a non-cleavable form of CD40L; 6, a non-cleavable form of TNF-a; 3-4 is a combination of 3 and 4; 3-4-5 is a combination of 3,4 and 5; and 3-4-6 is a combination of 3,4 and 6. FIG. 15E shows CyTOF monocyte cluster analysis of hPBMC indicating relative median expression levels of monocyte markers CD40 and CD86. FIG. 15E shows CyTOF monocyte cluster analysis of hPBMC indicating relative median expression levels of CD4 T cell markers CD69 and CD25.

[00119] FIG. 16 is a graph that shows the results of luminex multiplex cytokine profiling of human PBMC responses to SK parent line and genetically modified SK lines. Control cultures included SK cells alone, hPBMCs alone, and hPBMCs stimulated with a mixture of anti-CD3 and anti-CD28 antibodies (1 μg/ml final concentration). Symbols indicate cytokine levels in pg/ml as estimated from a standard curve using recombinant cytokines. Absence of symbols indicates the cytokine was not detected. SK lines are represented by a number code; SK, unmodified parent line; 3, secreted GM-CSF and membrane expressed FLT-3L; 4, secreted FLT3L and membrane expressed GM-CSF; 5, a non-cleavable form of CD40L; 6, a non-cleavable form of TNF-a; 3-4 is a combination of 3 and 4; 3-4-5 is a combination of 3,4 and 5; and 3-4-6 is a combination of 3,4 and 6.

DETAILED DESCRIPTION

Definitions

[00120] The term "activation" or "lymphocyte activation" refers to stimulation of lymphocytes by specific antigens, nonspecific mitogens, or allogeneic cells resulting in synthesis

of RNA, protein and DNA and production of lymphokines; it is followed by proliferation and differentiation of various effector and memory cells. For example, a mature B cell can be activated by an encounter with an antigen that expresses epitopes that are recognized by its cell surface immunoglobulin Ig). The activation process may be a direct one, dependent on cross-linkage of membrane Ig molecules by the antigen (cross-linkage-dependent B cell activation) or an indirect one, occurring most efficiently in the context of an intimate interaction with a helper T cell ("cognate help process"). T-cell activation is dependent on the interaction of the TCR/CD3 complex with its cognate ligand, a peptide bound in the groove of a class I or class II MHC molecule. The molecular events set in motion by receptor engagement are complex. Among the earliest steps appears to be the activation of tyrosine kinases leading to the tyrosine phosphorylation of a set of substrates that control several signaling pathways. These include a set of adapter proteins that link the TCR to the ras pathway, phospholipase Cγ1, the tyrosine phosphorylation of which increases its catalytic activity and engages the inositol phospholipid metabolic pathway, leading to elevation of intracellular free calcium concentration and activation of protein kinase C, and a series of other enzymes that control cellular growth and differentiation. Full responsiveness of a T cell requires, in addition to receptor engagement, an accessory cell-delivered costimulatory activity, e.g., engagement of CD28 on the T cell by CD80 and/or CD86 on the antigen presenting cell (APC). The soluble product of an activated B lymphocyte is immmunoglobulins (antibodies). The soluble product of an activated T lymphocyte is lymphokines.

[00121] As used herein, the term "administration" and its various grammatical forms as it applies to a mammal, 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. "Administration" also encompasses in vitro and ex vivo treatments, e.g., of a cell, by a reagent, diagnostic, binding composition, or by another cell.

[00122] The term “allogeneic” as used herein means that the donor and the recipient (host) are of different genetic makeup, but of the same species. As used herein, an "allogeneic cell" refers to a cell that is not derived from the individual to which the cell is to be administered, that is, it has a different genetic constitution than the individual. An allogeneic cell is generally obtained from the same species as the individual to which the cell is to be administered. For example, the allogeneic cell can be a human cell, as disclosed herein, for administering to a human patient such as a cancer patient. As used herein, an "allogeneic tumor cell" refers to a tumor cell that is not derived from the individual to which the allogeneic cell is to be administered. Generally, the allogeneic tumor cell expresses one or more tumor antigens that can stimulate an immune response against a tumor in an individual to which the cell is to be administered. As used herein, an "allogeneic cancer cell," for example, a lung cancer cell, refers to a cancer cell that is not derived from the individual to which the allogeneic cell is to be administered.

[00123] The terms “amino acid residue” or “amino acid” or “residue” are used interchangeably to refer to an amino acid that is incorporated into a protein, a polypeptide, or a peptide, including, but not limited to, a naturally occurring amino acid and known analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids. The amino acids may be L- or D-amino acids. An amino acid may be replaced by a synthetic amino acid, which is altered so as to increase the half-life of the peptide, increase the potency of

the peptide, or increase the bioavailability of the peptide. The single letter designation for amino acids is used predominately herein. Such single letter designations are as follows: A is alanine; C is cysteine; D is aspartic acid; E is glutamic acid; F is phenylalanine; G is glycine; H is histidine; I is isoleucine; K is lysine; L is leucine; M is methionine; N is asparagine; P is proline; Q is glutamine; R is arginine; S is serine; T is threonine; V is valine; W is tryptophan; and Y is tyrosine. The following represents groups of amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic Acid (D), Glutamic Acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

[00124] The term "autologous" as used herein means derived from the same individual.

[00125] The term "cancer" as used herein refers to diseases in which abnormal cells divide without control and are able to invade other tissues. There are more than 100 different types of cancer. Most cancers are named for the organ or type of cell in which they start - for example, cancer that begins in the colon is called colon cancer; cancer that begins in melanocytes of the skin is called melanoma. Cancer types can be grouped into broader categories. The main categories of cancer include: carcinoma (meaning a cancer that begins in the skin or in tissues that line or cover internal organs, and its subtypes, including adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma); sarcoma (meaning a cancer that begins in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue); leukemia (meaning a cancer that starts in blood-forming tissue (e.g., bone marrow) and causes large numbers of abnormal blood cells to be produced and enter the blood; lymphoma and myeloma (meaning cancers that begin in the cells of the immune system); and Central nervous

system cancers (meaning cancers that begin in the tissues of the brain and spinal cord). The term “myelodysplastic syndrome” refers to a type of cancer in which the bone marrow does not make enough healthy blood cells (white blood cells, red blood cells, and platelets) and there are abnormal cells in the blood and/or bone marrow. Myelodysplastic syndrome may become acute myeloid leukemia (AML).

[00126] The term “contact” and its various grammatical forms as used herein refers to a state or condition of touching or of immediate or local proximity. Contacting a composition to a target destination may occur by any means of administration known to the skilled artisan.

[00127] The term “costimulatory molecule” as used herein refers to one of two or more molecules that are displayed on the cell surface that have a role in activating a T cell to become an effector cell. For example MHC proteins, which present foreign antigen to the T cell receptor, also require costimulatory proteins which bind to complementary receptors on the T cell’s surface to result in activation of the T cell.

[00128] The term "cytokine" as used herein refers to small soluble protein substances secreted by cells which have a variety of effects on other cells. Cytokines mediate many important physiological functions including growth, development, wound healing, and the immune response. They act by binding to their cell-specific receptors located in the cell membrane, which allows a distinct signal transduction cascade to start in the cell, which eventually will lead to biochemical and phenotypic changes in target cells. Cytokines can act both locally and distantly from a site of release. They include type I cytokines, which encompass many of the interleukins, as well as several hematopoietic growth factors; type II cytokines, including the interferons and interleukin-10; tumor necrosis factor ("TNF")-related molecules, including TNFα

and lymphotoxin; immunoglobulin super-family members, including interleukin 1 ("IL-1"); and the chemokines, a family of molecules that play a critical role in a wide variety of immune and inflammatory functions. The same cytokine can have different effects on a cell depending on the state of the cell. Cytokines often regulate the expression of, and trigger cascades of other cytokines. Nonlimiting examples of cytokines include e.g., IL-1α., IL-β., IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12/IL-23 P40, IL13, IL-15, IL-17, IL-18, IL-21, IL-23, TGF-β, IFN-γ, GM-CSF, Gro.alpha., MCP-1 and TNF-α.

[00129] The term "derived from" as used herein encompasses any method for receiving, obtaining, or modifying something from a source of origin.

[00130] The term "derivative" or "variant" with respect to a peptide or DNA sequence (e.g. immune modulator peptide sequence) as used herein refers to a non-identical peptide or DNA sequence that is modified from its original sequence. The terms "derivative" or "variant" with respect to cells as used herein refers to a cell line that has been modified from its cell line of origin (e.g. modified to express recombinant DNA sequences).

[00131] The term "detectable marker" encompasses both selectable markers and assay markers. The term "selectable markers" refers to a variety of gene products to which cells transformed with an expression construct can be selected or screened, including drug-resistance markers, antigenic markers useful in fluorescence-activated cell sorting, adherence markers such as receptors for adherence ligands allowing selective adherence, and the like.

[00132] The term "detectable response" refers to any signal or response that may be detected in an assay, which may be performed with or without a detection reagent. Detectable responses include, but are not limited to, radioactive decay and energy (e.g., fluorescent, ultraviolet,

infrared, visible) emission, absorption, polarization, fluorescence, phosphorescence, transmission, reflection or resonance transfer. Detectable responses also include chromatographic mobility, turbidity, electrophoretic mobility, mass spectrum, ultraviolet spectrum, infrared spectrum, nuclear magnetic resonance spectrum and x-ray diffraction. Alternatively, a detectable response may be the result of an assay to measure one or more properties of a biologic material, such as melting point, density, conductivity, surface acoustic waves, catalytic activity or elemental composition. A "detection reagent" is any molecule that generates a detectable response indicative of the presence or absence of a substance of interest. Detection reagents include any of a variety of molecules, such as antibodies, nucleic acid sequences and enzymes. To facilitate detection, a detection reagent may comprise a marker.

[00133] The term “dose” as used herein refers to the quantity of a therapeutic substance prescribed to be taken at one time.

[00134] The term “enrich” as used herein refers to increasing the proportion of a desired substance, for example, to increase the relative frequency of a subtype of cell compared to its natural frequency in a cell population. Positive selection, negative selection, or both are generally considered necessary to any enrichment scheme. Selection methods include, without limitation, magnetic separation and FACS. Regardless of the specific technology used for enrichment, the specific markers used in the selection process are critical, since developmental stages and activation-specific responses can change a cell’s antigenic profile.

[00135] As used herein, the term "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.

[00136] The term "expression vector" refers a DNA molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements including, but not limited to, promoters, tissue specific regulatory elements, and enhancers. Such a gene is said to be "operably linked to" the regulatory elements.

[00137] The term “flow cytometry” as used herein refers to a tool for interrogating the phenotype and characteristics of cells. It senses cells or particles as they move in a liquid stream through a laser (light amplification by stimulated emission of radiation)/light beam past a sensing area. The relative light-scattering and color-discriminated fluorescence of the microscopic particles is measured. Flow Analysis and differentiation of the cells is based on size, granularity, and whether the cells is carrying fluorescent molecules in the form of either antibodies or dyes. As the cell passes through the laser beam, light is scattered in all directions, and the light scattered in the forward direction at low angles (0.5-10°) from the axis is proportional to the square of the radius of a sphere and so to the size of the cell or particle. Light may enter the cell; thus, the 90 ° light (right-angled, side) scatter may be labeled with fluorochrome-linked antibodies or stained with fluorescent membrane, cytoplasmic, or nuclear dyes. Thus, the differentiation of cell types, the presence of membrane receptors and antigens, membrane potential, pH, enzyme activity, and DNA content may be facilitated. Flow cytometers are multiparameter, recording several measurements on each cell; therefore, it is possible to identify a homogeneous subpopulation within a heterogeneous population (Marion G. Macey, Flow cytometry: principles and applications, Humana Press, 2007). Fluorescence-activated cell sorting (FACS), which allows isolation of distinct cell populations too similar in physical characteristics to be separated by size or density, uses fluorescent tags to detect surface proteins

that are differentially expressed, allowing fine distinctions to be made among physically homogeneous populations of cells.

[00138] The term "functional equivalent" or "functionally equivalent" are used interchangeably herein to refer to substances, molecules, polynucleotides, proteins, peptides, or polypeptides having similar or identical effects or use.

[00139] The term "heteroclitic" is used herein to refer to peptides of higher biological potency than the original peptide. A "heteroclitic immunogen" is an immunogen that elicits an immune response that cross-reacts to an original poorly immunogenic antigen.

[00140] The terms "immune response" and "immune-mediated" are used interchangeably herein to refer to any functional expression of a subject's immune system, against either foreign or self-antigens, whether the consequences of these reactions are beneficial or harmful to the subject.

[00141] The terms "immunomodulatory", "immune modulator" and "immune modulatory" are used interchangeably herein to refer to a substance, agent, or cell that is capable of augmenting or diminishing immune responses directly or indirectly by expressing chemokines, cytokines and other mediators of immune responses.

[00142] As used herein the term "immunostimulatory amount" of the disclosed compositions refers to an amount of an immunogenic composition that is effective to stimulate an immune response, for example, as measured by ELISPOT assay (cellular immune response), ICS (intracellular cytokine staining assay) and major histocompatibility complex (MHC) tetramer assay to detect and quantify antigen-specific T cells, quantifying the blood population of antigen-specific CD4+ T cells, or quantifying the blood population of antigen specific CD8+ T cells by a

measurable amount, or where the increase is by at least 10%, 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 96%, at least 97%, at least 98%, at least 99%, at least 100%, when compared to a suitable control (e.g., a control composition where dendritic cells are not loaded with tumor-specific cells, or not loaded with peptide derived from tumor-specific cells).

[00143] The term "integrate into the genome" as used herein refers to a recombinant DNA sequence being concomitantly joined to the genomic DNA comprising a host cell's genome.

[00144] The term "Kaplan Meier plot" or "Kaplan Meier survival curve" as used herein refers to the plot of probability of clinical study subjects surviving in a given length of time while considering time in many small intervals. The Kaplan Meier plot assumes that: (i) at any time subjects who are censored (i.e., lost) have the same survival prospects as subjects who continue to be followed; (ii) the survival probabilities are the same for subjects recruited early and late in the study; and (iii) the event (e.g., death) happens at the time specified. Probabilities of occurrence of events are computed at a certain point of time with successive probabilities multiplied by any earlier computed probabilities to get a final estimate. The survival probability at any particular time is calculated as the number of subjects surviving divided by the number of subjects at risk. Subjects who have died, dropped out, or have been censored from the study are not counted as at risk.

[00145] The term "labeling" as used herein refers to a process of distinguishing a compound, structure, protein, peptide, antibody, cell or cell component by introducing a traceable constituent. Common traceable constituents include, but are not limited to, a fluorescent

antibody, a fluorophore, a dye or a fluorescent dye, a stain or a fluorescent stain, a marker, a fluorescent marker, a chemical stain, a differential stain, a differential label, and a radioisotope.

[00146] The terms "marker" or "cell surface marker" are used interchangeably herein to refer to an antigenic determinant or epitope found on the surface of a specific type of cell. Cell surface markers can facilitate the characterization of a cell type, its identification, and eventually its isolation. Cell sorting techniques are based on cellular biomarkers where a cell surface marker(s) may be used for either positive selection or negative selection, i.e., for inclusion or exclusion, from a cell population.

[00147] The terms "mixed lymphocyte tumor reaction" or "MLTR" are used interchangeably herein to refer to a reaction similar to a mixed lymphocyte reaction but rather than using allogeneic lymphocytes to stimulate a response, allogeneic tumor cells are used instead. The MLTR method comprises contacting tumor cells being tested for immunogenic potential with mixed lymphocytes from peripheral blood mononuclear cells, followed by measuring one or more of cellular proliferation of the lymphocytes, cellular subset differentiation of the lymphocytes, cytokine release profile of the lymphocytes, and tumor cell death.

[00148] The term "modified" or "modulated" as used herein with respect to immune response to tumor cells refers to changing the form or character of the immune response to the tumor cells via one or more recombinant DNA techniques such that the immune cells are able to recognize and kill tumor cells.

[00149] The term "myeloid suppressor cells" or "myeloid-derived suppressor cells" as used herein refers to the heterogeneous population of cells characterized by myeloid origin, immature

state, and ability to potently suppress T cell responses. These cells regulate immune responses and tissue repair in healthy individuals and the population rapidly expands during inflammation.

[00150] The term "open reading frame" as used herein refers to a sequence of nucleotides in a DNA molecule that has the potential to encode a peptide or protein: it starts with a start triplet (ATG), is followed by a string of triplets each of which encodes an amino acid, and ends with a stop triplet (TAA, TAG or TGA).

[00151] The phrase "operably linked" refers (1) to a first sequence(s) or domain being positioned sufficiently proximal to a second sequence(s) or domain so that the first sequence(s) or domain can exert influence over the second sequence(s) or domain or a region under control of that second sequence or domain; and (2) to a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, are in the same reading frame. According to some embodiments, the phrase "operatively linked" refers to a linkage in which two or more protein domains or polypeptides are ligated or combined via recombinant DNA technology or chemical reaction such that each protein domain or polypeptide of the resulting fusion protein retains its original function.

[00152] The term "overall survival" (OS) as used herein refers to the length of time from either the date of diagnosis or the start of treatment for a disease, such as cancer, that patients diagnosed with the disease are still alive.

[00153] The term "parenteral" and its other grammatical forms as used herein refers to administration of a substance occurring in the body other than by the mouth or alimentary canal.

For example, the term “parenteral” as used herein refers to introduction into the body by way of an injection (i.e., administration by injection), including, for example, subcutaneously (i.e., an injection beneath the skin), intramuscularly (i.e., an injection into a muscle); intravenously (i.e., an injection into a vein), intrathecally (i.e., an injection into the space around the spinal cord or under the arachnoid membrane of the brain), intrasternal injection, or infusion techniques.

[00154] The terms "peripheral blood mononuclear cells" or "PBMCs" are used interchangeably herein to refer to blood cells having a single round nucleus such as, for example, a lymphocyte or a monocyte.

[00155] The term “pharmaceutical composition” as used herein refers to a composition that is employed to prevent, reduce in intensity, cure or otherwise treat a target condition, syndrome, disorder or disease.

[00156] The term “pharmaceutically acceptable carrier” as used herein refers to any substantially non-toxic carrier conventionally useable for administration of pharmaceuticals in which the isolated polypeptide of the present invention will remain stable and bioavailable. The pharmaceutically acceptable carrier must be of sufficiently high purity and of sufficiently low toxicity to render it suitable for administration to the mammal being treated. It further should maintain the stability and bioavailability of an active agent. The pharmaceutically acceptable carrier can be liquid or solid and is selected, with the planned manner of administration in mind, to provide for the desired bulk, consistency, etc., when combined with an active agent and other components of a given composition.

[00157] The term "pharmaceutically acceptable salt" as used herein refers to those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of

humans and lower animals without undue toxicity, irritation, allergic response and the like and are commensurate with a reasonable benefit/risk ratio. When used in medicine the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof. Such salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulphuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene sulphonic, tartaric, citric, methane sulphonic, formic, malonic, succinic, naphthalene-2-sulphonic, and benzene sulphonic. Also, such salts may be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group. By "pharmaceutically acceptable salt" is meant those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well-known in the art. For example, P. H. Stahl, et al. describe pharmaceutically acceptable salts in detail in "Handbook of Pharmaceutical Salts: Properties, Selection, and Use" (Wiley VCH, Zurich, Switzerland: 2002). The salts may be prepared in situ during the final isolation and purification of the compounds described within the present invention or separately by reacting a free base function with a suitable organic acid. Representative acid addition salts include, but are not limited to, acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsufonate, digluconate, glycerophosphate, hemisulfate, heptanoate, hexanoate, fumarate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethansulfonate(isethionate), lactate, maleate, methanesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, phosphate, glutamate, bicarbonate,

p-toluenesulfonate and undecanoate. Also, the basic nitrogen-containing groups may be quaternized with such agents as lower alkyl halides such as methyl, ethyl, propyl, and butyl chlorides, bromides and iodides; dialkyl sulfates like dimethyl, diethyl, dibutyl and diamyl sulfates; long chain halides such as decyl, lauryl, myristyl and stearyl chlorides, bromides and iodides; arylalkyl halides like benzyl and phenethyl bromides and others. Water or oil-soluble or dispersible products are thereby obtained. Examples of acids which may be employed to form pharmaceutically acceptable acid addition salts include such inorganic acids as hydrochloric acid, hydrobromic acid, sulphuric acid and phosphoric acid and such organic acids as oxalic acid, maleic acid, succinic acid and citric acid. Basic addition salts may be prepared in situ during the final isolation and purification of compounds described within the invention by reacting a carboxylic acid-containing moiety with a suitable base such as the hydroxide, carbonate or bicarbonate of a pharmaceutically acceptable metal cation or with ammonia or an organic primary, secondary or tertiary amine. Pharmaceutically acceptable salts include, but are not limited to, cations based on alkali metals or alkaline earth metals such as lithium, sodium, potassium, calcium, magnesium and aluminum salts and the like and nontoxic quaternary ammonia and amine cations including ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, diethylamine, ethylamine and the like. Other representative organic amines useful for the formation of base addition salts include ethylenediamine, ethanolamine, diethanolamine, piperidine, piperazine and the like. Pharmaceutically acceptable salts also may be obtained using standard procedures well known in the art, for example by reacting a sufficiently basic compound such as an amine with a suitable acid affording a physiologically acceptable anion. Alkali metal (for example, sodium, potassium

or lithium) or alkaline earth metal (for example calcium or magnesium) salts of carboxylic acids may also be made.

[00158] The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The essential nature of such analogues of naturally occurring amino acids is that, when incorporated into a protein, that protein is specifically reactive to antibodies elicited to the same protein but consisting entirely of naturally occurring amino acids.

[00159] The terms "polypeptide", "peptide" and "protein" also are inclusive of modifications including, but not limited to, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation, and ADP-ribosylation. It will be appreciated, as is well known and as noted above, that polypeptides may not be entirely linear. For instance, polypeptides may be branched as a result of ubiquitination, and they may be circular, with or without branching, generally as a result of posttranslational events, including natural processing event and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translation natural process and by entirely synthetic methods, as well. According to some embodiments, the peptide is of any length or size.

[00160] The terms "protein domain" and "domain" are used interchangably to refer to a portion of a protein that has its own tertiary structure. Large proteins are generally composed of several domains connected to one another via flexible regions of polypeptide chain.

[00161] The following terms are used herein to describe the sequence relationships between two or more nucleic acids or polynucleotides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity." (a) The term "reference sequence" refers to a sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. (b) The term "comparison window" refers to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence may be compared to a reference sequence and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be at least 30 contiguous nucleotides in length, at least 40 contiguous nucleotides in length, at least 50 contiguous nucleotides in length, at least 100 contiguous nucleotides in length, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence, a gap penalty typically is introduced and is subtracted from the number of matches. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman, *Adv. Appl. Math.* 2:482 (1981); by the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970); by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci.* 85:2444 (1988); by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, Calif.; GAP, BESTFIT, BLAST,

FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wis., USA; the CLUSTAL program is well described by Higgins and Sharp, *Gene* 73:237-244 (1988); Higgins and Sharp, *CABIOS* 5:151-153 (1989); Corpet, et al., Nucleic Acids Research 16:10881-90 (1988); Huang, et al., Computer Applications in the *Biosciences,* 8:155-65 (1992), and Pearson, et al., *Methods in Molecular Biology*, 24:307-331 (1994). The BLAST family of programs, which can be used for database similarity searches, includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, *Current Protocols in Molecular Biology*, Chapter 19, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995). Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using the BLAST 2.0 suite of programs using default parameters. Altschul et al., *Nucleic Acids Res.* 25:3389-3402 (1997). Software for performing BLAST analyses is publicly available, e.g., through the National Center for Biotechnology-Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul et al., supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits then are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for

nucleotide sequences, the parameters M (reward score for a pair of matching residues; always>0) and N (penalty score for mismatching residues; always<0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) of 10, a cutoff of 100, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915). In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. BLAST searches assume that proteins may be modeled as random sequences. However, many real proteins comprise regions of nonrandom sequences which may be homopolymeric tracts, short-period repeats, or regions enriched in one or more amino acids. Such low-complexity regions may be aligned between unrelated proteins even though other regions of the protein are entirely dissimilar. A number of low-complexity filter programs may be employed to reduce such low-complexity alignments. For example, the SEG (Wooten and Federhen, *Comput. Chem.*, 17:149-163 (1993)) and XNU (Claverie and States, *Comput. Chem.*,

17:191-201 (1993)) low-complexity filters may be employed alone or in combination. (c) The term "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences is used herein to refer to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, i.e., where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g. charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well-known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, *Computer Applic. Biol. Sci.*, 4:11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif., USA). (d) The term "percentage of sequence identity" is used herein mean the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by

determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. (e) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70% sequence identity, at least 80% sequence identity, at least 90% sequence identity and at least 95% sequence identity, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill will recognize that these values may be adjusted appropriately to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of at least 60%, or at least 70%, at least 80%, at least 90%, or at least 95%. Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. However, nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides that they encode are substantially identical. This may occur, e.g., when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is that the polypeptide that the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid. Mutations may also be made to the nucleotide sequences of the present proteins by reference to the genetic code, including taking into account codon degeneracy.

[00162] The term "prime" (or "priming") as used herein refers to the process of increasing sensitivity to. When used in an immunological sense it refers to a process whereby a specific antigen is presented to naïve lymphocytes causing them to differentiate.

[00163] The term "progression free survival" or "PFS" as used herein refers to length of time during and after the treatment of a disease, such as cancer, that a patient lives with the disease but it does not get worse. In a clinical trial, measuring the progression free survival is one way to determine how well a new treatment works.

[00164] The term “recurrence” as used herein with respect to cancer refers to a cancer that has recurred (come back), usually after a period of time during which the cancer could not be detected. The cancer may come back to the same place as the original (primary) tumor or to another place in the body.

[00165] The term "relapse-free survival (RFS)" as used herein refers to the length of time after primary treatment for a cancer during which the patient survives without any signs or symptoms of that cancer. Also called disease-free survival (DFS) and progression free survival (PFS).

[00166] The term “response rate” as used herein refers to the percentage of patients whose cancer shrinks or disappears after treatment.

[00167] The term “resistant cancer” as used herein refers to a cancer that does not respond to a treatment at the beginning of such treatment or sometime during such treatment.

[00168] The term “reporter gene” (“reporter”) or "assay marker" refers to a gene and/or peptide that can be detected, or easily identified and measured. The expression of the reporter may be measured at either the RNA level, or at the protein level. The gene product, which may

be detected in an experimental assay protocol, includes, but is not limited to, marker enzymes, antigens, amino acid sequence markers, cellular phenotypic markers, nucleic acid sequence markers, and the like. Researchers may attach a reporter gene to another gene of interest in cell culture, bacteria, animals, or plants. For example, some reporters are selectable markers, or confer characteristics upon on organisms expressing them allowing the organism to be easily identified and assayed. To introduce a reporter gene into an organism, researchers may place the reporter gene and the gene of interest in the same DNA construct to be inserted into the cell or organism. For bacteria or eukaryotic cells in culture, this may be in the form of a plasmid. Commonly used reporter genes may include, but are not limited to, fluorescent proteins, luciferase, beta-galactosidase, and selectable markers, such as chloramphenicol and kanomycin.

[00169] The term "stimulate" in any of its grammatical forms as used herein refers to inducing activation or increasing activity.

[00170] As used herein, the terms "subject" or "individual" or "patient" are used interchangeably to refer to a member of an animal species of mammalian origin, including humans.

[00171] The phrase "subject in need thereof" as used herein refers to a patient that (i) will be administered an immunogenic composition according to the described invention, (ii) is receiving an immunogenic composition according to the described invention; or (iii) has received an immunogenic composition according to the described invention, unless the context and usage of the phrase indicates otherwise.

[00172] The term “therapeutic agent” as used herein refers to a drug, molecule, nucleic acid, protein, metabolite, composition or other substance that provides a therapeutic effect. The term “active” as used herein refers to the ingredient, component or constituent of the compositions of the described invention responsible for the intended therapeutic effect. The terms “therapeutic agent” and “active agent” are used interchangeably herein. The term "therapeutic component" as used herein refers to a therapeutically effective dosage (i.e., dose and frequency of administration) that eliminates, reduces, or prevents the progression of a particular disease manifestation in a percentage of a population. An example of a commonly used therapeutic component is the ED50 which describes the dose in a particular dosage that is therapeutically effective for a particular disease manifestation in 50% of a population.

[00173] The terms “therapeutic amount”, "therapeutically effective amount", an "amount effective", or “pharmaceutically effective amount” of an active agent is used interchangeably to refer to an amount that is sufficient to provide the intended benefit of treatment. However, dosage levels are based on a variety of factors, including the type of injury, the age, weight, sex, medical condition of the patient, the severity of the condition, the route of administration, and the particular active agent employed. Thus the dosage regimen may vary widely, but can be determined routinely by a physician using standard methods. Additionally, the terms “therapeutic amount”, “therapeutically effective amounts” and “pharmaceutically effective amounts” include prophylactic or preventative amounts of the compositions of the described invention. In prophylactic or preventative applications of the described invention, pharmaceutical compositions or medicaments are administered to a patient susceptible to, or otherwise at risk of, a disease, disorder or condition in an amount sufficient to eliminate or reduce the risk, lessen the severity, or delay the onset of the disease, disorder or condition,

including biochemical, histologic and/or behavioral symptoms of the disease, disorder or condition, its complications, and intermediate pathological phenotypes presenting during development of the disease, disorder or condition. It is generally preferred that a maximum dose be used, that is, the highest safe dose according to some medical judgment. The terms "dose" and "dosage" are used interchangeably herein.

[00174] The term "therapeutic effect" as used herein refers to a consequence of treatment, the results of which are judged to be desirable and beneficial. A therapeutic effect can include, directly or indirectly, the arrest, reduction, or elimination of a disease manifestation. A therapeutic effect can also include, directly or indirectly, the arrest reduction or elimination of the progression of a disease manifestation.

[00175] For any therapeutic agent described herein the therapeutically effective amount may be initially determined from preliminary in vitro studies and/or animal models. A therapeutically effective dose may also be determined from human data. The applied dose may be adjusted based on the relative bioavailability and potency of the administered compound. Adjusting the dose to achieve maximal efficacy based on the methods described above and other well-known methods is within the capabilities of the ordinarily skilled artisan.

[00176] General principles for determining therapeutic effectiveness, which may be found in Chapter 1 of Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10th Edition, McGraw-Hill (New York) (2001), incorporated herein by reference, are summarized below.

[00177] Pharmacokinetic principles provide a basis for modifying a dosage regimen to obtain a desired degree of therapeutic efficacy with a minimum of unacceptable adverse effects. In

situations where the drug's plasma concentration can be measured and related to the therapeutic window, additional guidance for dosage modification can be obtained.

[00178] Drug products are considered to be pharmaceutical equivalents if they contain the same active ingredients and are identical in strength or concentration, dosage form, and route of administration. Two pharmaceutically equivalent drug products are considered to be bioequivalent when the rates and extents of bioavailability of the active ingredient in the two products are not significantly different under suitable test conditions.

[00179] The term "therapeutic window" refers to a concentration range that provides therapeutic efficacy without unacceptable toxicity. Following administration of a dose of a drug, its effects usually show a characteristic temporal pattern. A lag period is present before the drug concentration exceeds the minimum effective concentration ("MEC") for the desired effect. Following onset of the response, the intensity of the effect increases as the drug continues to be absorbed and distributed. This reaches a peak, after which drug elimination results in a decline in the effect's intensity that disappears when the drug concentration falls back below the MEC. Accordingly, the duration of a drug's action is determined by the time period over which concentrations exceed the MEC. The therapeutic goal is to obtain and maintain concentrations within the therapeutic window for the desired response with a minimum of toxicity. Drug response below the MEC for the desired effect will be subtherapeutic, whereas for an adverse effect, the probability of toxicity will increase above the MEC. Increasing or decreasing drug dosage shifts the response curve up or down the intensity scale and is used to modulate the drug's effect. Increasing the dose also prolongs a drug's duration of action but at the risk of increasing

the likelihood of adverse effects. Accordingly, unless the drug is nontoxic, increasing the dose is not a useful strategy for extending a drug's duration of action.

[00180] Instead, another dose of drug should be given to maintain concentrations within the therapeutic window. In general, the lower limit of the therapeutic range of a drug appears to be approximately equal to the drug concentration that produces about half of the greatest possible therapeutic effect, and the upper limit of the therapeutic range is such that no more than about 5% to about 10% of patients will experience a toxic effect. These figures can be highly variable, and some patients may benefit greatly from drug concentrations that exceed the therapeutic range, while others may suffer significant toxicity at much lower values. The therapeutic goal is to maintain steady-state drug levels within the therapeutic window. For most drugs, the actual concentrations associated with this desired range are not and need not be known, and it is sufficient to understand that efficacy and toxicity are generally concentration-dependent, and how drug dosage and frequency of administration affect the drug level. For a small number of drugs where there is a small (two- to three-fold) difference between concentrations resulting in efficacy and toxicity, a plasma-concentration range associated with effective therapy has been defined.

[00181] In this case, a target level strategy is reasonable, wherein a desired target steady-state concentration of the drug (usually in plasma) associated with efficacy and minimal toxicity is chosen, and a dosage is computed that is expected to achieve this value. Drug concentrations subsequently are measured and dosage is adjusted if necessary to approximate the target more closely.

[00182] In most clinical situations, drugs are administered in a series of repetitive doses or as a continuous infusion to maintain a steady-state concentration of drug associated with the therapeutic window. To maintain the chosen steady-state or target concentration ("maintenance dose"), the rate of drug administration is adjusted such that the rate of input equals the rate of loss. If the clinician chooses the desired concentration of drug in plasma and knows the clearance and bioavailability for that drug in a particular patient, the appropriate dose and dosing interval can be calculated.

[00183] As used herein the term “treating” includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical symptoms of a condition, or substantially preventing the appearance of clinical symptoms of a condition. Treating further refers to accomplishing one or more of the following: (a) reducing the severity of the disorder; (b) limiting development of symptoms characteristic of the disorder(s) being treated; (c) limiting worsening of symptoms characteristic of the disorder(s) being treated; (d) limiting recurrence of the disorder(s) in patients that have previously had the disorder(s); and (e) limiting recurrence of symptoms in patients that were previously asymptomatic for the disorder(s).

[00184] The term “vaccinated” as used herein refers to being treated with a vaccine.

[00185] The term “vaccination” as used herein refers to treatment with a vaccine.

[00186] The term “vaccine” as used herein refers a substance or group of substances meant to cause the immune system to respond to a tumor or to microorganisms, or help the body recognize and destroy cancer cells or microorganisms. The term vaccine also refers to an

artificial stimulus used to stimulate a robust immune response against that exposure (e.g. infectious agent, cancer cell).

[00187] The term "vaccine therapy" as used herein refers to a type of treatment that uses a substance or group of substances to stimulate the immune system to destroy a tumor or infectious microorganisms.

Allogeneic Vaccine

[00188] Vaccine proteins can induce immune responses that find use in the described invention. According to one aspect, the described invention comprises a tumor-type specific allogeneic tumor vaccine for the treatment of cancer. According to some embodiments, the cancer is prostate cancer. According to some embodiments, the vaccine comprises an allogeneic cancer cell line that is genetically modified by two or more immunomodulatory molecules. According to some embodiments, the tumor cell provides a broad array of tumor specific antigens, most of which are of unknown nature. According to some embodiments, the immunomodulatory molecules genetically engineered or added to the cells are selected from a group for their ability to either initiate or sustain an anti-tumor immune response, or alternatively for their ability to abrogate pre-existing immunosuppression characteristically present in cancer patients, or a combination of all three. According to some embodiments, combinations of immunomodulatory molecules are evaluated and selected by a human mixed lymphocyte tumor cell reaction.

[00189] According to some embodiments, the allogeneic vaccine composition is administered to a subject diagnosed with cancer in combination with an agent that inhibits immunosuppressive molecules produced by tumor cells.

[00190] According to some embodiments, the allogeneic vaccine further comprises one or more checkpoint inhibitors that are sufficient to prevent premature termination of an effective immune response once such an immune response is initiated.

[00191] According to some embodiments, a subject (i.e. a subject diagnosed with cancer) is treated by checkpoint inhibitor therapy prior to or concurrently with administration of the allogeneic vaccine composition. In certain embodiments, the cancer is a melanoma.

[00192] Checkpoint Blockade/Blockage of Tumor Immunosuppression

[00193] Some human tumors can be eliminated by a patient's immune system. For example, administration of a monoclonal antibody targeted to an immune "checkpoint" molecule can lead to complete response and tumor remission. A mode of action of such antibodies is through inhibition of an immune regulatory molecule that the tumors have co-opted as protection from an anti-tumor immune response. By inhibiting these "checkpoint" molecules (e.g., with an antagonistic antibody), a patient's CD8+ T cells may be allowed to proliferate and destroy tumor cells.

[00194] For example, administration of a monoclonal antibody targeted to by way of example, without limitation, CTLA-4 or PD-1 can lead to a complete response and tumor remission. The mode of action of such antibodies is through inhibition of CTLA-4 or PD-1 that the tumors have co-opted as protection from an anti-tumor immune response. By inhibiting these "checkpoint" molecules (e.g., with an antagonistic antibody), a patient's CD8+ T cells may be allowed to proliferate and destroy tumor cells.

[00195] Thus, the allogeneic vaccine compositions provided herein can be used in combination with one or more blocking antibodies targeted to an immune "checkpoint" molecule. For instance, in some embodiments, the allogeneic vaccine compositions provided

herein can be used in combination with one or more blocking antibodies targeted to a molecule such as CTLA-4 or PD-1. For example, the allogeneic vaccine compositions provided herein may be used in combination with an agent that blocks, reduces and/or inhibits PD-1 and PD-L1 or PD-L2 and/or the binding of PD-1 with PD-L1 or PD-L2 (by way of non-limiting example, one or more of nivolumab (ONO-4538/BMS-936558, MDX1106, OPDIVO, BRISTOL MYERS SQUIBB), pembrolizumab (KEYTRUDA, Merck), pidilizumab (CT-011, CURE TECH), MK-3475 (MERCK), BMS 936559 (BRISTOL MYERS SQUIBB), MPDL328OA (ROCHE)). In an embodiment, the allogeneic vaccine compositions provided herein may be used in combination with an agent that blocks, reduces and/or inhibits the activity of CTLA-4 and/or the binding of CTLA-4 with one or more receptors (e.g. CD80, CD86, AP2M1, SHP-2, and PPP2R5A). For instance, in some embodiments, the immune-modulating agent is an antibody such as, by way of non-limitation, ipilimumab (MDX-010, MDX-101, Yervoy, BMS) and/or tremelimumab (Pfizer). Blocking antibodies against these molecules can be obtained from, for example, Bristol Myers Squibb (New York, N.Y.), Merck (Kenilworth, N.J.), Medlmmune (Gaithersburg, Md.), and Pfizer (New York, N.Y.).

[00196] Further, the allogeneic immune compositions provided herein can be used in combination with one or more blocking antibodies targeted to an immune "checkpoint" molecule such as for example, BTLA, HVEM, TIM3, GALS, LAG3, VISTA, KIR, 2B4, CD160 (also referred to as BY55), CGEN-15049, CHK 1 and CHK2 kinases, A2aR, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), GITR, GITRL, galectin-9, CD244, CD160, TIGIT, SIRPα, ICOS, CD172a, and TMIGD2 and various B-7 family ligands (including, but are not limited to, B7-1, B7-2, B7-DC, B7-H1, B7-H2, B7-H3, B7-H4, B7-H5, B7-H6 and B7-H7).

[00197] According to some embodiments, the allogeneic vaccine is adapted for rapid in vitro evaluation using human peripheral blood mononuclear cells from healthy subjects and cancer patients to examine inter-individual variability as well as normal to patient differences, thus avoiding animal experimentation.

[00198] According to some embodiments, the described invention comprises an allogeneic tumor cell vaccine for an active immunotherapy that can be universally administered to all patients with a particular type of cancer. According to some embodiments, the allogeneic vaccine comprises a genetically modified allogeneic tumor-type specific cell, or a membrane lysate derived from modified allogeneic tumor-type specific cells, formulated in a pharmaceutically acceptable carrier. According to some embodiments, the modified allogeneic tumor-type specific cells are derived from previously established cell lines.

[00199] According to some embodiments, the allogeneic vaccine is adapted to treat patients with minimal residual disease and a functional immune system. For example, according to some embodiments, the allogeneic vaccine is adapted to treat a patient with minimal residual disease obtained shortly after a primary lesion is surgically removed. According to some embodiments, the allogeneic vaccine is adapted for subcutaneous administration of the vaccine. According to some embodiments, the dose and schedule for administering the allogeneic vaccine are determined by using immunologic responses to the vaccine as a guide for eventual enhancement of overall survival.

[00200] According to some embodiments, the allogeneic vaccine is adapted to provide clinical benefit in the short term by the induction of strong anti-allogeneic vaccine responses, and, in the long term, to provide a long lived and cross reactive response to the endogenous unmodified host

tumor. According to some embodiments, the immune response against the allogeneic tumor cell vaccine comprises a heteroclitic cross reaction between a peptide native to the tumor cell line and a peptide native to the tumor cells of a patient receiving the vaccine (See, e.g., FIG. 1). According to some embodiments, the heteroclitic cross reaction enhances immunogenicity via enhanced binding of a T cell receptor with a tumor cell peptide-MHC complex that normally provides a non-immunogenic surface. According to some embodiments, the allogeneic tumor cell vaccine comprises peptides altered relative to tumor cells of a subject with cancer, where the altered peptides provide an immunogenic surface that results in a heteroclitic cross-reaction to the non-immunogenic peptide of tumor cells from the subject with cancer. According to some embodiments, the heteroclitic recognition and alloreactive antigen recognition of the tumor cell vaccine provides a broad array of antigens useful to elicit an immune response against the tumor cells of a patient receiving the vaccine. According to some embodiments, the allogeneic vaccine is adapted to provide a clinical benefit, e.g., in the form of progression free survival, relapse-free survival, or overall survival. According to some embodiments, the allogeneic vaccine is effective to provide heteroclitic immunization induced tumor immunity (Dyall R., et al., Heteroclitic Immunization Induces Tumor Immunity, J. Exp. Med., Vol. 188, No. 9, November 2, 1998, incorporated by reference herein in its entirety).

[00201] According to some embodiments, the allogeneic vaccine is derived from tumor cell lines genetically modified to comprise recombinant immunomodulatory signals that are expressed in therapeutic amounts. According to some embodiments, the allogeneic vaccine is derived from a uniform starting material, such as a tumor cell line, wherein multiple discrete biologics are expressed in the starting material in either soluble or membrane bound form. According to some embodiments, expression and activity of the soluble and membrane bound

forms are confirmed, in vitro, by flow cytometry and mixed lymphocyte tumor assays using peripheral blood mononuclear cells, respectively. According to some embodiments, expression and activity of the soluble and membrane bound forms are confirmed, in vitro, by flow cytometry and mixed lymphocyte tumor assays using peripheral blood mononuclear cells of the vaccinated cancer patient against the allogeneic tumor cells used to immunize.

[00202] According to some embodiments, the allogeneic vaccine comprises genetically modified immunomodulatory molecules each encoding a membrane bound or secreted signaling molecule. According to some embodiments, each membrane bound immunomodulatory molecule is adapted to deliver a therapeutic amount in sub-pharmacologic doses that is active in a spatially and temporally restricted manner to provide signaling predominantly at the time and place of antigen presentation. According to some embodiments, the membrane bound immunomodulatory molecules are adapted to decrease the probability of systemic side effects. According to some embodiments, the secreted immunomodulatory molecules are adapted to deliver local, not systemic, signals.

[00203] According to some aspects, the allogeneic vaccine comprises genetic material that is effective to genetically introduce one or more immunomodulatory molecules into a tumor cell line. According to some embodiments, the genetic material can be introduced by viral transduction techniques and isolated by positive selection for the genetically introduced immune modulator. For example, according to some embodiments, the positive selection of the genetically introduced immune modulator molecule comprises selection using antibodies. According to some embodiments, the immunomodulatory molecules are diverse and complementary with respect to impact on key immune cell subsets such as dendritic cell,

lymphocyte sub-populations (e.g. T cells, Natural Killer cells, and T-regulatory cells). According to some embodiments, the allogeneic vaccine comprises a variety of immunomodulatory molecules directed to a variety of immunomodulatory pathways on various immune cell subsets, wherein not all pathways will equally contribute to immunogenic response in individual cancer patients. According to some embodiments, the immunomodulatory molecules genetically introduced into a tumor cell line are stably expressed.

Tumor Antigen Specificity

[00204] Immunologic antigenic specificity may arise from one or more of 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.

[00205] Immunologic antigen specificity to a certain type of cancer cell may also arise from one or more of a particular fingerprint of a plurality of tumor antigens, 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, 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, and by omitting one or more peptides that may provoke immunotolerance. The skilled artisan can locate the relevant nucleic acid and polypeptide sequences, e.g., on the U.S. government's web site, at ncbi.nlm.nih.

[00206] According to some embodiments, tumor antigen specificity of the present invention may be determined by the parental tumor cell line that is selected for modification with immune modulators.

Parent Cell Lines

[00207] According to some embodiments, tumor cell line variants may be derived from established cell lines from either public sources (e.g. NIH, DCTD Tumor Repository operated by Charles River Laboratories Inc.) or commercial sources (e.g. ATCC, Sigma Alrich, Thermo Fischer Scientific, Genescript, DSM2). According to some embodiments, new cell lines can be established de novo from tumor cells taken from the tumor of a cancer patient.

[00208] According to some embodiments, cancer tissues, cancer cells, cells infected with a cancer-causing agent, other preneoplastic cells, and cell lines of human origin can be used. In some embodiments, a cancer cell can be from an established tumor cell line such as, without limitation, an established non-small cell lung carcinoma (NSCLC), bladder cancer, melanoma, ovarian cancer, renal cell carcinoma, prostate carcinoma, sarcoma, breast carcinoma, squamous cell carcinoma, head and neck carcinoma, hepatocellular carcinoma, pancreatic carcinoma, or colon carcinoma cell line.

[00209] According to some embodiments, the established cell lines comprise the LNCaP clone FGC (ATCC CRL-1740), which itself is derived from a metastatic prostate cancer that had migrated to a lymph node. According to some embodiments, the established cell lines comprise the PC-3 (ATCC CRL-1435) cell line, which itself is derived from metastatic prostate cancer that migrated to bone. According to some embodiments, the tumor cell line variants are derived from one or more of the following ATCC cell lines: VCaP (ATCC CRL-2876); MDA PCa 2b (ATCC CRL-2422); or DU 145 (ATCC HTB-81).

[00210] According to some embodiments, the established cell lines comprise the SK-MEL-2 clone (ATCC HTB-68), which itself is derived from metastasis on skin of thigh.

[00211] According to some embodiments, the established cell lines comprise one or more of mammary carcinoma cell lines designated COO-G, DU4475, ELL-G, HIG-G, MCF/7, MDA-MB-436, MX-1, SW-613, and VAN-G. According to some embodiments, the established cell lines comprise one or more of alveolar soft part sarcoma cell lines designated ASPS, and ASPS-1. According to some embodiments, the established cell lines comprise one or more lung cell lines designated LX-1, COS-G, H-MESO-1, H-MESO-1A, NCI-H23, and NCI-H460. According to some embodiments, the established cell lines comprise one or more colon cancer cell lines designated CX-5, GOB-G, HCC-2998, HCT-15, KLO-G, KM20L2, MRI-H-194, LOVO I, LOVO II, and MRI-H-250. According to some embodiments, the established cell lines comprise one or more melanoma cell lines designated NIS-G, TRI-G, WIL-G, MRI-H-121B, MRI-H-187, MRI-H-221, and MRI-H-255. According to some embodiments, the established cell lines comprise one or more cervical cancer cell lines designated MRI-H-177, MRI-H-186, MRI-H-196, and MRI-H-215. According to some embodiments, the established cell lines comprise one or more kidney cancer cell lines designated MRI-H-121 and MRI-H-166. According to some embodiments, the established cell lines comprise one or more endometrium cancer cell lines designated MRI-H-147 and MRI-H-220. According to some embodiments, the established cell lines comprise one or more ovarian cancer cell lines designated MRI-H-258, MRI-H-273, MRI-H-1834, and SWA-G. According to some embodiments, the established cell lines comprise one or more sarcoma cell lines designated HS-1, OGL-G, and DEL-G. According to some embodiments, the established cell lines comprise the epidermoid cell line designated DEAC-1. According to some embodiments, the established cell line comprises the glioblastoma cell line designated SF 295. According to some embodiments, the established cell line comprises

the prostate cancer cell line designated CWR-22. According to some embodiments, the established cell line comprises the Burkitt's lymphoma cell line designated DAU.

[00212] According to some embodiments, exemplary established cell lines comprise one or more of the following cell lines:

Designation	**Tissue of Origin**	**Histologic Type**
786-0	Kidney	Renal Cell Carcinoma
A2780	Ovary	Adenocarcinoma
A498	Kidney	Renal Cell Carcinoma
A549	Lung	Non-small Cell
A704	Kidney	Renal Cell Carcinoma
ACHN	Kidney	Renal Cell Carcinoma
ASPS-1	Lymph Node	Alveolar Soft Part Sarcoma
BT-549	Breast	Adenocarcinoma
CAKI-1	Kidney	Renal Cell Carcinoma
CCRF-CEM	Lymph	Leukemia
CCRF-SB	Lymph	Leukemia
CHA-59	Bone	Osteosarcoma
COLO 205	Colon	Adenocarcinoma
DMS-114	Lung	Small Cell
DU-145	Prostate	Carcinoma
EKVX	Lung	Adenocarcinoma
HCC-2998	Colon	Adenocarcinoma

Designation	Tissue of Origin	Histologic Type
HCT-15	Colon	Carcinoma
HCT-116	Colon	Adenocarcinoma
HOP-18	Lung	Large Cell Carcinoma
HOP-62	Lung	Adenocarcinoma
HL-60	Ascites	Pro-myelocytic Leukemia
H-MESO-1		Mesothelioma
HS 578T	Breast	Adenocarcinoma
HS 913T	Lung	Mixed Cell
HT-29	Colon	Adenocarcinoma
IGR-OV1	Ovary	Adenocarcinoma
KM-12	Colon	Adenocarcinoma
KM 20L2	Colon	Adenocarcinoma
K-562	Lymph	Leukemia
LOVO	Colon	Adenocarcinoma
LOX IMVI	Lymph Node Metastisis	Amelanotic Melanoma
LXFL 529	Lung	Large Cell Carcinoma
NCI-H1299	Lung	Adenocarcinoma
NCI-H2887	Lung	Adenocarcinoma
NCI-H3122	Lung	Adenocarcinoma
NCI-H322M	Lung	Adenocarcinoma
NCI-H3255	Lung	Adenocarcinoma

Designation	Tissue of Origin	Histologic Type
NCI-H358M	Lung	Bronchioalveolar Carcinoma
NCI-H460	Lung	Large Cell
NCI-H522	Lung	Adenocarcinoma
NCI-H69	Lung	Small Cell Carcinoma
NCI-H82	Lung	Small Cell Carcinoma
NCI-H838	Lung	Adenocarcinoma
NCI/ADR-RES	Ovary	Adenocarcinoma
OVCAR-3	Ovary	Adenocarcinoma
OVCAR-4	Ovary	Adenocarcinoma
OVCAR-5	Ovary	Adenocarcinoma
OVCAR-8	Ovary	Adenocarcinoma
PC-3	Prostate	Carcinoma
PC-3/M	Prostate	Carcinoma
RPMI-7951	Skin	Melanoma
RPMI-8226	Lymph	Leukemia
RXF 393	Kidney	Renal Cell Carcinoma
RXF 631	Kidney	Renal Cell Carcinoma
TK-10	Kidney	Renal Cell Carcinoma
UACC-62	Skin	Melanoma
UACC-257	Skin	Melanoma
UCSD 242L	Skin	Melanoma

Designation	Tissue of Origin	Histologic Type
UCSD 354K	Skin	Melanoma
UO-31	Kidney	Renal Cell Carcinoma
U-251	CNS	Glioblastoma
WIDR	Colon	Adenocarcinoma
XF 498	CNS	Glioblastoma

[00213] According to some embodiments, the choice of the parental cell line from which the tumor cell line variant may be derived affects the specificity of the allogeneic vaccine. For example, the use of a tumor cell line variant derived from metastatic prostate cancer that migrated to the bone of a patient may result in an allogeneic vaccine that elicits an immune response specific for metastatic prostate cancer in the bone of a patient.

[00214] According to some embodiments, the tumor cell line variants may be derived from a parental cell that comprises a universal cancer specific antigen. For example, the use of a parental tumor cell line variant derived from metastatic prostate cancer that migrated to the bone of a patient may result in an allogeneic vaccine that elicits an immune response against all prostate cancer cells.

[00215] According to some embodiments, the tumor cell line variants are derived from patient derived cells derived from various cancers. According to some embodiments, fresh tissue surgically removed from a tumor is enzymatically digested by type IV collagenase, followed by collection of disaggregated cells. According to some embodiments, disaggregated cells may then be grown in vitro in growth media with 10% fetal bovine serum on an extracellular matrix substrate, such as collagen or fibronectin, to promote attachment. According to some

embodiments, adherent cells may then be passaged until the immortal cancer cells outgrow the non-cancerous fibroblast cells.

[00216] For example, according to some embodiments, the tumor cell line variants may be derived from a solid tumor comprising tumor cells, including cancer stem cells, a metastatic cancer comprising metastatic tumor cells, comprising cancer stem cells, or a non-metastatic cancer. According to some embodiments, the cancer may originate in the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, duodenum, small intestine, large intestine, colon, rectum, anus, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. According to some embodiments, the cancer may be of a histological type, e.g., a cancer that begins in the skin or tissues that line or cover internal organs (carcinoma); a cancer that begins in bone or in the soft tissue of the body including cartilage, fat, muscle, blood vessels, and fibrous tissue (sarcoma); a cancer that starts in blood-forming tissue (leukemia); a cancer that begins in cells of the immune system (lymphoma); a cancer that arises in plasma cells (myeloma), or a brain/spinal cord cancer.

[00217] Examples of carcinomas include, without limitation, giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; an adenocarcinoma; a gastrinoma, a cholangiocarcinoma; a hepatocellular carcinoma; a combined hepatocellular carcinoma and cholangiocarcinoma; a trabecular adenocarcinoma; an adenoid cystic carcinoma; an adenocarcinoma in adenomatous polyp; an adenocarcinoma, familial polyposis coli; a solid carcinoma; a carcinoid tumor; a branchiolo-alveolar adenocarcinoma; a papillary adenocarcinoma; a chromophobe carcinoma; an

acidophil carcinoma; an oxyphilic adenocarcinoma; a basophil carcinoma; a clear cell adenocarcinoma; a granular cell carcinoma; a follicular adenocarcinoma; a non-encapsulating sclerosing carcinoma; adrenal cortical carcinoma; an endometroid carcinoma; a skin appendage carcinoma; an apocrine adenocarcinoma; a sebaceous adenocarcinoma; a ceruminous adenocarcinoma; a mucoepidermoid carcinoma; a cystadenocarcinoma; a papillary cystadenocarcinoma; a papillary serous cystadenocarcinoma; a mucinous cystadenocarcinoma; a mucinous adenocarcinoma; a signet ring cell carcinoma; an infiltrating duct carcinoma; a medullary carcinoma; a lobular carcinoma; an inflammatory carcinoma; paget's disease, a mammary acinar cell carcinoma; an adenosquamous carcinoma; an adenocarcinoma w/squamous metaplasia; a sertoli cell carcinoma; embryonal carcinoma; choriocarcinoma.

[00218] Examples of sarcomas include, without limitation, glomangiosarcoma; sarcoma; fibrosarcoma; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; carcinosarcoma; synovial sarcoma; hemangiosarcoma; kaposi's sarcoma; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; myeloid sarcoma; mast cell sarcoma.

[00219] Examples of leukemias include, without limitation, leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; and hairy cell leukemia.

[00220] Examples of lymphomas and myelomas include, without limitation, malignant lymphoma; hodgkin's disease; hodgkin's; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-hodgkin's lymphomas; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malignant melanoma in giant pigmented nevus; epithelioid cell melanoma; multiple myeloma.

[00221] Examples of brain/spinal cord cancers include, without limitation, pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant.

[00222] Examples of other cancers include, without limitation, a thymoma; an ovarian stromal tumor; a thecoma; a granulosa cell tumor; an androblastoma; a leydig cell tumor; a lipid cell tumor; a paraganglioma; an extra-mammary paraganglioma; a pheochromocytoma; blue nevus, malignant; fibrous histiocytoma, malignant; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; mesothelioma, malignant; dysgerminoma; teratoma, malignant; struma ovarii, malignant; mesonephroma, malignant; hemangioendothelioma, malignant; hemangiopericytoma, malignant; chondroblastoma, malignant; granular cell tumor, malignant; malignant histiocytosis; immunoproliferative small intestinal disease.

[00223] For any given tumor type, several tumor cell lines may be commercially available. According to some embodiments, pooling of several of these cells lines, either as a mixture of whole cells or by making a membrane preparation out of the mixture of whole cells, may provide an array of cell surface tumor antigens for that tumor type.

Selection of Immune Modulators

[00224] According to some embodiments, the tumor cell line variants may be engineered to express two or more recombinant sequences of DNA and protein that are then presented on the tumor cell and are functional.

IgG Heavy Chain Constant and Variable Region

[00225] Immunoglobulins (Ig) are glycoproteins produced by immune cells. Antibodies are serum proteins, the molecules of which possess small areas of their surface that are complementary to small chemical groupings on their targets. These complementary regions (referred to as complementary determining regions (CDRs), or antibody combining sites, or antigen binding sites) of which there are at least two per antibody molecule, and in some types of antibody molecules ten, eight, or in some species as many as 12, may react with their corresponding complementary region on the antigen (the antigenic determinant or epitope) to link several molecules of multivalent antigen together to form a lattice. Immunoglobulins play a critical role in an immune response by binding to particular antigens, such as those exhibited by bacteria or viruses. According to some embodiments, the binding of immunoglobulins to antigens may target them for destruction by the subject's immune cells.

[00226] The basic structural unit of a whole antibody molecule consists of four polypeptide chains, two identical light (L) chains (each containing about 220 amino acids) and two identical

heavy (H) chains (each usually containing about 440 amino acids). The two heavy chains and two light chains are held together by a combination of noncovalent and covalent (disulfide) bonds. The molecule is composed of two identical halves, each with an identical antigen-binding site composed of the N-terminal region of a light chain and the N-terminal region of a heavy chain. Both light and heavy chains usually cooperate to form the antigen binding surface.

[00227] In mammals, there are five classes of antibodies, IgA, IgD, IgE, IgG, and IgM, each with its own class of heavy chain- α (for IgA), δ (for IgD), ε (for IgE), γ (for IgG) and μ (for IgM). In addition, there are four subclasses of IgG immunoglobulins (IgG1, IgG2, IgG3, IgG4) having γ1, γ2, γ3, and γ4 heavy chains respectively. In its secreted form, IgM is a pentamer composed of five four-chain units, giving it a total of 10 antigen binding sites. Each pentamer contains one copy of a J chain, which is covalently inserted between two adjacent tail regions.

[00228] Diverse libraries of immunoglobulin heavy (VH) and light (Vκ and Vλ) chain variable genes from peripheral blood lymphocytes also can be amplified by polymerase chain reaction (PCR) amplification. Genes encoding single polypeptide chains in which the heavy and light chain variable domains are linked by a polypeptide spacer can be made by randomly combining heavy and light chain V-genes using PCR.

[00229] According to some embodiments, the tumor cell line variants may be engineered to express an IgG1 heavy chain constant region. In nature, the Ig gamma-1 (IgG-1) chain C region is a protein encoded by the IGHG1 gene in humans. According to some embodiments, a tumor cell line variant may express a membrane bound form IgG-1 chain C protein of SEQ ID NO: 1. According to some embodiments, a tumor cell line variant may express a secreted form of IgG-1 chain C of SEQ ID NO: 2. According to some embodiments, a tumor cell line variant may

express a secreted form of IgG-1 chain C of SEQ ID NO: 3. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to one or more of proteins with an amino acid sequence SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to one or more of proteins with an amino acid sequence SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to one or more of proteins with an amino acid sequence SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 90% to one or more of proteins with an amino acid sequence SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 95% to one or more of proteins with an amino acid sequence SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to one or more of proteins with an amino acid sequence SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 97% to one or more of proteins with an amino acid sequence SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 98% to one or more of proteins with an amino acid sequence SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3. According to some embodiments, a tumor cell line variant may comprise one or

more proteins with a sequence identity of at least 99% to one or more of proteins with an amino acid sequence SEQ ID NO: 1, SEQ ID NO: 2, and SEQ ID NO: 3.

[00230] According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to one or more proteins with amino acid sequence SEQ ID NO: 12, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 45, and SEQ ID NO: 46. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to one or more proteins with amino acid sequence SEQ ID NO: 12, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 45, and SEQ ID NO: 46. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to one or more proteins with amino acid sequence SEQ ID NO: 12, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 45, and SEQ ID NO: 46. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 90% to one or more proteins with amino acid sequence SEQ ID NO: 12, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 45, and SEQ ID NO: 46. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 95% to one or more proteins with amino acid sequence SEQ ID NO: 12, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 45, and

SEQ ID NO: 46. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to one or more proteins with amino acid sequence SEQ ID NO: 12, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 45, and SEQ ID NO: 46. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 97% to one or more proteins with amino acid sequence SEQ ID NO: 12, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 45, and SEQ ID NO: 46. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 98% to one or more proteins with amino acid sequence SEQ ID NO: 12, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 45, and SEQ ID NO: 46. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 99% to one or more proteins with amino acid sequence SEQ ID NO: 12, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 45, and SEQ ID NO: 46.

[00231] According to some embodiments, the tumor cell line variant may be engineered to express an IgG protein that is capable of binding to tumor cell specific antigens. For example, the tumor cell line variant may be engineered to express an IgG protein capable of binding to a prostate cancer specific antigen; e.g., the extracellular region of prostate-specific membrane antigen (PSMA) (See Chang, S., Overview of Prostate-Specific Membrane Antigen, Reviews in Urology, Vol.6 Suppl. 10, S13 (2004)). According to some embodiments, the tumor cell line

variant may be engineered to express an IgG protein that is capable of binding to immune cell specific antigens. For example, the tumor cell line variant may be engineered to express an IgG protein capable of binding to T cell markers, e.g., CD3, CD4, or CD8. According to another example, the tumor cell line variant may be engineered to express an IgG protein capable of binding to dendritic cell markers, e.g. CD11c or CD123.

[00232] According to some embodiments, the tumor cell line variants may be engineered to express an IgG3 heavy chain constant region. In nature, the IgG3 heavy chain constant region comprises CH1-hinge-CH2-CH3 domains, and is encoded by the IGHG3 gene in humans; the IGHG3 gene comprises structural polymorphisms comprising different hinge lengths. According to some embodiments, a tumor cell line variant may express an IgG-3 heavy chain constant region of SEQ ID NO: 4. According to some embodiments, a tumor cell line variant may express a derivative of SEQ ID NO: 4 with amino acids 1-76 missing. According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 with amino acids 1-76 missing. According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 with amino acids 77-98 replaced with amino acids QMQGVNCTVSS. According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 comprising an E213Q variant (SEQ ID NO: 16). According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 comprising a P221L variant (SEQ ID NO: 17). According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 comprising an E224Q variant (SEQ ID NO: 18). According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 comprising a Y226F variant (SEQ ID NO: 19). According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 comprising a D242N variant (SEQ

ID NO: 20). According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 comprising a N245D variant (SEQ ID NO: 21). According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 comprising a T269A variant (SEQ ID NO: 22). According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 comprising a S314N variant (SEQ ID NO: 23). According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 comprising a deleted S314 (SEQ ID NO: 24). According to some embodiments, a tumor cell line variant may express the derivative of SEQ ID NO: 4 comprising F366Y variant (SEQ ID NO: 25).

[00233] According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to the protein of SEQ ID NO: 4. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to the protein of SEQ ID NO: 4. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to the protein of SEQ ID NO: 4. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 90% to the protein of SEQ ID NO: 4. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 95% to the protein of SEQ ID NO: 4. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to the protein of SEQ ID NO: 4. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 97% to the protein of SEQ ID NO: 4. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least

98% to the protein of SEQ ID NO: 4. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 99% to the protein of SEQ ID NO: 4.

[00234] According to some embodiments, a tumor cell line variant may be engineered to express one or more IgG heavy chain variable regions. According to some embodiments, a tumor cell line variant may be engineered to express a lambda/kappa light chain constant and/or light chain variable region. According to some embodiments, the hinge region of IgG binds to the FcyR receptors on immune cells. According to some embodiments, the IgG is effective to activate the FcyR and enhance presentation of antigens (e.g. PSA associated with prostate cancer cells).

[00235] According to some embodiments, a tumor cell line variant may be engineered to express an intact monoclonal or polyclonal antibody on the cell surface of the tumor cell. According to some embodiments, the intact monoclonal or polyclonal antibody may be designed to deliver a molecule that elicits an immunogenic response. For example, according to some embodiments, the intact monoclonal antibody may be designed to bind to DNA to deliver CpG motifs to immune cells.

[00236] According to some embodiments, the immunostimulatory activity of bacterial DNA may be mimicked by engineering an immunomodulator to deliver unmethylated CpG motifs to immune cells. For example, according to some embodiments, the IgG may be engineered to bind to biotin, which is then capable of delivering biotinylated CpG to cells of the immune system. According to some embodiments, CpG motifs may be bound directly or indirectly to the surface of the tumor cells of the tumor cell vaccine to prevent systemic effects. According to some

embodiments, CpG motifs may be conjugated to one or more antigens presented on the surface of tumor cells from the tumor cell line variant. According to some embodiments, the CpG is a class A CpG. According to some embodiments, the CpG is a class B CpG. According to some embodiments, the CpG is a class C CpG. According to some embodiments, the CpG is a CpG 30-mer of the sequence 5' EEAACCGTATCGGCGATATCGGTTEEEEEG 3'. As used herein with respect to CpG motifs, "E" is a G-phosphorothioate and this linkage refers to the 3' end of the nucleotide (i.e. the phosphorothioate bond substitutes a sulfur atom for a non-bridging oxygen in the nucleotide backbone). According to some embodiments, the CpG is a biotinylated 30-mer of the sequence 5'-biotin-EEAACCGTATCGGCGATATCGGTTEEEEEG-3'. According to some embodiments, the CpG is a CpG 30-mer of the sequence 5' EEAACCGTATGCGGCATATCGGTTEEEEEG 3'. According to some embodiments, the CpG is a biotinylated CpG 30-mer of the sequence 5'-biotin-EEAACCGTATGCGGCATATCGGTTEEEEEG-3'.

[00237] According to some embodiments, the IgG may be engineered as a hybrid of one or more IgG subclasses. For example, according to some embodiments, the IgG comprises sequences from IgG1 and IgG3. According to some embodiments, the IgG may be engineered to have an affinity for biotin. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to the protein of SEQ ID NO: 45. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to the protein of SEQ ID NO: 45. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to the protein of SEQ ID NO: 45. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least

90% to the protein of SEQ ID NO: 45. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 95% to the protein of SEQ ID NO: 45. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to the protein of SEQ ID NO: 45. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 97% to the protein of SEQ ID NO: 45. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 98% to the protein of SEQ ID NO: 45. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 99% to the protein of SEQ ID NO: 45.

[00238] According to some embodiments, the IgG comprises one or more mutations relative to wild type IgG that enhance affinity for FcyR. According to some embodiments, a tumor cell line variant may comprise one or more proteins of SEQ ID NO: 45 with one or more of mutations T323A and E325A. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to the proteins of one or more of SEQ ID NO: 41, SEQ ID NO: 30, and SEQ ID NO: 43. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to the proteins of one or more of SEQ ID NO: 41, SEQ ID NO: 30, and SEQ ID NO: 43. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to the proteins of one or more of SEQ ID NO: 41, SEQ ID NO: 30, and SEQ ID NO: 43. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 90% to the proteins of one or more of SEQ ID NO: 41, SEQ ID NO: 30, and SEQ ID NO: 43.

According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 95% to the proteins of one or more of SEQ ID NO: 41, SEQ ID NO: 30, and SEQ ID NO: 43. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to the proteins of one or more of SEQ ID NO: 41, SEQ ID NO: 30, and SEQ ID NO: 43. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 97% to the proteins of one or more of SEQ ID NO: 41, SEQ ID NO: 30, and SEQ ID NO: 43. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 98% to the proteins of one or more of SEQ ID NO: 41, SEQ ID NO: 30, and SEQ ID NO: 43. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 99% to the proteins of one or more of SEQ ID NO: 41, SEQ ID NO: 30, and SEQ ID NO: 43.

CD40L

[00239] The ligand of CD40, known as CD154 or CD40L, is a type II transmembrane protein, with a variable molecular weight between 32 and 39 kDa because of post-translation modifications (Elgueta R et al., Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunological reviews. 2009; 229(1):10.1111/j.1600-065X.2009.00782.x. doi:10.1111/j.1600-065X.2009.00782.x, citing van Kooten C et al., J. Leukoc Biol. 2000 Jan; 67(1):2-17.). A soluble form of CD40L has been reported that has activities similar to the transmembrane form (Id. citing Graf D et al., Eur J Immunol. 1995 Jun; 25(6):1749-54; Mazzei GJ et al., J Biol Chem. 1995 Mar 31; 270(13):7025-8.).

[00240] In nature, CD40L is a member of the TNF superfamily and is characterized by a sandwich extracellular structure that is composed of a β-sheet, α-helix loop, and a β-sheet, which allows for the trimerization of CD40L (Id. citing Karpusas M et al., Structure. 1995 Oct 15; 3(10):1031-9). CD40L is expressed primarily by activated T cells, as well as activated B cells and platelets; under inflammatory conditions it is also induced on monocytic cells, natural killer cells, mast cells, and basophils (Id. citing Carbone E et al., J Exp Med. 1997 Jun 16; 185(12):2053-60). The wide spread expression of the costimulatory pair of CD40L and CD40 indicates the pivotal roles they play in different cellular immune processes.

[00241] CD40L has three binding partners: CD40, α5β1 integrin and αIIbβ3 integrin. CD40L acts as a costimulatory molecule and is particularly important on a subset of T cells called T follicular helper cells (TFH cells), where it promotes B cell maturation and function by engaging CD40 on the B cell surface facilitating cell-cell communication. A defect in the CD40L gene results in an inability to undergo immunoglobulin class switching and is associated with hyper-IgM syndrome. Absence of CD40L also stops the formation of germinal centers thereby prohibiting antibody affinity maturation, an important process in the adaptive immune system.

[00242] CD40 has been found to be expressed on APCs, while its ligand, CD40L, has been found on activated T cells. CD40 has been found to play a critical role in the humoral immune response, and has been identified as enabling APCs to activate T cells. Several pathologies have been associated with the CD40/CD40L pathway including lupus and atherosclerosis, but anti-CD40L antibodies have been limited to clinical applications of thrombic complications from CD40 expression on activated platelets (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[00243] CD40 has also been found on several types of cancer, including solid tumors and hematologic malignancies. Signaling through CD40 in hematological cancer may mediate growth or regression, while CD40 signaling in solid tumors is only tumoricidal. These characteristics are found even in SCID mouse models, and therefore are likely due to TNF death domain signaling. There is also evidence of immune modulation, for example blockade of the CD40/CD40L pathway mitigates the protective effect of GM-CSF secreting melanoma vaccines (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[00244] Tumor cell vaccines expressing CD40L have proved useful in cancer models. For example, ligation of CD40 with CD40L or anti-CD40 antibodies has shown synergy with GM-CSF, IFN-gamma, IL-2, and CTLA-4 blockade. Furthermore, anti-CD40 antibodies have been reported to have anti-tumor activity in a pre-clinical mouse model (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)).

[00245] According to some embodiments of the disclosed invention, the tumor cell line variant may be engineered to express the cleavable CD40L peptide of SEQ ID NO: 6. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to the protein of SEQ ID NO: 6. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to the protein of SEQ ID NO: 6. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to the protein of SEQ ID NO: 6. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 90% to the protein of SEQ ID NO: 6. According to some embodiments, a tumor cell line variant may comprise one or

more proteins with a sequence identity of at least 95% to the protein of SEQ ID NO: 6. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to the protein of SEQ ID NO: 6. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 97% to the protein of SEQ ID NO: 6. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 98% to the protein of SEQ ID NO: 6. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 99% to the protein of SEQ ID NO: 6.

[00246] According to some embodiments, the tumor cell line variant may be engineered to express the non-cleavable membrane bound CD40L peptide of SEQ ID NO: 7 on the membrane surface of the tumor cell. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to the protein of SEQ ID NO: 7. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to the protein of SEQ ID NO: 7. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to the protein of SEQ ID NO: 7. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 90% to the protein of SEQ ID NO: 7. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 95% to the protein of SEQ ID NO: 7. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to the protein of SEQ ID NO: 7. According to some embodiments, a tumor cell line variant may comprise one or more proteins

with a sequence identity of at least 97% to the protein of SEQ ID NO: 7. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 98% to the protein of SEQ ID NO: 7. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 99% to the protein of SEQ ID NO: 7.

Tumor Necrosis Factor Alpha

[00247] Tumor necrosis factor (TNF; tumor necrosis factor alpha (TNFα); cachexin, cachectin) is a cytokine, primarily produced by activated macrophages and lymphocytes, which is involved in systemic inflammation. It is also one of the cytokines involved in the acute phase of an immunogenic response. TNF may be produced by other cell types such as, for example, CD4+ lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons.

[00248] In its primary role as a regulator of immune cells, TNF is capable of inducing fever, apoptotic cell death, cachexia, inflammation, and inhibition of tumorigenesis; of inhibiting viral replication; and of initiating a response to sepsis vial IL-1 and IL-6 producing cells. Dysregulated TNF production has been associated with a wide array of human diseases, including Alzheimer's disease, major depression, psoriasis, and inflammatory bowel disease (IBD). TNF can be produced ectopically in the setting of malignancy and parallels parathyroid hormone both in causing secondary hypercalcemia and in the cancers with which excessive production is associated.

[00249] TNF comprises a 26 kDa membrane bound form and 17 kDa soluble cytokine form. The soluble form of TNF is derived from proteolytic cleavage of the membrane bound form by TNF-alpha converting enzyme (TACE) (Grell M. et al., The Transmembrane Form of Tumor Necrosis Factor Is the Prime Activating Ligand of the 80 kDa Tumor Necrosis Factor Receptor,

Cell, Vol. 83, 793-802). TACE is a matrix metalloprotease that recognizes a cleavage site in the extracellular domain of full-length TNF (Rieger, R., Chimeric form of tumor necrosis factor-alpha has enhanced surface expression and antitumor activity, Cancer Gene Therapy, 2009, 16, 53-64). Deletion of the cleavage site on TNF results in enhanced membrane stability of TNF (Id.).

[00250] TNF has antiproliferative and cytotoxic effects on cells, is known to reduce tumor blood flow and tumor vascular damage, and is able to modulate immune response by stimulating macrophage and NK cell activity. However, the use of TNF as a therapeutic itself has been limited by dose-dependent hypotension and capillary leak that can cause a sepsis-like syndrome. For that reason, it must be delivered in a manner that limits systemic effects. TNF has been added to standard chemotherapy agents to improve response rates. Other approaches to administering TNF include injection of adenovirus altered to express TNF in gastrointestinal malignancies. A tumor vascular-targeted TNF compound has also been developed (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). Recombinant TNF has been used as an immunostimulant under the name tasonermin, while HUMIRA® is an antibody to TNF, useful for the treatment of inflammatory diseases (e.g. psoriasis and rheumatoid arthritis). In recognition of this role, molecules such as antibodies have been designed to interfere with TNF activity. However, such therapies pose the risk of initiating a cytokine storm caused by the inappropriate systemic release of cytokines, resulting in a positive feedback loop of white blood cell activation/cytokine release that potentially can be fatal.

[00251] According to some embodiments, a tumor cell line variant may express the membrane bound form of TNF on the membrane of the tumor cell. For example, according to some

embodiments, the cell line variants comprise the peptide of SEQ ID NO: 8. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to the protein of SEQ ID NO: 8. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to the protein of SEQ ID NO: 8. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to the protein of SEQ ID NO: 8. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 90% to the protein of SEQ ID NO: 8. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 95% to the protein of SEQ ID NO: 8. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to the protein of SEQ ID NO: 8. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 97% to the protein of SEQ ID NO: 8. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 98% to the protein of SEQ ID NO: 8. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 99% to the protein of SEQ ID NO: 8.

[00252] According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound form of TNF. For example, according to some embodiments, the tumor cell line variant may comprise the TNF protein of SEQ ID NO: 8 with one or more of amino acids VRSSSRTPSDKP deleted (see e.g. SEQ ID NO: 26).

[00253] According to some embodiments, a tumor cell line variant may express a soluble form of TNF. For example, according to some embodiments, the tumor cell line variant may

express the TNF protein of SEQ ID NO: 8 with part or the entire transmembrane region removed. For example, according to some embodiments, the tumor cell line variant may comprise a derivative TNF protein of SEQ ID NO: 8 with one or more of amino acids F, S, F, L, I, V, A, G, A, T, T, L, F, C, L, L, H, F, G, V, I deleted (see e.g. SEQ ID NO: 27).

[00254] According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound chimeric form of CD40L and TNF. For example, according to some embodiments, the ligand binding portion of a TNF molecule may be fused with the transmembrane and proximal extracellular domains of CD40L, such that the TNF lacks a defined TNF alpha cleaving enzyme (TACE) site. According to some embodiments, the intracellular, transmembrane, and partial extracellular portions CD40L may be fused with the extracellular region of TNF distal to the TACE cleavage site. According to some embodiments, the chimeric form of CD40L/TNF may comprise the CD40L sequence of SEQ ID NO: 9 and the TNF sequence of SEQ ID NO: 10. According to some embodiments, the CD40L/TNF sequences are operably linked via a linking peptide between 1 and 30 amino acids in length. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 60% to the proteins of SEQ ID NO: 9 and SEQ ID NO: 10. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 70% to the proteins of SEQ ID NO: 9 and SEQ ID NO: 10. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 80% to the proteins of SEQ ID NO: 9 and SEQ ID NO: 10. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 90% to the proteins of SEQ ID NO: 9 and SEQ ID NO: 10. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence

identity of at least 95% to the proteins of SEQ ID NO: 9 and SEQ ID NO: 10. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 96% to the proteins of SEQ ID NO: 9 and SEQ ID NO: 10. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 97% to the proteins of SEQ ID NO: 9 and SEQ ID NO: 10. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 98% to the proteins of SEQ ID NO: 9 and SEQ ID NO: 10. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 99% to the proteins of SEQ ID NO: 9 and SEQ ID NO: 10.

[00255] According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound form of TNF with a sequence identity of at least 60% to the protein of SEQ ID NO: 11. According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound form of TNF with a sequence identity of at least 70% to the protein of SEQ ID NO: 11. According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound form of TNF with a sequence identity of at least 80% to the protein of SEQ ID NO: 11. According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound form of TNF with a sequence identity of at least 90% to the protein of SEQ ID NO: 11. According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound form of TNF with a sequence identity of at least 95% to the protein of SEQ ID NO: 11. According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound form of TNF with a sequence identity of at least 96% to the protein of SEQ ID NO: 11. According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound form of TNF with a sequence identity

of at least 97% to the protein of SEQ ID NO: 11. According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound form of TNF with a sequence identity of at least 98% to the protein of SEQ ID NO: 11. According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound form of TNF with a sequence identity of at least 99% to the protein of SEQ ID NO: 11.

[00256] According to some embodiments, a tumor cell line variant may express a non-cleavable membrane bound chimeric form of CD40L and TNF. For example, according to some embodiments, the ligand portion of a TNF molecule may be fused with extracellular portions of CD40L, wherein CD40L comprises an extracellular portion that is non-cleavable and the TNF lacks a defined TACE site (e.g. cleavage site between amino acids 76 and 77). According to some embodiments, some or all of a CD40L peptide sequence is fused with the extracellular region of a TNF peptide sequence distal to the TACE cleavage site. According to some embodiments, the chimeric form of CD40L/TNF may comprise the sequence of SEQ ID NO: 31. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 60% to the protein of SEQ ID NO: 31. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 70% to the protein of SEQ ID NO: 31. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 80% to the protein of SEQ ID NO: 31. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 90% to the protein of SEQ ID NO: 31. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 95% to the protein of SEQ ID NO: 31. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of

at least 96% to the protein of SEQ ID NO: 31. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 97% to the protein of SEQ ID NO: 31. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 98% to the protein of SEQ ID NO: 31. According to some embodiments, a tumor cell line variant may comprise a fusion protein with a sequence identity of at least 99% to the protein of SEQ ID NO: 31.

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)

[00257] Granulocyte-macrophage colony-stimulating factor (GM-CSF; colony stimulating factor 2; CSF2) is found in monocytes/macrophages and activated T cells, and can act as a growth factor to stimulate and recruit dendritic cells. GM-CSF is a monomeric glycoprotein secreted by cells of the immune system, as well as endothelial cells and fibroblasts. Human GM-CSF is a 144 amino acid protein comprising a 17 amino acid signal peptide that can be cleaved to produce a mature 127 amino acid protein. Biological activity of GM-CSF occurs via binding to heteromeric cell surface receptors that are expressed on monocytes, macrophages, granulocytes, lymphocytes, endothelial cells and alveolar epithelial cells. The GM-CSF receptor (GM-CSFR) typically has a low expression (e.g. 20-200/cell), but has a high affinity (Shi Y et al., Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know, Cell Research (2006) 16: 126–133).

[00258] In some mouse models, vaccination with syngeneic mouse melanoma cells that secrete GM-CSF stimulates a more potent and long-lasting antitumor immunity than vaccines produced by other cytokines. Melanoma patients treated with soluble GM-CSF as an adjuvant therapy displayed an increase in disease free survival compared to controls. GM-CSF has been used as an immune adjuvant in various ways, including, without limitation, systemic and topical

application of soluble GM-CSF, GM-CSF fusion proteins, transfection of tumor cells with GM-CSF and injection of GM-CSF DNA. Recombinant GM-CSF has been used an adjuvant for various peptide, protein, and viral vaccines, and has been shown to be an effective adjuvant in patients with melanoma, breast, and ovarian cancer. A fusion protein comprising GM-CSF has also been shown to enhance immunogenicity of an antigen. GM-CSF has been tested for use in a gene therapy approach where allogeneic or autologous GM-CSF expressing cells are used as a vaccine (Kaufman and Wolchok eds., General Principles of Tumor Immunotherapy, Chpt 5, 67-121 (2007)). Such vaccines have had varying degrees of effectiveness among several different cancer types.

[00259] According to some embodiments, a tumor cell line variant may express the GM-CSF peptide of SEQ ID NO: 13. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to the protein of SEQ ID NO: 13. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to the protein of SEQ ID NO: 13. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to the protein of SEQ ID NO: 13. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 90% to the protein of SEQ ID NO: 13. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 95% to the protein of SEQ ID NO: 13. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to the protein of SEQ ID NO: 13. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 97% to the protein of SEQ ID NO: 13. According to some

embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 98% to the protein of SEQ ID NO: 13. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 99% to the protein of SEQ ID NO: 13.

[00260] According to some embodiments, a tumor cell line variant may comprise one or more proteins comprising a fusion between GM-CSF and HLA-I to enable membrane expression. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to the protein of SEQ ID NO: 42 or SEQ ID NO: 5. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to the protein of SEQ ID NO: 42 or SEQ ID NO: 5. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to the protein of SEQ ID NO: 42 or SEQ ID NO: 5. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 90% to the protein of SEQ ID NO: 42 or SEQ ID NO: 5. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 95% to the protein of SEQ ID NO: 42 or SEQ ID NO: 5. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to the protein of SEQ ID NO: 42 or SEQ ID NO: 5. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 97% to the protein of SEQ ID NO: 42 or SEQ ID NO: 5. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 98% to the protein of SEQ ID NO: 42 or SEQ ID NO: 5.

According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 99% to the protein of SEQ ID NO: 42 or SEQ ID NO: 5.

Fms-like tyrosine kinase-3 ligand (Flt-3L)

[00261] The human Flt3L protein is a membrane bound hematopoietic four helical bundle cytokine encoded by the FLT3LG gene. Flt3L acts as a growth factor that stimulates proliferation and differentiation of various blood cell progenitors, and is crucial for production and development of dendritic cells. Mice that lack Flt3L have low levels of dendritic cells, while Flt3L administered to mice or humans results in very high levels of dendritic cells (Shortman et al., Steady-state and inflammatory dendritic-cell development, Nature Reviews Immunology, Vol. 7. 19-30 (2007)).

[00262] According to some embodiments, a tumor cell line variant expresses the Flt3L peptide of SEQ ID NO: 14. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to the protein of SEQ ID NO: 14. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to the protein of SEQ ID NO: 14. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to the protein of SEQ ID NO: 14. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 90% to the protein of SEQ ID NO: 14. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 95% to the protein of SEQ ID NO: 14. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to the protein of SEQ ID NO: 14.

According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 97% to the protein of SEQ ID NO: 14. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 98% to the protein of SEQ ID NO: 14. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 99% to the protein of SEQ ID NO: 14.

[00263] According to some embodiments, a tumor cell line variant comprises a soluble form of Flt3L. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 60% to the protein of SEQ ID NO: 44. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 70% to the protein of SEQ ID NO: 44. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 80% to the protein of SEQ ID NO: 44. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 90% to the protein of SEQ ID NO: 44. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 95% to the protein of SEQ ID NO: 44. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 96% to the protein of SEQ ID NO: 44. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 97% to the protein of SEQ ID NO: 44. According to some embodiments, a tumor cell line variant may comprise one or more proteins with a sequence identity of at least 98% to the protein of SEQ ID NO: 44. According to some embodiments, a tumor cell line

variant may comprise one or more proteins with a sequence identity of at least 99% to the protein of SEQ ID NO: 44.

Vectors and Host Cells

[00264] The described invention provides nucleic acid constructs that encode two or more immune modulators that can be expressed in prokaryotic and eukaryotic cells. For example, the described invention provides expression vectors (e.g., DNA- or RNA-based vectors) containing nucleotide sequences that encode two or more immune modulators. In addition, the described invention provides methods for making the vectors described herein, as well as methods for introducing the vectors into appropriate host cells for expression of the encoded polypeptides. In general, the methods provided herein include constructing nucleic acid sequences encoding two or more immune modulators, and cloning the sequences into an expression vector. The expression vector can be introduced into host cells or incorporated into virus particles, either of which can be administered to a subject to, for example, treat cancer.

[00265] cDNA or DNA sequences encoding two or more immune modulators can be obtained (and, if desired, modified) using conventional DNA cloning and mutagenesis methods, DNA amplification methods, and/or synthetic methods. In general, a sequence encoding two or more immune modulators can be inserted into a cloning vector for genetic modification and replication purposes prior to expression. Each coding sequence can be operably linked to a regulatory element, such as a promoter, for purposes of expressing the encoded protein in suitable host cells in vitro and in vivo.

[00266] Expression vectors can be introduced into host cells for producing secreted immune modulators. There are a variety of techniques available for introducing nucleic acids into viable cells. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include

the use of liposomes, electroporation, microinjection, cell fusion, polymer-based systems, DEAE-dextran, viral transduction, the calcium phosphate precipitation method, etc. For in vivo gene transfer, a number of techniques and reagents may also be used, including liposomes; and natural polymer-based delivery vehicles, such as chitosan and gelatin; viral vectors are also suitable for in vivo transduction. In some situations it is desirable to provide a targeting agent, such as an antibody or ligand specific for a cell surface membrane protein. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g., capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al., J. Biol. Chem. 262, 4429-4432 (1987); and Wagner et al., Proc. Natl. Acad. Sci. USA 87, 3410-3414 (1990).

[00267] Where appropriate, gene delivery agents such as, e.g., integration sequences can also be employed. Numerous integration sequences are known in the art (see, e.g., Nunes-Duby et al., Nucleic Acids Res. 26:391-406, 1998; Sadwoski, J. Bacteriol., 165:341-357, 1986; Bestor, Cell, 122(3):322-325, 2005; Plasterk et al., TIG 15:326-332, 1999; Kootstra et al., Ann. Rev. Pharm. Toxicol., 43:413-439, 2003). These include recombinases and transposases. Examples include Cre (Sternberg and Hamilton, J. Mol. Biol., 150:467-486, 1981), lambda (Nash, Nature, 247, 543-545, 1974), FIp (Broach, et al., Cell, 29:227-234, 1982), R (Matsuzaki, et al., J. Bacteriology, 172:610-618, 1990), cpC31 (see, e.g., Groth et al., J. Mol. Biol. 335:667-678, 2004), sleeping beauty, transposases of the mariner family (Plasterk et al., supra), and components for integrating viruses such as AAV, retroviruses, and antiviruses having components that provide for virus integration such as the LTR sequences of retroviruses or

lentivirus and the ITR sequences of AAV (Kootstra et al., Ann. Rev. Pharm. Toxicol., 43:413-439, 2003).

[00268] Cells may be cultured in vitro or genetically engineered, for example. Host cells can be obtained from normal or affected subjects, including healthy humans, cancer patients, private laboratory deposits, public culture collections such as the American Type Culture Collection, or from commercial suppliers.

[00269] Cells that can be used for production and secretion of two or more immune modulators in vivo include, without limitation, epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, or granulocytes, various stem or progenitor cells, such as hematopoietic stem or progenitor cells (e.g., as obtained from bone marrow), umbilical cord blood, peripheral blood, fetal liver, etc., and tumor cells (e.g., human tumor cells). The choice of cell type depends on the type of tumor or infectious disease being treated or prevented, and can be determined by one of skill in the art.

[00270] Different host cells have characteristic and specific mechanisms for post-translational processing and modification of proteins. A host cell may be chosen which modifies and processes the expressed gene products in a specific fashion similar to the way the recipient processes its heat shock proteins (hsps).

[00271] In some embodiments, an expression construct as provided herein can be introduced into an antigenic cell. As used herein, antigenic cells can include preneoplastic cells that are infected with a cancer-causing infectious agent, such as a virus, but that are not yet neoplastic, or antigenic cells that have been exposed to a mutagen or cancer-causing agent, such as a DNA-damaging agent or radiation, for example. Other cells that can be used are preneoplastic cells that

are in transition from a normal to a neoplastic form as characterized by morphology or physiological or biochemical function.

[00272] Typically, the cancer cells and preneoplastic cells used in the methods provided herein are of mammalian origin. In some embodiments, cancer cells (e.g., human tumor cells) can be used in the methods described herein. Cell lines derived from a preneoplastic lesion, cancer tissue, or cancer cells also can be used. Cancer tissues, cancer cells, cells infected with a cancer-causing agent, other preneoplastic cells, and cell lines of human origin can be used. In some embodiments, a cancer cell can be from an established tumor cell line such as, without limitation, an established non-small cell lung carcinoma (NSCLC), bladder cancer, melanoma, ovarian cancer, renal cell carcinoma, prostate carcinoma, sarcoma, breast carcinoma, squamous cell carcinoma, head and neck carcinoma, hepatocellular carcinoma, pancreatic carcinoma, or colon carcinoma cell line.

[00273] Parent cell lines are described *supra*.

[00274] Further, in some embodiments, the allogeneic tumor cell vaccines provide for an adjuvant effect that further allows the immune system of a patient, when used in the various methods described herein, to be activated against a disease of interest.

[00275] Both prokaryotic and eukaryotic vectors can be used for expression of the two or more immune modulators in the methods provided herein. Prokaryotic vectors include constructs based on *E. coli* sequences (see, e.g., Makrides, Microbiol Rev 1996, 60:512-538). Non-limiting examples of regulatory regions that can be used for expression in *E. coli* include lac, trp, 1pp, phoA, recA, tac, T3, T7 and lamda P_L. Non-limiting examples of prokaryotic expression vectors may include the Agt vector series such as .lamda.gt11 (Huynh et al., in "DNA Cloning

Techniques, Vol. I: A Practical Approach," 1984, (D. Glover, ed.), pp. 49-78, IRL Press, Oxford), and the pET vector series (Studier et al., Methods Enzymol 1990, 185:60-89).

[00276] A variety of regulatory regions can be used for expression of the allogeneic tumor vaccines in mammalian host cells. For example, the SV40 early and late promoters, the cytomegalovirus (CMV) immediate early promoter, and the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter can be used. Inducible promoters that may be useful in mammalian cells include, without limitation, promoters associated with the metallothionein II gene, mouse mammary tumor virus glucocorticoid responsive long terminal repeats (MMTV-LTR), the n-interferon gene, and the hsp70 gene (see, Williams et al., Cancer Res 1989, 49:2735-42; and Taylor et al., Mol Cell Biol 1990, 10:165-75). Heat shock promoters or stress promoters also may be advantageous for driving expression of the fusion proteins in recombinant host cells.

[00277] Animal regulatory regions that exhibit tissue specificity and have been utilized in transgenic animals also can be used in tumor cells of a particular tissue type: the elastase I gene control region that is active in pancreatic acinar cells (Swift et al., Cell 1984, 38:639-646; Ornitz et al., Cold Spring Harbor Symp Quant Biol 1986, 50:399-409; and MacDonald, Hepatology 1987, 7:425-515); the insulin gene control region that is active in pancreatic beta cells (Hanahan, Nature 1985, 315:115-122), the immunoglobulin gene control region that is active in lymphoid cells (Grosschedl et al., Cell 1984, 38:647-658; Adames et al., Nature 1985, 318:533-538; and Alexander et al., Mol Cell Biol 1987, 7:1436-1444), the mouse mammary tumor virus control region that is active in testicular, breast, lymphoid and mast cells (Leder et al., Cell 1986, 45:485-495), the albumin gene control region that is active in liver (Pinkert et al., Genes Devel, 1987, 1:268-276), the alpha-fetoprotein gene control region that is active in liver (Krumlauf et al., Mol Cell Biol 1985, 5:1639-1648; and Hammer et al., Science 1987, 235:53-58); the alpha 1-

antitrypsin gene control region that is active in liver (Kelsey et al., Genes Devel 1987, 1:161-171), the beta-globin gene control region that is active in myeloid cells (Mogram et al., Nature 1985, 315:338-340; and Kollias et al., Cell 1986, 46:89-94); the myelin basic protein gene control region that is active in oligodendrocyte cells in the brain (Readhead et al., Cell 1987, 48:703-712); the myosin light chain-2 gene control region that is active in skeletal muscle (Sani, Nature 1985, 314:283-286), and the gonadotropic releasing hormone gene control region that is active in the hypothalamus (Mason et al., Science 1986, 234:1372-1378).

[00278] An expression vector also can include transcription enhancer elements, such as those found in SV40 virus, Hepatitis B virus, cytomegalovirus, immunoglobulin genes, metallothionein, and .beta.-actin (see, Bittner et al., Meth Enzymol 1987, 153:516-544; and Gorman, Curr Op Biotechnol 1990, 1:36-47). In addition, an expression vector can contain sequences that permit maintenance and replication of the vector in more than one type of host cell, or integration of the vector into the host chromosome. Such sequences include, without limitation, to replication origins, autonomously replicating sequences (ARS), centromere DNA, and telomere DNA.

[00279] In addition, an expression vector can contain one or more selectable or screenable marker genes for initially isolating, identifying, or tracking host cells that contain DNA encoding the immunogenic proteins as described herein. For long term, high yield production of gp96-Ig and T cell costimulatory fusion proteins, stable expression in mammalian cells can be useful. A number of selection systems can be used for mammalian cells. For example, the Herpes simplex virus thymidine kinase (Wigler et al., Cell 1977, 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalski and Szybalski, Proc Natl Acad Sci USA 1962, 48:2026), and adenine phosphoribosyltransferase (Lowy et al., Cell 1980, 22:817) genes can be employed

in tk^-, $hgprf^-$, or $aprf^-$ cells, respectively. In addition, antimetabolite resistance can be used as the basis of selection for dihydrofolate reductase (dhfr), which confers resistance to methotrexate (Wigler et al., Proc Natl Acad Sci USA 1980, 77:3567; O'Hare et al., Proc Natl Acad Sci USA 1981, 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan and Berg, Proc Natl Acad Sci USA 1981, 78:2072); neomycin phosphotransferase (neo), which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., J Mol Biol 1981, 150:1); and hygromycin phosphotransferase (hyg), which confers resistance to hygromycin (Santerre et al., Gene 1984, 30:147). Other selectable markers such as histidinol and Zeocin™ also can be used.

[00280] A number of viral-based expression systems also can be used with mammalian cells to produce the allogeneic tumor cell vaccines. Vectors using DNA virus backbones have been derived from simian virus 40 (SV40) (Hamer et al., Cell 1979, 17:725), adenovirus (Van Doren et al., Mol Cell Biol 1984, 4:1653), adeno-associated virus (McLaughlin et al., J Virol 1988, 62:1963), and bovine papillomas virus (Zinn et al., Proc Natl Acad Sci USA 1982, 79:4897). When an adenovirus is used as an expression vector, the donor DNA sequence may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This fusion gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) can result in a recombinant virus that is viable and capable of expressing heterologous products in infected hosts. (See, e.g., Logan and Shenk, Proc Natl Acad Sci USA 1984, 81:3655-3659).

[00281] Bovine papillomavirus (BPV) can infect many higher vertebrates, including man, and its DNA replicates as an episome. A number of shuttle vectors have been developed for recombinant gene expression, which exist as stable, multicopy (20-300 copies/cell)

extrachromosomal elements in mammalian cells. Typically, these vectors contain a segment of BPV DNA (the entire genome or a 69% transforming fragment), a promoter with a broad host range, a polyadenylation signal, splice signals, a selectable marker, and "poisonless" plasmid sequences that allow the vector to be propagated in *E. coli*. Following construction and amplification in bacteria, the expression gene constructs are transfected into cultured mammalian cells by, for example, calcium phosphate coprecipitation. For those host cells that do not manifest a transformed phenotype, selection of transformants is achieved by use of a dominant selectable marker, such as histidinol and G418 resistance.

[00282] Alternatively, the vaccinia 7.5K promoter can be used. (See, e.g., Mackett et al., Proc Natl Acad Sci USA 1982, 79:7415-7419; Mackett et al., J Virol 1984, 49:857-864; and Panicali et al., Proc Natl Acad Sci USA 1982, 79:4927-4931.) In cases where a human host cell is used, vectors based on the Epstein-Barr virus (EBV) origin (OriP) and EBV nuclear antigen 1 (EBNA-1; a trans-acting replication factor) can be used. Such vectors can be used with a broad range of human host cells, e.g., EBO-pCD (Spickofsky et al., DNA Prot Eng Tech 1990, 2:14-18); pDR2 and .lamda.DR2 (available from Clontech Laboratories).

[00283] Allogeneic tumor cell vaccines also can be made with retrovirus-based expression systems. Retroviruses, such as Moloney murine leukemia virus, can be used since most of the viral gene sequence can be removed and replaced with exogenous coding sequence while the missing viral functions can be supplied in trans. In contrast to transfection, retroviruses can efficiently infect and transfer genes to a wide range of cell types including, for example, primary hematopoietic cells. Moreover, the host range for infection by a retroviral vector can be manipulated by the choice of envelope used for vector packaging.

[00284] For example, a retroviral vector can comprise a 5' long terminal repeat (LTR), a 3' LTR, a packaging signal, a bacterial origin of replication, and a selectable marker. The gp96-Ig fusion protein coding sequence, for example, can be inserted into a position between the 5' LTR and 3' LTR, such that transcription from the 5' LTR promoter transcribes the cloned DNA. The 5' LTR contains a promoter (e.g., an LTR promoter), an R region, a U5 region, and a primer binding site, in that order. Nucleotide sequences of these LTR elements are well known in the art. A heterologous promoter as well as multiple drug selection markers also can be included in the expression vector to facilitate selection of infected cells. See, McLauchlin et al., Prog Nucleic Acid Res Mol Biol 1990, 38:91-135; Morgenstern et al., Nucleic Acid Res 1990, 18:3587-3596; Choulika et al., J Virol 1996, 70:1792-1798; Boesen et al., Biotherapy 1994, 6:291-302; Salmons and Gunzberg, Human Gene Ther 1993, 4:129-141; and Grossman and Wilson, Curr Opin Genet Devel 1993, 3:110-114.

[00285] Any of the cloning and expression vectors described herein may be synthesized and assembled from known DNA sequences using techniques that are known in the art. The regulatory regions and enhancer elements can be of a variety of origins, both natural and synthetic. Some vectors and host cells may be obtained commercially. Non-limiting examples of useful vectors are described in Appendix 5 of Current Protocols in Molecular Biology, 1988, ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, which is incorporated herein by reference; and the catalogs of commercial suppliers such as Clontech Laboratories, Stratagene Inc., and Invitrogen, Inc.

Recombinant Immunomodulators

[00286] According to some embodiments, two or more immune modulators may be cloned into plasmid constructs for transfection (via, e.g., lipids, calcium phosphate, cationic polymers, DEAE-dextran, activated dendrimers, magnetic beads, electroporation, biolistic technology, microinjection, laserfection/optoinjection) or transduction (via, e.g., retrovirus, lentivirus, adenovirus, adeno-associated virus) into cells of tumor cell lines. According to some embodiments, recombinant DNA encoding each immune modulator protein may be cloned into a lentiviral vector plasmid for integration into the genome of cells of tumor cell lines. According to some embodiments, recombinant DNA encoding the immune modulator protein may be cloned into a plasmid DNA construct encoding a selectable trait, such as an antibiotic resistance gene. According to some embodiments, recombinant DNA encoding the immune modulator protein may be cloned into a plasmid construct that is adapted to stably express each recombinant protein in the cells of the tumor cell line. According to some embodiments, the transfected or transduced tumor cells may be clonally expanded to achieve a cell line variant with a homogenous site of integration of the recombinant DNA encoding each immune modulator protein into the genome of the cells of the tumor cell line.

Lentiviral Constructs

[00287] According to some embodiments, the DNA sequences coding for immune modulator proteins may be cloned into a lentiviral vector for transduction into mammalian cells. According to some embodiments, the lentiviral system may comprise a lentiviral transfer plasmid encoding the two or more immune modulator sequences, packaging plasmids encoding the GAG, POL, TAT, and REV sequences, and an envelope plasmid encoding the ENV sequences. According to some embodiments, the lentiviral transfer plasmid uses a viral LTR promoter for gene expression. According to some embodiments, the lentiviral transfer plasmid uses a hybrid

promoter, or other specialized promoter. According to some embodiments, the promoter of the lentiviral transfer plasmid is selected to express the two or more immune modulator sequences at a desired level relative to other immunomodulatory sequences. According to some embodiments, the relative level is measured on the level of transcription as mRNA transcripts. According to some embodiments, the relative level is measured on the level of translation as protein expression.

Multicistronic plasmid constructs

[00288] According to some embodiments, one or more immune modulator sequence may be cloned in a multicistronic vector for co-expression of one immune modulator with a second immune modulator or other recombinant sequence. According to some embodiments, an immune modulator sequence may be cloned into a plasmid comprising an IRES element to promote translation of two or more proteins from a single transcript. According to some embodiments, one or more immune modulator sequences is cloned into a multicistronic vector comprising sequences for a self cleaving 2A peptide to produce two or more immune modulator proteins from a single transcript.

Genetic introduction of immune modulators

[00289] According to some embodiments, plasmid constructs comprising the recombinant immune modulator sequences may be transfected or transduced into tumor cell lines.

Lentiviral System

[00290] According to some embodiments, the lentiviral system may be employed where the transfer vector with immune modulator sequences, an envelope vector, and a packaging vector

are each transfected into host cells for virus production. According to some embodiments, the lentiviral vectors may be transfected into 293T cells by any of calcium phosphate precipitation transfection, lipid based transfection, or electroporation, and incubated overnight. For embodiments where the immune modulator sequence may be accompanied by a fluorescence reporter, inspection of the 293T cells for florescence may be checked after overnight incubation. The culture medium of the 293T cells comprising virus particles may be harvested 2 or 3 times every 8-12 hours and centrifuged to sediment detached cells and debris. The culture medium may then be used directly, frozen or concentrated as needed.

[00291] Tumor cell lines may be grown to a confluency of about 70% under standard tissue culture conditions. The cells may then be treated with hexadimethrine bromide (to enhance transduction of cells) and lentiviral particles comprising recombinant constructs in fresh media, and incubated for 18-20 hours followed by a media change.

Lipid Based Transfection

[00292] According to some embodiments, cells of tumor cell lines may be transfected with immune modulator sequences using a lipid based transfection method. According to some embodiments, established lipid based transfection reagents, such as LIPOFECTAMINE, may be used. Tumor cell lines may be grown to about 70-90% confluence in a tissue culture vessel. Appropriate amounts of Lipofectamine® and plasmid construct comprising the immune modulator sequences may be separately diluted in tissue culture media and briefly incubated at room temperature. The diluted Lipofectamine® and plasmid constructs in media may be mixed together and incubated briefly at room temperature. The plasmid LIPOFECTAMINE mixture

may then be added to the cells of the tumor cell lines in the tissue culture vessel and incubated for 1-3 days under standard tissue culture conditions.

Selection of Expressing Clones

[00293] According to some embodiments, tumor cells of the tumor cell line that have been transfected with immune modulator sequences may be selected for various levels of expression.

[00294] According to some embodiments, the immune modulator sequences may be accompanied by antibiotic resistance genes, which may be used to select for clones with stable integration of the recombinant DNA encoding the immune modulator sequences. According to some embodiments, the immune modulator sequences may be cloned into a plasmid construct comprising antibiotic resistance, such as the Neomycin/Kanamycin resistance gene. Transfected cells are treated with antibiotics according to the manufacturer's protocol for 1-2 weeks or more with daily media changes. At some point during antibiotic treatment, there is massive tumor cell death of all cells that have not stably integrated the antibiotic resistance gene, leaving behind small colonies of stably expressing clones. Each of the stably expressing clones may be picked, cultured in a separate tissue culture container, and tested for levels of immune modulator expression by any established method, such as western blot, flow cytometry, and fluorescence microscopy.

[00295] According to some embodiments, transfected tumor cells may be selected for high expression of the immune modulators by fluorescence activated cell sorting (FACS). According to some embodiments, immune modulator sequences may be accompanied by one or more fluorescent proteins (e.g. GFP), which can be used to quantify expression of immune modulator. For example, a bicistronic plasmid comprising an immune modulator sequence connected to a

GFP sequence via IRES sequence would result in both an immune modulator and GFP protein translated from the same transcript. Thus, the GFP expression level would act as a proxy for the expression level of immune modulator. Single cell suspensions of immune modulator/GFP transfected tumor cells could be selected for the desired level of expression by FACS based on the fluorescence intensity. Any fluorescent protein may be used in this regard. For example, any of the following recombinant fluorescent proteins may be used: EBFP, ECFP, EGFP, YFP, mHoneydew, mBanana, mOrange, tdTomato, mTangerine, mStrawberry, mCherry, mGrape, mRasberry, mGrape2, mPlum.

[00296] Alternatively, the expression of the recombinant immune modulator may be directly observed by fluorescent antibodies specific to each immune modulator or specific to a tag engineered onto each immune modulator. For example, according to some embodiments the extracellular region of an immune modulator sequence may be fused with a FLAG tag or HA tag. Anti-FLAG or anti-HA antibodies may be used, along with a fluorophore attached to the primary antibody or a secondary antibody) to detect the expression of the immune modulator on the surface of the transfected tumor cells. Tumor cells expressing the desired level of immune modulator may be selected by FACS sorting and cultured separately.

Testing of Clones for Immunogenic Potential

Mixed lymphocyte tumor cell reactivity

[00297] According to some embodiments, the genetically introduced immunomodulators may be assessed for their immunogenic potential by a mixed lymphocyte tumor cell reaction (MLTR). The MLTR assay comprises incubating mixed lymphocytes with tumor cell line variants (or controls) for several days to allow the tumor cells of the tumor cell line variant to elicit an

immune response from the mixed lymphocytes in vitro. This method may provide a rapid in vitro method to assess mixed lymphocyte responses (such as cellular proliferation of lymphocytes, cellular subset differentiation of lymphocytes, cytokine release profile of lymphocytes, and tumor cell death) to tumor cells or lysates. This approach may enable comprehensive monitoring of cellular, humoral, or both, immunity responses to phenotypically modified transfected tumor cells using human peripheral blood mononuclear cells. The MLTR also may provide an alternative to murine tumor survival studies, and may result in selection of optimal tumor cell line variants for anti-tumor response. A similar assay has been described by Hunter TB et al., (2007) Scandanavian J. Immunology 65, 479-486, which is incorporated herein by reference in its entirety.

[00298] According to some embodiments, tumor cell line variants may be tested for immunogenic potential by contacting transfected tumor cells with mixed lymphocytes from peripheral blood mononuclear cells, followed by measuring cellular proliferation, cellular subset differentiation, cytokine release profile, and tumor cell lysate.

[00299] According to some embodiments, mixed lymphocytes may be obtained from peripheral blood mononuclear cells isolated by a Ficoll-Paque gradient. Briefly, anticoagulant-treated blood may be diluted in the range of 1:2 to 1:4 with PBS/EDTA to reduce aggregation of erythrocytes. The diluted blood may then be layered above a Ficoll-Paque solution in a centrifuge tube, without mixing. The layered blood/Ficoll-Paque may be centrifuged for 40 minutes at 400 x g between 18° and 20° C, without the use of the centrifuge brake, resulting in the formation of blood fractions comprising, from top to bottom, a first fraction comprising

blood plasma; a second fraction comprising mononuclear cells; a third fraction comprising Ficoll-Paque media; and a fourth fraction comprising granulocytes and erythrocytes.

[00300] The fractions may be further processed to isolate specific fraction components. For example, to further process mononuclear cells, the second fraction comprising mononuclear cells may be carefully removed from the Ficoll-Paque gradient using a Pasteur pipet. Alternatively, the second fraction may be removed directly by puncturing the tube with a needle and directly withdrawing the second fraction. The second fraction may then be washed and centrifuged at 300 x g, 18° and 20° C, three times with PBS/EDTA, discarding the supernatant after each round.

[00301] According to some embodiments, tumor cell line variants may be co-cultured with the PBMCs comprising lymphocytes for seven days to allow for direct evaluation of activation of anti-tumor response in the presence of immune modulators from the tumor cell line variants.

[00302] According to some embodiments, one parameter used for measuring activation of lymphocytes may be cellular proliferation. According to some embodiments, proliferation may be detected by ^{3}H-thymidine incorporation. Briefly, approximately 5 x 10^{3} tumor cell line variant cells may be co-cultured with approximately 1 x 10^{6} mixed lymphocytes in round bottomed 96-well plates. After three days of culture, cells may be pulsed with 1 μCi of ^{3}H-thymidine for 18 hours. The cells may then be harvested onto filter mats, and ^{3}H-thymidine incorporation may be measured using a scintillation counter. Proliferation of tumor cell line variants compared to non-transfected tumor cell controls may be measured. An increase, a decrease, or no change in proliferation relative to controls, are possible outcomes.

[00303] According to some embodiments, another parameter for measuring activation of lymphocytes may be the cytokine release profile. For example, the number of responsive T cells

in the mixed lymphocyte population may be quantified by enzyme linked immunospot (ELISpot) analysis of IFN-gamma and/or IL-2 production by PBMCs. Briefly, PBMCs comprising mixed lymphocytes and a tumor cell line variant may be co-cultured between 3 and 7 days. Co-cultured cells may then be harvested and incubated on ELISpot plates pre-coated with anti-IFN-gamma and/or anti-IL-2 antibodies. After 20 hours, cells may be removed by washing 2 times in distilled water and two times in washing buffer. ELISpot plates may then be contacted with biotinylated anti-IFN-gamma and/or anti-IL-2 antibodies and streptavidin alkaline phosphatase in blocking buffer for 1-2 hours. After washing, plates may be contacted with alkaline phosphatase substrate until dark spot emerge. Plates may then be washed in tap water and air dried. Spots are then quantified manually or by plate reader and compared to non-transfected tumor cell line control group.

[00304] According to some embodiments, another parameter for measuring activation of lymphocytes may be by quantifying cellular subset differentiation. For example, the differentiation of CD45+/CD3+ T-lymphocytes to CD45+/CD3+/CD4+ helper T-lymphocytes, CD45+/CD3+/CD8+ cytotoxic T-lymphocytes, and CD45+/CD3+/CD25+ activated T-lymphocytes may be quantified by flow cytometry analysis.

[00305] According to some embodiments, another parameter for measuring activation of lymphocytes may be by quantifying tumor cell cytotoxicity. Cytotoxicity of tumor cells may be measured by any number of established methods. For example, according to some embodiments, an LDH-Cytotoxicity colorimetric assay kit (BioVision Cat. # K311-400) may be used to measure cytotoxicity of tumor cells by testing for lactate dehydrogenase (LDH) released from damaged cells into the growth media. Briefly, 100µl of media from each of the control group

(comprising untransfected tumor cells), the experimental group (comprising immune modulator transfected tumor cells), and media alone may be pipetted into the wells of a 96 well plate. 100µl of the LDH reaction mixture, comprising dye solution and catalyst solution, may then be added to the wells of the 96 well plate and incubated for 30 minutes at room temperature. Then the samples may be measured for light absorbance at 490-500 nm using a microtiter plate reader.

Sequentially add new plasmid constructs to the clones

[00306] According to some embodiments, tumor cell line variants that express one or more immune modulator sequences are transfected with additional immune modulators for stable expression in a sequential manner. By sequentially adding recombinant immune modulators in successive fashion, cells of a tumor cell line variant may be created that express several immune modulators simultaneously. According to some embodiments, a tumor cell line variant may be created that expresses two immune modulators simultaneously. According to some embodiments, a tumor cell line variant may be created that expresses three immune modulators simultaneously. According to some embodiments, a tumor cell line variant may be created that expresses four immune modulators simultaneously. According to some embodiments, a tumor cell line variant may be created that expresses five immune modulators simultaneously.

Variably Expressing Clones

[00307] According to one aspect of the disclosed invention, multiple recombinant immune modulator peptides may be expressed in a single clonally derived tumor cell line variant. According to some embodiments, the amount (or level) of each individual immune modulator expressed in each cell is the same as the level of expression of all other immune modulator peptides. According to some embodiments, however, the level of each individual immune

modulator expressed in each cell is different from the level of expression of the other immune modulators expressed in the cell. According to some embodiments, clonally derived tumor cell line variants that express the same complement of immune modulators stably express those immune modulators in varying amounts relative to each other.

[00308] The relative amount of recombinant immune modulator expressed within each clonally derived tumor cell line variant, and between tumor cell line variants, can be measured on the level of transcription or translation. For example, the relative amount of recombinant immune modulator can be quantified by western blot, RT-PCR, flow cytometry, immunofluorescence, and northern blot, among others.

[00309] According to some embodiments, the differences in the amount of expressed immune modulators relative to one another may be a result of random integration into more or less transcriptionally active regions of the genome of the tumor cell line variant. According to some embodiments, the relative differences in the amount of expressed immune modulator may be achieved by elements engineered into the transfected or transduced DNA used to create the tumor cell line variant.

[00310] For example, according to some embodiments, the level of expression of the immune modulator proteins may be achieved on the transcriptional level by engineering stronger or weaker gene promoter sequences to control expression of the immune modulator gene. According to some embodiments, one or more of the following promoters may be used to control expression of immune modulators: simian virus 40 early promoter (SV40), cytomegalovirus immediate-early promoter (CMV), human Ubiquitin C promoter (UBC), human elongation

factor 1α promoter (EF1A), mouse phosphoglycerate kinase 1 promoter (PGK), and chicken β-Actin promoter coupled with CMV early enhancer (CAGG).

[00311] According to some embodiments, the level of expression of the immune modulator proteins may be achieved on the translational level by engineering stronger or weaker Kozak consensus sequences around the start codon of the immune modulator transcript. According to some embodiments, the following nucleotide sequences may be provided to control immune modulator translation: GCCGCC(A/G)CCAUGG (SEQ ID NO: 15). According to some embodiments, a sequence that is at least 60% identical to SEQ ID NO: 15 may be provided to control immune modulator translation. According to some embodiments, a sequence that is at least 70% identical to SEQ ID NO: 15 may be provided to control immune modulator translation. According to some embodiments, a sequence that is at least 80% identical to SEQ ID NO: 15 may be provided to control immune modulator translation. According to some embodiments, a sequence that is at least 90% identical to SEQ ID NO: 15 may be provided to control immune modulator translation. According to some embodiments, a sequence that is at least 95% identical to SEQ ID NO: 15 may be provided to control immune modulator translation. According to some embodiments, a sequence that is at least 96% identical to SEQ ID NO: 15 may be provided to control immune modulator translation. According to some embodiments, a sequence that is at least 97% identical to SEQ ID NO: 15 may be provided to control immune modulator translation. According to some embodiments, a sequence that is at least 98% identical to SEQ ID NO: 15 may be provided to control immune modulator translation. According to some embodiments, a sequence that is at least 99% identical to SEQ ID NO: 15 may be provided to control immune modulator translation.

Therapeutic Compositions

[00312] According to another aspect of the disclosed invention, an immunogenic composition may comprise an amount of a tumor cell line variant comprising two or more genes encoding human immune modulators. According to some embodiments, clones of tumor cell line variants that maximally express the human immune modulators are identified and selected. According to some embodiments, expression of the human immune modulators by populations of the tumor cell line variants is determined by flow cytometry. According to some embodiments, flow cytometry is used to gate on the maximally expressing population(s) of tumor cell line variants.

[00313] According to some embodiments, the immunogenic amount may be effective to stimulate an anti-tumor immune response to one or more tumor specific antigens. According to some embodiments, the immunogenic amount may be titrated to provide both safety and efficacy.

[00314] According to some embodiments, the immunogenic composition comprises a pharmaceutically acceptable carrier.

[00315] According to some embodiments, the immunogenic composition further comprises an adjuvant.

[00316] According to some embodiments, the tumor cell line variant may comprise tumor cells derived from an established cell line. According to some embodiments, the tumor cell line variant comprises tumor cells derived from a patient with cancer, wherein the tumor cells are derived from a solid tumor.

[00317] According to some embodiments, the tumor cell line variant comprises an immunogenic amount of a disrupted tumor cell line variant. Examples of methods for physical

disruption include, without limitation, sonication, cavitation, dehydration, ion depletion, or by toxicity from exposure to one or more salts.

[00318] According to some embodiments, the immunogenic amount of the immunogenic composition may comprise at least 1 x 10^3 whole or disrupted tumor cell line variant cells. According to some embodiments, the amount of the immunogenic composition may comprises at least 1 x 10^4 whole or disrupted tumor cell line variant cells. According to some embodiments, the amount of the immunogenic composition may comprise at least 1 x 10^5 whole or disrupted tumor cell line variant cells. According to some embodiments, the amount of the immunogenic composition may comprise at least 1 x 10^6 whole or disrupted tumor cell line variant cells. According to some embodiments, the amount of the immunogenic composition may comprise at least 1 x 10^7 whole or disrupted tumor cell line variant cells. According to some embodiments, the amount of the immunogenic composition may comprise at least 1 x 10^8 whole or disrupted tumor cell line variant cells. According to some embodiments, the amount of the immunogenic composition may comprise at least 1 x 10^9 whole or disrupted tumor cell line variant cells. According to some embodiments, the immunogenic amount may be a therapeutic amount.

[00319] According to some embodiments, the immunogenic amount may be effective (1) to stimulate an effective immune response comprising one or more of cytotoxic T cells, natural killer cells, antibodies, APCs, T cells, B cells, and dendritic cells; and (2) to improve a clinical outcome parameter selected from one or more of progression-free survival, disease-free survival, time to progression, time to distant metastasis, and overall survival of the subject, when compared to a suitable control.

[00320] According to some embodiments, the immunogenic composition may be administered 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, once every seven months, once every eight months, once every nine months, once every ten months, once every eleven months, or once a year. According to some embodiments, administration occurs in one day or over 2 days, 3 days, 4, days, 5 days, 6 days, 7 days, 8, days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, or more. According to some embodiments, administration may involve two or more administrations on the same day.

Combination Therapies

[00321] According to some embodiments, the disclosure provides methods that further comprise administering an additional agent to a subject. In some embodiments, the invention pertains to co-administration and/or co-formulation.

[00322] In some embodiments, administration of the immunogenic composition acts synergistically when co-administered with another agent and is administered at doses that are lower than the doses commonly employed when such agents are used as monotherapy.

[00323] In some embodiments, inclusive of, without limitation, cancer applications, the present invention pertains to chemotherapeutic agents as additional agents. Examples of chemotherapeutic agents include, but are not limited to, alkylating agents such as thiotepa and CYTOXAN cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide,

triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (e.g., bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; cally statin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (e.g., cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB 1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, especially calicheamicin gammall and calicheamicin omegall; dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomy sins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN doxorubicin (including morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxy doxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine;

androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as minoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex; razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (e.g., T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g., TAXOL paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel, and TAXOTERE doxetaxel; chloranbucil; GEMZAR gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE. vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; combretastatin; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); lapatinib (TYKERB); inhibitors of PKC-.alpha., Raf, H-Ras, EGFR (e.g., erlotinib (Tarceva)) and VEGF-A that reduce cell proliferation and

pharmaceutically acceptable salts, acids or derivatives of any of the above. In addition, the methods of treatment can further include the use of radiation.

[00324] Other additional agents are described elsewhere herein, including the blocking antibodies targeted to an immune "checkpoint" molecule.

[00325] According to some embodiments, the treatment regimen may comprise a standard anti-tumor therapy (such as surgery, radiation therapy, a targeted therapy that precisely identifies and attacks cancer cells, a hormone therapy, or a combination thereof). According to some embodiments, the standard anti-tumor therapy is effective to treat the tumor while preserving any existing anti-tumor immune response. According to some embodiments, the immunogenic composition is not applied after chemotherapy. According to some embodiments, the immunogenic composition is applied after low-dose chemotherapy.

[00326] According to some embodiments, the immunogenic composition comprises two or more clonally derived tumor cell line variants. According to some embodiments, the two or more tumor cell line variants comprise the same complement of recombinant immune modulators. According to some embodiments, the two or more tumor cell line variants comprise different panels of recombinant immune modulators.

[00327] According to some embodiments, the tumor cell line variants are treated with an agent that prevents cell division prior to administration to a subject. According to some embodiments, the tumor cell line variants are irradiated. According to some embodiments, the tumor cell line variants are treated with a chemical agent that prevents proliferation.

[00328] According to some embodiments, the tumor cell line variants may be administered parenterally. According to some embodiments, the tumor cell line variants may be administered

locally into a surgical excision cavity. According to some embodiments, the tumor cell variants may be administered by intradermal injection. According to some embodiments, the tumor cell line variants may be administered by subcutaneous injection. According to some embodiments, the tumor cell line variants may be administered by intramuscular injection.

[00329] Methods of Treatment

[00330] Tumor cell line variants as provided herein can be incorporated into a composition for administration to a subject (e.g., a research animal or a mammal, such as a human, having a clinical condition such as cancer or an infection). For example, an allogeneic tumor cell vaccine comprising a tumor cell line variant comprising two or more stably expressed recombinant membrane bound immunomodulatory molecules selected from IgG1, CD40L, TNF-alpha, and Flt-3L peptides; and stably expressed recombinant soluble GM-CSF peptides; and a pharmaceutically acceptable carrier; can be administered to a subject for the treatment of cancer. In another example, an allogeneic tumor cell vaccine comprising a tumor-type specific cell line variant is used to deliver a broad array of tumor antigens in the context of immunomodulatory signals sufficient to elicit a potent anti-tumor response as reflected in improved progression free survival, overall survival, or both relative to placebo controls, wherein the immunomodulatory signals are comprised of two or more stably expressed recombinant membrane bound immunomodulatory molecules selected from membrane expressed IgG1, CD40L, TNF-alpha, as well as membrane and soluble forms of GM-CSF, and Flt-3L.

[00331] Thus, the described invention provides methods for treating clinical conditions such as cancer with the allogeneic tumor vaccines provided herein.

[00332] In various embodiments, the described invention pertains to cancers and/or tumors; for example, the treatment or prevention of cancers and/or tumors. The phrase "cancers or tumors" refers to an uncontrolled growth of cells and/or abnormal increased cell survival and/or inhibition of apoptosis which interferes with the normal functioning of the bodily organs and systems. Included are benign and malignant cancers, polyps, hyperplasia, as well as dormant tumors or micrometastases. Also included are cells having abnormal proliferation that is not impeded by the immune system (e.g. virus infected cells). The cancer may be a primary cancer or a metastatic cancer. The primary cancer may be an area of cancer cells at an originating site that becomes clinically detectable, and may be a primary tumor. In contrast, the metastatic cancer may be the spread of a disease from one organ or part to another non-adjacent organ or part. The metastatic cancer may be caused by a cancer cell that acquires the ability to penetrate and infiltrate surrounding normal tissues in a local area, forming a new tumor, which may be a local metastasis. The cancer may also be caused by a cancer cell that acquires the ability to penetrate the walls of lymphatic and/or blood vessels, after which the cancer cell is able to circulate through the bloodstream (thereby being a circulating tumor cell) to other sites and tissues in the body. The cancer may be due to a process such as lymphatic or hematogeneous spread. The cancer may also be caused by a tumor cell that comes to rest at another site, re-penetrates through the vessel or walls, continues to multiply, and eventually forms another clinically detectable tumor. The cancer may be this new tumor, which may be a metastatic (or secondary) tumor.

[00333] The cancer may be caused by tumor cells that have metastasized, which may be a secondary or metastatic tumor. The cells of the tumor may be like those in the original tumor. As an example, if a breast cancer or colon cancer metastasizes to the liver, the secondary tumor,

while present in the liver, is made up of abnormal breast or colon cells, not of abnormal liver cells. The tumor in the liver may thus be a metastatic breast cancer or a metastatic colon cancer, not liver cancer.

[00334] Illustrative cancers that may be treated include, but are not limited to, carcinomas, e.g. various subtypes, including, for example, adenocarcinoma, basal cell carcinoma, squamous cell carcinoma, and transitional cell carcinoma), sarcomas (including, for example, bone and soft tissue), leukemias (including, for example, acute myeloid, acute lymphoblastic, chronic myeloid, chronic lymphocytic, and hairy cell), lymphomas and myelomas (including, for example, Hodgkin and non-Hodgkin lymphomas, light chain, non-secretory, MGUS, and plasmacytomas), and central nervous system cancers (including, for example, brain (e.g. gliomas (e.g. astrocytoma, oligodendroglioma, and ependymoma), meningioma, pituitary adenoma, and neuromas, and spinal cord tumors (e.g. meningiomas and neurofibroma).

[00335] In certain embodiments, cancers/tumors that may be treated are those where the standard of care is no longer chemotherapy, since chemotherapy is known to interfere with immune responses, which are expected to occur during a successful vaccination protocol. Exemplary tumor types include tumor types treated with hormonal therapies such as prostate and breast cancers (e.g. Abiraterone® for prostate cancer and Tamoxifen® for breat cancer), tumor types treated with targeted therapies like antibodies (e.g. Rituxan® for B cell malignancies, Herceptin® for breast cancer), tumor types treated with kinase inhibitors such as GLEEVEC™ for chronic myelogenous leukemia and tumor types treated with other immune system sparing or enhancing modalities, such as checkpoint inhibitors, oncolytic viruses and CAR-T cells

[00336] Representative cancers and/or tumors of the present invention are described herein.

The described invention also provides compositions containing an allogeneic tumor cell vaccine comprising a tumor cell line variant comprising two or more stably expressed recombinant membrane bound immunomodulatory molecules selected from IgG1, CD40L, TNF-alpha, and Flt-3L peptides; and stably expressed recombinant soluble GM-CSF peptides; and a pharmaceutically acceptable carrier, as described herein, in combination with a physiologically and pharmaceutically acceptable carrier. The physiologically and pharmaceutically acceptable carrier can include any of the well-known components useful for immunization. The carrier can facilitate or enhance an immune response to an antigen administered in a vaccine. The cell formulations can contain buffers to maintain a preferred pH range, salts or other components that present an antigen to an individual in a composition that stimulates an immune response to the antigen. The physiologically acceptable carrier also can contain one or more adjuvants that enhance the immune response to an antigen. Pharmaceutically acceptable carriers include, for example, pharmaceutically acceptable solvents, suspending agents, or any other pharmacologically inert vehicles for delivering compounds to a subject. Pharmaceutically acceptable carriers can be liquid or solid, and can be selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, and other pertinent transport and chemical properties, when combined with one or more therapeutic compounds and any other components of a given pharmaceutical composition. Typical pharmaceutically acceptable carriers include, without limitation: water, saline solution, binding agents (e.g., polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose or dextrose and other sugars, gelatin, or calcium sulfate), lubricants (e.g., starch, polyethylene glycol, or sodium acetate), disintegrates (e.g., starch or sodium starch glycolate), and wetting agents (e.g., sodium

lauryl sulfate). Compositions can be formulated for subcutaneous, intramuscular, or intradermal administration, or in any manner acceptable for immunization.

[00337] An "adjuvant" refers to a substance which, when added to an immunogenic agent such as a tumor cell expressing secreted vaccine protein, nonspecifically enhances or potentiates an immune response to the agent in the recipient host upon exposure to the mixture. Adjuvants can include, for example, oil-in-water emulsions, water-in oil emulsions, alum (aluminum salts), liposomes and microparticles, such as, polysytrene, starch, polyphosphazene and polylactide/polyglycosides.

[00338] Adjuvants can also include, for example, squalene mixtures (SAF-I), muramyl peptide, saponin derivatives, mycobacterium cell wall preparations, monophosphoryl lipid A, mycolic acid derivatives, nonionic block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al., Nature 1990, 344:873-875. For veterinary use and for production of antibodies in animals, mitogenic components of Freund's adjuvant (both complete and incomplete) can be used. In humans, Incomplete Freund's Adjuvant (IFA) is a useful adjuvant. Various appropriate adjuvants are well known in the art (see, for example, Warren and Chedid, CRC Critical Reviews in Immunology 1988, 8:83; and Allison and Byars, in Vaccines: New Approaches to Immunological Problems, 1992, Ellis, ed., Butterworth-Heinemann, Boston). Additional adjuvants include, for example, bacille Calmett-Guerin (BCG), DETOX (containing cell wall skeleton of Mycobacterium phlei (CWS) and monophosphoryl lipid A from Salmonella minnesota (MPL)), and the like (see, for example, Hoover et al., J Clin Oncol 1993, 11:390; and Woodlock et al., J Immunother 1999, 22:251-259).

[00339] In some embodiments, an allogeneic tumor cell vaccine can be administered to a subject one or more times (e.g., once, twice, two to four times, three to five times, five to eight times, six to ten times, eight to 12 times, or more than 12 times). An allogeneic tumor cell vaccine as provided herein can be administered one or more times per day, one or more times per week, every other week, one or more times per month, once every two to three months, once every three to six months, or once every six to 12 months. An allogeneic tumor cell vaccine can be administered over any suitable period of time, such as a period from about 1 day to about 12 months. In some embodiments, for example, the period of administration can be from about 1 day to 90 days; from about 1 day to 60 days; from about 1 day to 30 days; from about 1 day to 20 days; from about 1 day to 10 days; from about 1 day to 7 days. In some embodiments, the period of administration can be from about 1 week to 50 weeks; from about 1 week to 50 weeks; from about 1 week to 40 weeks; from about 1 week to 30 weeks; from about 1 week to 24 weeks; from about 1 week to 20 weeks; from about 1 week to 16 weeks; from about 1 week to 12 weeks; from about 1 week to 8 weeks; from about 1 week to 4 weeks; from about 1 week to 3 weeks; from about 1 week to 2 weeks; from about 2 weeks to 3 weeks; from about 2 weeks to 4 weeks; from about 2 weeks to 6 weeks; from about 2 weeks to 8 weeks; from about 3 weeks to 8 weeks; from about 3 weeks to 12 weeks; or from about 4 weeks to 20 weeks.

[00340] In some embodiments, after an initial dose (sometimes referred to as a "priming" dose) of an allogeneic tumor cell vaccine has been administered and a maximal antigen-specific immune response has been achieved, one or more boosting doses can be administered. For example, a boosting dose can be administered about 10 to 30 days, about 15 to 35 days, about 20 to 40 days, about 25 to 45 days, or about 30 to 50 days after a priming dose.

[00341] In some embodiments, the methods provided herein can be used for controlling solid tumor growth and/or metastasis. The methods can include administering an effective amount of an allogeneic tumor cell vaccine as described herein to a subject in need thereof.

[00342] The vectors and methods provided herein can be useful for stimulating an immune response against a tumor. Such immune response is useful in treating or alleviating a sign or symptom associated with the tumor. A practitioner will appreciate that the methods described herein are to be used in concomitance with continuous clinical evaluations by a skilled practitioner (physician or veterinarian) to determine subsequent therapy. Such evaluations will aid and inform in evaluating whether to increase, reduce, or continue a particular treatment dose, mode of administration, etc.

[00343] The methods provided herein can thus be used to treat a tumor, including, for example, a cancer. The methods can be used, for example, to inhibit the growth of a tumor by preventing further tumor growth, by slowing tumor growth, or by causing tumor regression. Thus, the methods can be used, for example, to treat a cancer. It will be understood that the subject to which a compound is administered need not suffer from a specific traumatic state. Indeed, the allogeneic tumor cell vaccine described herein may be administered prophylactically, prior to development of symptoms (e.g., a patient in remission from cancer).

[00344] Anti-tumor and anti-cancer effects include, without limitation, modulation of tumor growth (e.g., tumor growth delay), tumor size, or metastasis, the reduction of toxicity and side effects associated with a particular anti-cancer agent, the amelioration or minimization of the clinical impairment or symptoms of cancer, extending the survival of the subject beyond that which would otherwise be expected in the absence of such treatment, and the prevention of

tumor growth in an animal lacking tumor formation prior to administration, i.e., prophylactic administration.

[00345] Therapeutically effective amounts can be determined by, for example, starting at relatively low amounts and using step-wise increments with concurrent evaluation of beneficial effects. The methods provided herein thus can be used alone or in combination with other well-known tumor therapies, to treat a patient having a tumor. One skilled in the art will readily understand advantageous uses of the allogeneic tumor cell vaccines and methods provided herein, for example, in prolonging the life expectancy of a cancer patient and/or improving the quality of life of a cancer patient (e.g., a lung cancer patient).

Subjects

[00346] The methods described herein are intended for use with any subject that may experience the benefits of these methods. Thus, "subjects," "patients," and "individuals" (used interchangeably) include humans as well as non-human subjects, particularly domesticated animals.

[00347] In some embodiments, the subject and/or animal is a mammal, e g., a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, rabbit, sheep, or non-human primate, such as a monkey, chimpanzee, or baboon. In other embodiments, the subject and/or animal is a non-mammal, such, for example, a zebrafish. In some embodiments, the subject and/or animal may comprise fluorescently-tagged cells (with e.g. GFP). In some embodiments, the subject and/or animal is a transgenic animal comprising a fluorescent cell.

[00348] In some embodiments, the subject and/or animal is a human In some embodiments, the human is a pediatric human In other embodiments, the human is an adult human. In other

embodiments, the human is a geriatric human In other embodiments, the human may be referred to as a patient.

[00349] In certain embodiments, the human has an age in a range of from about 0 months to about 6 months old, from about 6 to about 12 months old, from about 6 to about 18 months old, from about 18 to about 36 months old, from about 1 to about 5 years old, from about 5 to about 10 years old, from about 10 to about 15 years old, from about 15 to about 20 years old, from about 20 to about 25 years old, from about 25 to about 30 years old, from about 30 to about 35 years old, from about 35 to about 40 years old, from about 40 to about 45 years old, from about 45 to about 50 years old, from about 50 to about 55 years old, from about 55 to about 60 years old, from about 60 to about 65 years old, from about 65 to about 70 years old, from about 70 to about 75 years old, from about 75 to about 80 years old, from about 80 to about 85 years old, from about 85 to about 90 years old, from about 90 to about 95 years old or from about 95 to about 100 years old.

[00350] In other embodiments, the subject is a non-human animal, and therefore the invention pertains to veterinary use. In a specific embodiment, the non-human animal is a household pet. In another specific embodiment, the non-human animal is a livestock animal In certain embodiments, the subject is a human cancer patient that cannot receive chemotherapy, e.g. the patient is unresponsive to chemotherapy or too ill to have a suitable therapeutic window for chemotherapy (e.g. experiencing too many dose- or regimen-limiting side effects). In certain embodiments, the subject is a human cancer patient having advanced and/or metastatic disease.

[00351] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the

upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges which may independently be included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention.

[00352] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and described the methods and/or materials in connection with which the publications are cited.

[00353] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. All technical and scientific terms used herein have the same meaning.

[00354] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application and each is incorporated by reference in its entirety. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

EXAMPLES

[00355] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1.

[00356] Examples 2-5 make use of, but are not limited to, the methods described hereinbelow.

Western Blotting

[00357] Briefly, cells are lysed with cold lysis buffer and centrifuged to pellet cellular debris. Protein concentration of the supernatant is determined by a protein quantification assay (e.g., Bradford Protein Assay, Bio-Rad Laboratories). The lysate supernatant is then combined with an equal volume of 2X SDS sample buffer and boiled at 100° C for 5 minutes. Equal amounts of protein in sample buffer are loaded into the wells of an SDS-PAGE gel along with molecular weight marker and electrophoresed for 1-2 hours at 100 V. Proteins are then transferred to a nitrocellulose or PVDF membrane. The membrane is then blocked for 1 hour at room temperature using 5% non-fat dry milk in TBST blocking buffer. The membrane is then incubated with a 1:500 dilution of primary antibody in 5% non-fat dry milk in TBST blocking buffer, followed by three washes in 20 Mn Tris, Ph 7.5; 150 mM NaCl, 0.1% Tween 20 (TBST)

for 5 minutes. The membrane is then incubated with conjugated secondary antibody at a 1:2000 dilution in 5% non-fat dry milk in TBST blocking buffer for 1 hour at room temperature, followed by three washes in TBST for 5 minutes each. Images of the blot are obtained using dark room development techniques for chemiluminesence detection, or using image scanning techniques for colorimetric or fluorescent detection.

Real Time PCR

[00358] Real-time PCR techniques may be performed as described to analyze expression level of mRNAs (Zhao Y. et al., Biochemical and Biophysical Research Communications 360 (2007) 205-211). Briefly, total RNA is extracted from cells using the Quiagen kit (Valencia CA), followed by first strand cDNA synthesis using random hexamer primers (Fermentas, Hanover MD). Real-time PCR is performed on each sample using the Mx3000p Quantitative PCR system (Stratagene, La Jolla, CA), for 40 cycles using validated gene specific RT-PCR primer sets for each gene of interest. Relative expression level of each transcript is corrected for that of the house keeping gene beta-actin as an internal control.

Immunofluorescence

[00359] Briefly, adherent tumor cell line variant cells are fixed with 4% formaldehyde diluted in warm PBS for 15 minutes at room temperature. The fixative is aspirated and the cells washed three times with PBS for 5 minutes each. Cells are blocked in a 5% BSA blocking buffer for 60 minutes at room temperature. Blocking buffer is then aspirated and a solution of primary antibody (e.g. 1:100 dilution) is incubated with the cells overnight at 4° C. Cells are then rinsed three times with PBS for 5 minutes each, and subsequently incubated with a solution of fluorochrome conjugated secondary antibody (e.g. 1:1000 dilution) for 1-2 hours at room

temperature. Cells are then washed three times with PBS for 5 minutes each and visualized by fluorescence microscopy.

Flow Cytometry

[00360] Flow Cytomtery analysis may be performed as described (Zhao Y. et al., Exp. Cell Res., 312, 2454 (2006)). Briefly, tumor cell line variant cells that are either treated with trypsin/EDTA or left untreated are collected by centrifugation and re-suspended in PBS. The cells are fixed in 4% formaldehyde for 10 minutes at 37° C. For extracellular staining with antibodies, cells are not permeabilized. For intracellular staining, cells are permeabilized by adding ice-cold 100% methanol to pre-chilled cells to a final concentration of 90% methanol and incubated on ice for 30 minutes. Cells are immunostained by first resuspending cells in incubation buffer and adding dilutions of primary antibody. Cells are incubated with primary antibody for 1 hour at room temperature, followed by three washes with incubation buffer. Cells are then resuspended in incubation buffer with dilutions of conjugated secondary antibody for 30 minutes at room temperature, followed by three washes in incubation buffer. Stained cells are then analyzed by flow cytometry.

Enzyme-linked immunosorbent assay (ELISA)

[00361] Briefly, a capture antibody, specific for a protein of interest, is coated onto the wells of a microplate. Samples, including a standard containing protein of interest, control specimens, and unknowns, are pipetted into wells of the microplate, where the protein antigen binds to the capture antibody. After washing 4 times, a detection antibody is added to the wells for one hour, binding to the immobilized protein captured during the first incubation. After removal of excess detection antibody and washing 4 times, a horse radish peroxidase (HRP) conjugate (secondary

antibody or streptavidin) is added for 30 minutes to bind to the detection antibody. After washing 4 more times to remove the excess HRP conjugate, a substrate solution is added for 30 minutes in the dark to be converted by the enzyme to a detectable form (color signal). A stop solution is added to each well of the microplate and evaluated within 30 minutes of stopping the reaction. Intensity of the colored product may be directly proportional to the concentration of antigen present in the original specimen.

Human Mixed Lymphocyte Tumor Reaction (MLTR) Testing

[00362] The mixed lymphocyte tumor reaction (MLTR) is an all human, *in vitro* assay, designed to optimize lead candidates. In the MLTR, optimization is achieved through the qualitative and quantitative assessment of human peripheral blood mononuclear cell (PBMC) responses to engineered allogeneic tumor cells. The MLTR assay permits assessment of proliferation and differentiation by flow cytometry and mass cytometry (CyTOF). Cytotoxicity, can be measured by lactate dehydrogenase (LDH) release assay, and the cytokine profile can be measured by Luminex multiplex assay. In certain embodiments, allogeneic cell pools expressing a single immunomodulatory protein are used in the MLTR. In other embodiments, allogeneic cell pools expressing multiple immunomodulatory proteins are used in the MLTR.

[00363] The basic MLTR one day procedure is carried out as follows:

[00364] Frozen human PBMC are thawed. Cells are then washed in dPBS. PMBC cells are resuspended at 2.5 x 10^6 cell per ml stock in X-VIVO serum free media (Invitrogen). The cells are characterized by flow cytometry to document and confirm the phenotypic nature of the cell population.

[00365] The MLTR set up in the following fashion:

- 2.5 x 10^5 cell PBMC (100 µl of stock)
- 0.5 x 10^5 allogeneic cells (100 µl of stock), when used
- Positive control 50 µl of a 6x stock (anti-CD28/CD3)
- Total volume 300 µl in a 96-well flat bottom –total volume of a 96-well is 300 ul.
- Incubate for 1 day.
- 100 µl is removed for cytokine analysis by Luminex multiplex assay
- CyTOF is conducted on the cellular component.

[00366] CyTOF has been previously described, for example in Bendall et al. (Science, Vol. 332, 6 May 2011) and Bendall and Nolan (Nature Biotechnology, Vol. 30 No. 7, July 2012), both of which are incorporated by reference in their entireties herein. Human markers employed in CyTOF staining are shown below in Table 1.

Table 1.

Human Markers for CyTOF Staining			
*	denotes intracellular targets while all others are cell surface targets		
	Marker	**Clone**	**Metal**
	HLA-DR	L243	89Y
	CD3	UCHT1	115In
	CD27	O323	141Pr
	CD19	HIB19	142Nd
	CD134/OX40	Ber-ACT35	143Nd
*	Granzyme B	GB11	144Nd
	CD258/LIGHT	115520	145Nd
	CD8A	RPA t8	146Nd
	CD45RO	UCHL1	147Sm
	CD226/DNAM-1	11A8	149Sm
	CD194/CCR4	L291H4	150Nd
	PD1 (CD279)	EH12.2H7	151Eu
	CD170	1A5	152Sm

Human Markers for CyTOF Staining			
*	denotes intracellular targets while all others are cell surface targets		
	CD69	FN50	153Eu
	CD70	113-16	154Sm
	CD4	RPA T4	155Gd
	CD8b	SIDI8BEE	156Gd
	IL-17R	W15177A	158Gd
*	CTLA-4 CD152	L3D10	159Tb
	CD278/ICOS	C398.4A	160Gd
*	AHR	FF3399	161Dy
	CD56	NCAM16.2	162Dy
	CD195/CCR5	J418F1	163Dy
*	Ki67	8D5	164Dy
*	FoxP3	Use Ebio	165Ho
	CD40	5C3	166Er
*	Helios	22F6	168Er
*	PU.1	puph13	169Tm
*	RORgt	1181A	170Er
	CD127/IL-7R	40131	171Yb
	CD38	HIT2	172Yb
	CD25	M-A251	173Yb
	CD86	IT2.2	174Yb
*	T-bet	4B10	175Lu
*	Perforin	dG9	176Yb

Luminex Multiplex Assay

[00367] The Luminex xMAP technology (formerly LabMAP, FlowMetrix) uses digital signal processing capable of classifying polystyrene beads (microspheres) dyed with distinct proportions of red and near-infrared fluorophores. These proportions define 'spectral addresses' for each bead population. As a result, up to one hundred different detection reactions can be carried out simultaneously on the various bead populations in very small sample volumes (Earley et al. Report from a Workshop on Multianalyte Microsphere Arrays. Cytometry 2002;50:239–242; Oliver et al. Clin Chem 1998;44(9):2057–2060; Eishal and McCoy, Methods 38(4): 317-323, April 2006, all of which are incorporated by reference in their entireties herein).

The Luminex Multiplex Assay is commercially available and is described at thermofisher.com/us/en/home/life-science/protein-biology/protein-assays-analysis/luminex-multiplex-assays.html, incorporated by reference in its entirety herein. *Mitomycin C Preparation of Allogeneic Cells for MLTR Assay of Greater than 1 Day Duration.*

[00368] Mitomycin C is prepared from dry powder (2 mg per vial) using 400µl of DMSO (500x stock = 5 mg/ml), dissolved completely and aliquoted into 25 ul volumes, and stored at -80C. 20 µl of a single aliquot is used in 10 ml warmed C5 to yield 10 µg/ml final working solution. The solution is filter sterilized.

[00369] The solution can be used on resuspended cells or adherent cells in flasks.

[00370] Cells are incubated at 37C for 30 minutes in the dark, then washed in warm C5 cell culture media (RPMI, supplemented with non-essential amino acids, glutamine, antibiotics and 5% Fetal calf serum) three times. Cells are resuspended in 1 ml X-VIVO, counted and final concentration adjusted to $1x10^6$ /ml stock solution in X-VIVO (serum free media, Lonza).

Example 2

[00371] The described invention provides an approach for restoring immunologic balance in, for example, treating cancer, by targeting multiple immunomodulators with a single cellular platform. This approach enables the simultaneous modulation of multiple signals, and affords a spatially and temporally restricted method of modulating the immune response, an important feature that differentiates this methodology from traditional approaches using systemic administration of biologic agents to act on a single immunomodulatory pathway at a time.

[00372] According to one aspect of the disclosed invention, a tumor-type specific cell line variant expressing five or more recombinant peptides may be generated for use as a tumor cell vaccine to treat that cancer type. For example, a tumor cell line may be selected for modification and lentiviral transfection of recombinant immunomodulator sequences may be used to stably integrate immune modulators into the cell genome. Example 3 below describes 7 lentiviral vectors (vector 1, vector 2, vector 3, vector 4, vector 5, vector 6 and vector 7) that may be used to stably integrate immune modulators into the cell genome.

[00373] According to some embodiments, two recombinant immunomodulator proteins may be transfected simultaneously, followed by transfections of two more recombinant immunomodulator proteins simultaneously, followed by transfection of a single recombinant immunomodulator protein to achieve the total of five recombinant peptides for use as a tumor cell vaccine. According to some embodiments, two recombinant peptides may be transfected simultaneously, followed by transfection of a single recombinant peptide, followed by transfection of a single recombinant peptide, followed by transfection of a single recombinant peptide to achieve the total of five recombinant peptides for use as a tumor cell vaccine. According to some embodiments, a single recombinant peptide is transfected, followed by transfection of two recombinant peptides simultaneously, followed by transfection of two recombinant peptides simultaneously to achieve a total of five recombinant peptide for use as a tumor cell vaccine.

[00374] According to one embodiment of the disclosed invention, combinations of allogeneic cell pools, each expressing a single immunomodulatory protein, are used to model what a single

cell expressing multiple immunomodulatory proteins might do (*e.g.* additivity, synergy, interference).

[00375] According to one aspect of the disclosed invention, a tumor cell line variant expressing one, two, three, four, five or more recombinant peptides may be generated for use as a tumor cell vaccine to treat skin cancer. For example, the SK-MEL2 human melanoma cell line (ATCC HTB-68) may be selected for modification, and lentiviral transfection of recombinant immune modulator sequences may be used to stably integrate immune modulators into the cell genome.

[00376] According to one aspect of the disclosed invention, a tumor cell line variant expressing one, two, three, four, five or more recombinant peptides may be generated for use as a tumor cell vaccine to treat a prostate cancer. For example, the DU-145 human prostate carcinoma cell line may be selected for modification, and lentiviral transfection of recombinant immune modulator sequences may be used to stably integrate immune modulators into the cell genome. According to some embodiments, two recombinant immunomodulator proteins may be transfected simultaneously, followed by transfections of two more recombinant immunomodulator proteins simultaneously, followed by transfection of a single recombinant immunomodulator protein to achieve the total of five recombinant peptides for use as a tumor cell vaccine. According to some embodiments, two recombinant peptides may be transfected simultaneously, followed by transfection of a single recombinant peptide, followed by transfection of a single recombinant peptide, followed by transfection of a single recombinant peptide to achieve the total of five recombinant peptides for use as a tumor cell vaccine. According to some embodiments, a single recombinant peptide is transfected, followed by

transfection of two recombinant peptides simultaneously, followed by transfection of two recombinant peptides simultaneously to achieve a total of five recombinant peptide for use as a tumor cell vaccine.

[00377] According to another aspect of the present invention, two or more tumor cell line variants expressing one or more recombinant peptides may be generated for use as a tumor cell vaccine to treat a prostate cancer. For example, the DU-145 and PC-3 human prostate carcinoma cell line may be selected for modification, and lentiviral transfection of recombinant immune modulator sequences may be used to stably integrate immune modulators into the cell genome.

CD40L immunomodulator

[00378] The CD40L immune modulator cDNA sequence may be cloned into the lentiviral transfer plasmid construct pLenti-puro (Addgene Cat. No. 39481) driven by a CMV promoter with puromycin selectable marker. The CD40L immune modulator cDNA sequence may be engineered to be non-cleavable, which ultimately keeps the translated CD40L protein in a membrane bound state (e.g. SEQ ID NO: 7).

[00379] Each of the lentiviral transfer plasmid, packaging plasmid, and envelope plasmid may be transfected into log phase growth 293T cells using Lipofectamine 2000 (ThermoFisher Cat. No. 11668027). Briefly, cells are seeded at 70% to 90% confluence. On the day of transfection, 12 µl of Lipofectamine reagent is diluted in 150 µl of serum free cell media. 5 µg of DNA for transfection is also diluted in 150 µl of serum free media. The diluted DNA is then added to the diluted Lipofectamine and incubated for 5 minutes at room temperature. The total volume of the mixture is then added dropwise to the media of the seeded 293T cells while swirling. Cells are then incubated for one to three days at 37 degrees.

[00380] The 293T cell culture medium comprising virus particle is harvested 3 times every 8-12 hours and centrifuged to pellet detached cells and debris. The culture medium containing virus particles is used directly to infect the DU-145 cell line.

[00381] The DU-145 cell line is cultured in Eagle's Minimum Essential Medium (EMEM) with 10% fetal bovine serum to a confluency of about 70%. Hexadimethrine bromide (Sigma-Aldrich Cat No. H9268) is then combined with media containing virus particles to make a final concentration of 8μg/mL Hexadimethrine bromide. Culture media of the DU-145 cells is aspirated and replaced with media containing virus particles and 8μg/mL Hexadimethrine bromide. DU-145 cells are cultured for 18-20 hours followed by media change.

[00382] Infected DU-145 cells are then grown in media containing 1μg/mL Puromycin (ThermoFisher Cat. No. A1113802) until cell die off begins after about a week. Multiple surviving colonies of transfected cells are picked for expansion and tested for CD40L expression by Western blot. The Western blot is probed with mouse monoclonal anti-HA primary antibodies (Abcam Cat. No. ab18181) and goat anti-mouse HRP (Abcam Cat. No. ab205719) secondary antibodies to quantify the relative amounts of recombinant CD40L expressed in each clonal line. The highest stably expressing DU-145 line is labeled DU145-Gen1 and selected for further manipulation.

TNF-alpha/GM-CSF

[00383] The DU145-Gen1 cells transfected to express CD40L are further transfected with a bi-cistronic lentiviral vector comprising TNF-alpha and GM-CSF sequences. Each of TNF-alpha cDNA and GM-CSF cDNA is first cloned into the pEF1α-IRES bicistronic mammalian expression vector (Clontech Cat. No. 631970) under the control of the human elongation factor 1

alpha (EF1α) promoter. A variant of TNF-alpha that cannot be cleaved by TACE is used so that the translated protein remains in membrane bound form. The TNF-alpha sequence is provided with a FLAG tag sequence on the extracellular region of TNF-alpha for easy detection of translated protein. The FLAG tag peptide sequence is DYKDDDDK (SEQ ID NO: 29). GM-CSF sequences capable of forming soluble GM-CSF are used. The entirety of the pEF1 promoter, TNF-alpha sequences, IRES sequences, and GM-CSF sequences is then cloned into the pLenti-puro (Addgene Cat. No. 39481) lentiviral vector (the original CMV promoter from the vector is removed during this process). Packaging plasmid psPAX2 (AddGene Cat. No. 12260) and envelope plasmid pLTR-RD114A (AddGene Cat. No. 17576) are also selected.

[00384] Each of the lentiviral transfer plasmid, packaging plasmid, and envelope plasmid is transfected into log phase growth 293T cells using Lipofectamine 2000 (ThermoFisher Cat. No. 11668027). Briefly, cells are seeded at 70% to 90% confluence. On the day of transfection, 12 µl of Lipofectamine reagent is diluted in 150 µl of serum free cell media. 5 µg of DNA for transfection is also diluted in 150 µl of serum free media. The diluted DNA is then added to the diluted Lipofectamine and incubated for 5 minutes at room temperature. The total volume of the mixture is then added dropwise to the media of the seeded 293T cells while swirling. Cells are then incubated for one to three days at 37 degrees.

[00385] The 293T cell culture medium comprising virus particle is harvested 3 times every 8-12 hours and centrifuged to pellet detached cells and debris. The culture medium containing virus particles is used directly to infect the DU145-Gen1 cell line.

[00386] The DU145-Gen1 cell line is cultured to a confluency of about 70%. Hexadimethrine bromide (Sigma-Aldrich Cat No. H9268) is then combined with media

containing virus particles to make a final concentration of 8μg/mL Hexadimethrine bromide. Culture media of the DU145-Gen1 cells is aspirated and replaced with media containing virus particles and 8μg/mL Hexadimethrine bromide. DU145-Gen1 cells are cultured for 18-20 hours followed by media change.

[00387] The transduced DU145-Gen1 cells are then selected for clones that stably express the recombinant immune modulators. The selection process is performed by fluorescence activated cell sorting using the FLAG tag on the TNF-alpha to identify cells that have integrated the immune modulators. Live cells are probed with mouse monoclonal anti-FLAG antibody (Sigma Aldrich F3040) and rabbit anti-mouse FITC conjugated secondary antibody (Sigma Aldrich ASB3701170) in PBS with blocking buffer. The highest expressing cells are sorted, isolated, and cultured for further processing. After sorting based on the presence of the FLAG tag, expression of soluble GM-CSF is confirmed by Western blot. Concentrated media of sorted cultured cells is resolved by SDS-PAGE and probed by Western blot with mouse anti-GM-CSF antibody (ThermoFisher Cat. No. 3092) and goat anti-mouse HRP conjugated secondary antibody. Cell lysate may also be resovled by SDS-PAGE and probed for FLAG tag to verify the presence of TNF. Cell cultures that express high levels of recombinant GM-CSF and TNF-alpha are designated DU145-Gen2 and selected for further processing.

Flt-3L

[00388] The DU145-Gen2 cells transfected to express CD40L, GM-CSF, and TNF are further transfected with a lentiviral vector comprising Flt-3L immune modulator sequences. The Flt-3L cDNA is cloned into a pEF1α-IRES bicistronic mammalian expression vector (Clontech Cat. No. 631970), along with GFP protein sequences to be used as a marker for integration and

expression. The sequence of Flt-3L is translated into a membrane bound peptide, while the GFP remains cytoplasmic. The entirety of the pEF1 promoter, Flt-3L sequences, IRES sequences, and GFP sequences is then cloned into the pLenti-puro (Addgene Cat. No. 39481) lentiviral vector (the original CMV promoter from the vector is removed during this process). Packaging plasmid psPAX2 (AddGene Cat. No. 12260) and envelope plasmid pLTR-RD114A (AddGene Cat. No. 17576) are also selected.

[00389] Each of the lentiviral transfer plasmid, packaging plasmid, and envelope plasmid is transfected into log phase growth 293T cells using Lipofectamine 2000 (ThermoFisher Cat. No. 11668027). Briefly, cells are seeded at 70% to 90% confluence. On the day of transfection, 12 µl of Lipofectamine reagent is diluted in 150 µl of serum free cell media. 5 µg of DNA for transfection is also diluted in 150 µl of serum free media. The diluted DNA is then added to the diluted Lipofectamine and incubated for 5 minutes at room temperature. The total volume of the mixture is then added dropwise to the media of the seeded 293T cells while swirling. Cells are then incubated for one to three days at 37 degrees.

[00390] The 293T cell culture medium comprising virus particle is harvested 3 times every 8-12 hours and centrifuged to pellet detached cells and debris. The culture medium containing virus particles is used directly to infect the DU145-Gen2 cell line.

[00391] The DU145-Gen2 cell line is cultured to a confluency of about 70%. Hexadimethrine bromide (Sigma-Aldrich Cat No. H9268) is then combined with media containing virus particles to make a final concentration of 8µg/mL Hexadimethrine bromide. Culture media of the DU145-Gen2 cells is aspirated and replaced with media containing virus

particles and 8μg/mL Hexadimethrine bromide. DU145-Gen2 cells are cultured for 18-20 hours followed by media change.

[00392] The DU145-Gen2 cells are then selected for cells stably expressing the Flt-3L sequences using the GFP marker. The selection process is performed by fluorescence activated cell sorting (FACS) using the GFP marker to identify cells that have integrated the immune modulator. The highest expressing cells are sorted, isolated, and cultured for further processing. After sorting based on the presence of the GFP marker, the expression of Flt-3L is confirmed by Western blot. Cultured cell lysates are resolved by SDS-PAGE and probed by Western blot with rabbit polyclonal anti-Flt-3L antibody (AbCam Cat. No. ab9688) and goat anti-rabbit HRP conjugated secondary antibody (AbCam Cat. No. ab205718). Cell cultures that express high levels of recombinant Flt-3L are designated DU145-Gen3 and are selected for further processing.

IgG Heavy and Light chains

[00393] The DU145-Gen3 cells transfected to express CD40L, GM-CSF, TNF-alpha, and Flt-3L are further transfected with a lentiviral vector comprising IgG 1 (SEQ ID NO: 1), a membrane bound IgG1 heavy chain fragment. The IgG1 heavy chain cDNA is cloned into pEF1α-IRES bicistronic mammalian expression vector (Clontech Cat. No. 631970), along with RFP protein sequences to be used as a marker for integration and expression. The sequence of IgG1 heavy chain is translated into a membrane bound peptide, while the RFP remains cytoplasmic. The entirety of the pEF1 promoter, IgG1 heavy chain sequence, IRES sequence, and RFP sequence is then cloned into the pLenti-puro (Addgene Cat. No. 39481) lentiviral vector (the original CMV promoter from the vector is removed during this process). Packaging plasmid

psPAX2 (AddGene Cat. No. 12260) and envelope plasmid pLTR-RD114A (AddGene Cat. No. 17576) are also selected.

[00394] Each of the lentiviral transfer plasmid, packaging plasmid, and envelope plasmid is transfected into log phase growth 293T cells using Lipofectamine 2000 (ThermoFisher Cat. No. 11668027). Briefly, cells are seeded at 70% to 90% confluence. On the day of transfection, 12 µl of Lipofectamine reagent is diluted in 150 µl of serum free cell media. 5 µg of DNA for transfection is also diluted in 150 µl of serum free media. The diluted DNA is then added to the diluted Lipofectamine and incubated for 5 minutes at room temperature. The total volume of the mixture is then added dropwise to the media of the seeded 293T cells while swirling. Cells are then incubated for one to three days at 37 degrees.

[00395] The 293T cell culture medium comprising virus particle is harvested 3 times every 8-12 hours and centrifuged to pellet detached cells and debris. The culture medium containing virus particles is used directly to infect the DU145-Gen3 cell line.

[00396] The DU145-Gen3 cell line is cultured to a confluency of about 70%. Hexadimethrine bromide (Sigma-Aldrich Cat No. H9268) is then combined with media containing virus particles to make a final concentration of 8µg/mL Hexadimethrine bromide. Culture media of the DU145-Gen2 cells is aspirated and replaced with media containing virus particles and 8µg/mL Hexadimethrine bromide. DU145-Gen3 cells are cultured for 18-20 hours followed by media change.

[00397] The DU145-Gen3 cells are then selected for cells stably expressing the IgG1 heavy chain sequences using the RFP marker. The selection process is performed by fluorescence activated cell sorting (FACS) using the RFP marker to identify cells that have integrated the

immune modulator. The highest expressing cells are sorted, isolated, and cultured for further processing. After sorting based on the presence of the RFP marker, the expression of IgG1 heavy chain is confirmed by Western blot. Cell cultures that express high levels of recombinant IgG1 heavy chain are designated DU145-Gen4 and are selected for further processing.

[00398] The DU145-Gen4 tumor cell line transfected to express CD40L, GM-CSF, TNF, Flt-3L, and IgG1 heavy chain is characterized by RT-PCR, immunofluorescence, and Western blotting to confirm all recombinant immune modulators are expressed by the cells and are in the right location (e.g. on the membrane of the cell).

Human Mixed Lymphocyte Tumor Reaction (MLTR) Testing

[00399] The DU145-Gen4 cells are tested for their immunomodulatory potential by primary and secondary MLTR assay against each of the other generations (i.e. DU145-Gen2 and DU145-Gen3) of modified cells and unmodified DU145 cells.

[00400] Peripheral blood mononuclear cells (PBMCs) are obtained from the peripheral blood of healthy individuals and from prostate cancer patients, and the blood cells separated using a Ficoll-Paque gradient. Anticoagulant-treated blood is diluted in the range of 1:2 to 1:4 with PBS/EDTA to reduce aggregation of erythrocytes. The diluted blood is then layered above a Ficoll-Paque solution in a centrifuge tube, without mixing. The layered blood/Ficoll-Paque is centrifuged for 40 minutes at 400 x g between 18° and 20° C, without the use of the centrifuge brake, resulting in the formation of blood fractions. The fraction comprising mononuclear cells is selected for further processing.

[00401] Each of the cells from the transfected tumor cell line variants and from parental tumor cell line DU-145 (control) is co-cultured with PBMCs for seven days under standard tissue

culture conditions, followed by evaluation for immune cell proliferation, immune cell differentiation, measured by flow cytometry and CyTOF, cytokine release profile, and cytoxicity, measured by LDH release assay.

Example 3.

[00402] A schematic of the core lentiviral vectors employed in the experiments described herein is shown in FIG. 2A and a schematic of the encoded proteins is shown in FIG. 2B. The promoter is human elongation factor 1 alpha (EF1α) promoter and the internal ribosomal entry sequence (IRES) is derived from encephalomyocarditis virus (EMCV). The core vectors are described in detail hereinbelow as follows:

Vector 1. Immunomodulator: scFv-anti- biotin-G3hinge-mIgG1 (to generate surface IgG)

[00403] A schematic of the organization of vector 1, used for the immunomodulator scFv-anti-biotin-G3hinge-mIgG1 is shown in FIG. 3A. The nucleotide sequence of vector 1 (SEQ ID NO. 47) is shown in FIG. 3B. Table 2, below, shows the vector component name, the corresponding nucleotide position in SEQ ID NO. 47, the full name of the component and a description.

[00404] Table 2

Component Name	Nucleotide Position	Full Name	Description
RSV promoter	1-229	Rous sarcoma virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA.
Δ5' LTR	230-410	HIV-1 truncated 5' LTRT	Permits viral packaging and reverse transcription of the viral mRNA.
Ψ	521-565	HIV-1 psi packaging signal	Allows viral packaging.
RRET	1075-1308	HIV-1 Rev response element	Permits Rev-dependent nuclear export of unspliced viral mRNA.

Component Name	Nucleotide Position	Full Name	Description
cPPT	1803-1920	Central polypurine tract	Facilitates the nuclear import of HIV-1 cDNA through a central DNA flap.
EF1A	1959-3137	EF1A	Component entered by user
Kozak	3162-3167	Kozak	Component entered by user
{ORF1}	3168-5005	{ORF1}	Component entered by user
WPRE	5044-5641	Woodchuck hepatitis virus posttranscriptional regulatory element	Facilitates effective transcription termination at the 3'TLTR.T
ΔU3/3' LTR	5723-5957	HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5'LTR for biosafety purposes. The element also contains apolyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 early pA	6030-6164	SV40 early polyadenation signal	Allows transcription termination and polyadenylation of mRNA.
Ampicillin	7118-7978	Ampicillin resistance gene	Allows selection of the plasmid in E.coli.
pUC ori	8149-8737	pUC origin of replication	Permits high-copy replication and maintenance in E.coli.

[00405] When vector 1 is employed, anti-IgG is used for flow detection. A biotin + fluorescent labelled oligodeoxynucleotides (ODN) is used as a secondary detection method.

[00406] The following is a description of the immunomodulator scFv-anti-biotin-G3hinge-IgG1-Tm.

[00407] Type:

- Immunoglobulin

[00408] Annotation:

- H7 heavy chain leader

- Anti-biotin Variable Heavy chain (VH) allows for loading biotin labeled CpG
- Inter-domain disulfide linkage VH44 (G->C) and VL100 (G->C)
- IgG3 hinge to enhance FcyR interaction
- Linkage is standard
- IgG1 (CH2-CH3-Tm-Cyt) used for interaction with FcyR/FcRn and membrane anchoring
- T233A mutation to enhance FcRn and FcyR interaction

[00409] The sequences are shown as follows:

[00410] H7 heavy chain leader (SEQ ID NO. 54)

MEFGLSWVFLVALFRGVQC

[00411] anti-biotin murine vH with inserted Cys for inter-domain linkage (SEQ ID NO. 55)

QVKLQESGPG LVAPSQSLSI TCTVSGFSLT AYGVDWVRQP PGK**C**LEWLGV

IWGGGRTNYN SGLMSRLSIR KDNSKSQVFL TMNSLQTDDT AKYYCVKHTN

WDGGFAYWGQ GTTVTVSS

[00412] linker (SEQ ID NO. 56)

GGGGSGGGGS GGGGS

[00413] Light Chain Variable (human lambda variable) (SEQ ID NO. 57)

GSPGQSVSIS CSGSSSNIGN NYVYWYQHLP GTAPKLLIYS DTKRPSGVPD

RISGSKSGTS ASLAISGLQS EDEADYYCAS WDDSLDGPVF G**C**GTKLTVL

[00414] IgG3 hinge for greater accessibility to FcyR (SEQ ID NO. 58)

LKTPLGDTTHTCPR CPEPKSCDTP PPCPRCPEPK SCDTPPPCPR

CPEPKSCDTP PPCPRCP

[00415] IgG1 CH2, CH3 Tm and cytoplasmic tail (T256A) (SEQ ID NO. 59)

LLGGPSVFLF PPKPKDTLMI SRAPEVTCVV VDVSHEDPEV KFNWYVDGVE

VHNAKTKPRE EQYNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKALPAPIE

KTISKAKGQP REPQVYTLPP SRDELTKNQV SLTCLVKGFY PSDIAVEWES

NGQPENNYKT TPPVLDSDGS FFLYSKLTVD KSRWQQGNVF SCSVMHEALH

NHYTQKSLSL SPELQLEESC AEAQDGELDG LWTTITIFIT LFLLSVCYSA

TVTFFKVKWI FSSVVDLKQT IIPDYRNMIG QGA*

[00416] The following shows the sequence of scFv-anti-biotin-G3hinge-IgG1-Tm (598 ORF1) (SEQ ID NO. 60)

MEFGLSWVFLVALFRGVQCQVKLQESGPGLVAPSQSLSITCTVSGFSLTA

YGVDWVRQPPGKCLEWLGVIWGGGRTNYNSGLMSRLSIRKDNSKSQVFLT

MNSLQTDDTAKYYCVKHTNWDGGFAYWGQGTTVTVSSGGGGSGGGGSGGG

GSGSPGQSVSISCSGSSSNIGNNYVYWYQHLPGTAPKLLIYSDTKRPSGV

PDRISGSKSGTSASLAISGLQSEDEADYYCASWDDSLDGPVFGCGTKLTV

LLKTPLGDTTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPEPK

SCDTPPPCPRCPAPELLGGPSVFLFPPKPKDTLMISRAPEVTCVVVDVSH

EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE

YKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCL

VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ

QGNVFSCSVMHEALHNHYTQKSLSLSPELQLEESCAEAQDGELDGLWTTI

TIFITLFLLSVCYSATVTFFKVKWIFSSVVDLKQTIIPDYRNMIGQGA*

Vector 2. Immunomodulator: full anti-biotin – G3hinge-mIgG1 (using heavy chain/ires/light chain)

[00417] A schematic of the organization of vector 2, used for the immunomodulator full anti-biotin – G3hinge-mIgG1 is shown in FIG. 4A. Vector 2 is bicistronic. The nucleotide sequence of vector 2 (SEQ ID NO. 48) is shown in FIG. 4B. Table 3, below, shows the vector component name, the corresponding nucleotide position in SEQ ID NO. 48, the full name of the component and a description.

[00418] Table 3

Component Name	**Nucleotide Position**	**Full Name**	**Description**
RSV promoter	1-229	Rous sarcoma virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA.
Δ5' LTR	230-410	HIV-1 truncated 5' LTRT	Permits viral packaging and reverse transcription of the viral mRNA.
Ψ	521-56g	HIV-1 psi packaging signal	Allows viral packaging.
RRET	1075-1308	HIV-1 Rev response element	Permits Rev-dependent nuclear export of unspliced viral mRNA.
cPPT	1803-1920	Central polypurine tract	Facilitates the nuclear import of HIV-1 cDNA through a central DNA flap.
EF1A	1950-3128	EF1A	Component entered by user
Kozak	3153-3158	Kozak	Component entered by user
{ORF1}	3159-5342	{ORF2A}	Component entered by user
WPRE	6703-7300	Woodchuck hepatitis virus	Facilitates effective transcription

Component Name	Nucleotide Position	Full Name	Description
		posttranscriptional regulatory element	termination at the 3'TLTR.T
ΔU3/3' LTR	7382-7616	HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5'LTR for biosafety purposes. The element also contains apolyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 early pA	7689-7823	SV40 early polyadenation signal	Allows transcription termination and polyadenylation of mRNA.
Ampicillin	8777-9637	Ampicillin resistance gene	Allows selection of the plasmid in E.coli.
pUC ori	9808-10396	pUC origin of replication	Permits high-copy replication and maintenance in E.coli.

[00419] When vector 2 is employed, anti-IgG is used for flow detection. Biotin + fluorescent labelled ODN is used as a secondary detection method.

[00420] The following is a description of the immunomodulator full anti-biotin – G3hinge-mIgG1 (using heavy chain/ires/light chain).

[00421] Type:

- Membrane anchored Immunoglobulin

[00422] Annotation:

- H7 heavy chain leader
- IgG3 hinge to enhance FcyR interaction
- T233A mutation to enhance FcRn and FcyR interaction
- Anti-biotin Variable H allows for loading biotin labeled CpG
- CH1 (generic)

- LC Variable (human lambda variable)
- LC Constant Region 1 from Lambda (http://www.uniprot.org/uniprot/P0CG04)
- Interdomain disulfide linkage VH44 (G->C) and VL100 (G->C) (ref)
- Linkage is standard
- IgG1 (CH2-CH3-Tm-Cyt) for interaction with FcyR/FcRn and membrane anchoring
- L1 light chain leader (modified for improved IRES expression)

  MATDMRVPAQLLGLLLLWLSGARC (SEQ ID NO. 61)

[00423] The sequences are shown as follows:

[00424] H7 heavy chain leader (SEQ ID NO. 61)

MEFGLSWVFLVALFRGVQC

[00425] anti-biotin vH (murine) (SEQ ID NO. 62)

QVKLQESGPG LVAPSQSLSI TCTVSGFSLT AYGVDWVRQP PGKGLEWLGV

IWGGGRTNYN SGLMSRLSIR KDNSKSQVFL TMNSLQTDDT AKYYCVKHTN

WDGGFAYWGQ GTTVTVSS

[00426] CH1 (generic) (SEQ ID NO. 63)

PSVFP LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV

HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVE

[00427] IgG3 hinge for greater accessibility to FcyR (SEQ ID NO. 64)

LKTP LGDTTHTCPR CPEPKSCDTP PPCPRCPEPK SCDTPPPCPR

CPEPKSCDTP PPCPRCP

[00428] IgG1 CH2, CH3 Tm and cytoplasmic tail (T256A) (SEQ ID NO. 65)

APELLGGPSVFLF PPKPKDTLMI SRAPEVTCVV VDVSHEDPEV KFNWYVDGVE

VHNAKTKPRE EQYNSTYRVV SVLTVLHQDW LNGKEYKCKV SNKALPAPIE KTISKAKGQP REPQVYTLPP SRDELTKNQV SLTCLVKGFY PSDIAVEWES NGQPENNYKT TPPVLDSDGS FFLYSKLTVD KSRWQQGNVF SCSVMHEALH NHYTQKSLSL SPELQLEESC AEAQDGELDG LWTTITIFIT LFLLSVCYSA TVTFFKVKWI FSSVVDLKQT IIPDYRNMIG QGA*

[00429] Summary (578 ORF2a) (SEQ ID NO. 66)

MEFGLSWVFLVALFRGVQCQVKLQESGPGLVAPSQSLSITCTVSGFSLTA YGVDWVRQPPGKGLEWLGVIWGGGRTNYNSGLMSRLSIRKDNSKSQVFLT MNSLQTDDTAKYYCVKHTNWDGGFAYWGQGTTVTVSSPSVFPLAPSSKST SGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSV VTVPSSSLGTQTYICNVNHKPSNTKVDKKVELKTPLGDTTHTCPRCPEPK SCDTPPPCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPAPELLGGP SVFLFPPKPKDTLMISRAPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK TKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPE NNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPELQLEESCAEAQDGELDGLWTTITIFITLFLLSVCYSATVTFF KVKWIFSSVVDLKQTIIPDYRNMIGQGA*

[00430] IRES (SEQ ID NO. 67)

[00431] L1 Signal (modified to be IRES compatible) (SEQ ID NO. 68)

MATDMRVPAQLLGLLLLWLSGARC

[00432] LC Variable (human lambda variable) (SEQ ID NO. 69)

GSPGQSVSIS CSGSSSNIGN NYVYWYQHLP GTAPKLLIYS DTKRPSGVPD

RISGSKSGTS ASLAISGLQS EDEADYYCAS WDDSLDGPVF GGGTKLTVL

[00433] LC Constant Region 1 from Lambda (http://www.uniprot.org/uniprot/P0CG04) (irrelevant) (SEQ ID NO. 70)

GQPKANPTVT LFPPSSEELQ ANKATLVCLI SDFYPGAVTV AWKADGSPVK AGVETTKPSK QSNNKYAASS YLSLTPEQWK SHRSYSCQVT HEGSTVEKTV APTECS*

[00434] Summary (229 ORF2b) (SEQ ID NO. 71)

MATDMRVPAQLLGLLLLWLSGARCGSPGQSVSISCSGSSSNIGNNYVYWY QHLPGTAPKLLIYSDTKRPSGVPDRISGSKSGTSASLAISGLQSEDEADY YCASWDDSLDGPVFGGGTKLTVLGQPKANPTVTLFPPSSEELQANKATLV CLISDFYPGAVTVAWKADGSPVKAGVETTKPSKQSNNKYAASSYLSLTPE QWKSHRSYSCQVTHEGSTVEKTVAPTECS*

Vector 3. Immunomodulator: sGM-CSF/ires/mFLT3L

[00435] A schematic of the organization of vector 3, used for the immunomodulator sGM-CSF/ires/mFLT3L is shown in FIG. 5A. Vector 3 is bicistronic. The nucleotide sequence of vector 3 (SEQ ID NO. 49) is shown in FIG. 5B. Table 4, below, shows the vector component name, the corresponding nucleotide position in SEQ ID NO. 49, the full name of the component and a description.

[00436] Table 4

Component Name	Nucleotide Position	Full Name	Description
RSV promoter	1-229	Rous sarcoma virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA.
Δ5' LTR	230-410	HIV-1 truncated 5' LTRT	Permits viral packaging and reverse transcription of the viral mRNA.

Component Name	Nucleotide Position	Full Name	Description
Ψ	521-565	HIV-1 psi packaging signal	Allows viral packaging.
RRE	1075-1308	HIV-1 Rev response element	Permits Rev-dependent nuclear export of unspliced viral mRNA.
cPPT	1803-1920	Central polypurine tract	Facilitates the nuclear import of HIV-1 cDNA through a central DNA flap.
EF1A	1950-3128	EF1A	Component entered by user
Kozak	3153-3158	Kozak	Component entered by user
{ORF3A_wSPACER}	3159-4040	{ORF3A_wSPACER}	Component entered by user
IRES	4065-4652	IRES	Component entered by user
{ORF3B}	4653-5392	{ORF3B}	Component entered by user
WPRE	5422-6019	Woodchuck hepatitis virus posttranscriptional regulatory element	Facilitates effective transcription termination at the 3' LTR.
AU3/3' LTR	6101-6335	HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5'LTR for biosafety purposes. The element also contains apolyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 early pA	6408-6542	SV40 early polyadenation signal	Allows transcription termination and polyadenylation of mRNA.
Ampicillin	7496-8356	Ampicillin resistance gene	Allows selection of the plasmid in E.coli.
pUC ori	8527-9115	pUC origin of replication	Permits high-copy replication and maintenance in E.coli.

[00437] When vector 3 is employed, anti-FLT3L is used for flow detection. The highest surface FLT3L expressor will have the highest secreted GM-CSF expression.

[00438] The following is a description of the immunomodulator sGM-CSF/ires/mFLT3L.

[00439] Type:

- cytokine, growth and differentiation factor

[00440] Annotation:

- wild-type sequence

[00441] The sequences are shown as follows:

[00442] GM-CSF signal sequence (SEQ ID NO. 72)

MWLQSLLLLG TVACSIS

[00443] wild type GM-CSF sequence (SEQ ID NO. 73)

APA RSPSPSTQPW EHVNAIQEAR RLLNLSRDTA

AEMNETVEVI SEMFDLQEPT CLQTRLELYK QGLRGSLTKL KGPLTMMASH

YKQHCPPTPE TSCATQIITF ESFKENLKDF LLVIPFDCWE PVQE*

[00444] IRES (SEQ ID NO. 74)

[00445] FLT3L signal (modified to be IRES friendly) (SEQ ID NO. 75)

MATVLAPAWSP TTYLLLLLLL SSGLS

[00446] FLT3L (SEQ ID NO. 76)

GTQDC SFQHSPISSD FAVKIRELSD

YLLQDYPVTV ASNLQDEELC GGLWRLVLAQ RWMERLKTVA GSKMQGLLER

VNTEIHFVTK CAFQPPPSCL RFVQTNISRL LQETSEQLVA LKPWITRQNF

SRCLELQCQP DSSTLPPPWS PRPLEATAPT APQPPLLLLL LLPVGLLLLA

AAWCLHWQRT RRRTPRPGEQ VPPVPSPQDL LLVEH*

[00447] Summary (144 ORF3a) (SEQ ID NO. 77)

MWLQSLLLLGTVACSISAPARSPSPSTQPWEHVNAIQEARRLLNLSRDTA

AEMNETVEVISEMFDLQEPTCLQTRLELYKQGLRGSLTKLKGPLTMMASH

YKQHCPPTPETSCATQIITFESFKENLKDFLLVIPFDCWEPVQE*

[00448] Summary (236 ORF3b) (SEQ ID NO. 78)

MATVLAPAWSPTTYLLLLLLLSSGLSGTQDCSFQHSPISSDFAVKIRELS

DYLLQDYPVTVASNLQDEELCGGLWRLVLAQRWMERLKTVAGSKMQGLLE

RVNTEIHFVTKCAFQPPPSCLRFVQTNISRLLQETSEQLVALKPWITRQN

FSRCLELQCQPDSSTLPPPWSPRPLEATAPTAPQPPLLLLLLLPVGLLLL

AAAWCLHWQRTRRRTPRPGEQVPPVPSPQDLLLVEH*

Vector 4. Immunomodulator: sFLT3L/ires/(FLT3 signal-GM-CSF-Tm)

[00449] A schematic of the organization of vector 4, used for the immunomodulator sFLT3L/ires/(FLT3 signal-GM-CSF-Tm) is shown in FIG. 6A. Vector 4 is bicistronic. The nucleotide sequence of vector 4 (SEQ ID NO. 50) is shown in FIG. 6B. Table 5, below, shows the vector component name, the corresponding nucleotide position in SEQ ID NO. 50, the full name of the component and a description.

[00450] Table 5

Component Name	Nucleotide Position	Full Name	Description
RSV promoter	1-229	Rous sarcoma virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA.
Δ5' LTR	230-410	HIV-1 truncated 5' LTRT	Permits viral packaging and reverse transcription of the viral mRNA.
Ψ	521-565	HIV-1 psi packaging signal	Allows viral packaging.

Component Name	Nucleotide Position	Full Name	Description
RRE	1075-1308	HIV-1 Rev response element	Permits Rev-dependent nuclear export of unspliced viral mRNA.
cPPT	1803-1920	Central polypurine tract	Facilitates the nuclear import of HIV-1 cDNA through a central DNA flap.
EF1A	1950-3128	EF1A	Component entered by user
Kozak	3153-3158	Kozak	Component entered by user
{ORF4A_wSPACER}	3159-4157	{ORF4A_wSPACER}	Component entered by user
IRES	4182-4769	IRES	Component entered by user
{ORF4B}	4770-5557	{ORF4B}	Component entered by user
WPRE	5587-6184	Woodchuck hepatitis virus posttranscriptional regulatory element	Facilitates effective transcription termination at the 3' LTR.
AU3/3' LTR	6266-6500	HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5'LTR for biosafety purposes. The element also contains apolyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 early pA	6573-6707	SV40 early polyadenation signal	Allows transcription termination and polyadenylation of mRNA.
Ampicillin	7661-8521	Ampicillin resistance gene	Allows selection of the plasmid in E.coli.
pUC ori	8692-9280	pUC origin of replication	Permits high-copy replication and maintenance in E.coli.

[00451] When vector 4 is employed, anti-GM-CSF is used for flow detection. The highest surface GMCSF expressor will have highest secreted FLT3L expression.

[00452] The following is a description of the immunomodulator sFLT3L/ires/(FLT3 signal-GM-CSF-Tm)

[00453] Type:

- cytokine, growth and differentiation factor

[00454] Annotation:

- wild-type sequence

[00455] The sequences are shown as follows:

[00456] wild type FLT3L sequence with transmembrane deleted (SEQ ID NO. 79)

MTVLAPAWSP TTYLLLLLLL SSGLSGTQDC SFQHSPISSD FAVKIRELSD

YLLQDYPVTV ASNLQDEELC GGLWRLVLAQ RWMERLKTVA GSKMQGLLER

VNTEIHFVTK CAFQPPPSCL RFVQTNISRL LQETSEQLVA LKPWITRQNF

SRCLELQCQP DSSTLPPPWS PRPLEATAPT APQ*

[00457] IRES (SEQ ID NO. 80)

[00458] FLT3L signal (modified to be IRES friendly) (SEQ ID NO. 81)

MATVLAPAWSP TTYLLLLLLL SSGLS

[00459] wild type GM-CSF sequence (minus native signal) (SEQ ID NO. 82)

APA RSPSPSTQPW EHVNAIQEAR RLLNLSRDTA

AEMNETVEVI SEMFDLQEPT CLQTRLELYK QGLRGSLTKL KGPLTMMASH

YKQHCPPTPE TSCATQIITF ESFKENLKDF LLVIPFDCWE PVQE

[00460] CD8alpha transmembrane and cytoplasmic domain (SEQ ID NO. 83)

PTTTP APRPPTPAPTIASQPLSLRP EACRPAAGGA VHTRGLDFAC DIYIWAPLAG

TCGVLLLSLVITLYCNHRNR RRVCKCPRPV VKSGDKPSLS ARYV*

[00461] Summary (183 ORF4a) (SEQ ID NO. 84)

MTVLAPAWSPTTYLLLLLLLSSGLSGTQDCSFQHSPISSDFAVKIRELSD

YLLQDYPVTVASNLQDEELCGGLWRLVLAQRWMERLKTVAGSKMQGLLER

VNTEIHFVTKCAFQPPPSCLRFVQTNISRLLQETSEQLVALKPWITRQNF

SRCLELQCQPDSSTLPPPWSPRPLEATAPTAPQ*

[00462] Summary for CYAGEN (253 ORF4b) (SEQ ID NO. 85)

MATVLAPAWSPTTYLLLLLLLSSGLS APARSPSPSTQPWEHVNAIQEAR

RLLNLSRDTAAEMNETVEVISEMFDLQEPTCLQTRLELYKQGLRGSLTKL

KGPLTMMASHYKQHCPPTPETSCATQIITFESFKENLKDFLLVIPFDCWE

PVQEPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD

IYIWAPLAGTCGVLLLSLVITLYCNHRNRRRVCKCPRPVVKSGDKPSLSA

RYV*

Vector 5. Immunomodulator: mCD40L

[00463] A schematic of the organization of vector 5, used for the immunomodulator mCD40L is shown in FIG. 7A. Vector 5 is monocistronic. The nucleotide sequence of vector 5 (SEQ ID NO. 51) is shown in FIG. 7B. Table 6, below, shows the vector component name, the corresponding nucleotide position in SEQ ID NO. 51, the full name of the component and a description.

[00464] Table 6

Component Name	Nucleotide Position	Full Name	Description
RSV promoter	1-229	Rous sarcoma virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA.
Δ5' LTR	230-410	HIV-1 truncated 5' LTRT	Permits viral packaging and reverse transcription of the viral mRNA.
Ψ	521-565	HIV-1 psi packaging signal	Allows viral packaging.
RRE	1075-1308	HIV-1 Rev response element	Permits Rev-dependent nuclear export of unspliced viral mRNA.
cPPT	1803-1920	Central polypurine tract	Facilitates the nuclear import of HIV-1 cDNA through a central DNA flap.
EF1A	1959-3137	EF1A	Component entered by user
Kozak	3162-3167	Kozak	Component entered by user
{ORF5}	3168-3991	{ORF5}	Component entered by user
WPRE	4030-4627	Woodchuck hepatitis virus posttranscriptional regulatory element	Facilitates effective transcription termination at the 3' LTR.
AU3/3' LTR	4709-4943	HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5'LTR for biosafety purposes. The element also contains apolyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 early pA	5016-5150	SV40 early polyadenation signal	Allows transcription termination and polyadenylation of mRNA.
Ampicillin	6104-6964	Ampicillin resistance gene	Allows selection of the plasmid in E.coli.
pUC ori	7135-7723	pUC origin of replication	Permits high-copy replication and maintenance in E.coli.

[00465] When Vector 5 is employed, anti-CD40L is used for flow detection.

[00466] The following is a description of the immunomodulator mCD40L.

[00467] Type:

- TNF type II transmembrane protein

[00468] Annotation:

- Mutations (UNDERLINED) introduced to make a non-cleavable version

[00469] The sequences are shown as follows:

[00470] Modified sequence to stop cleavage (SEQ ID NO. 86)

MIETYNQTSP RSAATGLPIS MKIFMYLLTV FLITQMIGSA LFAVYLHRRL

DKIEDERNLH EDFVFMKTIQ RCNTGERSLS LLNCEEIKSQ FEGFVKDIML

NKEETKKENS FEM**PR**G**EEDS** QIAAHVISEA SSKTTSVLQW AEKGYYTMSN

NLVTLENGKQ LTVKRQGLYY IYAQVTFCSN REASSQAPFI ASLCLKSPGR

FERILLRAAN THSSAKPCGQ QSIHLGGVFE LQPGASVFVN VTDPSQVSHG

TGFTSFGLLK L*

[00471] Summary (261 ORF5) (SEQ ID NO. 87)

MIETYNQTSPRSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHRRL

DKIEDERNLHEDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIML

NKEETKKENSFEMPRGEEDSQIAAHVISEASSKTTSVLQWAEKGYYTMSN

NLVTLENGKQLTVKRQGLYYIYAQVTFCSNREASSQAPFIASLCLKSPGR

FERILLRAANTHSSAKPCGQQSIHLGGVFELQPGASVFVNVTDPSQVSHG

TGFTSFGLLKL*

Vector 6. Immunomodulator: mTNFalpha (TNFa)

[00472] A schematic of the organization of vector 6, used for the immunomodulator mTNFα is shown in FIG. 8A. Vector 6 is monocistronic. The nucleotide sequence of vector 6 (SEQ ID NO. 52) is shown in FIG. 8B. Table 7, below, shows the vector component name, the corresponding nucleotide position in SEQ ID NO. 52, the full name of the component and a description.

[00473] Table 7

Component Name	Nucleotide Position	Full Name	Description
RSV promoter	1-229	Rous sarcoma virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA.
Δ5' LTR	230-410	HIV-1 truncated 5' LTRT	Permits viral packaging and reverse transcription of the viral mRNA.
Ψ	521-565	HIV-1 psi packaging signal	Allows viral packaging.
RRE	1075-1308	HIV-1 Rev response element	Permits Rev-dependent nuclear export of unspliced viral mRNA.
cPPT	1803-1920	Central polypurine tract	Facilitates the nuclear import of HIV-1 cDNA through a central DNA flap.
EF1A	1959-3137	EF1A	Component entered by user
Kozak	3162-3167	Kozak	Component entered by user
{ORF6}	3168-3871	{ORF6}	Component entered by user
WPRE	3910-4507	Woodchuck hepatitis virus posttranscriptional regulatory element	Facilitates effective transcription termination at the 3' LTR.
AU3/3' LTR	4859-4823	HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5'LTR for biosafety purposes. The element also contains apolyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 early pA	4896-5030	SV40 early polyadenation signal	Allows transcription termination and polyadenylation of mRNA.
Ampicillin	5984-6844	Ampicillin resistance gene	Allows selection of the plasmid in E.coli.
pUC ori	7015-7603	pUC origin of replication	Permits high-copy replication and

Component Name	Nucleotide Position	Full Name	Description
			maintenance in E.coli.

[00474] When vector 6 is employed, anti-TNFα is used for flow detection.

[00475] The following is a description of the immunomodulator mTNFα.

[00476] Type:

- TNF type II transmembrane protein

[00477] Annotation:

- Mutations introduced to make a non-cleavable version, as described below in SEQ ID NO. 88

[00478] The sequences are shown as follows:

[00479] Modified to stop cleavage (SEQ ID NO. 88)

MSTESMIRDV ELAEEALPKK TGGPQGSRRC LFLSLFSFLI VAGATTLFCL

LHFGVIGPQR EEFPRDLSLI SPLAQA.... .....VA HVVANPQAEG

QLQWLNRRAN ALLANGVELR DNQLVVPSEG LYLIYSQVLF KGQGCPSTHV

LLTHTISRIA VSYQTKVNLL SAIKSPCQRE TPEGAEAKPW YEPIYLGGVF

QLEKGDRLSA EINRPDYLDF AESGQVYFGI IAL*

[00480] Summary (221 ORF6) (SEQ ID NO. 89)

MSTESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFLIVAGATTLFCL

LHFGVIGPQREEFPRDLSLISPLAQAVAHVVANPQAEGQLQWLNRRANAL

LANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVS

YQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEI

NRPDYLDFAESGQVYFGIIAL*

Vector 7. Immunomodulator: mRANKL/ires/FLT3 signal-V5- scFV anti-biotin-Tm

[00481] A schematic of the organization of vector 7, used for the immunomodulator mRANKL /ires/FLT3 signal-V5- scFV anti-biotin-Tm is shown in FIG. 9A. The nucleotide sequence of vector 7 (SEQ ID NO. 53) is shown in FIG. 9B. Table 8, below, shows the vector component name, the corresponding nucleotide position in SEQ ID NO. 53, the full name of the component and a description.

[00482] Table 8

Component Name	Nucleotide Position	Full Name	Description
RSV promoter	1-229	Rous sarcoma virus (RSV) enhancer/promoter	Allows Tat-independent production of viral mRNA.
Δ5' LTR	230-410	HIV-1 truncated 5' LTRT	Permits viral packaging and reverse transcription of the viral mRNA.
Ψ	521-565	HIV-1 psi packaging signal	Allows viral packaging.
RRE	1075-1308	HIV-1 Rev response element	Permits Rev-dependent nuclear export of unspliced viral mRNA.
cPPT	1803-1920	Central polypurine tract	Facilitates the nuclear import of HIV-1 cDNA through a central DNA flap.
EF1A	1950-3128	EF1A	Component entered by user
Kozak	3153-3158	Kozak	Component entered by user
{ORF7_wSPACER}	3159-4091	{ORF7_wSPACER}	Component entered by user
IRES	4116-4703	IRES	Component entered by user
{ORF7B}	4704-5878	{ORF7B}	Component entered by user
WPRE	5908-6505	Woodchuck hepatitis virus posttranscriptional regulatory element	Facilitates effective transcription termination at the 3' LTR.

Component Name	Nucleotide Position	Full Name	Description
AU3/3' LTR	6587-3821	HIV-1 truncated 3' LTR	Allows viral packaging but self-inactivates the 5'LTR for biosafety purposes. The element also contains apolyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells.
SV40 early pA	6894-7028	SV40 early polyadenation signal	Allows transcription termination and polyadenylation of mRNA.
Ampicillin	7982-8842	Ampicillin resistance gene	Allows selection of the plasmid in E.coli.
pUC ori	9013-9601	pUC origin of replication	Permits high-copy replication and maintenance in E.coli.

[00483] When vector 7 is employed, anti-RANKL is used for flow detection. Anti-V5 mAb is used as a secondary detection method.

[00484] The following is a description of the immunomodulator mRANKL /ires/FLT3 signal-V5- scFV anti-biotin-Tm.

[00485] Type:

- TNF type II transmembrane protein

[00486] Annotation:

- wild-type sequence

[00487] The sequences are shown as follows:

[00488] wild-type (SEQ ID NO. 90)

MDPNRISEDG THCIYRILRL HENADFQDTT LESQDTKLIP DSCRRIKQAF

QGAVQKELQH IVGSQHIRAE KAMVDGSWLD LAKRSKLEAQ PFAHLTINAT

DIPSGSHKVS LSSWYHDRGW AKISNMTFSN GKLIVNQDGF YYLYANICFR

HHETSGDLAT EYLQLMVYVT KTSIKIPSSH TLMKGGSTKY WSGNSEFHFY

SINVGGFFKL RSGEEISIEV SNPSLLDPDQ DATYFGAFKV RDID*"

[00489] IRES (SEQ ID NO. 91)

[00490] FLT3L signal (modified to be IRES friendly) (SEQ ID NO. 92)

M**A**TVLAPAWSP TTYLLLLLLL SSGLS

[00491] Linker (SEQ ID NO. 93)

GGGGS

[00492] V5 epitope tag for flow detection (SEQ ID NO. 94)

GKPIPNPLLGLDST

[00493] Linker (SEQ ID NO. 93)

GGGGS

[00494] anti-biotin murine vH with inserted Cys for intralinkage (SEQ ID NO. 95)

QVKLQESGPG LVAPSQSLSI TCTVSGFSLT AYGVDWVRQP PGK**C**LEWLGV

IWGGGRTNYN SGLMSRLSIR KDNSKSQVFL TMNSLQTDDT AKYYCVKHTN

WDGGFAYWGQ GTTVTVSS

[00495] linker (SEQ ID NO. 96)

GGGGSGGGGS GGGGS

[00496] LC Variable (human lambda variable) (SEQ ID NO. 97)

GSPGQSVSIS CSGSSSNIGN NYVYWYQHLP GTAPKLLIYS DTKRPSGVPD

RISGSKSGTS ASLAISGLQS EDEADYYCAS WDDSLDGPVF GCGTKLTVL

[00497] CD8alpha transmembrane and cytoplasmic domain (SEQ ID NO. 98)

PTTTP APRPPTPAPTIASQPLSLRP EACRPAAGGA VHTRGLDFAC DIYIWAPLAG

TCGVLLLSLVITLYCNHRNR RRVCKCPRPV VKSGDKPSLS ARYV*

[00498] Summary (244 ORF7a) (SEQ ID NO. 99)

MDPNRISEDGTHCIYRILRLHENADFQDTTLESQDTKLIPDSCRRIKQAF

QGAVQKELQHIVGSQHIRAEKAMVDGSWLDLAKRSKLEAQPFAHLTINAT

DIPSGSHKVSLSSWYHDRGWAKISNMTFSNGKLIVNQDGFYYLYANICFR

HHETSGDLATEYLQLMVYVTKTSIKIPSSHTLMKGGSTKYWSGNSEFHFY

SINVGGFFKLRSGEEISIEVSNPSLLDPDQDATYFGAFKVRDID*"

[00499] Summary (381aa ORF7b) (SEQ ID NO. 100)

MATVLAPAWSPTTYLLLLLLLSSGLSGGGGSGKPIPNPLLGLDSTGGGGS

QVKLQESGPGLVAPSQSLSITCTVSGFSLTAYGVDWVRQPPGKCLEWLGV

IWGGGRTNYNSGLMSRLSIRKDNSKSQVFLTMNSLQTDDTAKYYCVKHTN

WDGGFAYWGQGTTVTVSSGGGGSGGGGSGGGGSGSPGQSVSISCSGSSSN

IGNNYVYWYQHLPGTAPKLLIYSDTKRPSGVPDRISGSKSGTSASLAISG

LQSEDEADYYCASWDDSLDGPVFGCGTKLTVLPTTTPAPRPPTPAPTIAS

QPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITL

YCNHRNRRRVCKCPRPVVKSGDKPSLSARYV*

[00500] According to one embodiment, a tumor cell line is selected for modification, and vector 2 is used to stably integrate immune modulators into the cell genome.

[00501] According to one embodiment, a tumor cell line is selected for modification, and vector 3 is used to stably integrate immune modulators into the cell genome.

[00502] According to one embodiment, a tumor cell line is selected for modification, and vector 4 is used to stably integrate immune modulators into the cell genome.

[00503] According to one embodiment, a tumor cell line is selected for modification, and vector 5 is used to stably integrate immune modulators into the cell genome.

[00504] According to one embodiment, a tumor cell line is selected for modification, and vector 6 is used to stably integrate immune modulators into the cell genome.

[00505] According to one embodiment, a tumor cell line is selected for modification, and vector 2 and vector 3 are used to stably integrate immune modulators into the cell genome.

[00506] According to one embodiment, a tumor cell line is selected for modification, and vector 2 and vector 4 are used to stably integrate immune modulators into the cell genome.

[00507] According to one embodiment, a tumor cell line is selected for modification, and vector 2 and vector 5 are used to stably integrate immune modulators into the cell genome.

[00508] According to one embodiment, a tumor cell line is selected for modification, and vector 2 and vector 6 are used to stably integrate immune modulators into the cell genome.

[00509] According to one embodiment, a tumor cell line is selected for modification, and vector 3 and vector 4 are used to stably integrate immune modulators into the cell genome.

[00510] According to one embodiment, a tumor cell line is selected for modification, and vector 3 and vector 5 are used to stably integrate immune modulators into the cell genome.

[00511] According to one embodiment, a tumor cell line is selected for modification, and vector 3 and vector 6 are used to stably integrate immune modulators into the cell genome.

[00512] According to one embodiment, a tumor cell line is selected for modification, and vector 2, vector 3 and vector 4 are used to stably integrate immune modulators into the cell genome.

[00513] According to one embodiment, a tumor cell line is selected for modification, and vector 2, vector 3 and vector 5 are used to stably integrate immune modulators into the cell genome.

[00514] According to one embodiment, a tumor cell line is selected for modification, and vector 2, vector 3 and vector 6 are used to stably integrate immune modulators into the cell genome.

[00515] According to one embodiment, a tumor cell line is selected for modification, and vector 2, vector 3 and vector 6 are used to stably integrate immune modulators into the cell genome.

[00516] According to one embodiment, a tumor cell line is selected for modification, and vector 3, vector 4 and vector 5 are used to stably integrate immune modulators into the cell genome.

[00517] According to one embodiment, a tumor cell line is selected for modification, and vector 3, vector 4 and vector 6 are used to stably integrate immune modulators into the cell genome.

[00518] According to one embodiment, a tumor cell line is selected for modification, and vector 2, vector 3, vector 4 and vector 5 are used to stably integrate immune modulators into the cell genome.

[00519] According to one embodiment, a tumor cell line is selected for modification, and vector 2, vector 3, vector 4 and vector 6 are used to stably integrate immune modulators into the cell genome.

[00520] According to one embodiment, a tumor cell line is selected for modification, and vector 2, vector 3, vector 5 and vector 6 are used to stably integrate immune modulators into the cell genome.

[00521] According to one embodiment, a tumor cell line is selected for modification, and vector 3, vector 4, vector 5 and vector 6 are used to stably integrate immune modulators into the cell genome.

[00522] According to one embodiment, a tumor cell line is selected for modification, and vector 2, vector 3, vector 4, vector 5 and vector 6 are used to stably integrate immune modulators into the cell genome.

Example 4.

[00523] Experiments were carried out to demonstrate that the immunomodulators described herein, expressed on the tumor cell line SK-MEL2 differentially impact the proliferation and

differentiation of human PBMC. FIG. 10 is a schematic that shows the general experimental design. The following allogeneic cell lines are tested:

- SK-MEL (Parental line) ("SK")
- SK modified with Vector 2 only ("2")
- SK modified with Vector 3 only ("3")
- SK modified with Vector 4 only ("4")
- SK modified with Vector 6 only (6")
- SK modified with Vector 3 and Vector 4 ("3-4")
- SK modified with Vector 3, Vector 4 and Vector 5 ("3-4-5")
- SK modified with Vector 3, Vector 5 and Vector 6 ("3-5-6")

[00524] Functional characterization of the allogeneic cell lines was performed using a primary MLTR assay, as described herein. The MLTR assay was set up with 250,000 freshly thawed PBMC and 50,000 of select engineered allogeneic cell lines. The following outputs were measured: 1) Proliferation is measured by flow on CFSE labeled PMBC; 2) Differentiation is measured by CyTOF on unlabeled PMBC; 3) Cytokine profiling is performed by Luminex.

Flow Cytometry Data

[00525] The experiments described herein detect hPBMC activation from direct allorecognition of allogeneic cells versus a pan-T-cell activation using anti-CD3 and anti-CD28 mAbs. It was found that hPBMC activation via direct allorecognition of allogeneic cells displays a fundamentally different response compared to pan-T-cell activation with anti-CD3/CD28

treatment. Three key observations were made with regard to this differential hPBMC activation: 1) that ~10% of hPBMC proliferate in response to incubation with allogeneic cells compared to ~50% with anti-CD3/CD28 treatment; 2) that hPBMC proliferate through more cell divisions in response to activation with allogeneic cells compared to activation with anti-CD3/CD28 treatment ; 3) that hPBMC take on a more highly varied morphology as measured by side scatter as compared to the the more uniform cell morphology when hPBMC are stimulated with anti-CD3/CD28 treatment.

[00526] FIG. 11 is a panel of graphs that show the results of flow cytometry experiments. Forward (FSC) and side scatter (SSC) plots for size and granularity are shown. SK lines are represented by a number code; SK, unmodified parent line; 3, secreted GM-CSF and membrane expressed FLT-3L; 4, secreted FLT3L and membrane expressed GM-CSF; 5, a non-cleavable form of CD40L; 6, a non-cleavable form of TNF-alpha; 3-4 is a combination of 3 and 4; 3-4-5 is a combination of 3,4 and 5; and 3-4-6 is a combination of 3,4 and 6. Cell lines 6, 3-4-5 and 3-4-6 display a larger and more granular phenotype likely owing to the presence of receptors for TNF-alpha and CD40L on cells of epithelial origin.

[00527] FIG. 12 is a panel of graphs that show representative flow cytometry stains for the indicated engineered surface markers; GM-CSF, FLT3L, TNF-a and CD40L. SK lines are represented by a number code; SK, unmodified parent line; 3, secreted GM-CSF and membrane expressed FLT-3L; 4, secreted FLT3L and membrane expressed GM-CSF ; 5, a non-cleavable form of CD40L; 6, a non-cleavable form of TNF-alpha; 3-4 is a combination of 3 and 4; 3-4-5 is a combination of 3,4 and 5; and 3-4-6 is a combination of 3, 4 and 6.

[00528] FIG. 13 is a panel of graphs that show representative flow cytometry stains for the indicated engineered surface markers; GM-CSF, FLT3L, TNF-a and CD40L. SK lines are represented by a number code; SK, unmodified parent line; 3, secreted GM-CSF and membrane expressed FLT-3L; 4, secreted FLT3L and membrane expressed GM-CSF; 5, a non-cleavable form of CD40L; 6, a non-cleavable form of TNF-a; 3-4 is a combination of 3 and 4; 3-4-5 is a combination of 3,4 and 5; and 3-4-6 is a combination of 3,4 and 6.

CyTOF Data

[00529] CyTOF mass cytometry single-cell phenotype analysis of hPBMC response to SK melanoma cells with modification by expression of immunomodulatory factors is shown in FIG. 14A and FIG. 14B. The SK melanoma cell line and hPBMCs were cultured for 24 hours. Cells were harvested from cultures and stained with a 32-marker CyTOF antibody panel to detect multiple immune cell subsets as well as cell-surface and intracellular phenotyping markers. CyTOF mass cytometry data was generated on a Helios instrument. The data were normalized for signal using equilibration beads. Cell staining data were analyzed using Cytobank – a cloud computing suite for CyTOF data analysis that includes cell gating functions and an array of data visualization methods.

[00530] The data shown in FIG. 14A and FIG. 14B were plotted using viSNE, which is a dimensional reduction method that converts multidimensional staining signals from single cells into plots for visualization. FIG. 14A shows viSNE density contour plots of CyTOF staining data showing relative changes in immune cell subset abundance and phenotype. FIG. 14B shows single-cell phenotype analysis. viSNE density contour plots were generated by viSNE from ungated total PBMCs that were cultured with SK melanoma cells or modified SK melanoma

cells. The plots illustrate relative changes in cell density for hPBMC immune cell subsets. The inserted viSNE plot identifies the immune cell subsets that are found within the clusters of the viSNE density plots. The arrows in the density contour plots point to the obvious changes in immune cell subsets between hPBMCs, SK cells, and the modified SK cells.

[00531] FIG. 15A- FIG. 15D show CyTOF monocyte cluster analysis of hPBMC indicating changes in the activation markers CD40 (FIG. 15A), CD86 (FIG. 15B), CD69 (FIG. 15C) and CD25 (FIG. 15D) expression following 1 day stimulation with the indicated genetically modified SK lines at a 1:5 cell ratio. FIG. 15E shows CyTOF monocyte cluster analysis of hPBMC indicating relative median expression levels of monocyte markers CD40 and CD86. FIG. 15E shows CyTOF monocyte cluster analysis of hPBMC indicating relative median expression levels of CD4 T cell markers CD69 and CD25.

Cytokine Data

[00532] Luminex multiplex cytokine profiling of human PBMC responses to SK parent line and genetically modified SK lines is shown in FIG 16. SK cells or the indicated modified cell lines were cultured for 24 hours with human PBMCs at a 1:5 cell ratio. Control cultures included SK cells alone, hPBMCs alone, and hPBMCs stimulated with a mixture of anti-CD3 and anti-CD28 antibodies (1 μg/ml final concentration). Supernatants were screened for cytokine levels using a multiplexed Luminex bead array assay to detect IL-1a, IL-1b, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, IL-23, TNFa, IFNg, G-CSF, GM-CSF, MIP1b, MCP-1, Rantes, Tweak, and TREM-1. Those cytokines found to be specifically induced by the SK parent line and modified SK lines are shown in the plots. Symbols indicate cytokine levels in pg/ml as estimated from a standard curve using recombinant cytokines. Absence of

symbols indicates the cytokine was not detected. SK lines are represented by a number code; SK, unmodified parent line; 3, secreted GM-CSF and membrane expressed FLT-3L; 3, secreted FLT3L and membrane expressed GM-CSF; 5, a non-cleavable form of CD40L; 6, a non-cleavable form of TNF-alpha; 3-4 is a combination of 3 and 4; 3-4-5 is a combination of 3,4 and 5; and 3-4-6 is a combination of 3,4 and 6.

[00533] The described study provides a proof of concept that the complex combinatorial space of immunomodulators can be rapidly and efficiently assessed using an all human *in vitro* MLTR assay.

[00534] "Allorecognition" is a term used to define immunological recognition of histoincompatible antigens between genetically disparate individuals within the same species. "Direct allorecognition" is a mechanism by which recipient T cells recognize determinants on MHC-molecule-peptide complexes displayed on the surface of transplanted cells without the requirement for antigen processing by recipient APCs. The direct allorecognition response is detected early in the course of the MLTR assay (up to 1 day duration) during which time no antigen processing by host APC is required.

[00535] "Indirect allorecognition" refers to recognition of processed antigens of allogeneic cell origin presented by self-HLA on the surface of host antigen presenting cells. The indirect allorecognition response can be deteted later in the course of the MLTR assay (greater than 3 days duration) during which time antigen processing by host APC has occurred.

[00536] Approximately 10% of peripheral blood T cells bear a TCR capable of allorecognition of the allogeneic tumor type specific cells used for vaccination. This is called "direct allorecognition" and occurs early in the course of events post vaccination. Direct

allorecognition targets a T cell mediated immune response against the allogeneic cells resulting in their death and release of tumor type specific neoantigens (and shared normal antigens. These tumor neoantigens (and normal antigens) are taken up by host antigen presenting cells, processed and presented in the context of host HLA. This "indirect allorecognition" occurs late in the course of events post vaccination. The TCRs activated during indirect allorecognition are different from those involved earlier during direct allorecognition, but both processes occur in a local environment after exposure to allogeneic cells. The presence of genetically introduced immunomodulators on the allogeneic cells altered the allorecognition response in qualitative and quantitative ways.

[00537] Epitope spreading is a process of expanding an immune response to include distinct but closely related T cell epitopes. This is generally described as a maturation of the immune response. The differential maturation of the immune response against tumor neoantigens versus self-antigens is driven by the fact that tolerance mechanisms are in place to differentially protect against immune responses against self-antigens. While self-tolerance can be broken, it is more difficult than the response against a tumor neoantigen.

[00538] Without being limited by theory, since all tumors of a given type share many antigens, the T-cell mediated response initially driven by indirect allorecognition of the immune response will cross react against the host tumor of the same type. According to some embodiments, since the tumor microenvironment may provide an insurmountable negative immunomodulatory hurdle, this approach may best be used in combination with checkpoint inhibitors in the setting of minimal residual disease after a debulking therapy (e.g. surgery, radiation or oncolytic viruses).

[00539] While the present invention has been described with reference to the specific embodiments thereof it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention. In addition, many modifications may be made to adopt a particular situation, material, composition of matter, process, process step or steps, to the objective spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

What is claimed is:

1. A method of treating a melanoma cancer in a patient comprising the steps of:

(a) preparing an allogeneic melanoma tumor cell line variant transfected to express two or more immunomodulator peptides, wherein the immunomodulator peptides are GM-CSF and Flt-3L, by:

(1) providing an allogeneic parental tumor cell line;

(2) transfecting or transducing recombinant DNA sequences coding for the two or more of immunomodulator peptides selected;

(3) generating the melanoma tumor cell line variants by selecting for tumor cell clones that stably express an immunogenic amount of the two or more immune modulator peptides, the clonally derived melanoma cell line variants comprise:

(a) a soluble form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 13 and a membrane bound form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 14;

(b) a membrane bound form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 5 and a soluble form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 44; or

(c) a combination of (i) and (ii); (i) a combination of a soluble form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 13 and a membrane bound form of Flt-3L comprising an amino acid sequence that

has at least 80% identity to SEQ ID NO: 14; and (ii) a combination of a membrane bound form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 5 and a soluble form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 44,

(4) selecting in a mixed lymphocyte tumor cell reaction the clonally derived melanoma cell line variants from step (3) that expresses an immunostimulatory amount of the immunomodulators as measured by one or more of the following parameters of lymphocyte activation selected from cellular proliferation, cellular subset differentiation, cytokine release profile, and tumor cell lysis; wherein the selected clonally derived melanoma cell line variant is effective to stimulate activation of one or more of T cells, B cells, and dendritic cells; and

(b) administering to the patient that has melanoma an immunostimulatory amount of the melanoma tumor cell line variant from step (4), wherein the immunostimulatory amount is effective to improve clinical outcome.

2. The method of claim 1, wherein the tumor cell line variant vaccine is effective to improve overall survival of cancer patients relative to placebo controls.

3. The method of claim 1 or 2, wherein the GM-CSF immune modulator comprises an amino acid sequence that has at least 95% identity to SEQ ID NO: 13 or SEQ ID NO: 5.

4. The method of any one of claims 1 to 3, wherein the Flt-3L immune modulator comprises an amino acid sequence that has at least 95% identity to SEQ ID NO: 14 or SEQ ID NO: 44.

5. An allogeneic tumor cell vaccine comprising:

(1) a tumor cell line variant comprising

(a) two or more stably expressed recombinant membrane bound immunomodulatory molecules, wherein the immunomodulator peptides are GM-CSF and Flt-3L; and

(b) stably expressed recombinant soluble GM-CSF peptides; and

(2) a pharmaceutically acceptable carrier;

wherein an immune stimulatory amount of the melanoma tumor cell line variant is effective to elicit an immune response that improves progression free survival, overall survival, or both relative to placebo controls.

6. The allogeneic tumor cell vaccine of claim 5, wherein the tumor cell line variant expresses:

(a) a membrane bound form of Flt-3L peptide with at least 80% identity to SEQ ID NO: 14; and

(b) a soluble GM-CSF peptide with at least 80% identity to SEQ ID NO: 13.

7. The method of any one of claims 1 to 4, wherein the tumor cell line variants comprise soluble GM-CSF and membrane bound Flt-3L.

8. The method of any one of claims 1 to 4, wherein the tumor cell line variant comprises membrane and soluble forms of GM-CSF and membrane and soluble forms of Flt-3L.

9. Use of a melanoma tumor cell line variant in the manufacture of a medicament for treating a melanoma cancer in a patient, wherein said melanoma tumor cell line variant is an allogeneic melanoma tumor cell line variant transfected to express two or more immunomodulator peptides, wherein the immunomodulator peptides are GM-CSF and Flt-3L, prepared by:

(1) providing an allogeneic melanoma parental tumor cell line;

(2) transfecting or transducing recombinant DNA sequences coding for the two or more of immunomodulator peptides selected;

(3) generating the melanoma tumor cell line variants by selecting for melanoma tumor cell clones that stably express an immunogenic amount of the two or more immune modulator peptides, the clonally derived melanoma cell line variants comprise:

(a) a soluble form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 13 and a membrane bound form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 14;

(b) a membrane bound form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 5 and a soluble form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 44; or

(c) a combination of (i) and (ii); (i) a combination of a soluble form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 13 and a membrane bound form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 14; and (ii) a combination of a membrane bound form of GM-CSF comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 5 and a soluble form of Flt-3L comprising an amino acid sequence that has at least 80% identity to SEQ ID NO: 44;

(4) selecting in a mixed lymphocyte tumor cell reaction the clonally derived melanoma cell line variants from step (3) that expresses an immunostimulatory amount of the immunomodulators as measured by one or more of the following parameters of lymphocyte activation selected from cellular proliferation, cellular subset differentiation, cytokine release

profile, and tumor cell lysis; wherein the selected clonally derived melanoma cell line variant is effective to stimulate activation of one or more of T cells, B cells, and dendritic cells; and wherein the medicament is to be administered to the patient in an immunostimulatory amount effective to improve clinical outcome.

10. The use of claim 9, wherein the tumor cell line variant vaccine is effective to improve overall survival of cancer patients relative to placebo controls.

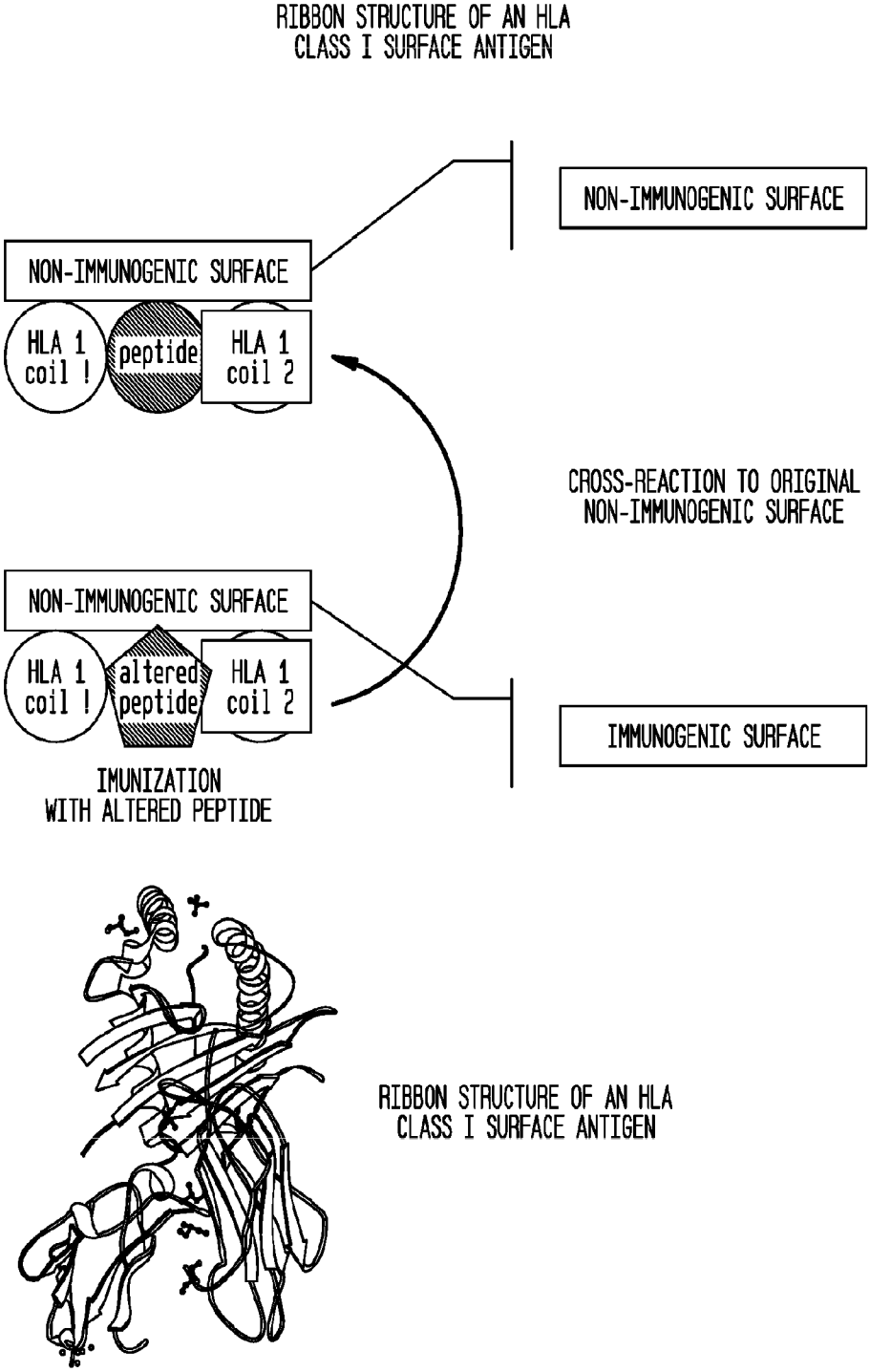
11. The use of claim 9 or 10, wherein the GM-CSF immune modulator comprises an amino acid sequence that has at least 95% identity to SEQ ID NO: 13 or SEQ ID NO: 5.

12. The use of any one of claims 9 to 11, wherein the Flt-3L immune modulator comprises an amino acid sequence that has at least 95% identity to SEQ ID NO: 14 or SEQ ID NO: 44.

13. The method of any one of claims 9 to 12, wherein the tumor cell line variants comprise soluble GM-CSF and membrane bound Flt-3L.

14. The method of any one of claims 9 to 12, wherein the tumor cell line variant comprises membrane and soluble forms of GM-CSF and membrane and soluble forms of Flt-3L.

FIG. 1


RIBBON STRUCTURE OF AN HLA
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NON-IMMUNOGENIC SURFACE
NON-IMMUNOGENIC SURFACE
HLA 1
coil !
peptide
HLA 1
coil 2
CROSS-REACTION TO ORIGINAL
NON-IMMUNOGENIC SURFACE
NON-IMMUNOGENIC SURFACE
HLA 1
coil !
altered
peptide
HLA 1
coil 2
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IMUNIZATION
WITH ALTERED PEPTIDE
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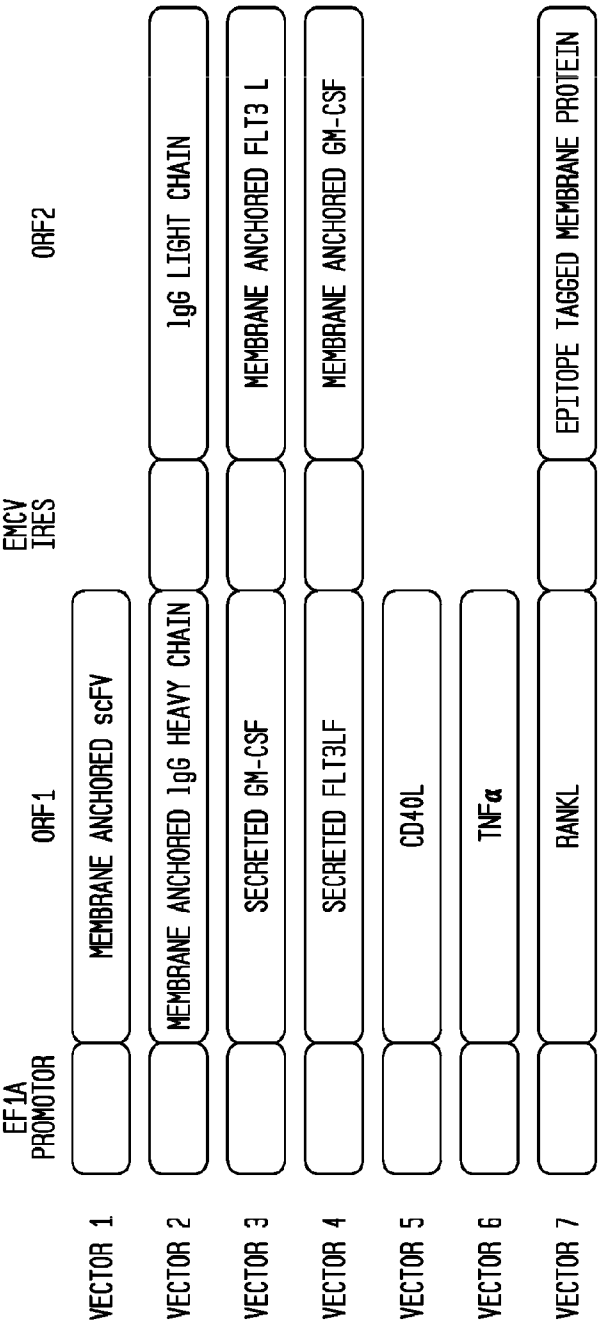
CLASS I SURFACE ANTIGEN

FIG. 2A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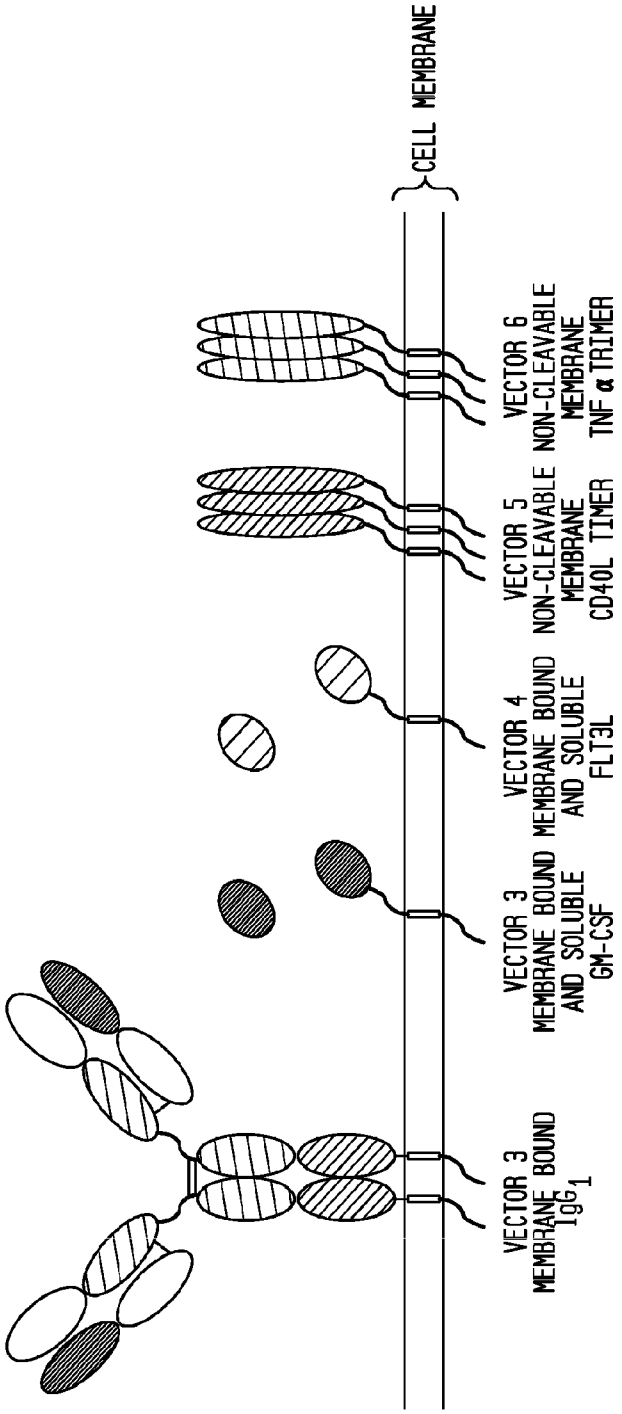
ORF1
EMCV IRES
ORF2
VECTOR 1
MEMBRANE ANCHORED scFV
VECTOR 2
MEMBRANE ANCHORED IgG HEAVY CHAIN
IgG LIGHT CHAIN
VECTOR 3
SECRETED GM-CSF
MEMBRANE ANCHORED FLT3 L
VECTOR 4
SECRETED FLT3LF
MEMBRANE ANCHORED GM-CSF
VECTOR 5
CD40L
VECTOR 6
TNFα
VECTOR 7
RANKL
EPITOPE TAGGED MEMBRANE PROTEIN

FIG. 2B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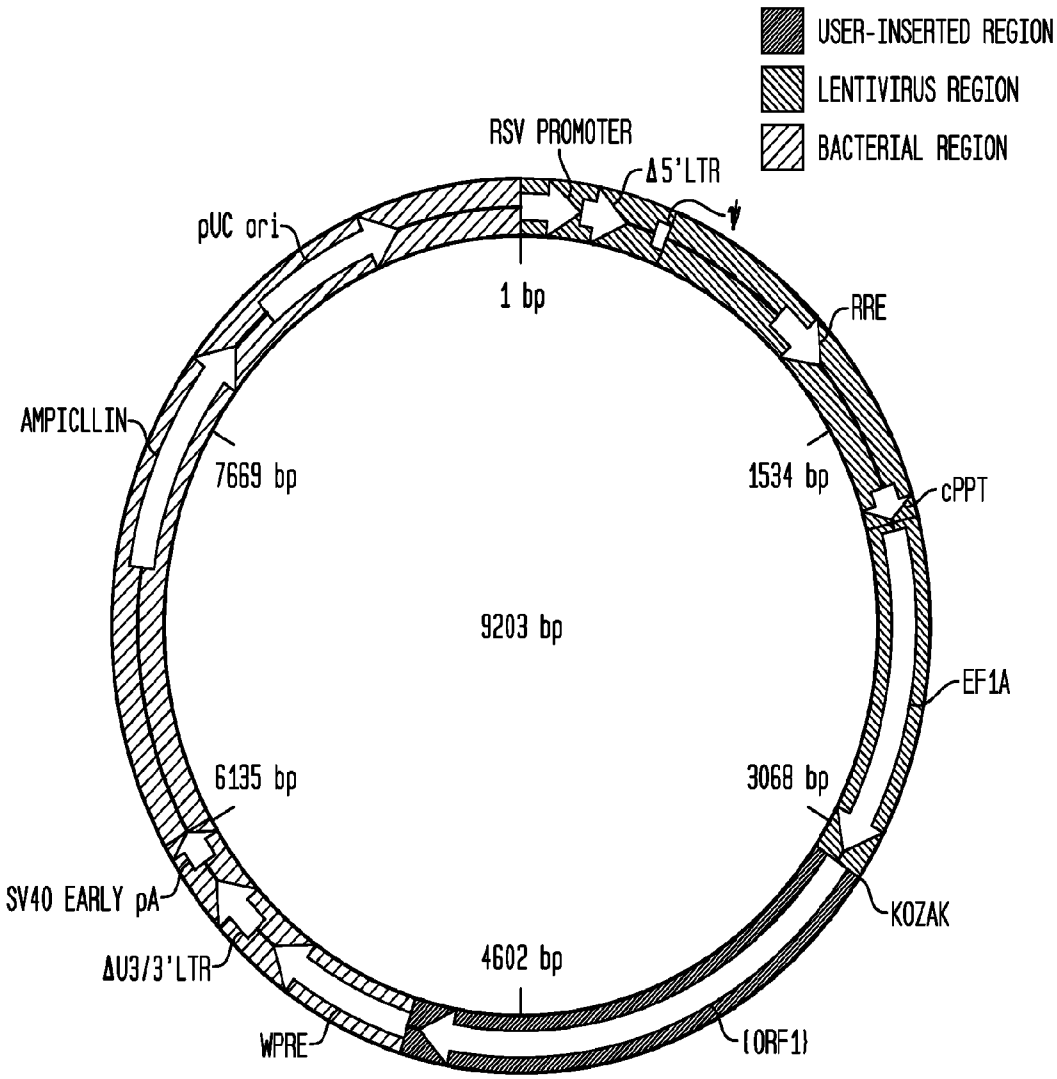
MEMBRANE BOUND
IgG1
VECTOR 3
MEMBRANE BOUND
AND SOLUBLE
GM-CSF
VECTOR 4
MEMBRANE BOUND
AND SOLUBLE
FLT3L
VECTOR 5
NON-CLEAVABLE
MEMBRANE
CD40L TIMER
VECTOR 6
NON-CLEAVABLE
MEMBRANE
TNF α TRIMER

FIG. 3A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LENTIVIRUS REGION
BACTERIAL REGION
RSV PROMOTER
Δ5'LTR
pUC ori
1 bp
RRE
AMPICLLIN
7669 bp
1534 bp
cPPT
9203 bp
EF1A
6135 bp
3068 bp
SV40 EARLY pA
KOZAK
ΔU3/3'LTR
4602 bp
WPRE
(ORF1)

FIG. 3B
SEQ ID NO. 49

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 181 GCCGCATTGC AGAGATATTG TATTTAAGTG CCTAGCTCGA TACATAAACG GGTCTCTCTG
 241 GTTAGACCAG ATCTGAGCCT GGGAGCTCTC TGGCTAACTA GGGAACCCAC TGCTTAAGCC
 301 TCAATAAAGC TTGCCTTGAG TGCTTCAAGT AGTGTGTGCC CGTCTGTTGT GTGACTCTGG
 361 TAACTAGAGA TCCCTCAGAC CCTTTTAGTC AGTGTGGAAA ATCTCTAGCA GTGGCGCCCG
 421 AACAGGGACT TGAAAGCGAA AGGGAAACCA GAGGAGCTCT CTCGACGCAG GACTCGGCTT
 481 GCTGAAGCGC GCACGGCAAG AGGCGAGGGG CGGCGACTGG TGAGTACGCC AAAAATTTTG
 541 ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG TCAGTATTAA GCGGGGGAGA
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FIG. 3B-1

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FIG. 3B-2

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6961 TTAACAAAAT ATTAACGCTT ACAATTTAGG TGGCACTTTT CGGGGAAATG TGCGCGGAAC
7021 CCCTATTTGT TTATTTTTCT AAATACATTC AAATATGTAT CCGCTCATGA GACAATAACC
7081 CTGATAAATG CTTCAATAAT ATTGAAAAAG GAAGAGTATG AGTATTCAAC ATTTCCGTGT
7141 CGCCCTTATT CCCTTTTTTG CGGCATTTTG CCTTCCTGTT TTTGCTCACC CAGAAACGCT
7201 GGTGAAAGTA AAAGATGCTG AAGATCAGTT GGGTGCACGA GTGGGTTACA TCGAACTGGA
7261 TCTCAACAGC GGTAAGATCC TTGAGAGTTT TCGCCCCGAA GAACGTTTTC CAATGATGAG
7321 CACTTTTAAA GTTCTGCTAT GTGGCGCGGT ATTATCCCGT ATTGACGCCG GGCAAGAGCA
7381 ACTCGGTCGC CGCATACACT ATTCTCAGAA TGACTTGGTT GAGTACTCAC CAGTCACAGA
7441 AAAGCATCTT ACGGATGGCA TGACAGTAAG AGAATTATGC AGTGCTGCCA TAACCATGAG
7501 TGATAACACT GCGGCCAACT TACTTCTGAC AACGATCGGA GGACCGAAGG AGCTAACCGC
7561 TTTTTTGCAC AACATGGGGG ATCATGTAAC TCGCCTTGAT CGTTGGGAAC CGGAGCTGAA
7621 TGAAGCCATA CCAAACGACG AACTACTTAC CACGATGCCT GTAGCAATGG CAACAACGTT
7681 GCGCAAACTA TTAACTGGCG AACTACTTAC TCTAGCTTCC CGGCAACAAT TAATAGACTG
7741 GATGGAGGCG GATAAAGTTG CAGGACCACT TCTGCGCTCG GCCCTTCCGG CTGGCTGGTT
7801 TATTGCTGAT AAATCTGGAG CCGGTGAGCG TGGGTCTCGC GGTATCATTG CAGCACTGGG
7861 GCCAGATGGT AAGCCCTCCC GTATCGTAGT TATCTACACG ACGGGGAGTC AGGCAACTAT
7921 GGATGAACGA AATAGACAGA TCGCTGAGAT AGGTGCCTCA CTGATTAAGC ATTGGTAACT
7981 GTCAGACCAA GTTTACTCAT ATATACTTTA GATTGATTTA AAACTTCATT TTTAATTTAA
8041 AAGGATCTAG GTGAAGATCC TTTTTGATAA TCTCATGACC AAAATCCCTT AACGTGAGTT
8101 TTCGTTCCAC TGAGCGTCAG ACCCCGTAGA AAAGATCAAA GGATCTTCTT GAGATCCTTT
8161 TTTTCTGCGC GTAATCTGCT GCTTGCAAAC AAAAAAACCA CCGCTACCAG CGGTGGTTTG
8221 TTTGCCGGAT CAAGAGCTAC CAACTCTTTT TCCGAAGGTA ACTGGCTTCA GCAGAGCGCA
8281 GATACCAAAT ACTGTTCTTC TAGTGTAGCC GTAGTTAGGC CACCACTTCA AGAACTCTGT
8341 AGCACCGCCT ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTGCTG CCAGTGGCGA
8401 TAAGTCGTGT CTTACCGGGT TGGACTCAAG ACGATAGTTA CCGGATAAGG CGCAGCGGTC
8461 GGGCTGAACG GGGGGTTCGT GCACACAGCC CAGCTTGGAG CGAACGACCT ACACCGAACT
8521 GAGATACCTA CAGCGTGAGC TATGAGAAAG CGCCACGCTT CCCGAAGAGA GAAAGGCGGA
8581 CAGGTATCCG GTAAGCGGCA GGGTCGGAAC AGGAGAGCGC ACGAGGGAGC TTCCAGGGGG
8641 AAACGCCTGG TATCTTTATA GTCCTGTCGG GTTTCGCCAC CTCTGACTTG AGCGTCGATT
8701 TTTGTGATGC TCGTCAGGGG GGCGGAGCCT ATGGAAAAAC GCCAGCAACG CGGCCTTTTT
8761 ACGGTTGGTG GCCTTTTGCT GGCCTTTTGC TCACATGTTC TTTCCTGCGT TATCCCCTGA
8821 TTCTGTGGAT AACCGTATTA CCGCCTTTGA GTGAGCTGAT ACCGCTCGCC GCAGCCGAAC
8881 GACCGAGCGC AGCGAGTCAG TGAGCGAGGA AGCGGAAGAG CGCCCAATAC GCAAACCGCC
8941 TCTCCCCGCG CGTTGGCCGA TTCATTAATG CAGCTGGCAC GACAGGTTTC CCGACTGGAA
9001 AGCGGGCAGT GAGCGCAACG CAATTAATGT GAGTTAGCTC ACTCATTAGG CACCCCAGGC
9061 TTTACACTTT ATGCTTCCGG CTCGTATGTT GTGTGGAATT GTGAGCGGAT AACAATTTCA
9121 CACAGGAAAC AGCTATGACC ATGATTACGC CAAGCGCGCA ATTAACCCTC ACTAAAGGGA
9181 ACAAAAGCTG GAGCTGCAAG CTT
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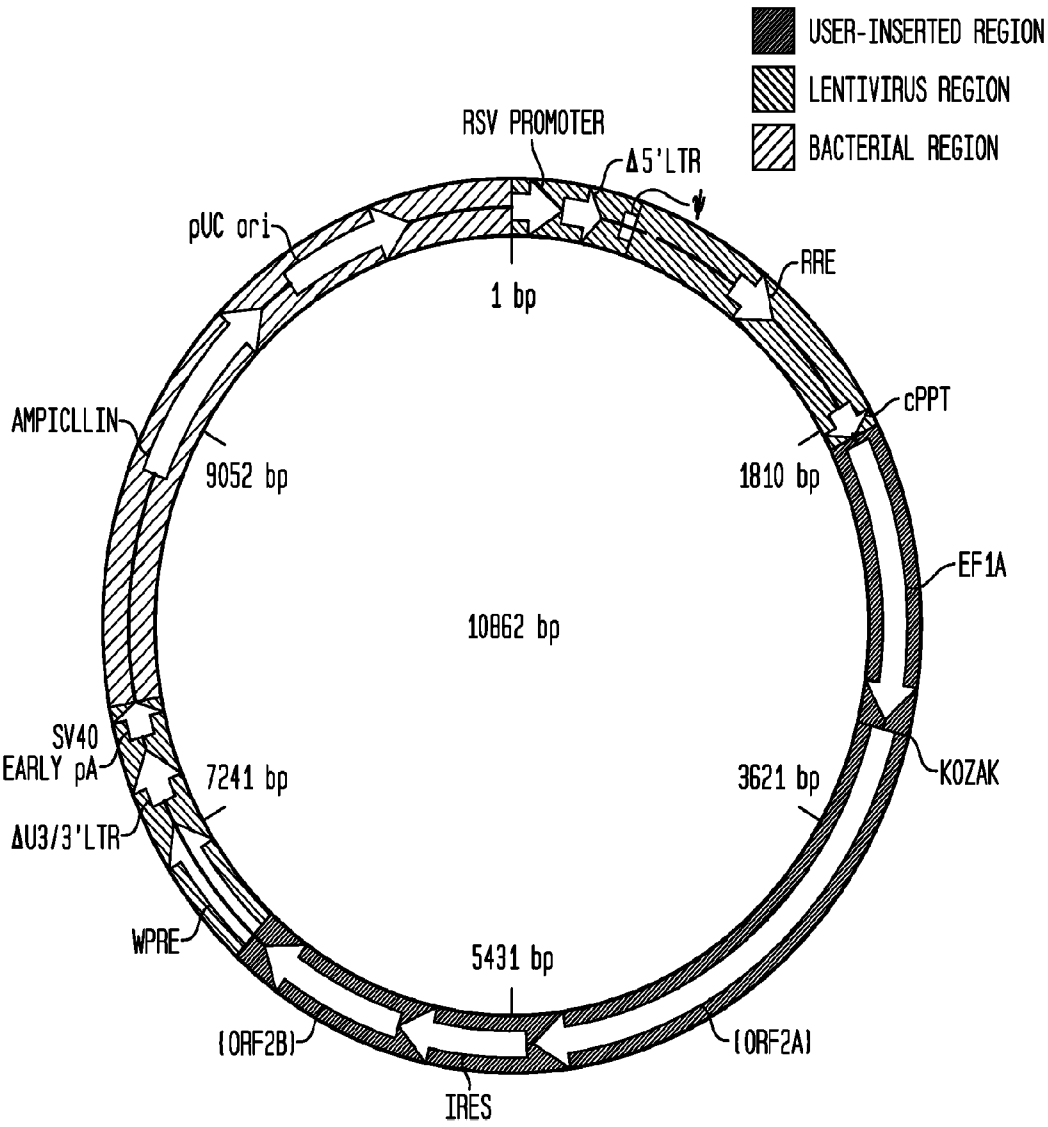
FIG. 4A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EARLY pA
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KOZAK
ΔU3/3'LTR
WPRE
5431 bp
(ORF2B)
(ORF2A)
IRES

FIG. 4B

SEQ ID NO. 48

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  61  TGCCTTACAA GGAGAGAAAA AGCACCGTGC ATGCCGATTG GTGGAAGTAA GGTGGTACGA
 121  TCGTGCCTTA TTAGGAAGGC AACAGACGGG TCTGACATGG ATTGGACGAA CCACTGAATT
 181  GCCGCATTGC AGAGATATTG TATTTAAGTG CCTAGCTCGA TACATAAACG GGTCTCTCTG
 241  GTTAGACCAG ATCTGAGCCT GGGAGCTCTC TGGCTAACTA GGGAACCCAC TGCTTAAGCC
 301  TCAATAAAGC TTGCCTTGAG TGCTTCAAGT AGTGTGTGCC CGTCTGTTGT GTGACTCTGG
 361  TAACTAGAGA TCCCTCAGAC CTTTTAGTC AGTGTGGAAA ATCTCTAGCA ATATAAATTA
 421  AACAGGGACT TGAAAGCGAA AGGGAAACCA GAGGAGCTCT CTCGACGCAG GACTCGGCTT
 481  GCTGAAGCGC GCACGGCAAG AGGCGAGGGG CGGCGACTGG TGAGTACGCC AAAAATTTTG
 541  ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG TCAGTATTAA GCGGGGGAGA
 601  ATTAGATCGC GATGGGAAAA AATTCGGTTA AGGCCAGGGG GAAAGAAAAA ATATAAATTA
 661  AAACATATAG TATGGGCAAG CAGGGAGCTA GAACGATTCG CAGTTAATCC TGGCCTGTTA
 721  GAAACATCAG AAGGCTGTAG ACAAATACTG GGACAGCTAC AACCATCCCT TCAGACAGGA
 781  TCAGAAGAAC TTAGATCATT ATATAATACA GTAGCAACCC TCTATTGTGT GCATCAAAGG
 841  ATAGAGATAA AAGACACCAA GGAAGCTTTA GACAAGATAG AGGAAGAGCA AAACAAAAGT
 901  AAGACCACCG CACAGCAAGC GGCCGCTGAT CTTCAGACCT GGAGGAGGAG ATATGAGGGA
 961  CAATTGGAGA AGTGAATTAT ATAAATATAA AGTAGTAAAA ATTGAACCAT TAGGAGTAGC
1021  ACCCACCAAG GCAAAGAGAA GAGTGGTGCA GAGAGAAAAA AGAGCAGTGG ATATGAGGGA
1081  TTTGTTCCTT GGGTTCTTGG GAGCAGCAGG AAGCACTATG GGCGCAGCGT CAATGACGCT
1141  GACGGTACAG GCCAGACAAT TATTGTCTGG TATAGTGCAG CAGCAGAACA ATTTGCTGAG
1201  GGCTATTGAG GCGCAACAGC ATCTGTTGCA ACTCACAGTC TGGGGCATCA AGCAGCTCCA
1261  GGCAAGAATC CTGGCTGTGG AAAGATACCT AAAGGATCAA CAGCTCCTGG GGATTTGGGG
1321  TTGCTCTGGA AAACTCATTT GCACCACTGC TGTGCCTTGG AATGCTAGTT GGAGTAATAA
1381  ATCTCTGGAA CAGATTTGGA ATCACACGAC CTGGATGGAG TGGGACAGAG AAATTAACAA
1441  TTACACAAGC TTAATACACT CCTTAATTGA AGAATCGCAA AACCAGCAAG AAAAGAATGA
1501  ACAAGAATTA TTGGAATTAG ATAAATGGGC AAGTTTGTGG AATTGGTTTA ACATAACAAA
1561  TTGGCTGTGG TATATAAAAT TATTCATAAT GATAGTAGGA GGCTTGGTAG GTTTAAGAAT
1621  AGTTTTTGCT GTACTTTCTA TAGTGAATAG AGTTAGGCAG GGATATTCAC CATTATCGTT
1681  TCAGACCCAC CTCCCAACCC CGAGGGGACC CGACAGGCCC GAAGGAATAG AAGAAGAAGG
1741  TGGAGAGAGA GACAGAGACA GATCCATTCG ATTAGTGAAC GGATCTCGAC GGTATCGCTA
1801  GCTTTTAAAA GAAAAGGGGG GATTGGGGGG TACAGTGCAG GGGAAAGAAT AGTAGACATA
1861  ATAGCAACAG ACATACAAAC TAAAGAATTA CAAAAACAAA TTACAAAAAT TCAAAATTTT
1921  ACTAGTATCA ACTTTGTATA GAAAAGTTGG GCTCCGGTGC CCGTCAGTGG GCAGAGCGCA
1981  CATCGCCCAC CATCGCCCAC AAGTTGGGGG GAGGGGTCGG CAATTGAACC GGTGCCTAGA
2041  GAAGGTGGCG CGGGGTAAAC TGGGAAAGTG ATGTCGTGTA CTGGCTCCGC CTTTTTCCCG
2101  AGGGTGGGGG AGAACCGTAT ATAAGTGCAG TAGTCGCCGT GAACGTTCTT TTTCGCAACG
2161  GGTTTGCCGC CAGAACACAG GTAAGTGCCG TGTGTGGTTC CCGCGGGCCT GGCCTCTTTA
2221  CGGGTTATGG CCCTTCGCTG CCTTGAATTA CTTCCACCTG GCTGCAGTAC GTGATTCTTG
2281  ATCCCGAGCT TCGGGTTGGA AGTGGGTGGG AGAGTTCGAG GCCTTGCGCT TAAGGAGCCC
2341  CTTCGCCTCG TGCTTGAGTT GAGGCCTGGC CTGGGCGCTG GGGCCGCCGC CTGCGAATCT
2401  GGTGGCACCT TCGCGCCTGT CTCGCTGCTT TCGATAAGTC TCTAGCCATT TAAAATTTTT
2461  GATGACCTGC TGCGACGCTT TTTTCTGGC AAGATAGTCT TGTAAATGCG GGCCAAGATC
2521  TGCACACTGG TATTTCGGTT TTTGGGGCCG CGGGCGGCGA CGGGGCCCGT GCGTCCCAGC
2581  GCACATGTTC GGCGAGGCGG GGCCTGCGAG CGCGGCCACC GAGAATCGGA CGGGGGTAGT
2641  CTCAAGCTGG CCGGCCTGCT CTGGTGCCTG GTCTCGCGCC GCCGTGTATC GCCCCGCCCT
2701  GGGCGGCAAG GCTGGCCCGG TCGGCACCAG TTGCGTGAGC GGAAAGATGG CCGCTTCCCG
2761  GCCCTGCTGC AGGGAGCTCA AAATGGAGGA CGCGGCGCTC GGGAGAGCGG GCGGGTGAGT
```

FIG. 4B-1

```
2821 CACCCACACA AAGGAAAAGG GCCTTTCCGT CCTCAGCCGT CGCTTCATGT GACTCCACGG
2881 AGTACCGGGC GCCGTCCAGG CACCTCGATT AGTTCTCGAG CTTTTGGAGT ACGTCGTCTT
2941 TAGGTTGGGG GGAGGGGTTT TATGCGATGG AGTTTCCCCA CACTGAGTGG GTGGAGACTG
3001 AAGTTAGGCC AGCTTGGCAC TTGATGTAAT TCTCCTTGGA ATTTGCCCTT TTTGAGTTTG
3061 GATCTTGGTT CATTCTCAAG CCTCAGACAC TGGTTCAAAG TTTTTTTCTT CCATTTCAGG
3121 TGTCGTGACA AGTTTGTACA AAAAAGCAGG CTGCCACCAT GGAGTTCGGC CTGAGCTGGG
3181 TGTTCCTGGT GGCCCTGTTC AGAGGCGTGC AGTGCCAGGT GAAGCTGCAG GAGAGCGGCC
3241 CCGGCCTGGT GGCCCCCAGC CAGAGCCTGA GCATCACCTG CACCGTGAGC GGCTTCAGCC
3301 TGACCGCCTA CGGCGTGGAC TGGGTGAGAC AGCCCCCCGG CAAGGGCCTG GAGTGGCTGG
3361 GCGTGATCTG GGGCGGCGGC AGAACCAACT ACAACAGCGG CCTGATGAGC AGACTGAGCA
3421 TCAGAAAGGA CAACAGCAAG AGCCAGGTGT TCCTGACCAT GAACAGCCTG CAGACCGACG
3481 ACACCGCCAA GTACTACTGC GTGAAGCACA CCAACTGGGA CGGCGGCTTC GCCTACTGGC
3541 GCCAGGGCAC CACCGTGACC GTGAGCAGCC CCAGCGTGTT CCCCCTGGCC CCCAGCAGCA
3601 AGAGCACCAG CGGCGGCACC GCCGCCCTGG GCTGCCTGGT GAAGGACTAC TTCCCCGAGC
3661 CCGTGACCGT GAGCTGGAAC AGCGGCGCCC TGACCAGCGG CGTGCACACC TTCCCCGCCG
3721 TGCTGCAGAG CAGCGGCCTG TACAGCCTGA GCAGGGTGGT GACCGTGCCC AGGAGCAGCC
3781 TGGGCACCCA GACCTACATC TGCAACGTGA ACCACAAGCC CAGCAACACC AAGGTGGACA
3841 AGAAGGTGGA GCTGAAGACC CCCCTGGGCG ACACCACCCA CACCTGCCCC AGATGCCCCG
3901 AGCCCAAGAG CTGCGACACC CCCCCCCCCT GCCCCAGATG CCCCGAGCCC AAGAGCTGCG
3961 ACACCCCCCC CCCCTGCCCC AGATGCCCCG AGCCCAAGAG CTGCGACACC CCCCCCCCCT
4021 GCCCCAGATG CCCCGCCCCC GAGCTGCTGG GCGGCCCCAG CGTGTTCCTG TTCCCCCCCA
4081 AGCCCAAGGA CACCCTGATG ATCAGCAGAG CCCCCGAGGT GACCTGCGTG GTGGTGGACG
4141 TGAGCCACGA GGACCCCGAG GTGAAGTTCA ACTGGTACGT GGACGGCGTG GAGGTGCACA
4201 ACGCCAAGAC CAAGCCCAGA GAGGAGCAGT ACAACAGCAC CTACAGAGTG GTGAGCGTGC
4261 TGACCGTGCT GCACCAGGAC TGGCTGAACG GCAAGGACTA CAAGTGCAAG GTGAGCAACA
4321 AGGCCCTGCC CGCCCCCATC GAGAAGACCA TCAGCAAGGC CAAGGGCCAG CCCAGAGAGC
4381 CCCAGGTGTA CACCCTGCCC CCCAGCAGAG ACGAGCTGAC CAAGAACCAG GTGAGCCTGA
4441 CCTGCCTGGT GAAGGGCTTC TACCCCAGCG ACATCGCCGT GGAGTGGGAG AGCAACGGCC
4501 AGCCCGAGAA CAACTACAAG GACAAGAGCA GATGGCAGCA GGGCAACGTG TTCAGCTGCA
4621 GCGTGATGCA CGAGGCCCTG CACAACCACT ACACCCAGAA GAGCCTGAGC CTGAGCCCCG
4681 AGCTGCAGCT GGAGGAGAGC TGCGCCGAGG CCCAGGACGG CGAGCTGGAC GGCCTGTGGA
4741 CCACCATCAC CATCTTCATC ACCCTGTTCC TGCTGAGCGT GTGCTACAGC GCCACCGTGA
4801 CCTTCTTCAA GGTGAAGTGG ATCTTCAGCA GCGTGGTGGA CCTGAAGCAG ACCATCATCC
4861 CCGACTACAG AAACATGATC GGCCAGGGCG CCTAAAACAA CAACAATTGC ATTCATTTTA
4921 TGTTTCAGGT TCAGGGGGAG GTGTGGGAGG TTTTTTAAAG CAAGTAAAAC CTCTACAAAT
4981 GTGGTACGCG TTAACAACAA CAATTGCATT CATTTTATGT TTCAGGTTCA GGGGGAGGTG
5041 TGGGAGGTTT TTTAAACGAA GTAAAACCTC TACAAATGTG GTACGCGTTA CCCAGCTTTC
5101 TTGTACAAAG TGGTAAATAG ATAGAACAAC AACAATTGCA TTCATTTTTG ATTTCAGGTT
5161 CAGGGGGAGG TGTGGGAGGT TTTTTAAAGC AAGTAAAACC TCTACACTGA CGGTACGCGT
5221 TAACAACAAC AATTGCATTC ATTTGTAGTT TCAGGTTCAG GGGGAGGTGT GGGAGGTTTT
5281 TTAAAGCAAG TTAAACCTCT AAAATAGTGG TACGCGTTAC CCAGCTTTCT TGTACAAAGT
5341 GGACCCAGCT TTCTTGTACA AAGTGGGCCC CTCTCCCTCC CCCCCCCCTA ACGTTACTGG
5401 CCGAAGCCGC TTGGAATAAG GCCGGTGTGC GTTTGTCTAT ATGTTATTTT CCACCATATT
5461 GCCGTCTTTT GGCAATGTGA GGGCCCGGAA ACCTGGCCCT GTCTTCTTGA ACGTTACTGG
5521 TAGGGGTCTT TCCCCTCTCG CCAAAGGAAT GCAAGGTCTG TTGAATGTCG TGAAGGAAGC
5581 AGTTCCTCTG GAAGCTTCTT GAAGACAAAC AACGTCTGTA GCGACCCTTT GCAGGCAGCG
5641 GAACCCCCCA CCTGGCGACA GGTGCCTCTG CGGCCAAAAG CCACGTGTAT AAGATACACC
5701 TGCAAAGGCG GCACAACCCC AGTGCCACGT TGTGAGTTGG ATAGTTGTGG AAAGAGTCAA
5761 ATGGCTCTCC TCAAGCGTAT TCAACAAGGG GCTGAAGGAT GCCCAGAAGG TACCCCATTG
5821 TATGGGATCT GATCTGGGGC CTCGGTGCAC ATGCTTTACA TGTGTTTAGT CGAGGTTAAA
```

FIG. 4B-2

```
5881  AAAACGTCTA GGCCCCCCGA ACCACGGGGA CGTGGTTTTC CTTTGAAAAA CACGATGATA
5941  ATATGGCCAC AACCATGGCC ACCGACATGA GAGTGCCCGC CCAGCTGCTG GGCCTGCTGC
6001  TGCTGTGGCT GAGCGGCGCC AGATGCGGCA GCCCCGGCCA GAGCGTGAGC ATCAGCTGCA
6061  GCGGCAGCAG CAGCAACATC GGCAACAACT ACGTGTACTG GTACCAGCAC CTGCCCGGCA
6121  CCGGGCCCAA GCTGCTGATC TACAGCGACA CCAAGAGACC CAGCGGCGTG CCCGACAGAA
6181  TCAGCGGCAG CAAGAGCGGC ACCAGCGCCA GCCTGGCCAT CAGCGGCCTG CAGAGCGAGG
6241  ACGAGGCCGA CTACTACTGC GCCAGCTGGG ACGACAGGCT GGACGGCCCC GTGTTCGGGG
6301  GCGGCACCAA GCTGACCGTG CTGGGCCAGC CCAAGGCCAA CCCCACCGTG ACCCTGTTCC
6361  CCCCCAGCAG CGAGGAGCTG CAGGCCAACA AGGCCACCCT GGTGTGCCTG ATCAGCGACT
6421  TCTACCCCGG CGCCGTGACC GTGGCCTGGA AGGCCGACGG CAGCCCCGTG AAGGCCGGCG
6481  TGGAGACCAC CAAGCCCAGC AAGCAGAGCA ACAACAAGTA CGCCGCCAGC AGCTACCTGA
6541  GCCTGACCCC CGAGCAGTGG AAGAGCCACA GAAGCTACAG CTGCCAGGTG ACCCACGAGG
6601  GCAGCACCGT GGAGAAGACC GTGGCCCCCA CCGAGTGCAG CTAACAACTT TATTATACAT
6661  AGTTGATCAA TTCCAACTTT ATTATACATA GTTGATCAAT TCCGATAATC AACCTCTGGA
6721  TTACAAAATT TGTGAAAGAT TGACTGGTAT TCTTAACTAT GTTGCTCCTT TTACGCTATG
6781  TGGATACGCT GCTTTAATGC CTTTGTATCA TGCTATTGCT TCCCGTATGG CTTTCATTTT
6841  CTCCTCCTTG TATAAATCCT GGTTGCTGTC TCTTTATGAG GAGTTGTGGC CCGTTGTCAG
6901  GCAACGTGGC GTGGTGTGCA CTGTGTTTGC TGACGCAACC CCCACTGGTT CGGGCATTGC
6961  CACCACCTGT CAGCTCCTTT CCGGGACTTT CGCTTTCCCC CTCCCTATTG CCACGGCGGA
7021  ACTCATCGCG GCCTGCGTTG CCCGCTGCTG GACAGGGCCT CGGCTGTTGG GCACTGACAA
7081  TTCCGTGGTG TTGTCGGGGA AGCTGACGTC CTTTCCATGG CTGCTCGCCT GTGTTGCCAC
7141  CTGGATTCTG CGCGGGACGT CCTTCTGCTA CCTCCCTTCG GCCCTCAATC GCCCTCAATC
7201  TCCTTCCCGC CGGCTCTGCG GCCTCTTCCG GCCTCTTCCG CGTCTTCGCC TTCGCCCTCA
7261  GACGACTCGG ATCTCCCTTT GGGCCGCCTC CCCGCATCGG GAATTCCCGC GGTTCGCTTT
7321  AAGACCAATG ACTTACAAGG CAGCTGTAGA TCTTAGCCAC TTTTTAAAAG AAAAGGGGGG
7381  ACTGGAAGGG CTAATTCACT CCCAACGAAG ACAAGATCTG CTTTTTGCTT GTACTGGGTC
7441  TCTCTGGTTA GACCAGATCT GAGCCTGGGA GCTCTCTGGC TAACTAGGGA ACCCACTGCT
7501  TAAGCCTCAA TAAAGCTTGC CTTGAGTGCT TCAAGTAGTG TGTGCCCGTC TGTTGTGTGA
7561  CTCTGGTAAC TAGAGATCCC TCAGACCCTT TTAGTCACTG TGGAAAATCT CTAGCAGTAG
7621  TAGTTCATGT CATCTTATTA TTCAGTATTT ATAACTTGCA AAGAAATGAA TATCAGAGAG
7681  TGAGAGGAAC TTGTTTATTG CAGCTTATAA TGGTTACAAA TAAAGCAATA GCATCACAAA
7741  TTTCACAAAT AAAGCATTTT TTTCACTGCA TTCTAGTTGT GGTTTGTCCA AACTCATCAA
7801  TGTATCTTAT CATGTGTGGC TCTAGCTATC CCGCCCCTAA CTCCGCCCAT CCCGCCCCTA
7861  ACTCCGCCCA GTTCCGCCCA TTCTCCGCCC CATGGCTGAC TATTTTTTTT TATTTATGCA
7921  GAGGCCGAGG CCGCCTCGGC CTCTGAGCTA TTCCAGAAGT ACTGAGGAGG CTTTTTTGCA
7981  GGCCTAGGGA CGTACCCAAT TCGCCCTATA GTGAGTCGTA TTACGCGCGC TCACTGGCCG
8041  TCGTTTTACA ACGTCGTGAC TGGGAAAACC CTGGCGTTAC CCAACTTAAT CGCCTTGCAG
8101  CACATCCCCC TTTCGCCAGC TGGCGTAATA GCGAAGAGGC CCGCACCGAT CGCCCTTCCC
8161  AACAGTTGCG CAGCCTGAAT GGCGAATGGG ACGCGCCCTG TAGCGGCGCA TTAAGCGCGG
8221  CGGGTGTGGT GGTTACGCGC AGCGTGACCG CTACACTTGC CAGCGCCCTA GCGCCCGCTC
8281  CTTTCGCTTT CTTCCCTTCC TTTCTCGCCA CGTTCGCCGG CTTTCCCCGT CAAGCTCTAA
8341  ATCGGGGGCT CCCTTTAGGG TTCCGATTTA GTGCTTTACG GCACCTCGAC CCCAAAAAAC
8401  TTGATTAGGG TGATGGTTCA CGTAGTGGGC CATCGCCCTG ATAGACGGTT TTTCGCCCTT
8461  TGACGTTGGA GTCCACGTTC TTTAATAGTG GACTCTTGTT CCAAACTGGA ACAACACTCA
8521  ACCCTATCTC GGTCTATTCT TTTGATTTAT AAGGGATTTT GCCGATTTCG GCCTATTGGT
8581  TAAAAAATGA GCTGATTTAA CAAAAATTTA ACGCGAATTT TAACAAAATA TTAACGCTTA
8641  CAATTTAGGT GGCACTTTTC GGGGAAATGT GCGCGGAACC CCTATTTGTT TATTTTTCTA
8701  AATACATTCA AATATGTATC CGCTCATGAG ACAATAACCC TGATAAATGC TTCAATAATA
8761  TTGAAAAAGG AAGAGTATGA GTATTCAACA TTTCCGTGTC GCCCTTATTC CCTTTTTTGC
8821  GGCATTTTGC CTTCCTGTTT TTGCTCACCC AGAAACGCTG GTGAAAGTAA AAGATGCTGA
8881  AGATCAGTTG GGTGCACGAG TGGGTTACAT CGAACTGGAT CTCAACAGCG GTAAGATCCT
```

FIG. 4B-3

```
8941  TGAGAGTTTT CGCCCCGAAG AACGTTTTCC AATGATGAGC ACTTTTAAAG TTCTGCTATG
9001  TGGCGCGGTA TTATCCCGTA TTGACGCCGG GCAAGAGCAA CTCGGTCGCC GCATACACTA
9061  TTCTCAGAAT GACTTGGTTG AGTACTCACC AGTCACAGAA AAGCATCTTA CGGATGGCAT
9121  GACAGTAAGA GAATTATGCA GTGCTGCCAT AACCATGAGT GATAACACTG CGGCCAACTT
9181  ACTTCTGACA ACGATCGGAG GACCGAAGGA GCTAACCCCT TTTTTGCACA ACATGGGGGA
9241  TCATGTAACT CGCCTTGATC GTTGGGAACC GGAGCTGAAT GAAGCCATAC CAAACGACGA
9301  GCGTGACACC ACGATGCCTG TAGCAATGGC AACAACGTTG CGCAAACTAT TAACTGGCGA
9361  ACTACTTACT CTAGCTTCCC GGCAACAATT AATAGACTGG ATGGAGGCGG ATAAAGTTGC
9421  AGGACCACTT CTGCGCTCGG CCCTTCCGGC TGGCTGGTTT ATTCGTGATA AATCTGGAGC
9481  CGGTGAGCGT GGGTCTCGCG GTATCATTGC AGCACTGGGG CCAGATGGTA AGCCCTCCCG
9541  TATCGTAGTT ATCTACACGA CGGGGAGTCA GGCAACTATG GATGAACGAA ATAGACAGAT
9601  CGCTGAGATA GGTGCCTCAC TGATTAAGCA TTGGTAACTG TCAGACCAAG TTTACTCATA
9661  TATACTTTAG ATTGATTTAA AACTTCATTT TTAATTTAAA AGGATCTAGG TGAAGATCCT
9721  TTTTGATAAT CTCATGACCA AAATCCCTTA ACGTGAGTTT TCGTTCCACT GAGCGTCAGA
9781  CCCCGTAGAA AAGATCAAAG GATCTTCTTG AGATCCTTTT TTTCTGCGCG TAATCTGCTG
9841  CTTCCAAACA AAAAAACCAC CGCTACCAGC GGTGGTTTGT TTGCCGGATC AAGAGCTACC
9901  AACTCTTTTT CCGAAGGTAA CTGGCTTCAG CAGAGCGCAG ATACCAAATA CTGTTCTTCT
9961  AGTGTAGCCG TAGTTAGGCC ACCACTTCAA GAACTCTGTA GCACCGCCTA CATACCTCGC
10021 TCTGCTAATC CTGTTACCAG TGGCTGCTGC CAGTGGCGAT AAGTCGTGTC TTACCGGGTT
10081 GGACTCAAGA CGATAGTTAC CGGATAAGGC GCAGCGGTCG GGCTGAACGG GGGGTTCGTG
10141 CACACAGCCC AGCTTGGAGC GAACGACCTA CACCGAACTG AGATACCTAC AGCGTGAGCT
10201 ATGAGAAAGC GCCACGCTTC CCGAAGAGAG AAAGGCGGAC AGGTATCCGG TAAGCGGCAG
10261 GGTCGGAACA GGAGAGCGCA CGAGGGAGCT TCCAGGGGGA AACGCCTGGT ATCTTTATAG
10321 TCCTGTCGGG TTTCGCCACC TCTGACTTGA GCGTCGATTT TTGTGATGCT CGTCAGGGGG
10381 GCGGAGCCTA TGGAAAAACG CCAGCAACGC GGCCTTTTTA CGGTTCCTGG CCTTTTGCTG
10441 GCCTTTTGCT CACATGTTCT TTCCTGCGTT ATCCCCTGAT TCTGTGGATA ACCGTATTAC
10501 CGCCTTTGAG TGAGCTGATA CCGCTCGCCG CAGCCGAACG ACCGAGCGCA GCGAGTCAGT
10561 GAGCGAGGAA GCGGAAGAGC GCCCAATACG CAAACCGCCT CTCCCCGCGC GTTGGCCGAT
10621 TCATTAATGC AGCTGGCACG ACAGGTTTCC CGACTGGAAA GCGGGCAGTG AGCGCAACGC
10681 AATTAATGTG AGTTAGCTCA CTCATTAGGC ACCCCAGGCT TTACACTTTA TGCTTCCGGC
10741 TCGTATGTTG TGTGGAATTG TGAGCGGATA ACAATTTCAC ACAGGAAACA GCTATGACCA
10801 TGATTACGCC AAGCGCGCAA TTAACCCTCA CTAAAGGGAA CAAAAGCTGG AGCTGCAAGC
10861 TT
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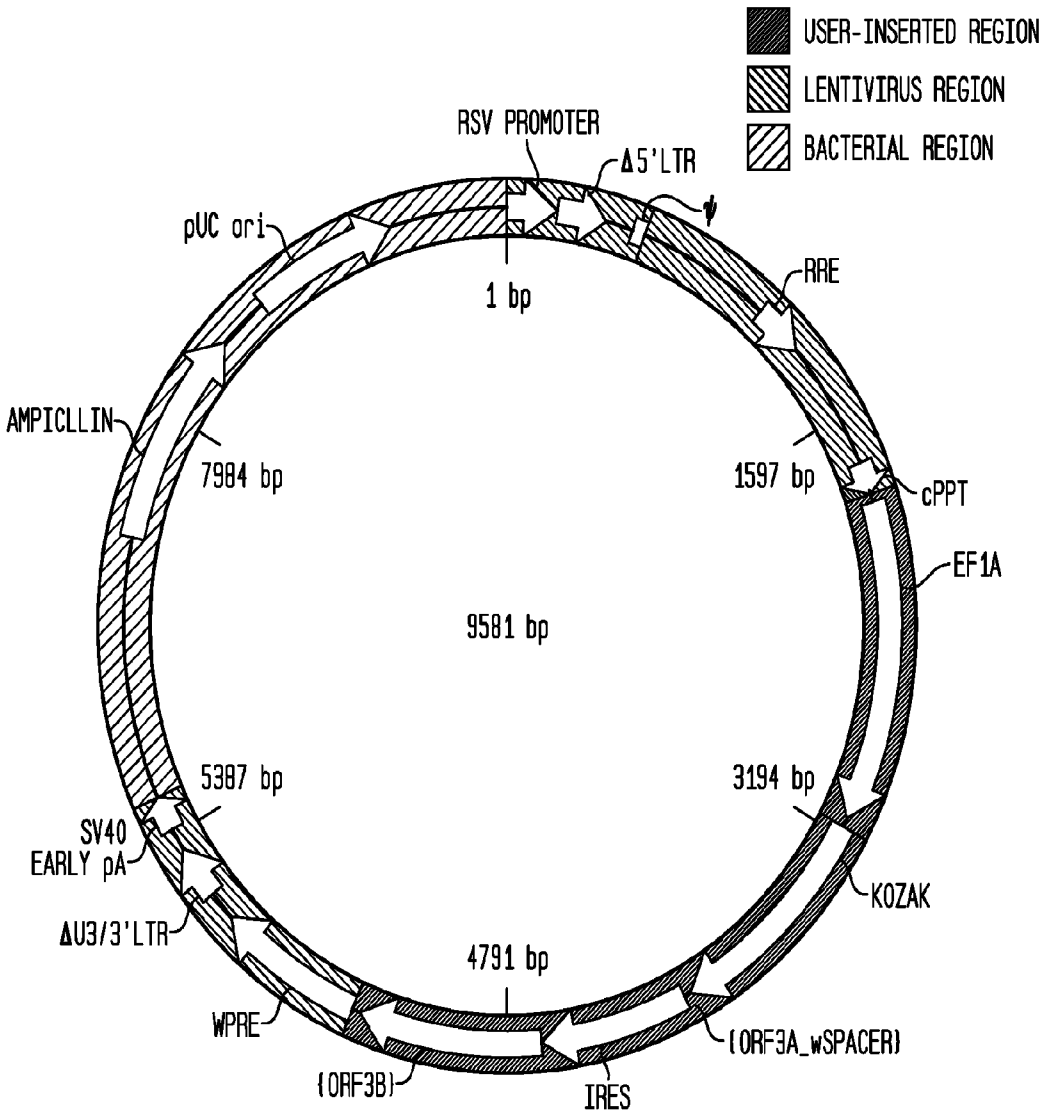
FIG. 5A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IRES
(ORF3B)
WPRE
ΔU3/3'LTR
SV40
EARLY pA
AMPICLLIN
pUC ori
1 bp
1597 bp
3194 bp
4791 bp
5387 bp
7984 bp
9581 bp

FIG. 5B
SEQ ID NO. 49

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   1 AATGTAGTCT TATGCAATAC TCTTGTAGTC TTGCAACATG GTAACGATGA GTTAGCAACA
  61 TGCCTTACAA GGAGAGAAAA AGCACCGTGC ATGCCGATTG GTGGAAGTAA GGTGGTACGA
 121 TCGTGCCTTA TTAGGAAGGC AACAGACGGG TCTGACATGG ATTGGACGAA CCACTGAATT
 181 GCCGCATTGC AGAGATATTG TATTTAAGTG CCTAGCTCGA TACATAAACG GGTCTCTCTG
 241 GTTAGACCAG ATCTGAGCCT GGGAGCTCTC TGGCTAACTA GGGAACCCAC TGCTTAAGCC
 301 TCAATAAAGC TTGCCTTGAG TGCTTCAAGT AGTGTGTGCC CGTCTGTTGT GTGACTCTGG
 361 TAACTAGAGA TCCCTCAGAC CCTTTTAGTC AGTGTGGAAA ATCTCTAGCA GTGGCGCCCG
 421 AACAGGGACT TGAAAGCGAA AGGGAAACCA GAGGAGCTCT CTCGACGCAG GACTCGGCTT
 481 GCTGAAGCGC GCACGGCAAG AGGCGAGGGG CGGCGACTGG TGAGTACGCC AAAAATTTTG
 541 ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG TCAGTATTAA GCGGGGGAGA
 601 ATTAGATCGC GATGGGAAAA AATTCGGTTA AGGCCAGGGG GAAAGAAAAA ATATAAATTA
 661 AAACATATAG TATGGGCAAG CAGGGAGCTA GAACGATTCG CAGTTAATCC TGGCCTGTTA
 721 GAAACATCAG AAGGCTGTAG ACAAATACTG GGACAGCTAC AACCATCCCT TCAGACAGGA
 781 TCAGAAGAAC TTAGATCATT ATATAATACA GTAGCAACCC TCTATTGTGT GCATCAAAGG
 841 ATAGAGATAA AAGACACCAA GGAAGCTTTA GACAAGATAG AGGAAGAGCA AAACAAAAGT
 901 AAGACCACCG CACAGCAAGC GGCCGCTGAT CTTCAGACCT GGAGGAGGAG ATATGAGGGA
 961 CAATTGGAGA AGTGAATTAT ATAAATATAA AGTAGTAAAA ATTGAACCAT TAGGAGTAGC
1021 ACCCACCAAG GCAAAGAGAA GAGTGGTGCA GAGAGAAAAA AGAGCAGTGG GAATAGGAGC
1081 TTTGTTCCTT GGGTTCTTGG GAGCAGCAGG AAGCACTATG GGCGCAGCGT CAATGACGCT
1141 GACGGTACAG GCCAGACAAT TATTGTCTGG TATAGTGCAG CAGCAGAACA ATTTGCTGAG
1201 GGCTATTGAG GCGCAACAGC ATCTGTTGCA ACTCACAGTC TGGGGCATCA AGCAGCTCCA
1261 GGCAAGAATC CTGGCTGTGG AAAGATACCT AAAGGATCAA CAGCTCCTGG GGATTTGGGG
1321 TTGCTCTGGA AAACTCATTT GCACCACTGC TGTGCCTTGG AATGCTAGTT GGAGTAATAA
1381 ATCTCTGGAA CAGATTTGGA ATCACACGAC CTGGATGGAG TGGGACAGAG AAATTAACAA
1441 TTACACAAGC TTAATACACT CCTTAATTGA AGAATCGCAA AACCAGCAAG AAAAGAATGA
1501 ACAAGAATTA TTGGAATTAG ATAAATGGGC AAGTTTGTGG AATTGGTTTA ACATAACAAA
1561 TTGGCTGTGG TATATAAAAT TATTCATAAT GATAGTAGGA GGCTTGGTAG GTTTAAGAAT
1621 AGTTTTTGCT GTACTTTCTA TAGTGAATAG AGTTAGGCAG GGATATTCAC CATTATCGTT
1681 TCAGACCCAC CTCCCAACCC CGAGGGGACC CGACAGGCCC GAAGGAATAG AAGAAGAAGG
1741 TGGAGAGAGA GACAGAGACA GATCCATTCG ATTAGTGAAC GGATCTCGAC GGTATCGCTA
1801 GCTTTTAAAA GAAAAGGGGG GATTGGGGGG TACAGTGCAG GGGAAAGAAT AGTAGACATA
1861 ATAGCAACAG ACATACAAAC TAAAGAATTA CAAAAACAAA TTACAAAAAT TCAAAATTTT
1921 ACTAGTATCA ACTTTGTATA GAAAAGTTGG GCTCCGGTGC CCGTCAGTGG GCAGAGCGCA
1981 CATCGCCCAC AGTCCCCGAG AAGTTGGGGG GAGGGGTCGG CAATTGAACC GGTGCCTAGA
2041 GAAGGTGGCG CGGGGTAAAC TGGGAAAGTG ATGTCGTGTA CTGGCTCCGC CTTTTTCCCG
2101 AGGGTGGGGG AGAACCGTAT ATAAGTGCAG TAGTCGCCGT GAACGTTCTT TTTCGCAACG
2161 GGTTTGCCGC CAGAACACAG GTAAGTGCCG TGTGTGGTTC CCGCGGGCCT GGCCTCTTTA
2221 CGGGTTATGG CCCTTGCGTG CCTTGAATTA CTTCCACCTG GCTGCAGTAC GTGATTCTTG
2281 ATCCCGAGCT TCGGGTTGGA AGTGGGTGGG AGAGTTCGAG GCCTTGCGCT TAAGGAGCCC
2341 CTTCGCCTCG TGCTTGAGTT GAGGCCTGGC CTGGGCGCTG GGGCCGCCGC GTGCGAATCT
2401 GGTGGCACCT TCGCGCCTGT CTCGCTGCTT TCGATAAGTC TCTAGCCATT TAAAATTTTT
2461 GATGACCTGC TGCGACGCTT TTTTTCTGGC AAGATAGTCT TGTAAATGCG GGCCAAGATC
2521 TGCACACTGG TATTTCGGTT TTTGGGGCCG CGGGCGGCGA CGGGGCCCGT GCGTCCCAGC
2581 GCACATGTTC GGCGAGGCGG GGCCTGCGAG CGCGGCCACC GAGAATCGGA CGGGGGTAGT
2641 CTCAAGCTGG CCGGCCTGCT CTGGTGCCTG GTCTCGCGCC GCCGTGTATC GCCCCGCCCT
2701 GGGCGGCAAG GCTGGCCCGG TCGGCACCAG TTGCGTGAGC GGAAAGATGG CCGCTTCCCG
2761 GCCCTGCTGC AGGGAGCTCA AAATGGAGGA CGCGGCGCTC GGGAGAGCGG GCGGGTGAGT
2821 CACCCACACA AAGGAAAAGG GCCTTTCCGT CCTCAGCCGT CGCTTCATGT GACTCCACGG
2881 AGTACCGGGC GCCGTCCAGG CACCTCGATT AGTTCTCGAG CTTTTGGAGT ACGTCGTCTT
```

FIG. 5B-1

2941 TAGGTTGGGG GGAGGGGTTT TATGCGATGG AGTTTCCCCA CACTGAGTGG GTGGAGACTG
3001 AAGTTAGGCC AGCTTGGCAC TTGATGTAAT TCTCCTTGGA ATTTGCCCTT TTTGAGTTTG
3061 GATCTTGGTT CATTCTCAAG CCTCAGACAG TGGTTCAAAG TTTTTTTCTT CCATTTCAGG
3121 TGTCGTGACA AGTTTGTACA AAAAAGCAGG CTGCCACCAT GTGGCTGCAG AGCCTGCTGC
3181 TGCTGGGCAC CGTGGCCTGC AGCATCAGCG CCCCCGCCAG AAGCCCCAGC CCCAGCACCC
3241 AGCCCTGGGA GCACGTGAAC GCCATCCAGG AGGCCAGAAG ACTGCTGAAC CTGAGCAGAG
3301 ACACCGCCGC CGAGATGAAC GAGACCGTGG AGGTGATCAG CGAGATGTTC GACCTGCAGG
3361 AGCCCACCTG CCTGCAGACC AGACTGGAGC TGTACAAGCA GGGCCTGAGA GGCAGCCTGA
3421 CCAAGCTGAA GGGCCCCCTG ACCATGATGG CCAGCCACTA CAAGCAGCAC TGCCCCCCCA
3481 CCCCCGAGAC CAGCTGCGCC ACCCAGATCA TCACCTTCGA GAGCTTCAAG GAGAACCTGA
3541 AGGACTTCCT GCTGGTGATC CCCTTCGACT GCTGGGAGCC CGTGCAGGAG TAAAACAACA
3601 ACAATTGCAT TCATTTTATG TTTCAGGTTC AGGGGGAGGT GTGGGAGGTT TTTTAAAGCA
3661 AGTAAAACCT CTACAAATGT GGTACGCGTT AACAACAACA ATTGCATTCA TTTTATGTTT
3721 CAGGTTCAGG GGGAGGTGTG GGAGGTTTTT TAAAGCAAGT AAAACCTCTA CAAATGTGGT
3781 ACGCGTTACC CAGCTTTCTT GTACAAAGTG GTAAATAGAT AGAACAACAA CAATTGCATT
3841 CATTTTTGAT TTCAGGTTCA GGGGGAGGTG TGGGAGGTTT TTTAAAGCAA GTAAAACCTC
3901 TACACTGACG GTACGCGTTA ACAACAACAA TTGCATTCAT TTGTAGTTTC AGGTTCAGGG
3961 GGAGGTGTGG GAGGTTTTTT AAAGCAAGTT AAACCTCTAA AATAGTGGTA CGCGTTACCC
4021 AGCTTTCTTG TACAAAGTGG ACCCAGCTTT CTTGTACAAA GTGGGCCCCT CTCCCTCCCC
4081 CCCCCCTAAC GTTACTGGCC GAAGCCGCTT GGAATAAGGC CGGTGTGCGT TTGTCTATAT
4141 GTTATTTTCC ACCATATTGC CGTCTTTTGG CAATGTGAGG GCCCGGAAAC CTGGCCCTGT
4201 CTTCTTGACG AGCATTCCTA GGGGTCTTTC CCCTCTCGCC AAAGGAATGC AAGGTCTGTT
4261 GAATGTCGTG AAGGAAGCAG TTCCTCTGGA AGCTTCTTGA AGACAAACAA CGTCTGTAGC
4321 GACCCTTTGC AGGCAGCGGA ACCCCCCACC TGGCGACAGG TGCCTCTGCG GCCAAAAGCC
4381 ACGTGTATAA GATACACCTG CAAAGGCGGC ACAACCCCAG TGCCACGTTG TGAGTTGGAT
4441 AGTTGTGGAA AGAGTCAAAT GGCTCTCCTC AAGCGTATTC AACAAGGGGC TGAAGGATGC
4501 CCAGAAGGTA CCCCATTGTA TGGGATCTGA TCTGGGGCCT CGGTGCACAT GCTTTACATG
4561 TGTTTAGTCG AGGTTAAAAA AACGTCTAGG CCCCCCGAAC CACGGGGACG TGGTTTTCCT
4621 TTGAAAAACA CGATGATAAT ATGGCCACAA CCATGGCCAC CGTGCTGGCC CCCGCCTGGA
4681 GCCCCACCAC CTACCTGCTG CTGCTGCTGC TGCTGAGCAG CGGCCTGAGC GGCACCCAGG
4741 ACTGCAGCTT CCAGCACAGC CCCATCAGCA GCGACTTCGC CGTGAAGATC AGAGAGCTGA
4801 GCGACTACCT GCTGCAGGAC TACCCCGTGA CCGTGGCCAG CAACCTGCAG GACGAGGAGC
4861 TGTGCGGCGG CCTGTGGAGA CTGGTGCTGG CCCAGAGATG GATGGAGAGA CTGAAGACCG
4921 TGGCCGGCAG CAAGATGCAG GGCCTGCTGG AGAGAGTGAA CACCGAGATC CACTTCGTGA
4981 CCAAGTGCGC CTTCCAGCCC CCCCCCAGCT GCCTGAGATT CGTGCAGACC AACATCAGCA
5041 GACTGCTGCA GGAGACCAGC GAGCAGCTGG TGGCCCTGAA GCCCTGGATC ACCAGACAGA
5101 ACTTCAGCAG ATGCCTGGAG CTGCAGTGCC AGCCCGACAG CAGCACCCTG CCCCCCCCCT
5161 GGAGCCCCAG ACCCCTGGAG GCCACCGCCC CCACCGCCCC CCAGCCCCCC CTGCTGCTGC
5221 TGCTGCTGCT GCCCGTGGGC CTGCTGCTGC TGGCCGCCGC CTGGTGCCTG CACTGGCAGA
5281 GAACCAGAAG AAGAACCCCC AGACCCGGCG AGCAGGTGCC CCCCGTGCCC AGCCCCCAGG
5341 ACCTGCTGCT GGTGGAGCAC TAACAACTTT ATTATACATA GTTGATCAAT TCCAACTTTA
5401 TTATACATAG TTGATCAATT CCGATAATCA ACCTCTGGAT TACAAAATTT GTGAAAGATT
5461 GACTGGTATT CTTAACTATG TTGCTCCTTT TACGCTATGT GGATACGCTG CTTTAATGCC
5521 TTTGTATCAT GCTATTGCTT CCCGTATGGC TTTCATTTTC TCCTCCTTGT ATAAATCCTG
5581 GTTGCTGTCT CTTTATGAGG AGTTGTGGCC CGTTGTCAGG CAACGTGGCG TGGTGTGCAC
5641 TGTGTTTGCT GACGCAACCC CCACTGGTTG GGGCATTGCC ACCACCTGTC AGCTCCTTTC
5701 CGGGACTTTC GCTTTCCCCC TCCCTATTGC CACGGCGGAA CTCATCGCCG CCTGCCTTGC
5761 CCGCTGCTGG ACAGGGGCTC GGCTGTTGGG CACTGACAAT TCCGTGGTGT TGTCGGGGAA
5821 GCTGACGTCC TTTCCATGGC TGCTCGCCTG TGTTGCCACC TGGATTCTGC GCGGGACGTC
5881 CTTCTGCTAC GTCCCTTCGG CCCTCAATCC AGCGGACCTT CCTTCCCGCG GCCTGCTGCC
5941 GGCTCTGCGG CCTCTTCCGC GTCTTCGCCT TCGCCCTCAG ACGAGTCGGA TCTCCCTTTG
6001 GGCCGCCTCC CCGCATCGGG AATTCCCGCG GTTCGCTTTA AGACCAATGA CTTACAAGGC
6061 AGCTGTAGAT CTTAGCCACT TTTTAAAAGA AAAGGGGGGA CTGGAAGGGC TAATTCACTC

FIG. 5B-2

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6121 CCAACGAAGA CAAGATCTGC TTTTTGCTTG TACTGGGTCT CTCTGGTTAG ACCAGATCTG
6181 AGCCTGGGAG CTCTCTGGCT AACTAGGGAA CCCACTGCTT AAGCCTCAAT AAAGCTTGCC
6241 TTGAGTGCTT CAAGTAGTGT GTGCCCGTCT GTTGTGTGAC TCTGGTAACT AGAGATCCCT
6301 CAGACCCTTT TAGTCAGTGT GGAAAATCTC TAGCAGTAGT AGTTCATGTC ATCTTATTAT
6361 TCAGTATTTA TAACTTGCAA AGAAATGAAT ATCAGAGAGT GAGAGGAACT TGTTTATTGC
6421 AGCTTATAAT GGTTACAAAT AAAGCAATAG CATCACAAAT TTCACAAATA AAGCATTTTT
6481 TTCACTGCAT TCTAGTTGTG GTTTGTCCAA ACTCATCAAT GTATCTTATC ATGTCTGGCT
6541 CTAGCTATCC CGCCCCTAAC TCCGCCCATC CCGCCCCTAA CTCCGCCCAG TTCCGCCCAT
6601 TCTCCGCCCC ATGGCTGACT AATTTTTTTT ATTTATGCAG AGGCCGAGGC CGCCTCGGCC
6661 TCTGAGCTAT TCCAGAAGTA GTGAGGAGGC TTTTTTGGAG GCCTAGGGAC GTACCCAATT
6721 CGCCCTATAG TGAGTCGTAT TACGCGCGCT CACTGGCCGT CGTTTTACAA CGTCGTGACT
6781 GGGAAAACCC TGGCGTTACC CAACTTAATC GCCTTGCAGC ACATCCCCCT TTCGCCAGCT
6841 GGCGTAATAG CGAAGAGGCC CGCACCGATC GCCCTTCCCA ACAGTTGCGC AGCCTGAATG
6901 GCGAATGGGA CGCGCCCTGT AGCGGCGCAT TAAGCGCGGC GGGTGTGGTG GTTACGCGCA
6961 GCGTGACCGC TACACTTGCC AGCGCCCTAG CGCCCGCTCC TTTCGCTTTC TTCCCTTCCT
7021 TTCTCGCCAC GTTCGCCGGC TTTCCCCGTC AAGCTCTAAA TCGGGGGCTC CCTTTAGGGT
7081 TCCGATTTAG TGCTTTACGG CACCTCGACC CCAAAAAACT TGATTAGGGT GATGGTTCAC
7141 GTAGTGGGCC ATCGCCCTGA TAGACGGTTT TTCGCCCTTT GACGTTGGAG TCCACGTTCT
7201 TTAATAGTGG ACTCTTGTTC CAAACTGGAA CAACACTCAA CCCTATCTCG GTCTATTCTT
7261 TTGATTTATA AGGGATTTTG CCGATTTCGG CCTATTGGTT AAAAAATGAG CTGATTTAAC
7321 AAAAATTTAA CGCGAATTTT AACAAAATAT TAACGCTTAC AATTTAGGTG GCACTTTTCG
7381 GGGAAATGTG CGCGGAACCC CTATTTGTTT ATTTTTCTAA ATACATTCAA ATATGTATCC
7441 GCTCATGAGA CAATAACCCT GATAAATGCT TCAATAATAT TGAAAAAGGA AGAGTATGAG
7501 TATTCAACAT TTCCGTGTCG CCCTTATTCC CTTTTTTGCG GCATTTTGCC TTCCTGTTTT
7561 TGCTCACCCA GAAACGCTGG TGAAAGTAAA AGATGCTGAA GATCAGTTGG GTGCACGAGT
7621 GGGTTACATC GAACTGGATC TCAACAGCGG TAAGATCCTT GAGAGTTTTC GCCCCGAAGA
7681 ACGTTTTCCA ATGATGAGCA CTTTTAAAGT TCTGCTATGT GGCGCGGTAT TATCCCGTAT
7741 TGACGCCGGG CAAGAGCAAC TCGGTCGCCG CATACACTAT TCTCAGAATG ACTTGGTTGA
7801 GTACTCACCA GTCACAGAAA AGCATCTTAC GGATGGCATG ACAGTAAGAG AATTATGCAG
7861 TGCTGCCATA ACCATGAGTG ATAACACTGC GGCCAACTTA CTTCTGACAA CGATCGGAGG
7921 ACCGAAGGAG CTAACCGCTT TTTTGCACAA CATGGGGGAT CATGTAACTC GCCTTGATCG
7981 TTGGGAACCG GAGCTGAATG AAGCCATACC AAACGACGAG CGTGACACCA CGATGCCTGT
8041 AGCAATGGCA ACAACGTTGC GCAAACTATT AACTGGCGAA CTACTTACTC TAGCTTCCCG
8101 GCAACAATTA ATAGACTGGA TGGAGGCGGA TAAAGTTGCA GGACCACTTC TGCGCTCGGC
8161 CCTTCCGGCT GGCTGGTTTA TTGCTGATAA ATCTGGAGCC GGTGAGCGTG GGTCTCGCGG
8221 TATCATTGCA GCACTGGGGC CAGATGGTAA GCCCTCCCGT ATCGTAGTTA TCTACACGAC
8281 GGGGAGTCAG GCAACTATGG ATGAACGAAA TAGACAGATC GCTGAGATAG GTGCCTCACT
8341 GATTAAGCAT TGGTAACTGT CAGACCAAGT TTACTCATAT ATACTTTAGA TTGATTTAAA
8401 ACTTCATTTT TAATTTAAAA GGATCTAGGT GAAGATCCTT TTTGATAATC TCATGACCAA
8461 AATCCCTTAA CGTGAGTTTT CGTTCCACTG AGCGTCAGAC CCCGTAGAAA AGATCAAAGG
8521 ATCTTCTTGA GATCCTTTTT TTCTGCGCGT AATCTGCTGC TTGCAAACAA AAAAACCACC
8581 GCTACCAGCG GTGGTTTGTT TGCCGGATCA AGAGCTACCA ACTCTTTTTC CGAAGGTAAC
8641 TGGCTTCAGC AGAGCGCAGA TACCAAATAC TGTTCTTCTA GTGTAGCCGT AGTTAGGCCA
8701 CCACTTCAAG AACTCTGTAG CACCGCCTAC ATACCTCGCT CTGCTAATCC TGTTACCAGT
8761 GGCTGCTGCC AGTGGCGATA AGTCGTGTCT TACCGGGTTG GACTCAAGAC GATAGTTACC
8821 GGATAAGGCG CAGCGGTCGG GCTGAACGGG GGGTTCGTGC ACACAGCCCA GCTTGGAGCG
8881 AACGACCTAC ACCGAACTGA GATACCTACA GCGTGAGCTA TGAGAAAGCG CCACGCTTCC
8941 CGAAGAGAGA AAGGCGGACA GGTATCCGGT AAGCGGCAGG GTCGGAACAG GAGAGCGCAC
9001 GAGGGAGCTT CCAGGGGGAA ACGCCTGGTA TCTTTATAGT CCTGTCGGGT TTCGCCACCT
9061 CTGACTTGAG CGTCGATTTT TGTGATGCTC GTCAGGGGGG CGGAGCCTAT GGAAAAACGC
9121 CAGCAACGCG GCCTTTTTAC GGTTCCTGGC CTTTTGCTGG CCTTTTGCTC ACATGTTCTT
9181 TCCTGCGTTA TCCCCTGATT CTGTGGATAA CCGTATTACC GCCTTTGAGT GAGCTGATAC
9241 CGCTCGCCGC AGCCGAACGA CCGAGCGCAG CGAGTCAGTG AGCGAGGAAG CGGAAGAGCG
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FIG. 5B-3

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9301  CCCAATACGC AAACCGCCTC TCCCCGCGCG TTGGCCGATT CATTAATGCA GCTGGCACGA
9361  CAGGTTTCCC GACTGGAAAG CGGGCAGTGA GCGCAACGCA ATTAATGTGA GTTAGCTCAC
9421  TCATTAGGCA CCCCAGGCTT TACACTTTAT GCTTCCGGCT CGTATGTTGT GTGGAATTGT
9481  GAGCGGATAA CAATTTCACA CAGGAAACAG CTATGACCAT GATTACGCCA AGCGCGCAAT
9541  TAACCCTCAC TAAAGGGAAC AAAAGCTGGA GCTGCAAGCT T
```

FIG. 6A

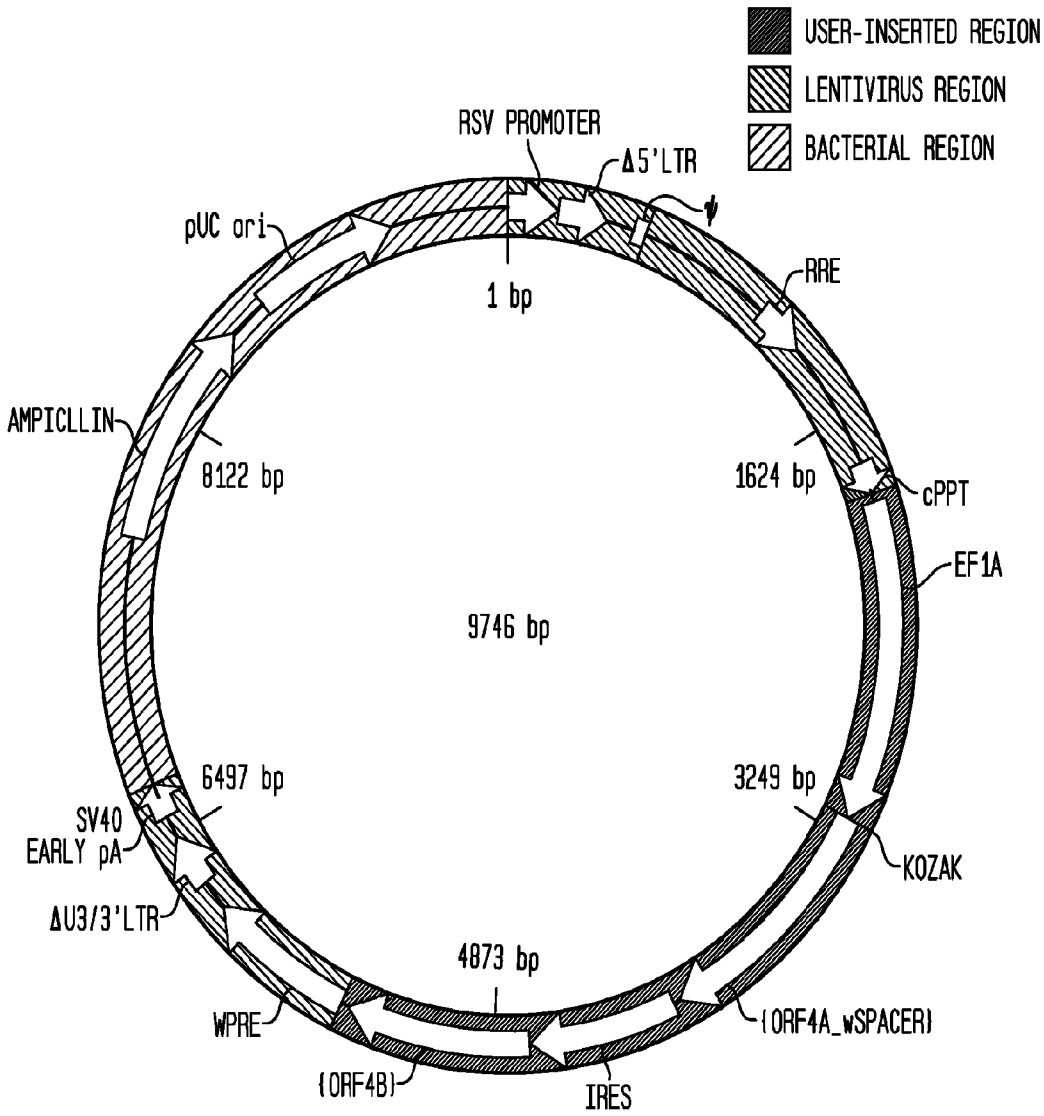
USER-INSERTED REGION
LENTIVIRUS REGION
BACTERIAL REGION
RSV PROMOTER
Δ5'LTR
ψ
RRE
cPPT
EF1A
KOZAK
(ORF4A_wSPACER)
IRES
(ORF4B)
WPRE
ΔU3/3'LTR
SV40
EARLY pA
AMPICLLIN
pUC ori
1 bp
1624 bp
3249 bp
4873 bp
6497 bp
8122 bp
9746 bp

FIG. 6B

SEQ ID NO. 50

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   1  AATGTAGTCT TATGCAATAC TCTTGTAGTC TTGCAACATG GTAACGATGA GTTAGCAACA
  61  TGCCTTACAA GGAGAGAAAA AGCACCGTGC ATGCCGATTG GTGGAAGTAA GGTGGTACGA
 121  TCGTGCCTTA TTAGGAAGGC AACAGACGGG TCTGACATGG ATTGGACGAA CCACTGAATT
 181  GCCGCATTGC AGAGATATTG TATTTAAGTG CCTAGCTCGA TACATAAACG GGTCTCTCTG
 241  GTTAGACCAG ATCTGAGCCT GGGAGCTCTC TGGCTAACTA GGGAACCCAC TGCTTAAGCC
 301  TCAATAAAGC TTGCCTTGAG TGCTTCAAGT AGTGTGTGCC CGTCTGTTGT GTGACTCTGG
 361  TAACTAGAGA TCCCTCAGAC CTTTTAGTC AGTGTGGAAA ATCTCTAGCA GTGGCGCCCG
 421  AACAGGGACT TGAAAGCGAA AGGGAAACCA GAGGAGCTCT CTCGACGCAG GACTCGGCTT
 481  GCTGAAGCGC GCACGGCAAG AGGCGAGGGG CGGCGACTGG TGAGTACGCC AAAAATTTTG
 541  ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG TCAGTATTAA GCGGGGGAGA
 601  ATTAGATCGC GATGGGAAAA AATTCGGTTA AGGCCAGGGG GAAAGAAAAA ATATAAATTA
 661  AAACATATAG TATGGGCAAG CAGGGAGCTA GAACGATTCG CAGTTAATCC TGGCCTGTTA
 721  GAAACATCAG AAGGCTGTAG ACAAATACTG GGACAGCTAC AACCATCCCT TCAGACAGGA
 781  TCAGAAGAAC TTAGATCATT ATATAATACA GTAGCAACCC TCTATTGTGT GCATCAAAGG
 841  ATAGAGATAA AAGACACCAA GGAAGCTTTA GACAAGATAG AGGAAGAGCA AAACAAAAGT
 901  AAGACCACCG CACAGCAAGC GGCCGCTGAT CTTCAGACCT GGAGGAGGAG ATATGAGGGA
 961  CAATTGGAGA AGTGAATTAT ATAAATATAA AGTAGTAAAA AGAGCAGTGG GAATAGGAGC
1021  ACCCACCAAG GCAAAGAGAA GAGTGGTGCA GAGAGAAAAA AGAGCAGTGG GAATAGGAGC
1081  TTTGTTCCTT GGGTTCTTGG GAGCAGCAGG AAGCACTATG GGCGCAGCGT CAATGACGCT
1141  GACGGTACAG GCCAGACAAT TATTGTCTGG TATAGTGCAG CAGCAGAACA ATTTGCTGAG
1201  GGCTATTGAG GCGCAACAGC ATCTGTTGCA ACTCACAGTC TGGGGCATCA AGCAGCTCCA
1261  GGCAAGAATC CTGGCTGTGG AAAGATACCT AAAGGATCAA CAGCTCCTGG GGATTTGGGG
1321  TTGCTCTGGA AAACTCATTT GCACCACTGC TGTGCCTTGG AATGCTAGTT GGAGTAATAA
1381  ATCTCTGGAA CAGATTTGGA ATCACACGAC CTGGATGGAG TGGGACAGAG AAATTAACAA
1441  TTACACAAGC TTAATACACT CCTTAATTGA AGAATCGCAA AACCAGCAAG AAAAGAATGA
1501  ACAAGAATTA TTGGAATTAG ATAAATGGGC AAGTTTGTGG AATTGGTTTA ACATAACAAA
1561  TTGGCTGTGG TATATAAAAT TATTCATAAT GATAGTAGGA GGCTTGGTAG GTTTAAGAAT
1621  AGTTTTTGCT GTACTTTCTA TAGTGAATAG AGTTAGGCAG GGATATTCAC CATTATCGTT
1681  TCAGACCCAC CTCCCAACCC CGAGGGGACC CGACAGGCCC GAAGGAATAG AAGAAGAAGG
1741  TGGAGAGAGA GACAGAGACA GATCCATTCG ATTAGTGAAC GGATCTCGAC GGTATCGCTA
1801  GCTTTTAAAA GAAAAGGGGG GATTGGGGGG TACAGTGCAG GGGAAAGAAT AGTAGACATA
1861  ATAGCAACAG ACATACAAAC TAAAGAATTA CAAAAACAAA TTACAAAAAT TCAAAATTTT
1921  ACTAGTATCA ACTTTGTATA GAAAAGTTGG GCTCCGGTGC CCGTCAGTGG GCAGAGCGCA
1981  CATCGCCCAC AGTCCCCGAG AAGTTGGGGG GAGGGGTCGG CAATTGAACC GGTGCCTAGA
2041  GAAGGTGGCG CGGGGTAAAC TGGGAAAGTG ATGTCGTGTA CTGGCTCCGC CTTTTTCCCG
2101  AGGGTGGGGG AGAACCGTAT ATAAGTGCAG TAGTCGCCGT GAACGTTCTT TTTCGCAACG
2161  GGTTTGCCGC CAGAACACAG GTAAGTGCCG TGTGTGGTTC CCGCGGGCCT GGCCTCTTTA
2221  CGGGTTATGG CCCTTGCGTG CCTTGAATTA CTTCCACCTG GCTGCAGTAC GTGATTCTTG
2281  ATCCCGAGCT TCGGGTTGGA AGTGGGTGGG AGAGTTCGAG GCCTTGCGCT TAAGGAGCCC
2341  CTTCGCCTCG TGCTTGAGTT GAGGCCTGGC CTGGGCGCTG GGGCCGCCGC GTGCGAATCT
2401  GGTGGCACCT TCGCGCCTGT CTCGCTGCTT TCGATAAGTC TCTAGCCATT TAAAATTTTT
2461  GATGACCTGC TGCGACGCTT TTTTCTGGCC AAGATAGTCT TGTAAATGCG GGCCAAGATC
2521  TGCACACTGG TATTTCGGTT TTTGGGGCCG CGGGCGGCGA CGGGGCCCGT GCGTCCCAGC
2581  GCACATGTTC GGCGAGGCGG GGCCTGCGAG CGCGGCCACC GAGAATCGGA CGGGGGTAGT
2641  CTCAAGCTGG CCGGCCTGCT CTGGTGCCTG GTCTCGCGCC GCCGTGTATC GCCCCGCCCT
2701  GGGCGGCAAG GCTGGCCCGG TCGGCACCAG TTGCGTGAGC GGAAAGATGG CCGCTTCCCG
2761  GCCCTGCTGC AGGGAGCTCA AAATGGAGGA CGCGGCGCTC GGGAGAGCGG GCGGGTGAGT
2821  CACCCACACA AAGGAAAAGG GCCTTTCCGT CCTCAGCCGT CGCTTCATGT GACTCCACGG
2881  AGTACCGGGC GCCGTCCAGG CACCTCGATT AGTTCTCGAG CTTTTGGAGT ACGTCGTCTT
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FIG. 6B-1

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2941 TAGGTTGGGG GGAGGGGTTT TATGCGATGG AGTTTCCCCA CACTGAGTGG GTGGAGACTG
3001 AAGTTAGGCC AGCTTGGCAC TTGATGTAAT TCTCCTTGGA ATTTGCCCTT TTTGAGTTTG
3061 GATCTTGGTT CATTCTCAAG CCTCAGACAG TGGTTCAAAG TTTTTTTCTT CCATTTCAGG
2121 TGTCGTGACA AGTTTGTACA AAAAAGCAGG CTGCCACCAT GACCGTGCTG GCCCCCGCCT
3181 GGAGCCCCAC CACCTACCTG CTGCTGCTGC TGCTGCTGAG CAGCGGCCTG AGCGGCACCC
3241 AGGACTGCAG CTTCCAGCAC AGCCCCATCA GCAGCGACTT CGCCGTGAAG ATCAGAGAGC
3301 TGAGCGACTA CCTGCTGCAG GACTACCCCG TGACCGTGGC CAGCAACCTG CAGGACGAGG
3361 AGCTGTGCGG CGGCCTGTGG AGACTGGTGC TGGCCCAGAG ATGGATGGAG AGACTGAAGA
3421 CCGTGGCCGG CAGCAAGATG CAGGGCCTGC TGGAGAGAGT GAACACCGAG ATCCACTTCG
3481 TGACCAAGTG CGCCTTCCAG CCCCCCCCCA GCTGCCTGAG ATTCGTGCAG ACCAACATCA
3541 GCAGACTGCT GCAGGAGACC AGCGAGCAGC TGGTGGCCCT GAAGCCCTGG ATCACCAGAC
3601 AGAACTTCAG CAGATGCCTG GAGCTGCAGT GCCAGCCCGA CAGCAGCACC CTGCCCCCCC
3661 CCTGGAGCCC CAGACCCCTG GAGCTGCAGT CCCCCACCGC CCCCCAGTAA AACAACAACA
3721 ATTGCATTCA TTTTATGTTT CAGGTTCAGG GGGAGGTGTG GGAGGTTTTT TAAAGCAAGT
3781 AAAACCTCTA CAAATGTGGT ACGCGTTAAC AACAACAATT GCATTCATTT TAAAGCAAGT
3841 GTTCAGGGGG AGGTGTGGGA GGTTTTTTAA AGCAAGTAAA ACCTCTACAA ATGTGGTACG
3901 CGTTACCCAG CTTTCTTGTA CAAAGTGGTA AATAGATAGA ACAACAACAA TTGCATTCAT
3961 TTTTGATTTC AGGTTCAGGG GGAGGTGTGG GAGGTTTTTT AAAGCAAGTA AAACCTCTAC
4021 ACTGACGGTA CGCGTTAACA ACAACAATTG CATTCATTTG TAGTTTCAGG TTCAGGGGGA
4081 GGTGTGGGAG GTTTTTTAAA GCAAGTTAAA CCTCTAAAAT AGTGGTACGC GTTACCCAGC
4141 TTTCTTGTAC AAAGTGGACC CAGCTTTCTT GTACAAAGTG GGCCCCTCTC CCTCCCCCCC
4201 CCCTAACGTT ACTGGCCGAA GCCGCTTGGA ATAAGGCCGG TGTGCGTTTG TCTATATGTT
4261 ATTTTCCACC ATATTGCCGT CTTTTGGCAA TGTGAGGGCC CGGAAACCTG GCCCTGTCTT
4321 CTTGACGAGC ATTCCTAGGG GTCTTTCCCC TCTCGCCAAA GGAATGCAAG GTCTGTTGAA
4381 TGTCGTGAAG GAAGCAGTTC CTCTGGAAGC TTCTTGAAGA CAAACAACGT CTGTAGCGAC
4441 CCTTTGCAGG CAGCGGAACC CCCCACCTGG CGACAGGTGC CTCTGCGGCC CCCGCCAGAA
4501 TGTATAAGAT ACACCTGCAA AGGCGGCACA TGGCCACCGT GCTGGCCCCC GCCTGGAGCC
4561 TGTGGAAAGA GTCAAATGGC TCTCCTCAAG CGTATTCAAC AAGGGGCTGA AGGATGCCCA
4621 GAAGGTACCC CATTGTATGG GATCTGATCT GGGGCCTCGG TGCACATGCT TTACATGTGT
4681 TTAGTCGAGG TTAAAAAAAC GTCTAGGCCC CCCGAACCAC GGGGACGTGG TTTTCCTTTG
4741 AAAAACACGA TGATAATATG GCCACAACCA TGGCCACCGT GCTGGCCCCC GCCTGGAGCC
4801 CCACCACCTA CCTGCTGCTG CTGCTGCTGC TGAGCAGCGG CCTGAGCGCC CCCGCCAGAA
4861 GCCCCAGCCC CAGCACCCAG CCCTGGGAGC ACGTGAACGC CATCCAGGAG GCCAGAAGAC
4921 TGCTGAACCT GAGCAGAGAC ACCGCCGCCG AGATGAACGA GACCGTGGAG GCCAGAAGAC
4981 AGATGTTCGA CCTGCAGGAG CCCACCTGCC TGCAGACCAG ACTGGAGCTG TACAAGCAGG
5041 GCCTGAGAGG CAGCCTGACC AAGCTGAAGG GCCCCCTGAC CATGATGGCC AGCCACTACA
5101 AGCAGCACTG CCCCCCCACC CCCGAGACCA GCTGCGCCAC CCAGATCATC ACCTTCGAGA
5161 GCTTCAAGGA GAACCTGAAG GACTTCCTGC TGGTGATCCC CTTCGACTGC TGGGAGCCCG
5221 TGCAGGAGCC CACCACCACC CCCGCCCCCA GACCCCCCAC CCCCGCCCCC ACCATCGCCA
5281 GCCAGCCCCT GAGCCTGAGA CCCGAGGCCT GCAGACCCGC CGCCGGCGGC GCCGTGCACA
5341 CCAGAGGCCT GGACTTCGCC TGCGACATCT ACATCTGGGC CCCCCTGGCC GGCACCTGCG
5401 GCGTGCTGCT GCTGAGCCTG GTGATCACCC TGTACTGCAA CCACAGAAAC AGAAGAAGAG
5461 TGTGCAAGTG CCCCAGACCC GTGGTGAAGA GCGGCGACAA GCCCAGCCTG AGCGCCAGAT
5521 ACGTGTAACA ACTTTATTAT ACATAGTTGA TCAATTCCAA CTTTATTATA CATAGTTGAT
5581 CAATTCCGAT AATCAACCTC TGGATTACAA AATTTGTGAA AGATTGACTG GTATTCTTAA
5641 CTATGTTGCT CCTTTTACGC TATGTGGATA CGCTGCTTTA ATGCCTTTGT ATCATGCTAT
5701 TGCTTCCCGT ATGGCTTTCA TTTTCTCCTC CTTGTATAAA TCCTGGTTGC TGTCTCTTTA
5761 TGAGGAGTTG TGGCCCGTTG TCAGGCAACG TGGCGTGGTG TGCACTGTGT TTGCTGACGC
5821 AACCCCCACT GGTTGGGGCA TTGCCACCAC CTGTCAGCTC CTTTCCGGGA CTTTCGCTTT
5881 CCCCCTCCCT ATTGCCACGG CGGAACTCAT CGCCGCCTGC CTTGCCCGCT GCTGGACAGG
5941 GGCTCGGCTG TTGGGCACTG ACAATTCCGT GGTGTTGTCG CTTGCCCGCT GCTGGACAGG
6001 ATGGCTGCTC GCCTGTGTTG CCACCTGGAT TCTGCGCGGG ACGTCCTTCT GCTACGTCCC
6061 TTCGGCCCTC AATCCAGCGG ACCTTCCTTC CCGCGGCCTG CTGCCGGCTC TGCGGCCTCT
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FIG. 6B-2

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6121 TCCGCGTCTT CGCCTTCGCC CTCAGACGAG TCGGATCTCC CTTTGGGCCG CCTCCCCGCA
6181 TCGGGAATTC CCGCGGTTCG CTTTAAGACC AATGACTTAC AAGGCAGCTG TAGATCTTAG
6241 CCACTTTTTA AAAGAAAAGG GGGGACTGGA AGGGCTAATT CACTCCCAAC GAAGACAAGA
6301 TCTGCTTTTT GCTTGTACTG GGTCTCTCTG GTTAGACCAG ATCTGAGCCT GGGAGCTCTC
6361 TGGCTAACTA GGGAACCCAC TGCTTAAGCC TCAATAAAGC TTGCCTTGAG TGCTTCAAGT
6421 AGTGTGTGCC CGTCTGTTGT GTGACTCTGG TAACTAGAGA TCCCTCAGAC CCTTTTAGTC
6481 AGTGTGGAAA ATCTCTAGCA GTAGTAGTTC ATGTCATCTT ATTATTCAGT ATTTATAACT
6541 TGCAAAGAAA TGAATATCAG AGAGTGAGAG GAACTTGTTT ATTGCAGCTT ATAATGGTTA
6601 CAAATAAAGC AATAGCATCA CAAATTTCAC AAATAAAGCA TTTTTTTCAC TGCATTCTAG
6661 TTGTGGTTTG TCCAAACTCA TCAATGTATC TTATCATGTC TGGCTCTAGC TATCCCGCCC
6721 CTAACTCCGC CCATCCCGCC CCTAACTCCG CCCAGTTCCG CCCATTCTCC GCCCCATGGC
6781 TGACTAATTT TTTTTATTTA TGCAGAGGCC GAGGCCGCCT CGGCCTCTGA GCTATTCCAG
6841 AAGTAGTGAG GAGGCTTTTT TGGAGGCCTA GGGACGTACC CAATTCGCCC TATAGTGAGT
6901 CGTATTACGC GCGCTCACTG GCCGTCGTTT TACAACGTCG TGACTGGGAA AACCCTGGCG
6961 TTACCCAACT TAATCGCCTT GCAGCACATC CCCCTTTCGC CAGCTGGCGT AATAGCGAAC
7021 AGGCCCGCAC CGATCGCCCT TCCCAACAGT TGCGCAGCCT GAATGGCGAA TGGGACGCGC
7081 CCTGTAGCGG CGCATTAAGC GCGGCGGGTG TGGTGGTTAC GCGCAGCGTG ACCGCTACAC
7141 TTGCCAGCGC CCTAGCGCCC GCTCCTTTCG CTTTCTTCCC TTCCTTTCTC GCCACGTTCG
7201 CCGGCTTTCC CCGTCAAGCT CTAAATCGGG GGCTCCCTTT AGGGTTCCGA TTTAGTGCTT
7261 TACGGCACCT CGACCCCAAA AAACTTGATT AGGGTGATGG TTCACGTAGT GGGCCATCGC
7321 CCTGATAGAC GGTTTTTCGC CCTTTGACGT TGGAGTCCAC GTTCTTTAAT AGTGGACTCT
7381 TGTTCCAAAC TGGAACAACA CTCAACCCTA TCTCGGTCTA TTCTTTTGAT TTATAAGGGA
7441 TTTTGCCGAT TTCGGCCTAT TGGTTAAAAA ATGAGCTGAT TTAACAAAAA TTTAACGCGA
7501 ATTTTAACAA AATATTAACG CTTACAATTT AGGTGGCACT TTTCGGGGAA ATGTGCGCGG
7561 AACCCCTATT TGTTTATTTT TCTAAATACA TTCAAATATG TATCCGCTCA TGAGACAATA
7621 ACCCTGATAA ATGCTTCAAT AATATTGAAA AAGGAAGAGT ATGAGTATTC AACATTTCCG
7681 TGTCGCCCTT ATTCCCTTTT TTGCGGCATT TTGCCTTCCT GTTTTTGCTC ACCCAGAAAC
7741 GCTGGTGAAA GTAAAAGATG CTGAAGATCA GTTGGGTGCA CGAGTGGGTT ACATCGAACT
7801 GGATCTCAAC AGCGGTAAGA TCCTTGAGAG TTTTCGCCCC GAAGAACGTT TTCCAATGAT
7861 GAGCACTTTT AAAGTTCTGC TATGTGGCGC GGTATTATCC CGTATTGACG CCGGGCAAGA
7921 GCAACTCGGT CGCCGCATAC ACTATTCTCA GAATGACTTG GTTGAGTACT CACCAGTCAC
7981 AGAAAAGCAT CTTACGGATG GCATGACAGT AAGAGAATTA TGCAGTGCTG CCATAACCAT
8041 GAGTGATAAC ACTGCGGCCA ACTTACTTCT GACAACGATC GGAGGACCGA AGGAGCTAAC
8101 CGCTTTTTTG CACAACATGG GGGATCATGT AACTCGCCTT GATCGTTGGG AACCGGAGCT
8161 GAATGAAGCC ATACCAAACG ACGAGCGTGA CACCACGATG CCTGTAGCAA TGGCAACAAC
8221 GTTGCGCAAA CTATTAACTG GAGCCGGTGA GCGTGGGTCT CGCGGTATCA TTGCAGCACT
8281 CTGGATGGAG GCGGATAAAG TTGCAGGACC ACTTCTGCGC TCGGCCCTTC CGGCTGGCTG
8341 GTTTATTGCT GATAAATCTG GAGCCGGTGA GCGTGGGTCT CGCGGTATCA TTGCAGCACT
8401 GGGGCCAGAT GGTAAGCCCT CCCGTATCGT AGTTATCTAC ACGACGGGGA GTCAGGCAAC
8461 TATGGATGAA CGAAATAGAC AGATCGCTGA GATAGGTGCC TCACTGATTA AGCATTGGTA
8521 ACTGTCAGAC CAAGTTTACT CATATATACT TTAGATTGAT TTAAAACTTC ATTTTTAATT
8581 TAAAAGGATC TAGGTGAAGA TCCTTTTTGA TAATCTCATG ACCAAAATCC CTTAACGTGA
8641 GTTTTCGTTC CACTGAGCGT CAGACCCCGT AGAAAAGATC AAAGGATCTT CTTGAGATCC
8701 TTTTTTTCTG CGCGTAATCT GCTGCTTGCA AACAAAAAAA CCACCGCTAC CAGCGGTGGT
8761 TTGTTTGCCG GATCAAGAGC TACCAACTCT TTTTCCGAAG GTAACTGGCT TCAGCAGAGC
8821 GCAGATACCA AATACTGTTC TTCTAGTGTA GCCGTAGTTA GGCCACCACT TCAAGAACTC
8881 TGTAGCACCG CCTACATACC TCGCTCTGCT AATCCTGTTA CCAGTGGCTG CTGCCAGTGG
8941 CGATAAGTCG TGTCTTACCG GGTTGGACTC AAGACGATAG TTACCGGATA AGGCGCAGCG
9001 GTCGGGCTGA ACGGGGGGTT CGTGCACACA GCCCAGCTTG GAGCGAACGA CCTACACCGA
9061 ACTGAGATAC CTACAGCGTG AGCTATGAGA AAGCGCCACG CTTCCCGAAG AGAGAAAGGC
9121 GGACAGGTAT CCGGTAAGCG GCAGGGTCGG AACAGGAGAG CGCACGAGGG AGCTTCCAGG
9181 GGGAAACGCC TGGTATCTTT ATAGTCCTGT CGGGTTTCGC CACCTCTGAC TTGAGCGTCG
9241 ATTTTTGTGA TGCTCGTCAG GGGGGCGGAG CCTATGGAAA AACGCCAGCA ACGCGGCCTT
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FIG. 6B-3

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9301  TTTACGGTTC CTGGCCTTTT GCTGGCCTTT TGCTCACATG TTCTTTCCTG CGTTATCCCC
9361  TGATTCTGTG GATAACCGTA TTACCGCCTT TGAGTGAGCT GATACCGCTC GCCGCAGCCG
9421  AACGACCGAG CGCAGCGAGT CAGTGAGCGA GGAAGCGGAA GAGCGCCCAA TACGCAAACC
9481  GCCTCTCCCC GCGCGTTGGC CGATTCATTA ATGCAGCTGG CACGACAGGT TTCCCGACTG
9541  GAAAGCGGGC AGTGAGCGCA ACGCAATTAA TGTGAGTTAG CTCACTCATT AGGCACCCCA
9601  GGCTTTACAC TTTATGCTTC CGGCTCGTAT GTTGTGTGGA ATTGTGAGCG GATAAGAATT
9661  TCACACAGGA AACAGCTATG ACCATGATTA CGCCAAGCGC GCAATTAACC CTCACTAAAG
9721  GGAACAAAAG CTGGAGCTGC AAGCTT
```

FIG. 7A

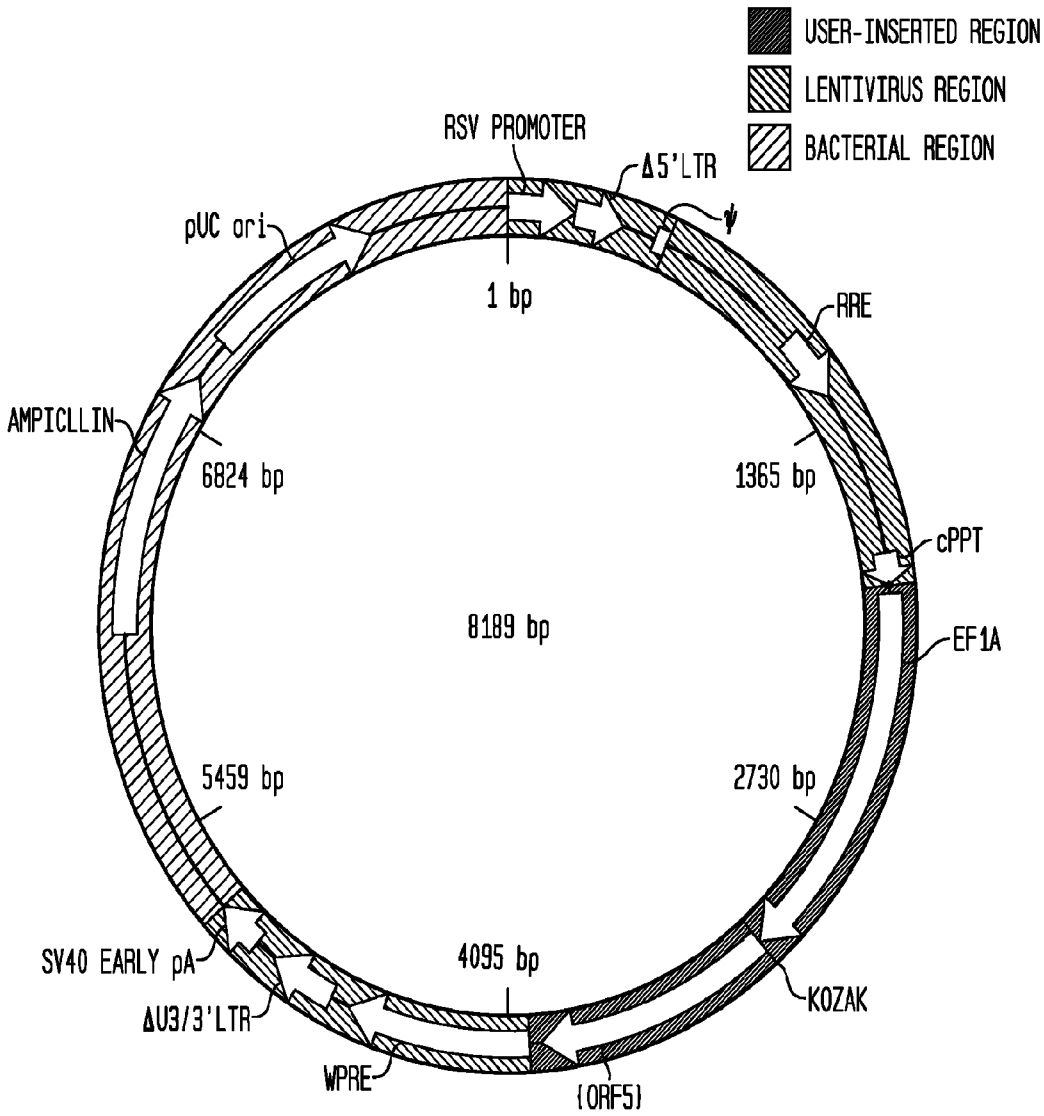
USER-INSERTED REGION
LENTIVIRUS REGION
BACTERIAL REGION
RSV PROMOTER
Δ5'LTR
ψ
pUC ori
1 bp
RRE
AMPICLLIN
6824 bp
1365 bp
cPPT
8189 bp
EF1A
5459 bp
2730 bp
SV40 EARLY pA
4095 bp
KOZAK
ΔU3/3'LTR
WPRE
(ORF5)

FIG. 7B
SEQ ID NO. 51

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   1 AATGTAGTCT TATGCAATAC TCTTGTAGTC TTGCAACATG GTAACGATGA GTTAGCAACA
  61 TGCCTTACAA GGAGAGAAAA AGCACCGTGC ATGCCGATTG GTGGAAGTAA GGTGGTACGA
 121 TCGTGCCTTA TTAGGAAGGC AACAGACGGG TCTGACATGG ATTGGACGAA CCACTGAATT
 181 GCCGCATTGC AGAGATATTG TATTTAAGTG CCTAGCTCGA TACATAAACG GGTCTCTCTG
 241 GTTAGACCAG ATCTGAGCCT GGGAGCTCTC TGGCTAACTA GGGAACCCAC TGCTTAAGCC
 301 TCAATAAAGC TTGCCTTGAG TGCTTCAAGT AGTGTGTGCC CGTCTGTTGT GTGACTCTGG
 361 TAACTAGAGA TCCCTCAGAC CCTTTTAGTC AGTGTGGAAA ATCTCTAGCA GTGGCGCCCG
 421 AACAGGGACT TGAAAGCGAA AGGGAAACCA GAGGAGCTCT CTCGACGCAG GACTCGGCTT
 481 GCTGAAGCGC GCACGGCAAG AGGCGAGGGG CGGCGACTGG TGAGTACGCC AAAAATTTTG
 541 ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG TCAGTATTAA GCGGGGGAGA
 601 ATTAGATCGC GATGGGAAAA ATTCGGTTA AGGCCAGGGG GAAAGAAAAA ATATAAATTA
 661 AAACATATAG TATGGGCAAG CAGGGAGCTA GAACGATTCG CAGTTAATCC TGGCCTGTTA
 721 GAAACATCAG AAGGCTGTAG ACAAATACTG GGACAGCTAC AACCATCCCT TCAGACAGGA
 781 TCAGAAGAAC TTAGATCATT ATATAATACA GTAGCAACCC TCTATTGTGT GCATCAAAGG
 841 ATAGAGATAA AAGACACCAA GGAAGCTTTA GACAAGATAG AGGAAGAGCA AAACAAAAGT
 901 AAGACCACCG CACAGCAAGC GGCCGCTGAT CTTCAGACCT GGAGGAGGAG ATATGAGGGA
 961 CAATTGGAGA AGTGAATTAT ATAAATATAA AGTAGTAAAA ATTGAACCAT TAGGAGTAGC
1021 ACCCACCAAG GCAAAGAGAA GAGTGGTGCA GAGAGAAAAA AGAGCAGTGG GAATAGGAGC
1081 TTTGTTCCTT GGGTTCTTGG GAGCAGCAGG AAGCACTATG GGCGCAGCGT CAATGACGCT
1141 GACGGTACAG GCCAGACAAT TATTGTCTGG TATAGTGCAG CAGCAGAACA ATTTGCTGAG
1201 GGCTATTGAG GCGCAACAGC ATCTGTTGCA ACTCACAGTC TGGGGCATCA AGCAGCTCCA
1261 CGCAAGAATC CTGGCTGTGG AAAGATACCT AAAGGATCAA CAGCTCCTGG GGATTTGGGG
1321 TTGCTCTGGA AAACTCATTT GCACCACTGC TGTGCCTTGG AATGCTAGTT GGAGTAATAA
1381 ATCTCTGGAA CAGATTTGGA ATCACACGAC CTGGATGGAG TGGGACAGAG AAATTAACAA
1441 TTACACAAGC TTAATACACT CCTTAATTGA AGAATCGCAA AACCAGCAAG AAAAGAATGA
1501 ACAAGAATTA TTGGAATTAG ATAAATGGGC AAGTTTGTGG AATTGGTTTA ACATAACAAA
1561 TTGGCTGTGG TATATAAAAT TATTCATAAT GATAGTAGGA GGCTTGGTAG GTTTAAGAAT
1621 AGTTTTTGCT GTACTTTCTA TAGTGAATAG AGTTAGGCAG GGATATTCAC CATTATCGTT
1681 TCAGACCCAC CTCCCAACCC CGAGGGGACC CGACAGGCCC GAAGGAATAG AAGAAGAAGG
1741 TGGAGAGAGA GACAGAGACA GATCCATTCG ATTAGTGAAC GGATCTCGAC GGTATCGCTA
1801 GCTTTTAAAA GAAAAGGGGG GATTGGGGGG TACAGTGCAG GGGAAAGAAT AGTAGACATA
1861 ATAGCAACAG ACATACAAAC TAAAGAATTA CAAAAACAAA TTACAAAAAT TCAAAATTTT
1921 ACTAGTGATT ATCGGATCAA CTTTGTATAG AAAAGTTGGG CTCCGGTGCC CGTCAGTGGG
1981 CAGAGCGCAC ATCGCCCACA GTCCCCGAGA AGTTGGGGGG AGGGGTCGGC AATTGAACCG
2041 GTGCCTAGAG AAGGTGGCGC GGGGTAAACT GGGAAAGTGA GTGCGTGTAC TGGCTCCGCC
2101 TTTTTCCCGA GGGTGGGGGA GAACCGTATA TAAGTGCAGT AGTCGCCGTG AACGTTCTTT
2161 TTCGCAACGG GTTTGCCGCC AGAACACAGG TAAGTGCCGT GTGTGGTTCC CGCGGGCCTG
2221 GCCTCTTTAC GGGTTATGGC CCTTGCGTGC CTTGAATTAC TTCCACCTGG CTGCAGTACG
2281 TGATTCTTGA TCCCGAGCTT CGGGTTGGAA GTGGGTGGGA GAGTTCGAGG CCTTGCGCTT
2341 AAGGAGCCCC TTCGCCTCGT GCTTGAGTTG AGGCCTGGCC TGGGCGCTGG GGCCGCCGCG
2401 TGCGAATCTG GTGGCACCTT CGCGCCTGTC TCGCTGCTTT CGATAAGTCT CTAGCCATTT
2461 AAAATTTTTG ATGACCTGCT GCGACGCTTT TTTTCTGGCA AGATAGTCTT GTAAATGCGG
2521 GCCAAGATCT GCACACTGGT ATTTCGGTTT TTGGGGCCGC GGGCGGCGAC GGGGCCCGTG
2581 CGTCCCAGCG CACATGTTCG GCGAGGCGGG GCCTGCGAGC GCGGCCACCG AGAATCGGAC
2641 GGGGGTAGTC TCAAGCTGGC CGGCCTGCTC TGGTGCCTGG TCTCGCGCCG CCGTGTATCG
2701 CCCCGCCCTG GGCGGCAAGG CTGGCCCGGT CGGCACCAGT TGCGTGAGCG GAAAGATGGC
2761 CGCTTCCCGG CCCTGCTGCA GGGAGCTCAA AATGGAGGAC GCGGCGCTCG GGAGAGCGGG
2821 CGGGTGAGTC ACCCACACAA AGGAAAAGGG CCTTTCCGTC CTCAGCCGTC GCTTCATGTG
2881 ACTCCACGGA GTACCGGGCG CCGTCCAGGC ACCTCGATTA GTTCTCGAGC TTTTGGAGTA
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FIG. 7B-1

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2941 CGTCGTCTTT AGGTTGGGGG GAGGGGTTTT ATGCGATGGA GTTTCCCCAC ACTGAGTGGG
3001 TGGAGACTGA AGTTAGGCCA GCTTGGCACT TGATGTAATT CTCCTTGGAA TTTGCCCTTT
3061 TTGAGTTTGG ATCTTGGTTC ATTCTCAAGC CTCAGACAGT GGTTCAAAGT TTTTTTCTTC
3121 CATTTCAGGT GTCGTGACAA GTTTGTACAA AAAAGCAGGC TGCCACCATG AAGATCTTCA
3181 ACAACCAGCA CAGCCCCAGA AGCGCCGCCA CCGGCCTGCC CATCAGCATG AAGATCTTCA
3241 TGTACCTGCT GACCGTGTTC CTGATCACCC AGATGATCGG CAGCGCCCTG TTCGCCGTGT
3301 ACCTGCACAG AAGACTGGAC AAGATCGAGG ACGAGAGAAA CCTGCACGAG GACTTCGTGT
3361 TCATGAAGAC CATCCAGAGA TGCAACACCG GCCAGAGAAG CCTGAGCCTG CTGAACTGCG
3421 AGGAGATCAA GAGCCAGTTC GAGGGCTTCG TGAAGGACAT CATGCTGAAC AAGGAGGAGA
3481 CCAAGAAGGA GAACAGCTTC GAGATGCCCA GAGGCGAGGA GGACAGCCAG ATCGCCGCCC
3541 ACGTGATCAG CGAGGCCAGC AGCAAGACCA CCAGCGTGCT GCAGTGGGCC GAGAAGGGCT
3601 ACTACACCAT GAGCAACAAC CTGGTGACCC TGGAGAACGG CAAGCAGCTG ACCGTGAAGA
3661 GACAGGGCCT GTACTACATC TACGCCCAGG TGACCTTCTG CAGCAACAGA GAGGCCAGCA
3721 GCCAGGCCCC CTTCATCGCC CACAGCAGCG CCAAGCCCTG CGGCCAGCAG AGCATCCACC
3781 TGCTGAGAGC CGCCAACACC CACAGCAGCG CCAAGCCCTG CGGCCAGCAG AGCATCCACC
3841 TGGGCGGCGT GTTCGAGCTG CAGCCCGGCG CCAGCGTGTT CGTGAACGTG ACCGACCCCA
3901 GCCAGGTGAG CCACGGCACC GGCTTCACCA GCTTCGGCCT GCTGAAGCTG TAAACCCAGC
3961 TTTCTTGTAC AAAGTGGTGA TAATCGAATT CACCCAGCTT TCTTGTACAA AGTGGTGATA
4021 ATCGAATTCC GATAATCAAC CTCTGGATTA CAAAATTTGT GAAAGATTGA CTGGTATTCT
4081 TAACTATGTT GCTCCTTTTA CGCTATGTGG ATACGCTGCT TTAATGCCTT TGTATCATGC
4141 TATTCGTTCC CGTATGGCTT TCATTTTCTC CTCCTTGTAT AAATCCTGGT TGCTGTCTCT
4201 TTATGAGGAG TTGTGGCCCG TTGTCAGGCA ACGTGGCGTG GTGTGCACTG TGTTTGCTGA
4261 CGCAACCCCC ACTGGTTGGG GCATTGCCAC CACCTGTCAG CTCCTTTCCG GGACTTTCGC
4321 TTTCCCCCTC CCTATTGCCA CGGCGGAACT CATCGCCGCC TGCCTTGCCC GCTGCTGGAC
4381 AGGGGCTCGG CTGTTGGGCA CTGACAATTC CGTGGTGTTG TCGGGGAAGC TGACGTCCTT
4441 TCCATGGCTG CTCGCCTGTG TTGCCACCTG GATTCTGCGC GGGACGTCCT TCTGCTACGT
4501 CCCTTCGGCC CTCAATCCAG CGGACCTTCC TTCCCGCGGC CTGCTGCCGG CTCTGCGGCC
4561 TCTTCCGCGT CTTCGCCTTC GCCCTCAGAC GAGTCGGATC TCCCTTTGGG CCGCCTCCCC
4621 GCATCGGGAA TTCCCGCGGT TCGCTTTAAG ACCAATGACT TACAAGGCAG CTGTAGATCT
4681 TAGCCACTTT TTAAAAGAAA AGGGGGGACT GGAAGGGCTA ATTCACTCCC AACGAAGACA
4741 AGATCTGCTT TTTGCTTGTA CTGGGTCTCT CTGGTTAGAC CAGATCTGAG CCTGGGAGCT
4801 CTCTGGCTAA CTAGGGAACC CACTGCTTAA GCCTCAATAA AGCTTGCCTT GAGTGCTTCA
4861 AGTAGTGTGT GCCCGTCTGT TGTGTGACTC TGGTAACTAG AGATCCCTCA GACCCTTTTA
4921 GTCAGTGTGG AAAATCTCTA GCAGTAGTAG TTCATGTCAT CTTATTATTC AGTATTTATA
4981 ACTTGCAAAG AAATGAATAT CAGAGAGTGA GAGGAACTTG TTTATTGCAG CTTATAATGG
5041 TTACAAATAA AGCAATAGCA TCACAAATTT CACAAATAAA GCATTTTTTT CACTGCATTC
5101 TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT GTCTGGCTCT AGCTATCCCG
5161 CCCCTAACTC CGCCCATCCC GCCCCTAACT CCGCCCAGTT CCGCCCATTC TCCGCCCCAT
5221 GGCTGACTAA TTTTTTTTAT TTATGCAGAG GCCGAGGCCG CCTCGGCCTC TGAGCTATTC
5281 CAGAAGTAGT GAGGAGGCTT TTTTGGAGGC CTAGGGACGT ACCCAATTCG CCCTATAGTG
5341 AGTCGTATTA CGCGCGCTCA CTGGCCGTCG TTTTACAACG TCGTGACTGG GAAAACCCTG
5401 GCGTTACCCA ACTTAATCGC CTTGCAGCAC ATCCCCCTTT CGCCAGCTGG CGTAATAGCG
5461 AAGAGGCCCG CACCGATCGC CCTTCCCAAC AGTTGCGCAG CCTGAATGGC GAATGGGACG
5521 CGCCCTGTAG CGGCGCATTA AGCGCGGCGG GTGTGGTGGT TACGCGCAGC GTGACCGCTA
5581 CACTTGCCAG CGCCCTAGCG CCCGCTCCTT TCGCTTTCTT CCCTTCCTTT CTCGCCACGT
5641 TCGCCGGCTT TCCCCGTCAA GCTCTAAATC GGGGGCTCCC TTTAGGGTTC CGATTTAGTG
5701 CTTTACGGCA CCTCGACCCC AAAAAACTTG ATTAGGGTGA TGGTTCACGT AGTGGGCCAT
5761 CGCCCTGATA GACGGTTTTT CGCCCTTTGA CGTTGGAGTC CACGTTCTTT AATAGTGGAC
5821 TCTTGTTCCA AACTGGAACA ACACTCAACC CTATCTCGGT CTATTCTTTT GATTTATAAG
5881 GGATTTTGCC GATTTCGGCC TATTGGTTAA AAAATGAGCT GATTTAACAA AAATTTAACG
5941 CGAATTTTAA CAAAATATTA ACGCTTACAA TTTAGGTGGC ACTTTTCGGG GAAATGTGCG
6001 CGGAACCCCT ATTTGTTTAT TTTTCTAAAT ACATTCAAAT ATGTATCCGC TCATGAGACA
6061 ATAACCCTGA TAAATGCTTC AATAATATTG AAAAAGGAAG AGTATGAGTA TTCAACATTT
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FIG. 7B-2

```
6121  CCGTGTCGCC CTTATTCCCT TTTTTGCGGC ATTTTGCCTT CCTGTTTTTG CTCACCCAGA
6181  AACGCTGGTG AAAGTAAAAG ATGCTGAAGA TCAGTTGGGT GCACGAGTGG GTTACATCGA
6241  ACTGGATCTC AACAGCGGTA AGATCCTTGA GAGTTTTCGC CCCGAAGAAC GTTTTCCAAT
6301  GATGAGCACT TTTAAAGTTC TGCTATGTGG CGCGGTATTA TCCCGTATTG ACGCCGGGCA
6361  AGAGCAACTC GGTCGCCGCA TACACTATTC TCAGAATGAC TTGGTTGAGT ACTCACCAGT
6421  CACAGAAAAG CATCTTACGG ATGGCATGAC AGTAAGAGAA TTATGCAGTG CTGCCATAAC
6481  CATGAGTGAT AACACTGCGG CCAACTTACT TCTGACAACG ATCGGAGGAC CGAAGGAGCT
6541  AACCGCTTTT TTGCACAACA TGGGGGATCA TGTAACTCGC CTTGATCGTT GGGAACCGGA
6601  GCTGAATGAA GCCATACCAA ACGACGAGCG TGACACCACG ATGCCTGTAG CAATGGCAAC
6661  AACGTTGCGC AAACTATTAA CTGGCGAACT ACTTACTCTA GCTTCCCGGC AACAATTAAT
6721  AGACTGGATG GAGGCGGATA AAGTTGCAGG ACCACTTCTG CGCTCGGCCC TTCCGGCTGG
6781  CTGGTTTATT GCTGATAAAT CTGGAGCCGG TGAGCGTGGG TCTCGCGGTA TCATTGCAGC
6841  ACTGGGGCCA GATGGTAAGC CCTCCCGTAT CGTAGTTATC TACACGACGG GGAGTCAGGC
6901  AACTATGGAT GAACGAAATA GACGATCGC TGAGATAGGT GCCTCACTGA TTAAGCATTG
6961  GTAACTGTCA GACCAAGTTT ACTCATATAT ACTTTAGATT GATTTAAAAC TTCATTTTTA
7021  ATTTAAAAGG ATCTAGGTGA AGATCCTTTT TGATAATCTC ATGACCAAAA TCCCTTAACG
7081  TGAGTTTTCG TTCCACTGAG CGTCAGACCC CGTAGAAAAG ATCAAAGGAT CTTCTTGAGA
7141  TCCTTTTTTT CTGCGCGTAA TCTGCTGCTT GCAAACAAAA AAACCACCGC TACCAGCGGT
7201  GGTTTGTTTG CCGGATCAAG AGCTACCAAC TCTTTTTCCG AAGGTAACTG GCTTCAGCAG
7261  AGCGCAGATA CCAAATACTG TTCTTCTAGT GTAGCCGTAG TTAGGCCACC ACTTCAAGAA
7321  CTCTGTAGCA CCGCCTACAT ACCTCGCTCT GCTAATCCTG TTACCAGTGG CTGCTGCCAG
7381  TGGCGATAAG TCGTGTCTTA CCGGGTTGGA CTCAAGACGA TAGTTACCGG ATAAGGCGCA
7441  GCGGTCGGGC TGAACGGGGG GTTCGTGCAC ACAGCCCAGC TTGGAGCGAA CGACCTACAC
7501  CGAACTGAGA TACCTACAGC GTGAGCTATG AGAAAGCGCC ACGCTTCCCG AAGAGAGAAA
7561  GGCGGACAGG TATCCGGTAA GCGGCAGGGT CGGAACAGGA GAGCGCACGA GGGAGCTTCC
7621  AGGGGGAAAC GCCTGGTATC TTTATAGTCC TGTCGGGTTT CGCCACCTCT GACTTGAGCG
7681  TCGATTTTTG TGATGCTCGT CAGGGGGGCG GAGCCTATGG AAAAACGCCA GCAACGCGGC
7741  CTTTTTACGG TTCCTGGCCT TTTGCTGGCC TTTTGCTCAC ATGTTCTTTC CTGCGTTATC
7801  CCCTGATTCT GTGGATAACC GTATTACCGC CTTTGAGTGA GCTGATACCG CTCGCCGCAG
7861  CCGAACGACC GAGCGCAGCG AGTCAGTGAG CGAGGAAGCG GAAGAGCGCC CAATACGCAA
7921  ACCGCCTCTC CCCGCGCGTT GGCCGATTCA TTAATGCAGC TGGCACGACA GGTTTCCCGA
7981  ACCGCCTCTC GGCAGTGAGC GCAACGCAAT TAATGTGAGT TAGCTCACTC ATTAGGCACC
8041  CCAGGCTTTA CACTTTATGC TTCCGGCTCG TATGTTGTGT GGAATTGTGA GCGGATAACA
8101  ATTTCACACA GGAAACAGCT ATGACCATGA TTACGCCAAG AGAGCAATTA ACCCTCACTA
8161  AAGGGAACAA AAGCTGGAGC TGCAAGCTT
```

FIG. 8A

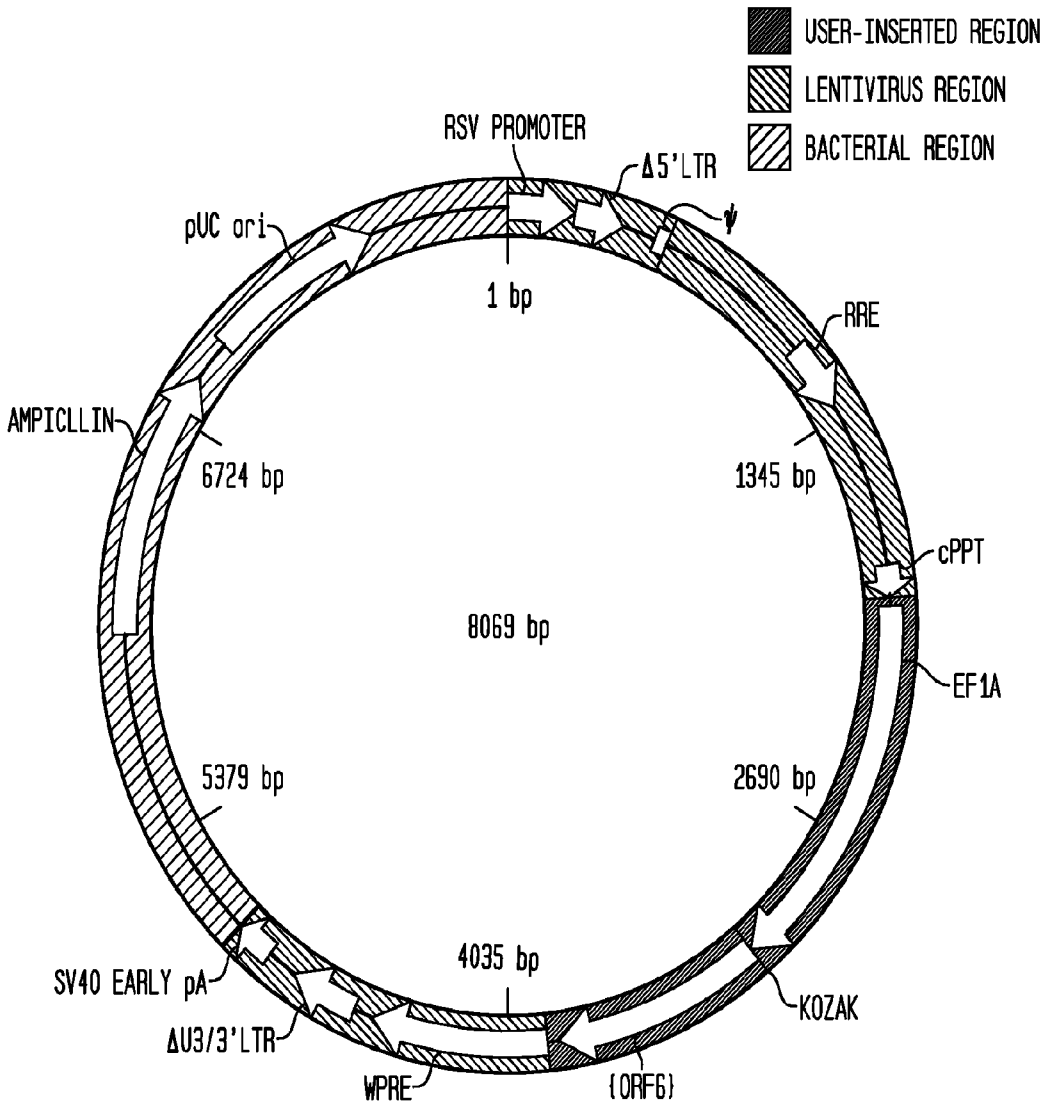

USER-INSERTED REGION
LENTIVIRUS REGION
BACTERIAL REGION
RSV PROMOTER
Δ5'LTR
ψ
pUC ori
1 bp
RRE
AMPICLLIN
6724 bp
1345 bp
cPPT
8069 bp
EF1A
5379 bp
2690 bp
4035 bp
SV40 EARLY pA
KOZAK
ΔU3/3'LTR
WPRE
(ORF6)

FIG. 8B

SEQ ID NO. 52

```
   1  AATGTAGTCT TATGCAATAC TCTTGTAGTC TTGCAACATG GTAACGATGA GTTAGCAACA
  61  TGCCTTACAA GGAGAGAAAA AGCACCGTGC ATGCCGATTG GTGGAAGTAA GGTGGTACGA
 121  TCGTGCCTTA TTAGGAAGGC AACAGACGGG TCTGACATGG ATTGGACGAA CCACTGAATT
 181  GCCGCATTGC AGAGATATTG TATTTAAGTG CCTAGCTCGA TACATAAACG GGTCTCTCTG
 241  GTTAGACCAG ATCTGAGCCT GGGAGCTCTC TGGCTAACTA GGGAACCCAC TGCTTAAGCC
 301  TCAATAAAGC TTGCCTTGAG TGCTTCAAGT AGTGTGTGCC CGTCTGTTGT GTGACTCTGG
 361  TAACTAGAGA TCCCTCAGAC CCTTTTAGTC AGTGTGGAAA ATCTCTAGCA GTGGCGCCCG
 421  AACAGGGACT TGAAAGCGAA AGGGAAACCA GAGGAGCTCT CTCGACGCAG GACTCGGCTT
 481  GCTGAAGCGC GCACGGCAAG AGGCGAGGGG CGGCGACTGG TGAGTACGCC AAAAATTTTG
 541  ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG TCAGTATTAA GCGGGGGAGA
 601  ATTAGATCGC GATGGGAAAA AATTCGGTTA AGGCCAGGGG GAAAGAAAAA ATATAAATTA
 661  AAACATATAG TATGGGCAAG CAGGGAGCTA GAACGATTCG CAGTTAATCC TGGCCTGTTA
 721  GAAACATCAG AAGGCTGTAG ACAAATACTG GGACAGCTAC AACCATCCCT TCAGACAGGA
 781  TCAGAAGAAC TTAGATCATT ATATAATACA GTAGCAACCC TCTATTGTGT GCATCAAAGG
 841  ATAGAGATAA AAGACACCAA GGAAGCTTTA GACAAGATAG AGGAAGAGCA AAACAAAAGT
 901  AAGACCACCG CACAGCAAGC GGCCGCTGAT CTTCAGACCT GGAGGAGGAG ATATGAGGGA
 961  CAATTGGAGA AGTGAATTAT ATAAATATAA AGTAGTAAAA ATTGAACCAT TAGGAGTAGC
1021  ACCCACCAAG GCAAAGAGAA GAGTGGTGCA GAGAGAAAAA AGAGCAGTGG GAATAGGAGC
1081  TTTGTTCCTT GGGTTCTTGG GAGCAGCAGG AAGCACTATG GGCGCAGCGT CAATGACGCT
1141  GACGGTACAG GCCAGACAAT TATTGTCTGG TATAGTGCAG CAGCAGAACA ATTTGCTGAG
1201  GGCTATTGAG GCGCAACAGC ATCTGTTGCA ACTCACAGTC TGGGGCATCA AGCAGCTCCA
1261  GGCAAGAATC CTGGCTGTGG AAAGATACCT AAAGGATCAA CAGCTCCTGG GGATTTGGGG
1321  TTGCTCTGGA AAACTCATTT GCACCACTGC TGTGCCTTGG AATGCTAGTT GGAGTAATAA
1381  ATCTCTGGAA CAGATTTGGA ATCACACGAC CTGGATGGAG TGGGACAGAG AAATTAACAA
1441  TTACACAAGC TTAATACACT CCTTAATTGA AGAATCGCAA AACCAGCAAG AAAAGAATGA
1501  ACAAGAATTA TTGGAATTAG ATAAATGGGC AAGTTTGTGG AATTGGTTTA ACATAACAAA
1561  TTGGCTGTGG TATATAAAAT TATTCATAAT GATAGTAGGA GGCTTGGTAG GTTTAAGAAT
1621  AGTTTTTGCT GTACTTTCTA TAGTGAATAG AGTTAGGCAG GGATATTCAC CATTATCGTT
1681  TCAGACCCAC CTCCCAACCC CGAGGGGACC CGACAGGCCC GAAGGAATAG AAGAAGAAGG
1741  TGGAGAGAGA GACAGAGACA GATCCATTCG ATTAGTGAAC GGATCTCGAC GGTATCGCTA
1801  GCTTTTAAAA GAAAAGGGGG GATTGGGGGG TACAGTGCAG GGGAAAGAAT AGTAGACATA
1861  ATAGCAACAG ACATACAAAC TAAAGAATTA CAAAAACAAA TTACAAAAAT TCAAAATTTT
1921  ACTAGTGATT ATCGGATCAA CTTTGTATAG AAAAGTTGGG CTCCGGTGCC CGTCAGTGGG
1981  CAGAGCGCAC ATCGCCCACA GTCCCCGAGA AGTTGGGGGG AGGGGTCGGC AATTGAACCG
2041  GTGCCTAGAG AAGGTGGCGC GGGGTAAACT GGGAAAGTGA TGTCGTGTAC TGGCTCCGCC
2101  TTTTTCCCGA GGGTGGGGGA GAACCGTATA TAAGTGCAGT AGTCGCCGTG AACGTTCTTT
2161  TTCGCAACGG GTTTGCCGCC AGAACACAGG TAAGTGCCGT GTGTGGTTCC CGCGGGCCTG
2221  GCCTCTTTAC GGGTTATGGC CCTTGCGTGC CTTGAATTAC TTCCACCTGG CTGCAGTACG
2281  TGATTCTTGA TCCCGAGCTT CGGGTTGGAA GTGGGTGGGA GAGTTCGAGG CCTTGCGCTT
2341  AAGGAGCCCC TTCGCCTCGT GCTTGAGTTG AGGCCTGGCC TGGGCGCTGG GGCCGCCGCG
2401  TGCGAATCTG GTGGCACCTT CGCGCCTGTC TCGCTGCTTT CGATAAGTCT CTAGCCATTT
2461  AAAATTTTTG ATGACCTGCT GCGACGCTTT TTTTCTGGCA AGATAGTCTT GTAAATGCGG
2521  GCCAAGATCT GCACACTGGT ATTTCGGTTT TTGGGGCCGC GGGCGGCGAC GGGGCCCGTG
2581  CGTCCCAGCG CACATGTTCG GCGAGGCGGG GCCTGCGAGC GCGGCCACCG AGAATCGGAC
2641  GGGGGTAGTC TCAAGCTGGC CGGCCTGCTC TGGTGCCTGG TCTCGCGCCG CCGTGTATCG
2701  CCCCGCCCTG GGCGGCAAGG CTGGCCCGGT CGGCACCAGT TGCGTGAGCG GAAAGATGGC
2761  CGCTTCCCGG CCTGCTGCA GGGAGCTCAA AATGGAGGAC GCGGCGCTCG GGAGAGCGGG
2821  CGGGTGAGTC ACCCACACAA AGGAAAAGGG CCTTTCCGTC CTCAGCCGTC GCTTCATGTG
```

FIG. 8B-1

```
2881  ACTCCACGGA GTACCGGGCG CCGTCCAGGC ACCTCGATTA GTTCTCGAGC TTTTGGAGTA
2941  CGTCGTCTTT AGGTTGGGGG GAGGGGTTTT ATGCGATGGA GTTTCCCCAC ACTGAGTGGG
3001  TGGAGACTGA AGTTAGGCCA GCTTGGCACT TGATGTAATT CTCCTTGGAA TTTGCCCTTT
3061  TTGAGTTTGG ATCTTGGTTC ATTCTCAAGC CTCAGACAGT GGTTCAAAGT TTTTTTCTTC
3121  CATTTCAGGT GTCGTGACAA GTTTGTACAA AAAAGCAGGC TGCCACCATG AGCACCGAGA
3181  GCATGATCAG AGACGTGGAG CTGGCCGAGG AGGCCCTGCC CAAGAAGACC GGCGGCCCCC
3241  AGGGCAGCAG AAGATGCCTG TTCCTGAGCC TGTTCAGCTT CCTGATCGTG GCCGGCGCCA
3301  CCACCCTGTT CTGCCTGCTG CACTTCGGCG TGATCGGCCC CCAGAGAGAG GAGTTCCCCA
3361  GAGACCTGAG CCTGATCAGC CCCCTGGCCC AGGCCGTGGC CCACGTGGTG GCCAACCCCC
3421  AGGCCGAGGG CCAGCTGCAG TGGCTGAACA GAAGAGCCAA CGCCCTGCTG GCCAACGGCG
3481  TGGAGCTGAG AGACAACCAG CTGGTGGTGC CCAGCGAGGG CCTGTACCTG ATCTACAGCC
3541  AGGTGCTGTT CAAGGGCCAG GGCTGCCCCA GCACCCACGT GCTGCTGACC CACACCATCA
3601  GCAGAATCGC CGTGAGCTAC CAGACCAAGG TGAACCTGCT GAGCGCCATC AAGAGCCCCT
3661  GCCAGAGAGA GACCCCCGAG GGCGCCGAGG CCAAGCCCTG GTACGAGCCC ATCTACCTGG
3721  GCGGCGTGTT CCAGCTGGAG AAGGGCGACA GACTGAGCGC CGAGATCAAG AGACCCGACT
3781  ACCTGGACTT CGCCGAGAGC GGCCAGGTGT ACTTCGGCAT CATCGCCCTG TAAACCCAGC
3841  TTTCTTGTAC AAAGTGGTGA TAATCGAATT CACCCAGCTT TCTTGTACAA AGTGGTGATA
3901  ATCGAATTCC GATAATCAAC CTCTGGATTA CAAAATTTGT GAAAGATTGA CTGGTATTCT
3961  TAACTATGTT GCTCCTTTTA CGCTATGTGG ATACGCTGCT TTAATGCCTT TGTATCATGC
4021  TATTGCTTCC CGTATGGCTT TCATTTTCTC CTCCTTGTAT AAATCCTGGT TGCTGTCTCT
4081  TTATGAGGAG TTGTGGCCCG TTGTCAGGCA ACGTGGCGTG GTGTGCACTG TGTTTGCTGA
4141  CGCAACCCCC ACTGGTTGGG GCATTGCCAC CACCTGTCAG CTCCTTTCCG GGACTTTCGC
4201  TTTCCCCCTC CCTATTGCCA CGGCGGAACT CATCGCCGCC TGCCTTGCCC GCTGCTGGAC
4261  AGGGGCTCGG CTGTTGGGCA CTGACAATTC CGTGGTGTTG TCGGGGAAGC TGACGTCCTT
4321  TCCATGGCTG CTCGCCTGTG TTGCCACCTG GATTCTGCGC GGGACGTCCT TCTGCTACGT
4381  CCCTTCGGCC CTCAATCCAG CGGACCTTCC TTCCCGCGGC CTGCTGCCGG CTCTGCGGCC
4441  TCTTCCGCGT CTTCGCCTTC GCCCTCAGAC GAGTCGGATC TCCCTTTGGG CCGCCTCCCC
4501  GCATCGGGAA TTCCCGCGGT TCGCTTTAAG ACCAATGACT TACAAGGCAG CTGTAGATCT
4561  TAGCCACTTT TTAAAAGAAA AGGGGGGACT GGAAGGGCTA ATTCACTCCC AACGAAGACA
4621  AGATCTGCTT TTTGCTTGTA CTGGGTCTCT CTGGTTAGAC CAGATCTGAG CCTGGGAGCT
4681  CTCTGGCTAA CTAGGGAACC CACTGCTTAA GCCTCAATAA AGCTTGCCTT GAGTGCTTCA
4741  AGTAGTGTGT GCCCGTCTGT TGTGTGACTC TGGTAACTAG AGATCCCTCA GACCCTTTTA
4801  GTCAGTGTGG AAAATCTCTA GCAGTAGTAG TTCATGTCAT CTTATTATTC AGTATTTATA
4861  ACTTGCAAAG AAATGAATAT CAGAGAGTGA GAGGAACTTG TTTATTGCAG CTTATAATGG
4921  TTACAAATAA AGCAATAGCA TCACAAATT  CACAAATAAA GCATTTTTTT CACTGCATTC
4981  TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT GTCTGGCTCT AGCTATCCCG
5041  CCCCTAACTC CGCCCATCCC GCCCCTAACT CCGCCCAGTT CCGCCCATTC TCCGCCCCAT
5101  GGCTGACTAA TTTTTTTTAT TTATGCAGAG GCCGAGGCCG CCTCGGCCTC TGAGCTATTC
5161  CAGAAGTAGT GAGGAGGCTT TTTTGGAGGC CTAGGGACGT ACCCAATTCG CCCTATAGTG
5221  AGTCGTATTA CGCGCGCTCA CTGGCCGTCG TTTTACAACG TCGTGACTGG GAAAACCCTG
5281  GCGTTACCCA ACTTAATCGC CTTGCAGCAC ATCCCCCTTT CGCCAGCTGG CGTAATAGCG
5341  AAGAGGCCCG CACCGATCGC CCTTCCCAAC AGTTGCGCAG CCTGAATGGC GAATGGGACG
5401  CGCCCTGTAG CGGCGCATTA AGCGCGGCGG GTGTGGTGGT TACGCGCAGC GTGACCGCTA
5461  CACTTGCCAG CGCCCTAGCG CCCGCTCCTT TCGCTTTCTT CCCTTCCTTT CTCGCCACGT
5521  TCGCCGGCTT TCCCCGTCAA GCTCTAAATC GGGGGCTCCC TTTAGGGTTC CGATTTAGTG
5581  CTTTACGGCA CCTCGACCCC AAAAAACTTG ATTAGGGTGA TGGTTCACGT AGTGGGCCAT
5641  CGCCCTGATA GACGGTTTTT CGCCCTTTGA CGTTGGAGTC CACGTTCTTT AATAGTGGAC
5701  TCTTGTTCCA AACTGGAACA ACACTCAACC CTATCTCGGT CTATTCTTTT GATTTATAAG
5761  GGATTTTGCC GATTTCGGCC ATTTGGTTAA AAAATGAGCT GATTTAACAA AAATTTAACG
5821  CGAATTTTAA CAAAATATTA ACGCTTACAA TTTAGGTGGC ACTTTTCGGG GAAATGTGCG
5881  CGGAACCCCT ATTTGTTTAT TTTTCTAAAT ACATTCAAT ATGTATCCGC TCATGAGACA
5941  ATAACCCTGA TAAATGCTTC AATAATATTG AAAAAGGAAG AGTATGAGTA TTCAACATTT
```

FIG. 8B-2

```
6001  CCGTGTCGCC CTTATTCCCT TTTTTGCGGC ATTTTGCCTT CCTGTTTTTG CTCACCCAGA
6061  AACGCTGGTG AAAGTAAAAG ATGCTGAAGA TCAGTTGGGT GCACGAGTGG GTTACATCGA
6121  ACTGGATCTC AACAGCGGTA AGATCCTTGA GAGTTTTCGC CCCGAAGAAC GTTTTCCAAT
6181  GATGAGCACT TTTAAAGTTC TGCTATGTGG CGCGGTATTA TCCCGTATTG ACGCCGGGCA
6241  AGAGCAACTC GGTCGCCGCA TACACTATTC TCAGAATGAC TTGGTTGAGT ACTCACCAGT
6301  CACAGAAAAG CATCTTACGG ATGGCATGAC AGTAAGAGAA TTATGCAGTG CTGCCATAAC
6361  CATGAGTGAT AACACTGCGG CCAACTTACT TCTGACAACG ATCGGAGGAC CGAAGGAGCT
6421  AACCGCTTTT TTGCACAACA TGGGGGATCA TGTAACTCGC CTTGATCGTT GGGAACCGGA
6481  GCTGAATGAA GCCATACCAA ACGACGAGCG TGACACCACG ATGCCTGTAG CAATGGCAAC
6541  AACGTTGCGC AAACTATTAA CTGGCGAACT ACTTACTCTA GCTTCCCGGC AACAATTAAT
6601  AGACTGGATG GAGGCGGATA AAGTTGCAGG ACCACTTCTG CGCTCGGCCC TTCCGGCTGG
6661  CTGGTTTATT GCTGATAAAT CTGGAGCCGG GTAGCGTGGG TCTCGCGGTA TCATTGCAGC
6721  ACTGGGGCCA GATGGTAAGC CCTCCCGTAT CGTAGTTATC TACACGACGG GGAGTCAGGC
6781  AACTATGGAT GAACGAAATA GACAGATCGC TGAGATAGGT GCCTCACTGA TTAAGCATTG
6841  GTAACTGTCA GACCAAGTTT ACTCATATAT ACTTTAGATT GATTTAAAAC TTCATTTTTA
6901  TGAGTTTTCG TTCCACTGAG CGTCAGACCC CGTAGAAAAG ATCAAAGGAT CTTCTTGAGA
6961  TGAGTTTTCG TTCCACTGAG CGTCAGACCC CGTAGAAAAG ATCAAAGGAT CTTCTTGAGA
7021  TCCTTTTTTT CTGCGCGTAA TCTGCTGCTT GCAAACAAAA AAACCACCGC TACCAGCGGT
7081  GGTTTGTTTG CCGGATCAAG AGCTACCAAC CTCTTTTTCCG AAGGTAACTG GCTTCAGCAG
7141  AGCGCAGATA CCAAATACTG TTCTTCTAGT GTAGCCGTAG TTAGGCCACC ACTTCAAGAA
7201  CTCTGTAGCA CCGCCTACAT ACCTCGCTCT GCTAATCCTG TTACCAGTGG CTGCTGCCAG
7261  TGGCGATAAG TCGTGTCTTA CCGGGTTGGA CTCAAGACGA TAGTTACCGG ATAAGGCGCA
7321  GCGGTCGGGC TGAACGGGGG GTTCGTGCAC ACAGCCCAGC TTGGAGCGAA CGACCTACAC
7381  CGAACTGAGA TACCTACAGC GTGAGCTATG AGAAAGCGCC ACGCTTCCCG AAGAGAGAAA
7441  GGCGGACAGG TATCCGGTAA GCGGCAGGGT CGGAACAGGA GAGCGCACGA GGGAGCTTCC
7501  AGGGGGAAAC GCCTGGTATC TTTATAGTCC TGTCGGGTTT CGCCACCTCT GACTTGAGCG
7561  TCGATTTTTG TGATGCTCGT CAGGGGGGCG GAGCCTATGG AAAAACGCCA GCAACGCGGC
7621  CTTTTTACGG TTCCTGGCCT TTTCGTGGCC TTTTGCTCAC ATGTTCTTTC CTGCGTTATC
7681  CCCTGATTCT GTGGATAACC GTATTACCGC CTTTGAGTGA GCTGATACCG CTCGCCGCAG
7741  CCGAACGACC GAGCGCAGCG AGTCAGTGAG CGAGGAAGCG GAAGAGCGCC CAATACGCAA
7801  ACCGCCTCTC CCCGCGCGTT GGCCGATTCA TTAATGCAGC TGGCACGACA GGTTTCCCGA
7861  CTGGAAAGCG GGCAGTGAGC GCAACGCAAT TAATGTGAGT TAGCTCACTC ATTAGGCACC
7921  CCAGGCTTTA CACTTTATGC TTCCGGCTCG TATGTTGTGT GGAATTGTGA GCGGATAACA
7981  ATTTCACACA GGAAACAGCT ATGACCATGA TTACGCCAAG CGCGCAATTA ACCCTCACTA
8041  AAGGGAACAA AAGCTGGAGC TGCAAGCTT
```

FIG. 9A

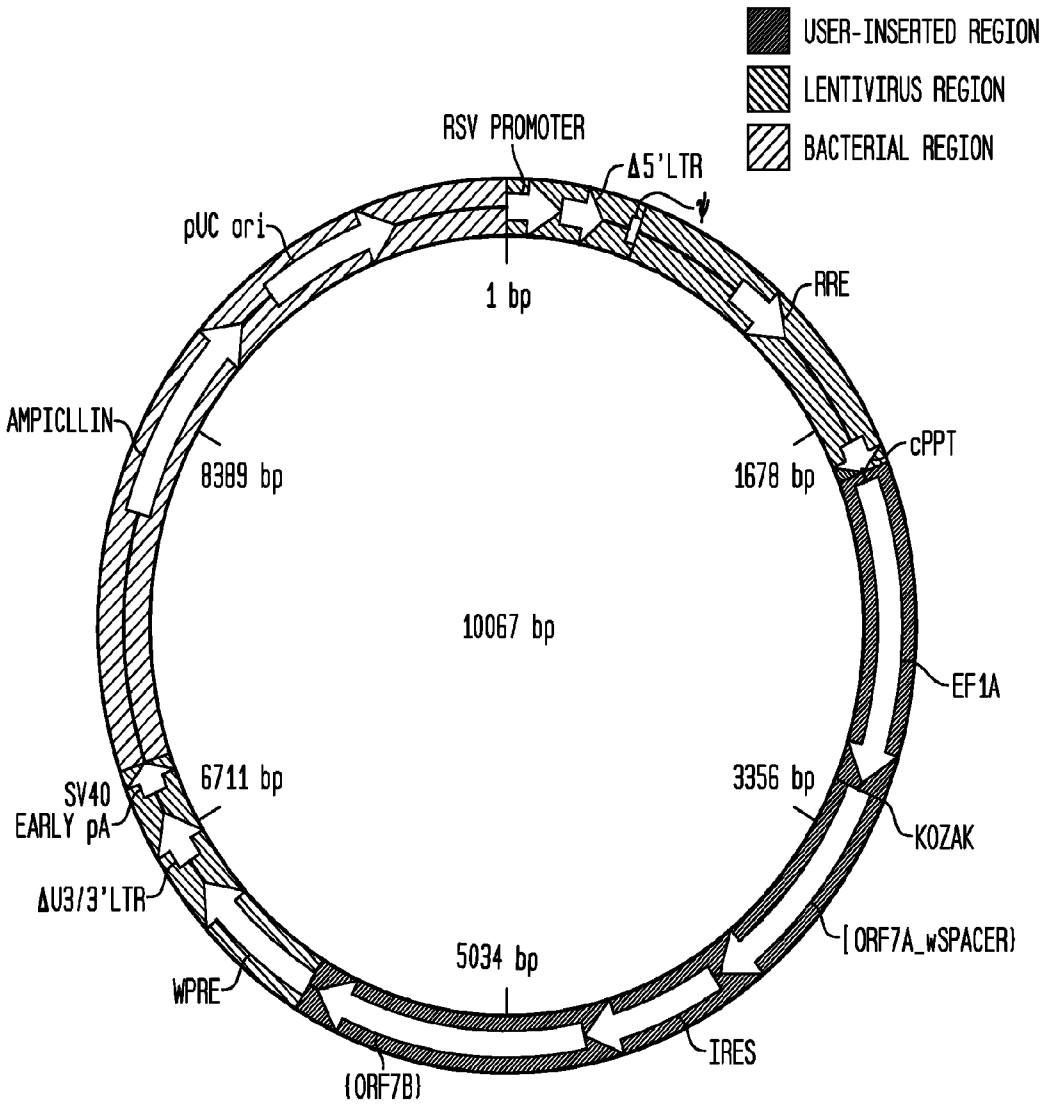

USER-INSERTED REGION
LENTIVIRUS REGION
BACTERIAL REGION
RSV PROMOTER
Δ5'LTR
ψ
RRE
cPPT
EF1A
KOZAK
(ORF7A_wSPACER)
IRES
(ORF7B)
WPRE
ΔU3/3'LTR
SV40
EARLY pA
AMPICLLIN
pUC ori
1 bp
1678 bp
3356 bp
5034 bp
6711 bp
8389 bp
10067 bp

FIG. 9B
SEQ ID NO. 53

```
   1 AATGTAGTCT TATGCAATAC TCTTGTAGTC TTGCAACATG GTAACGATGA GTTAGCAACA
  61 TGCCTTACAA GGAGAGAAAA AGCACCGTGC ATGCCGATTG GTGGAAGTAA GGTGGTACGA
 121 TCGTGCCTTA TTAGGAAGGC AACAGACGGG TCTGACATGG ATTGGACGAA CCACTGAATT
 181 GCCGCATTGC AGAGATATTG TATTTAAGTG CCTAGCTCGA TACATAAACG GGTCTCTCTG
 241 GTTAGACCAG ATCTGAGCCT GGGAGCTCTC TGGCTAACTA GGGAACCCAC TGCTTAAGCC
 301 TCAATAAAGC TTGCCTTGAG TGCTTCAAGT AGTGTGTGCC CGTCTGTTGT GTGACTCTGG
 361 TAACTAGAGA TCCCTCAGAC CCTTTTAGTC AGTGTGGAAA ATCTCTAGCA GTGGCGCCCG
 421 AACAGGGACT TGAAAGCGAA AGGGAAACCA GAGGAGCTCT CTCGACGCAG GACTCGGCTT
 481 GCTGAAGCGC GCACGGCAAG AGGCGAGGGG CGGCGACTGG TGAGTACGCC AAAAATTTTG
 541 ACTAGCGGAG GCTAGAAGGA GAGAGATGGG TGCGAGAGCG TCAGTATTAA GCGGGGGAGA
 601 ATTAGATCGC GATGGGAAAA AATTCGGTTA AGGCCAGGGG GAAAGAAAAA ATATAAATTA
 661 AAACATATAG TATGGGCAAG CAGGGAGCTA GAACGATTCG CAGTTAATCC TGGCCTGTTA
 721 GAAACATCAG AAGGCTGTAG ACAAATACTG GGACAGCTAC AACCATCCCT TCAGACAGGA
 781 TCAGAAGAAC TTAGATCATT ATATAATACA GTAGCAACCC TCTATTGTGT GCATCAAAGG
 841 ATAGAGATAA AAGACACCAA GGAAGCTTTA GACAAGATAG AGGAAGAGCA AAACAAAAGT
 901 AAGACCACCG CACAGCAAGC GGCCGCTGAT CTTCAGACCT GGAGGAGGAG ATATGAGGGA
 961 CAATTGGAGA AGTGAATTAT ATAAATATAA AGTAGTAAAA ATTGAACCAT TAGGAGTAGC
1021 ACCCACCAAG GCAAAGAGAA GAGTGGTGCA GAGAGAAAAA AGAGCAGTGG GAATAGGAGC
1081 TTTGTTCCTT GGGTTCTTGG GAGCAGCAGG AAGCACTATG GGCGCAGCGT CAATGACGCT
1141 GACGGTACAG GCCAGACAAT TATTGTCTGG TATAGTGCAG CAGCAGAACA ATTTGCTGAG
1201 GGCTATTGAG GCGCAACAGC ATCTGTTGCA ACTCACAGTC TGGGGCATCA AGCAGCTCCA
1261 GGCAAGAATC CTGGCTGTGG AAAGATACCT AAAGGATCAA CAGCTCCTGG GGATTTGGGG
1321 TTGCTCTGGA AAACTCATTT GCACCACTGC TGTGCCTTGG AATGCTAGTT GGAGTAATAA
1381 ATCTCTGGAA CAGATTTGGA ATCACACGAC CTGGATGGAG TGGGACAGAG AAATTAACAA
1441 TTACACAAGC TTAATACACT CCTTAATTGA AGAATCGCAA AACCAGCAAG AAAAGAATGA
1501 ACAAGAATTA TTGGAATTAG ATAAATGGGC AAGTTTGTGG AATTGGTTTA ACATAACAAA
1561 TTGGCTGTGG TATATAAAAT TATTCATAAT GATAGTAGGA GGCTTGGTAG GTTTAAGAAT
1621 AGTTTTTGCT GTACTTTCTA TAGTGAATAG AGTTAGGCAG GGATATTCAC CATTATCGTT
1681 TCAGACCCAC CTCCCAACCC CGAGGGGACC CGACAGGCCC GAAGGAATAG AAGAAGAAGG
1741 TGGAGAGAGA GACAGAGACA GATCCATTCG ATTAGTGAAC GGATCTCGAC GGTATCGCTA
1801 GCTTTTAAAA GAAAAGGGGG GATTGGGGGG TACAGTGCAG GGGAAAGAAT AGTAGACATA
1861 ATAGCAACAG ACATACAAAC TAAAGAATTA CAAAAACAAA TTACAAAAAT TCAAAATTTT
1921 ACTAGTATCA ACTTTGTATA GAAAAGTTGG GCTCCGGTGC CCGTCAGTGG GCAGAGCGCA
1981 CATCGCCCAC AGTCCCCGAG AAGTTGGGGG GAGGGGTCGG CAATTGAACC GGTGCCTAGA
2041 GAAGGTGGCG CGGGGTAAAC TGGGAAAGTG ATGTCGTGTA CTGGCTCCGC CTTTTTCCCG
2101 AGGGTGGGGG AGAACCGTAT ATAAGTGCAG TAGTCGCCGT GAACGTTCTT TTTCGCAACG
2161 GGTTTGCCGC CAGAACACAG GTAAGTGCCG TGTGTGGTTC CCGCGGGCCT GGCCTCTTTA
2221 CGGGTTATGG CCCTTGCGTG CCTTGAATTA CTTCCACCTG GCTGCAGTAC GTGATTCTTG
2281 ATCCCGAGCT TCGGGTTGGA AGTGGGTGGG AGAGTTCGAG GCCTTGCGCT TAAGGAGCCC
2341 CTTCGCCTCG TGCTTGAGTT GAGGCCTGGC CTGGGCGCTG GGGCCGCCGC GTGCGAATCT
2401 GGTGGCACCT TCGCGCCTGT CTCGCTGCTT TCGATAAGTC TCTAGCCATT TAAAATTTTT
2461 GATGACCTGC TGCGACGCTT TTTTTCTGGC AAGATAGTCT TGTAAATGCG GGCCAAGATC
2521 TGCACACTGG TATTTCGGTT TTTGGGGCCG CGGGCGGCGA CGGGGCCCGT GCGTCCCAGC
2581 GCACATGTTC GGCGAGGCGG GGCCTGCGAG CGCGGCCACC GAGAATCGGA CGGGGGTAGT
2641 CTCAAGCTGG CCGGCCTGCT CTGGTGCCTG GTCTCGCGCC GCCGTGTATC GCCCCGCCCT
2701 GGGCGGCAAG GCTGGCCCGG TCGGCACCAG TTGCGTGAGC GGAAAGATGG CCGCTTCCCG
```

FIG. 9B-1

2761 GCCCTGCTGC AGGGAGCTCA AAATGGAGGA CGCGGCGCTC GGGAGAGCGG GCGGGTGAGT
2821 CACCCACACA AAGGAAAAGG GCCTTTCCGT CCTCAGCCGT CGCTTCATGT GACTCCACGG
2881 AGTACCGGGC GCCGTCCAGG CACCTCGATT AGTTCTCGAG CTTTTGGAGT ACGTCGTCTT
2941 TAGGTTGGGG GGAGGGGTTT TATGCGATGG AGTTTCCCCA CACTGAGTGG GTGGAGACTG
3001 AAGTTAGGCC AGCTTGGCAC TTGATGTAAT TCTCCTTGGA ATTTGCCCTT TTTGAGTTTG
3061 GATCTTGGTT CATTCTCAAG CCTCAGACAG TGGTTCAAAG TTTTTTTCTT CCATTTCAGG
3121 TGTCGTGACA AGTTTGTACA AAAAAGCAGG CTGCCACCAT GAGCACCGAG AGCATGATCA
3181 GAGACGTGGA GCTGGCCGAG GAGGCCCTGC CCAAGAAGAC CGGCGGCCCC CAGGGCAGCA
3241 GAAGATGCCT GTTCCTGAGC CTGTTCAGCT TCCTGATCGT GGCCGGCGCC ACCACCCTGT
3301 TCTGCCTGCT GCACTTCGGC GTGATCGGCC CCCAGAGAGA GGAGTTCCCC AGAGACCTGA
3361 GCCTGATCAG CCCCCTGGCC CAGGCCGTGG CCCACGTGGT GGCCAACCCC CAGGCCGAGG
3421 GCCAGCTGCA GTGGCTGAAC AGAAGAGCCA ACGCCCTGCT GGCCAACGGC GTGGAGCTGA
3481 GAGACAACCA GCTGGTGGTG CCCAGCGAGG GCCTGTACCT GATCTACAGC CAGGTGCTGT
3541 TCAAGGGCCA GGGCTGCCCC AGCACCCACG TGCTGCTGAC CCACACCATC AGCAGAATCG
3601 CCGTGAGCTA CCAGACCAAG GTGAACCTGC TGAGCGCCAT CAAGAGCCCC TGCCAGAGAG
3661 AGACCCCCGA GGGCGCCGAG GCCAAGCCCT GGTACGAGCC CATCTACCTG GGCGGCGTGT
3721 TCCAGCTGGA GAAGGGCGAC AGACTGAGCG CCGAGATCAA CAGACCCGAC TACCTGGACT
3781 TCGCCGAGAG CGGCCAGGTG TACTTCGGCA TCATCGCCCT GTAAACCCAG CTTTCTTGTA
3841 CAAAGTGGTG ATAATCGAAT TCTAAATAGA TAGAACAACA ACAATTGCAT TCATTTTTGA
3901 TTTCAGGTTC AGGGGGAGGT GTGGGAGGTT TTTTAAAGCA AGTAAAACCT CTACACTGAC
3961 GGTACGCGTT AACAACAACA ATTGCATTCA TTTGTAGTTT CAGGTTCAGG GGGAGGTGTG
4021 GGAGGTTTTT TAAAGCAAGT TAAACCTCTA AAATAGTGGT ACGCGTTACC CAGCTTTCTT
4081 GTACAAAGTG GACCCAGCTT TCTTGTACAA AGTGGGCCCC TCTCCCTCCC CCCCCCCTAA
4141 CGTTACTGGC CGAAGCCGCT TGGAATAAGG CCGGTGTGCG TTTGTCTATA TGTTATTTTC
4201 CACCATATTG CCGTCTTTTG GCAATGTGAG GGCCCGGAAA CCTGGCCCTG TCTTCTTGAC
4261 GAGCATTCCT AGGGGTCTTT CCCCTCTCGC CAAAGGAATG CAAGGTCTGT TGAATGTCGT
4321 GAAGGAAGCA GTTCCTCTGG AAGCTTCTTG AAGACAAACA ACGTCTGTAG CGACCCTTTG
4381 CAGGCAGCGG AACCCCCCAC CTGGCGACAG GTGCCTCTGC GGCCAAAAGC CACGTGTATA
4441 AGATACACCT GCAAAGGCGG CACAACCCCA GTGCCACGTT GTGAGTTGGA TAGTTGTGGA
4501 AAGAGTCAAA TGGCTCTCCT CAAGCGTATT CAACAAGGGG CTGAAGGATG CCCAGAAGGT
4561 ACCCCATTGT ATGGGATCTG ATCTGGGGCC TCGGTGCACA TGCTTTACAT GTGTTTAGTC
4621 GAGGTTAAAA AAACGTCTAG GCCCCCCGAA CCACGGGGAC GTGGTTTTCC TTTGAAAAAC
4681 ACGATGATAA TATGGCCACA ACCATGGCCA CCGTGCTGGC CCCCGCCTGG AGCCCCACCA
4741 CCTACCTGCT GCTGCTGCTG CTGCTGAGCA GCGGCCTGAG CGGCGGCGGC GGCAGCGGCA
4801 AGCCCATCCC CAACCCCCTG CTGGGCCTGG ACAGCACCGG CGGCGGCGGC AGCCAGGTGA
4861 AGCTGCAGGA GAGCGGCCCC GGCCTGGTGG CCCCCAGCCA GAGCCTGAGC ATCACCTGCA
4921 CCGTGAGCGG CTTCAGCCTG ACCGCCTACG GCGTGGACTG GGTGAGACAG CCCCCCGGCA
4981 AGTGCCTGGA GTGGCTGGGC GTGATCTGGG GCGGCGGCAG AACCAACTAC AACAGCGGCC
5041 TGATGAGCAG ACTGAGCATC AGAAAGGACA ACAGCAAGAG CCAGGTGTTC CTGACCATGA
5101 ACAGCCTGCA GACCGACGAC ACCGCCAAGT ACTACTGCGT GAAGCACACC AACTGGGACG
5161 GCGGCTTCGC CTACTGGGGC CAGGGCACCA CCGTGACCGT GAGCAGCGGC GGCGGCGGCA
5221 GCGGCGGCGG CGGCAGCGGC GGCGGCGGCA GCGGCAGCCC CGGCCAGAGC GTGAGCATCA
5281 GCTGCAGCGG CAGCAGCAGC AACATCGGCA ACAACTACGT GTACTGGTAC CAGCACCTGC
5341 CCGGCACCGC CCCCAAGCTG CTGATCTACA GCGACACCAA GAGACCCAGC GGCGTGCCCG
5401 ACAGAATCAG CGGCAGCAAG AGCGGCACCA GCGCCAGCCT GGCCATCAGC GGCCTGCAGA
5461 GCGAGGACGA GGCCGACTAC TACTGCGCCA GCTGGGACGA CAGCCTGGAC GGCCCCGTGT
5521 TCGGCTGCGG CACCAAGCTG ACCGTGCTGC CCACCACCAC CCCCGCCCCC AGACCCCCCA
5581 CCCCCGCCCC CACCATCGCC AGCCAGCCCC TGAGCCTGAG ACCCGAGGCC TGCAGACCCG
5641 CCGCCGGCGG CGCCGTGCAC ACCAGAGGCC TGGACTTCGC CTGCGACATC TACATCTGGG
5701 CCCCCCTGGC CGGCACCTGC GGCGTGCTGC TGCTGAGCCT GGTGATCACC CGTGGTGAAG
5761 ACCACAGAAA CAGAAGAAGA GTGTGCAAGT GCCCCAGACC CGTGGTGAAG AGCGGCGACA

FIG. 9B-2

```
5821  AGCCCAGCCT  GAGCGCCAGA  TACGTGTAAC  AACTTTATTA  TACATAGTTG  ATCAATTCCA
5881  ACTTTATTAT  ACATAGTTGA  TCAATTCCGA  TAATCAACCT  CTGGATTACA  AAATTTGTGA
5941  AAGATTGACT  GGTATTCTTA  ACTATGTTGC  TCCTTTTACG  CTATGTGGAT  ACGCTGCTTT
6001  AATGCCTTTG  TATCATGCTA  TTGCTTCCCG  TATGGCTTTC  ATTTTCTCCT  CCTTGTATAA
6061  ATCCTGGTTG  CTGTCTCTTT  ATGAGGAGTT  GTGGCCCGTT  GTCAGGCAAC  GTGGCGTGGT
6121  GTGCACTGTG  TTTGCTGACG  CAACCCCCAC  TGGTTGGGGC  ATTGCCACCA  CCTGTCAGCT
6181  CCTTTCCGGG  ACTTTCGCTT  TCCCCCTCCC  TATTGCCACG  GCGGAACTCA  TCGCCGCCTG
6241  CCTTGCCCGC  TGCTGGACAG  GGGCTCGGCT  GTTGGGCACT  GACAATTCCG  TGGTGTTGTC
6301  GGGGAAGCTG  ACGTCCTTTC  CATGGCTGCT  CGCCTGTGTT  GCCACCTGGA  TTCTGCGCGG
6361  GACGTCCTTC  TGCTACGTCC  CTTCGGCCCT  CAATCCAGCG  GACCTTCCTT  CCCGCGGCCT
6421  GCTGCCGGCT  CTGCGGCCTC  TTCCGCGTCT  TCGCCTTCGC  CCTCAGACGA  GTCGGATCTC
6481  CCTTTGGGCC  GCCTCCCCGC  ATCGGGAATT  CCCGCGGTTC  GCTTTAAGAC  CAATGACTTA
6541  CAAGGCAGCT  GTAGATCTTA  GCCACTTTTT  AAAAGAAAAG  GGGGGACTGG  AAGGGCTAAT
6601  TCACTCCCAA  CGAAGACAAG  ATCTGCTTTT  TGCTTGTACT  GGGTCTCTCT  GGTTAGACCA
6661  GATCTGAGCC  TGGGAGCTCT  CTGGCTAACT  AGGGAACCCA  CTGCTTAAGC  CTCAATAAAG
6721  CTTGCCTTGA  GTGCTTCAAG  TAGTGTGTGC  CCGTCTGTTG  TGTGACTCTG  GTAACTAGAG
6781  ATCCCTCAGA  CCCTTTTAGT  CAGTGTGGAA  AATCTCTAGC  AGTAGTAGTT  CATGTCATCT
6841  TATTATTCAG  TATTTATAAC  TTGCAAAGAA  ATGAATATCA  GAGAGTGAGA  GGAACTTGTT
6901  TATTGCAGCT  TATAATGGTT  ACAAATAAAG  CAATAGCATC  ACAAATTTCA  CAAATAAAGC
6961  ATTTTTTTCA  CTGCATTCTA  GTTGTGGTTT  GTCCAAACTC  ATCAATGTAT  CTTATCATGT
7021  CTGGCTCTAG  CTATCCCGCC  CCTAACTCCG  CCCATCCCGC  CCCTAACTCC  GCCCAGTTCC
7081  GCCCATTCTC  CGCCCCATGG  CTGACTAATT  TTTTTTATTT  ATGCAGAGGC  CGAGGCCGCC
7141  TCGGCCTCTG  AGCTATTCCA  GAAGTAGTGA  GGAGGCTTTT  TTGGAGGCCT  AGGGACGTAC
7201  CCAATTCGCC  CTATAGTGAG  TCGTATTACG  CGCGCTCACT  GGCCGTCGTT  TTACAACGTC
7261  GTGACTGGGA  AAACCCTGGC  GTTACCCAAC  TTAATCGCCT  TGCAGCACAT  CCCCCTTTCG
7321  CCAGCTGGCG  TAATAGCGAA  GAGGCCCGCA  CCGATCGCCC  TTCCCAACAG  TTGCGCAGCC
7381  TGAATGGCGA  ATGGGACGCG  CCCTGTAGCG  GCGCATTAAG  CGCGGCGGGT  GTGGTGGTTA
7441  CGCGCAGCGT  GACCGCTACA  CTTGCCAGCG  CCCTAGCGCC  CGCTCCTTTC  GCTTTCTTCC
7501  CTTCCTTTCT  CGCCACGTTC  GCCGGCTTTC  CCCGTCAAGC  TCTAAATCGG  GGGCTCCCTT
7561  TAGGGTTCCG  ATTTAGTGCT  TTACGGCACC  TCGACCCCAA  AAAACTTGAT  TAGGGTGATG
7621  GTTCACGTAG  TGGGCCATCG  CCCTGATAGA  CGGTTTTTCG  CCCTTTGACG  TTGGAGTCCA
7681  CGTTCTTTAA  TAGTGGACTC  TTGTTCCAAA  CTGGAACAAC  ACTCAACCCT  ATCTCGGTCT
7741  ATTCTTTTGA  TTTATAAGGG  ATTTTGCCGA  TTTCGGCCTA  TTGGTTAAAA  AATGAGCTGA
7801  TTTAACAAAA  ATTTAACGCG  AATTTTAACA  AAATATTAAC  GCTTACAATT  TAGGTGGCAC
7861  TTTTCGGGGA  AATGTGCGCG  GAACCCCTAT  TTGTTTATTT  TTCTAAATAC  ATTCAAATAT
7921  GTATCCGCTC  ATGAGACAAT  AACCCTGATA  AATGCTTCAA  TAATATTGAA  AAAGGAAGAG
7981  TATGAGTATT  CAACATTTCC  GTGTCGCCCT  TATTCCCTTT  TTTGCGGCAT  TTTGCCTTCC
8041  TGTTTTTGCT  CACCCAGAAA  CGCTGGTGAA  AGTAAAAGAT  GCTGAAGATC  AGTTGGGTGC
8101  ACGAGTGGGT  TACATCGAAC  TGGATCTCAA  CAGCGGTAAG  ATCCTTGAGA  GTTTTCGCCC
8161  CGAAGAACGT  TTTCCAATGA  TGAGCACTTT  TAAAGTTCTG  CTATGTGGCG  CGGTATTATC
8221  CCGTATTGAC  GCCGGGCAAG  AGCAACTCGG  TCGCCGCATA  CACTATTCTC  AGAATGACTT
8281  GGTTGAGTAC  TCACCAGTCA  CAGAAAAGCA  TCTTACGGAT  GGCATGACAG  TAAGAGAATT
8341  ATGCAGTGCT  GCCATAACCA  TGAGTGATAA  CACTGCGGCC  AACTTACTTC  TGACAACGAT
8401  CGGAGGACCG  AAGGAGCTAA  CCGCTTTTTT  GCACAACATG  GGGGATCATG  TAACTCGCCT
8461  TGATCGTTGG  GAACCGGAGC  TGAATGAAGC  CATACCAAAC  GACGAGCGTG  ACACCACGAT
8521  GCCTGTAGCA  ATGGCAACAA  CGTTGCGCAA  ACTATTAACT  GGCGAACTAC  TTACTCTAGC
8581  TTCCCGGCAA  CAATTAATAG  ACTGGATGGA  GGCGGATAAA  GTTGCAGGAC  CACTTCTGCG
8641  CTCGGCCCTT  CCGGCTGGCT  GGTTTATTGC  TGATAAATCT  GGAGCCGGTG  AGCGTGGGTC
8701  TCGCGGTATC  ATTGCAGCAC  TGGGGCCAGA  TGGTAAGCCC  TCCCGTATCG  TAGTTATCTA
8761  CACGACGGGG  AGTCAGGCAA  CTATGGATGA  ACGAAATAGA  CAGATCGCTG  AGATAGGTGC
8821  CTCACTGATT  AAGCATTGGT  AACTGTCAGA  CCAAGTTTAC  TCATATATAC  TTTAGATTGA
```

FIG. 9B-3

```
8881 TTTAAAACTT CATTTTTAAT TTAAAAGGAT CTAGGTGAAG ATCCTTTTTG ATAATCTCAT
8941 GACCAAAATC CCTTAACGTG AGTTTTCGTT CCACTGAGCG TCAGACCCCG TAGAAAAGAT
9001 CAAAGGATCT TCTTGAGATC CTTTTTTTCT GCGCGTAATC TGCTGCTTGC AAACAAAAAA
9061 ACCACCGCTA CCAGCGGTGG TTTGTTTGCC GGATCAAGAG CTACCAACTC TTTTTCCGAA
9121 GGTAACTGGC TTCAGCAGAG CGCAGATACC AAATACTGTT CTTCTAGTGT AGCCGTAGTT
9181 AGGCCACCAC TTCAAGAACT CTGTAGCACC GCCTACATAC CTGGCTCTGC TAATCCTGTT
9241 ACCAGTGGCT GCTGCCAGTG GCGATAAGTC GTGTCTTACC GGGTTGGACT CAAGACGATA
9301 GTTACCGGAT AAGGCGCAGC GGTCGGGCTG AACGGGGGGT TCGTGCACAC AGCCCAGCTT
9361 GGAGCGAACG ACCTACACCG AACTGAGATA CCTACAGCGT GAGCTATGAG AAAGCGCCAC
9421 GCTTCCCGAA GAGAGAAAGG CGGACAGGTA TCCGGTAAGC GGCAGGGTCG GAACAGGAGA
9481 GCGCACGAGG GAGCTTCCAG GGGGAAACGC CTGGTATCTT TATAGTCCTG TCGGGTTTCG
9541 CCACCTCTGA CTTGAGCGTC GATTTTTGTG ATGCTCGTCA GGGGGGCGGA GCCTATGGAA
9601 AAACGCCAGC AACGCGGCCT TTTTACGGTT CCTGGCCTTT TGCTGGCCTT TTGCTCACAT
9661 GTTCTTTCCT GCGTTATCCC CTGATTCTGT GGATAACCGT ATTACCGCCT TTGAGTGAGC
9721 TGATACCGCT CGCCGCAGCC GAACGACCGA GCGCAGCGAG TCAGTGAGCG AGGAAGCGGA
9781 AGAGCGCCCA ATACGCAAAC CGCCTCTCCC CGCGCGTTGG CCGATTCATT AATGCAGCTG
9841 GCACGACAGC TTTCCCGACT GGAAAGCGGG CAGTGAGCGC AACGCAATTA ATGTGAGTTA
9901 GCTCACTCAT TAGGCACCCC AGGCTTTACA CTTTATGCTT CCGGCTCGTA TGTTGTGTGG
9961 AATTGTGAGC GGATAACAAT TTCACACAGG AAACAGCTAT GACCATGATT ACGCCAAGCG
10021 CGCAATTAAC CCTCACTAAA GGGAACAAAA GCTGGAGCTG CAAGCTT
```

FIG. 10

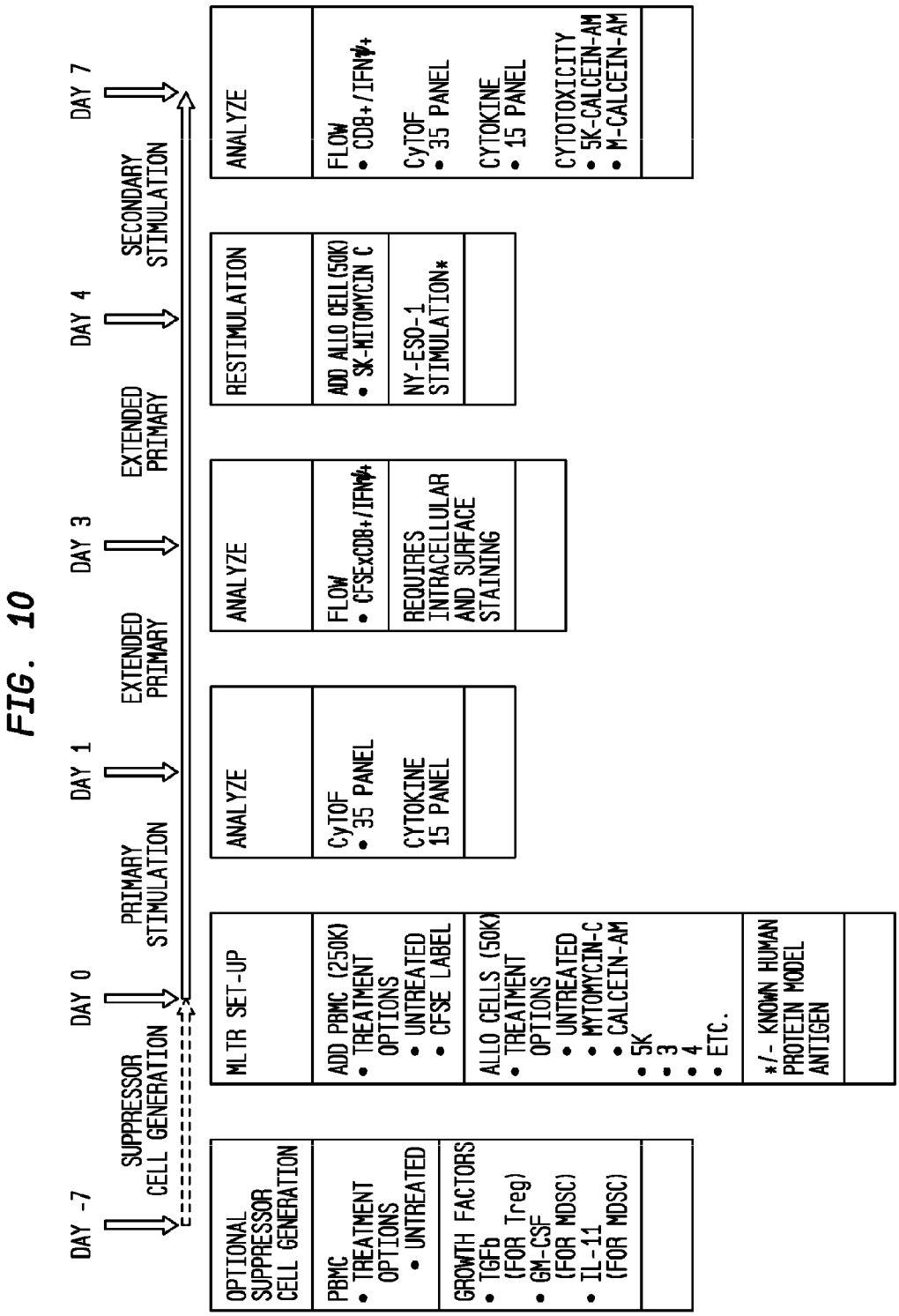
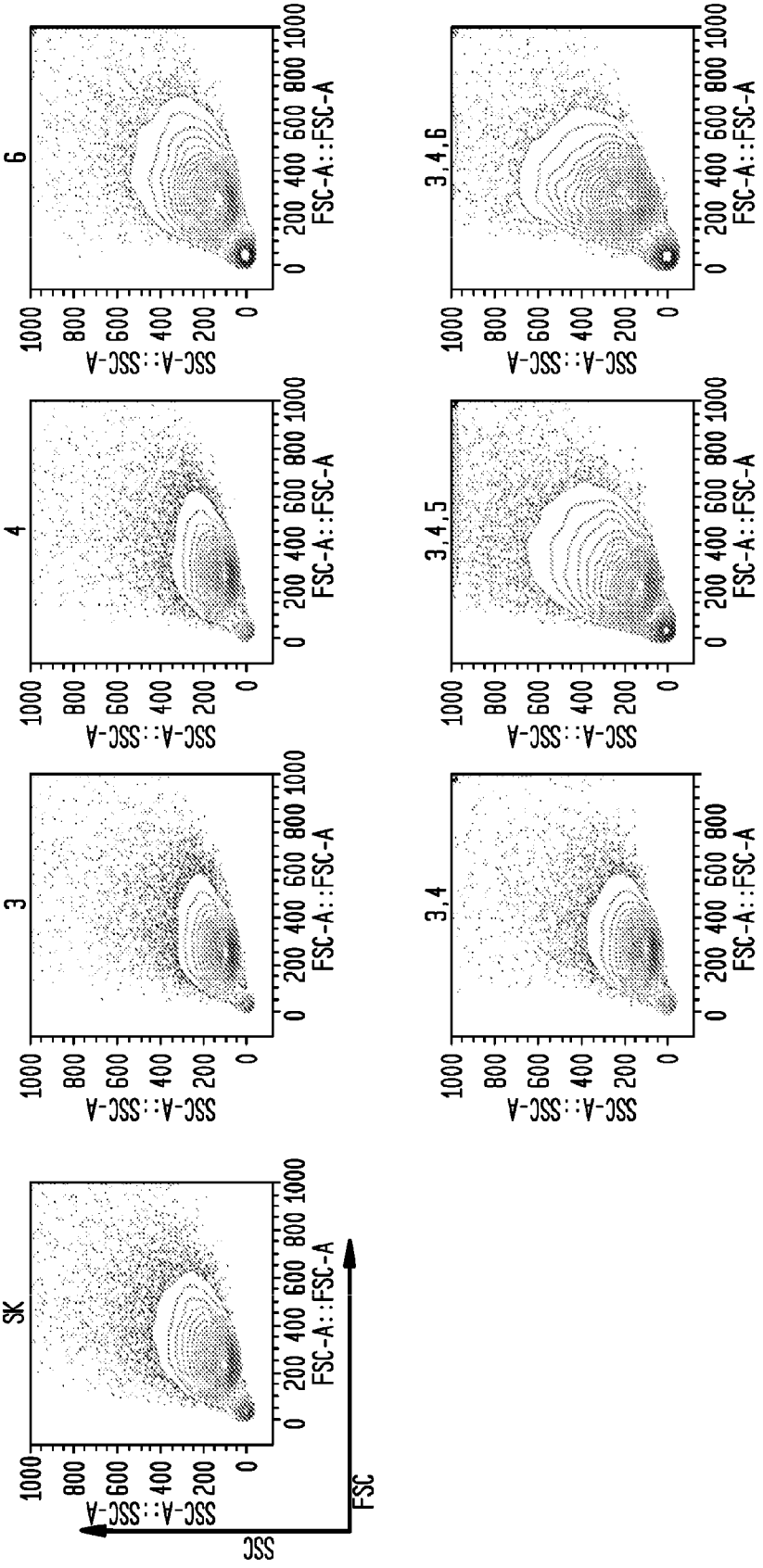

DAY -7
DAY 0
DAY 1
DAY 3
DAY 4
DAY 7
SUPPRESSOR CELL GENERATION
PRIMARY STIMULATION
EXTENDED PRIMARY
EXTENDED PRIMARY
SECONDARY STIMULATION
OPTIONAL SUPPRESSOR CELL GENERATION
PBMC
• TREATMENT OPTIONS
• UNTREATED
GROWTH FACTORS
• TGFb (FOR Treg)
• GM-CSF (FOR MDSC)
• IL-11 (FOR MDSC)
MLTR SET-UP
ADD PBMC (250K)
• TREATMENT OPTIONS
• UNTREATED
• CFSE LABEL
ALLO CELLS (50K)
• TREATMENT OPTIONS
• UNTREATED
• MYTOMYCIN-C
• CALCEIN-AM
• 5K
• 3
• 4
• ETC.
*/- KNOWN HUMAN PROTEIN MODEL ANTIGEN
ANALYZE
CyTOF
• 35 PANEL
CYTOKINE
15 PANEL
ANALYZE
FLOW
• CFSExCD8+/IFN
REQUIRES INTRACELLULAR AND SURFACE STAINING
RESTIMULATION
ADD ALLO CELL (50K)
• SK-MITOMYCIN C
NY-ESO-1 STIMULATION*
ANALYZE
FLOW
• CD8+/IFN
CyTOF
• 35 PANEL
CYTOKINE
• 15 PANEL
CYTOTOXICITY
• 5K-CALCEIN-AM
• M-CALCEIN-AM

FIG. 11


SK
3
4
6
3,4
3,4,5
3,4,6
SSC-A::SSC-A
FSC-A::FSC-A
SSC
FSC
0
200
400
600
800
1000

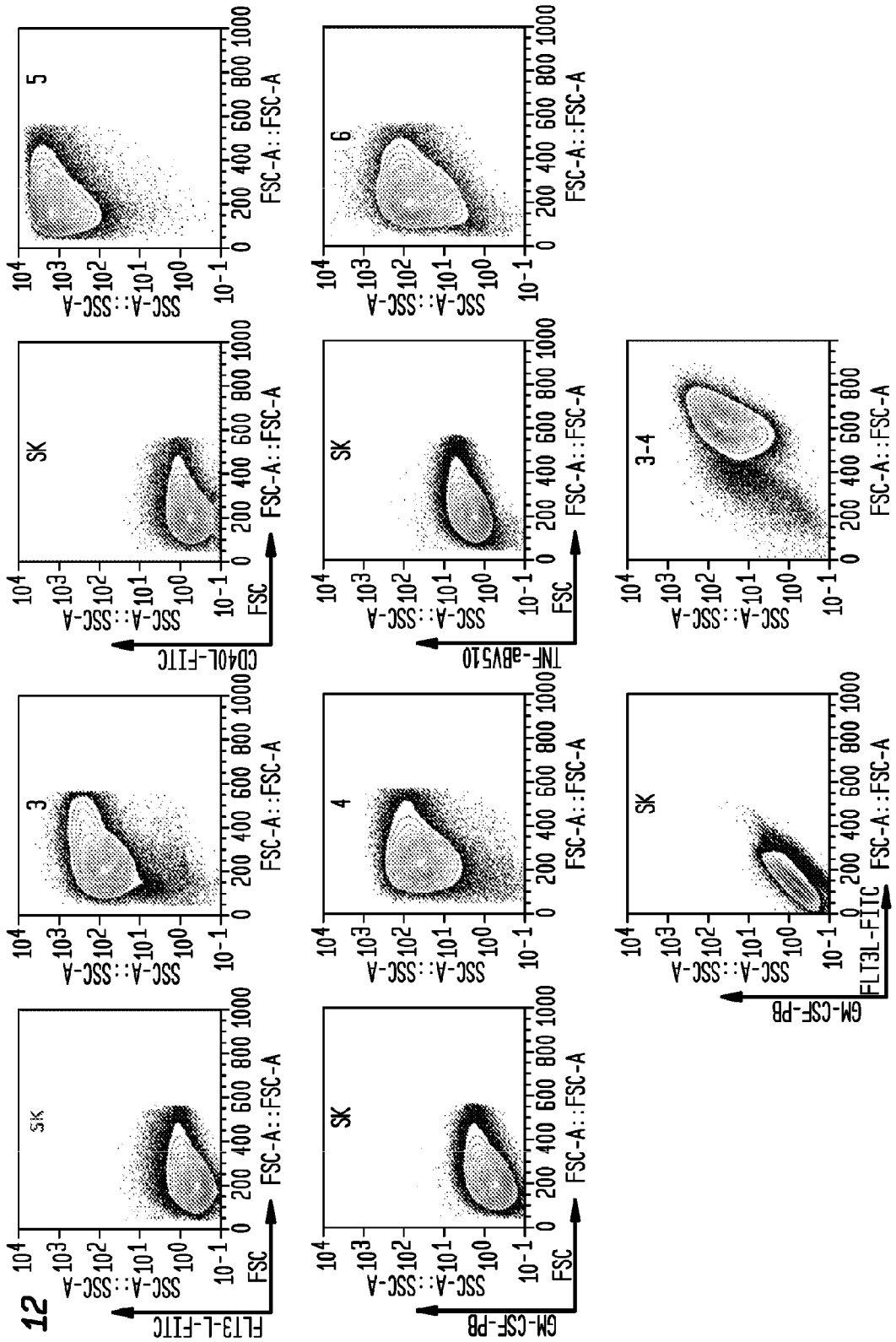
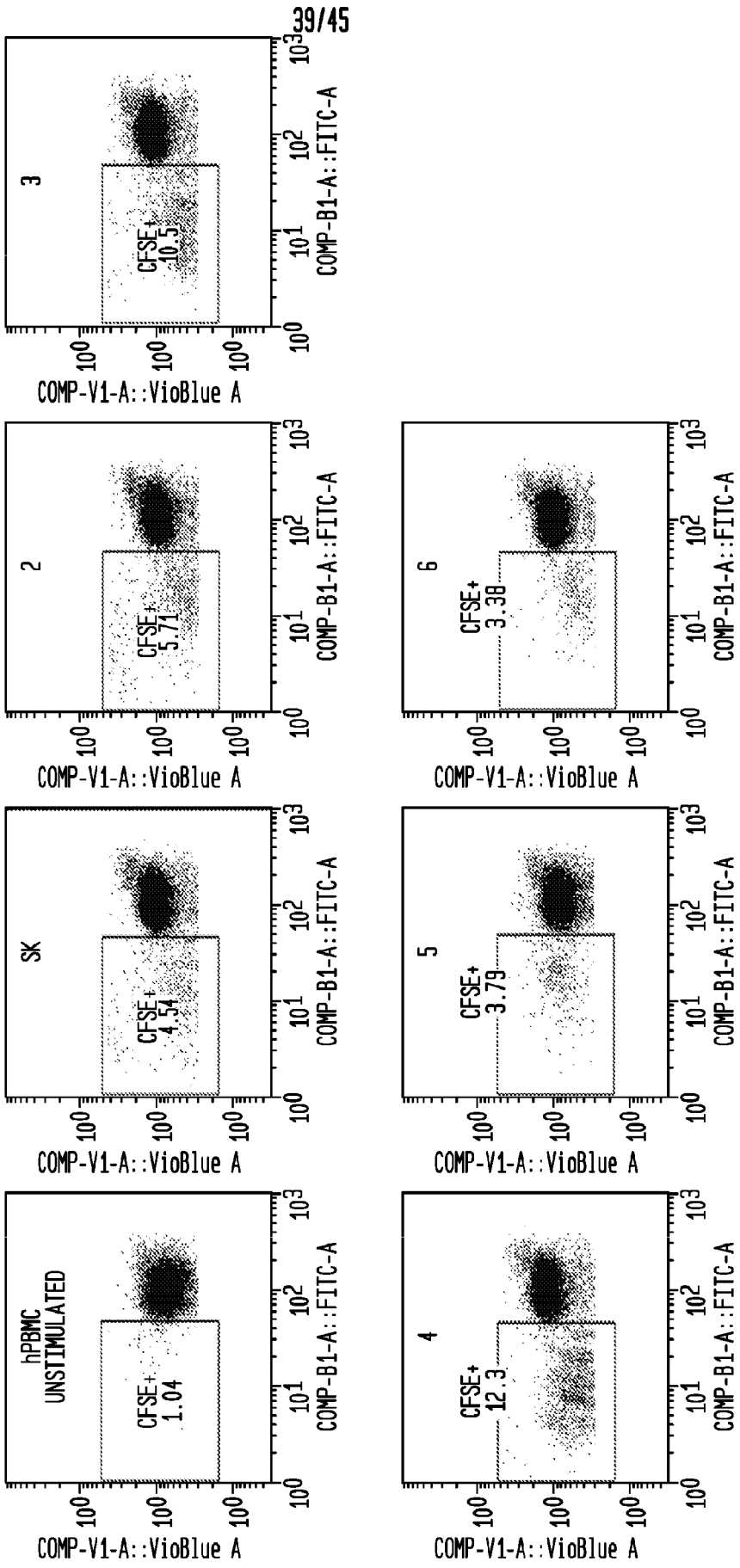

SK
FLT3L-FITC
GM-CSF-PB
3
4
SK
SK
CD40L-FITC
TNF-aBV510
5
6
3-4
SSC-A::SSC-A
FSC-A::FSC-A
FSC

FIG. 12

FIG. 13


hPBMC
UNSTIMULATED
CFSE+
1.04
SK
CFSE+
4.54
2
CFSE+
5.71
3
CFSE+
10.5
4
CFSE+
12.3
5
CFSE+
3.79
6
CFSE+
3.38
COMP-V1-A::VioBlue A
COMP-B1-A::FITC-A

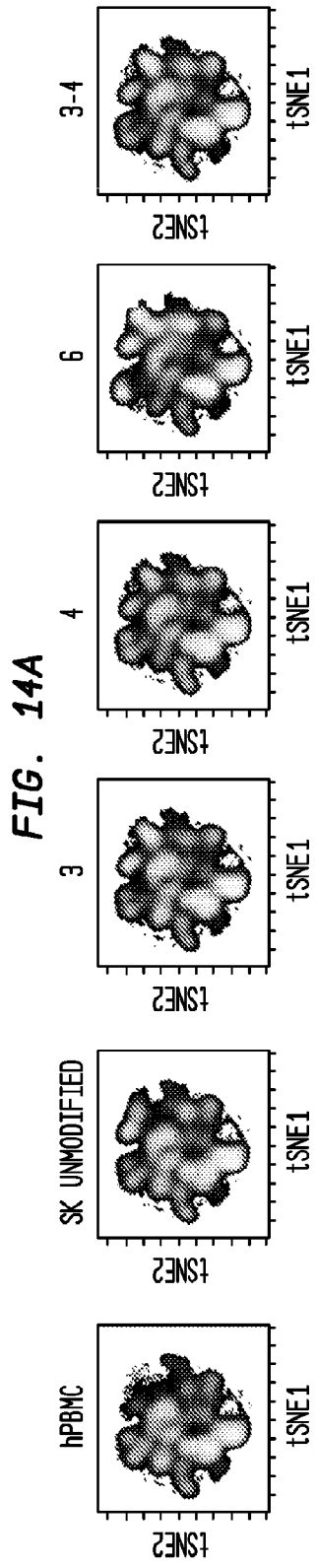


FIG. 14A

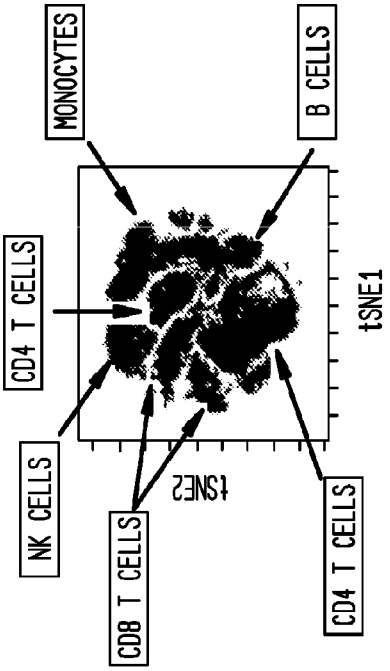


FIG. 14B

FIG. 15A

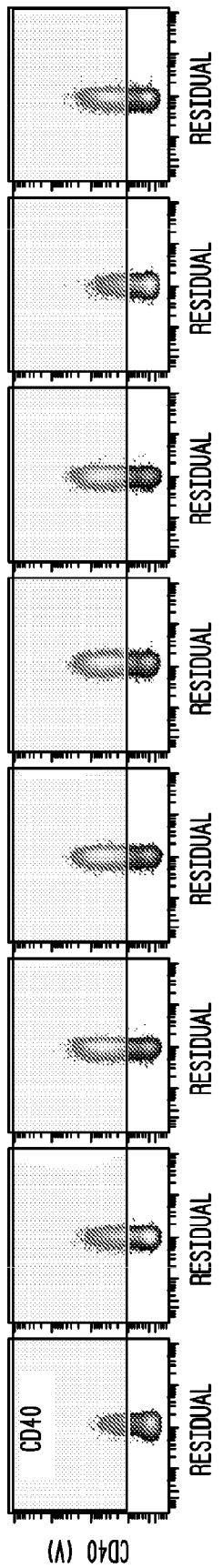
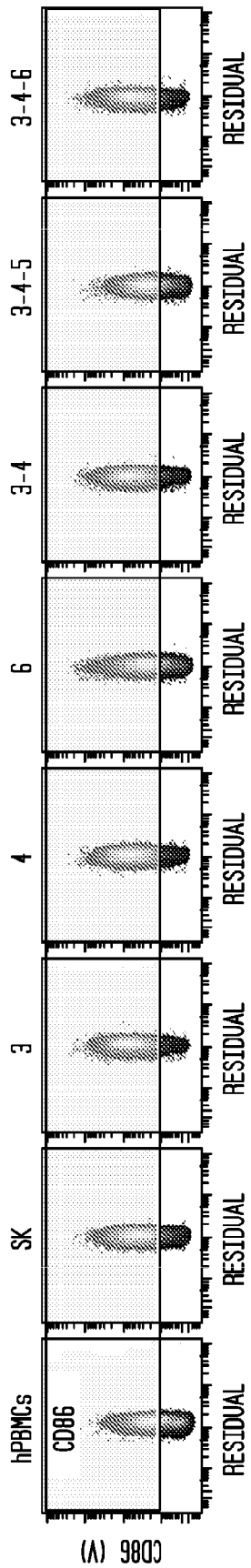


FIG. 15B



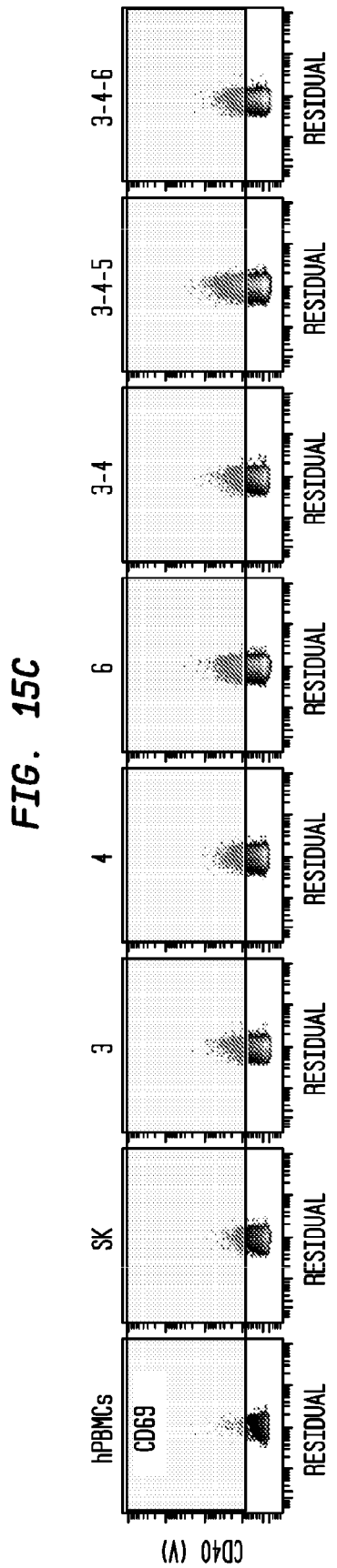


FIG. 15C

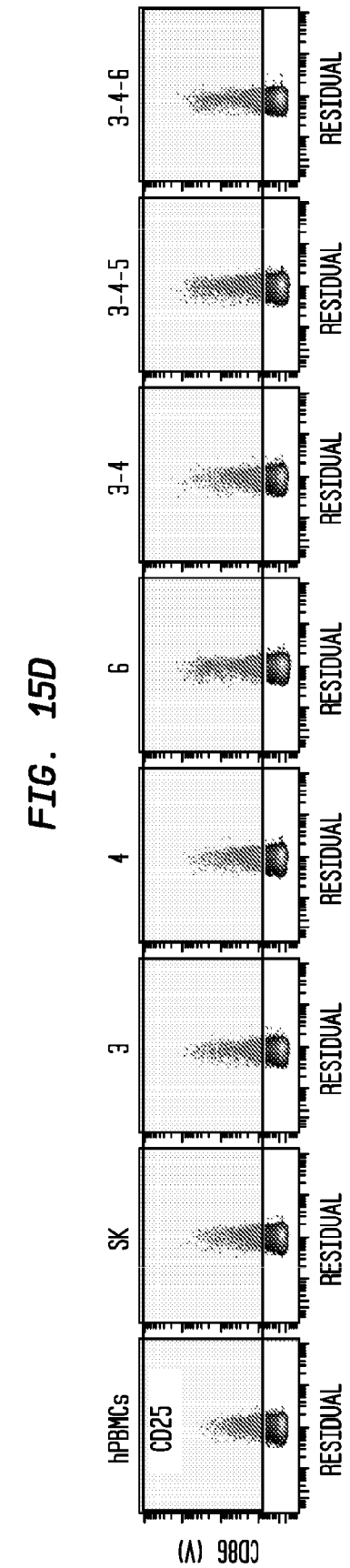


FIG. 15D

FIG. 15E

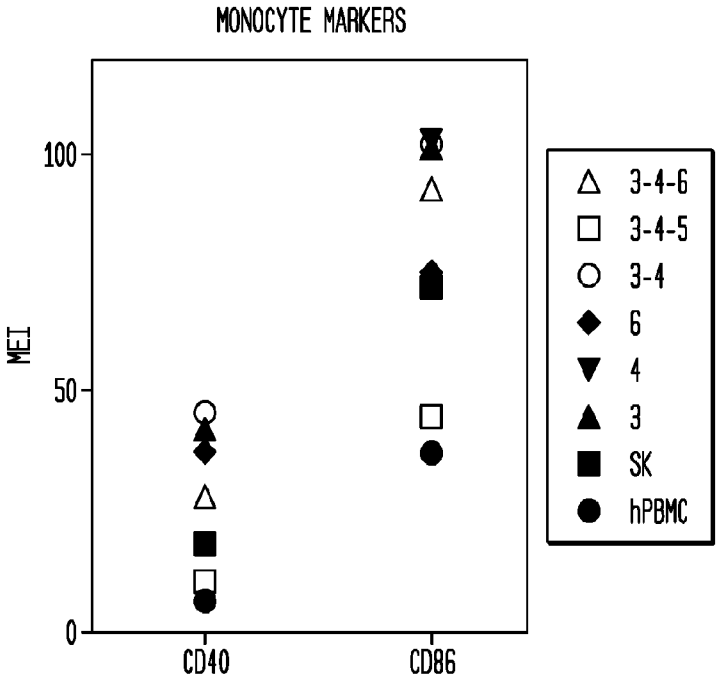

MONOCYTE MARKERS
MEI
100
50
0
CD40
CD86
3-4-6
3-4-5
3-4
6
4
3
SK
hPBMC

FIG. 15F

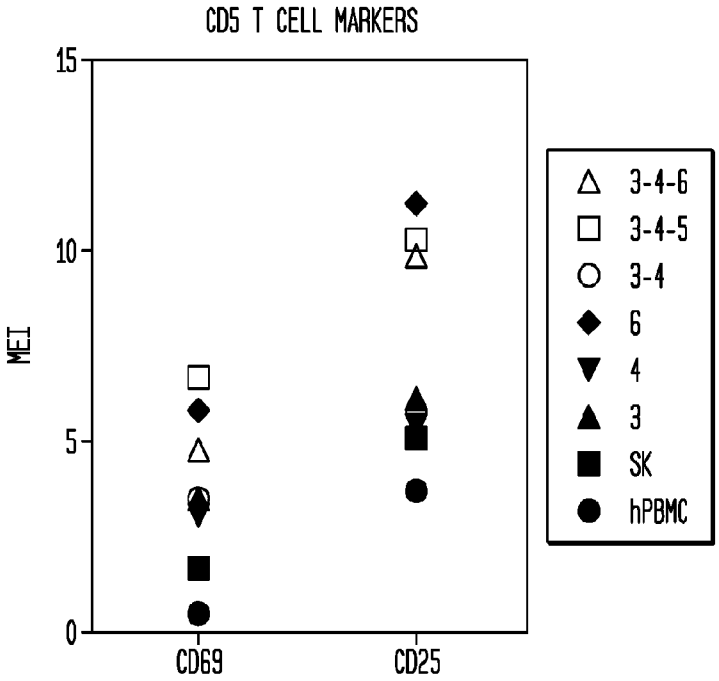
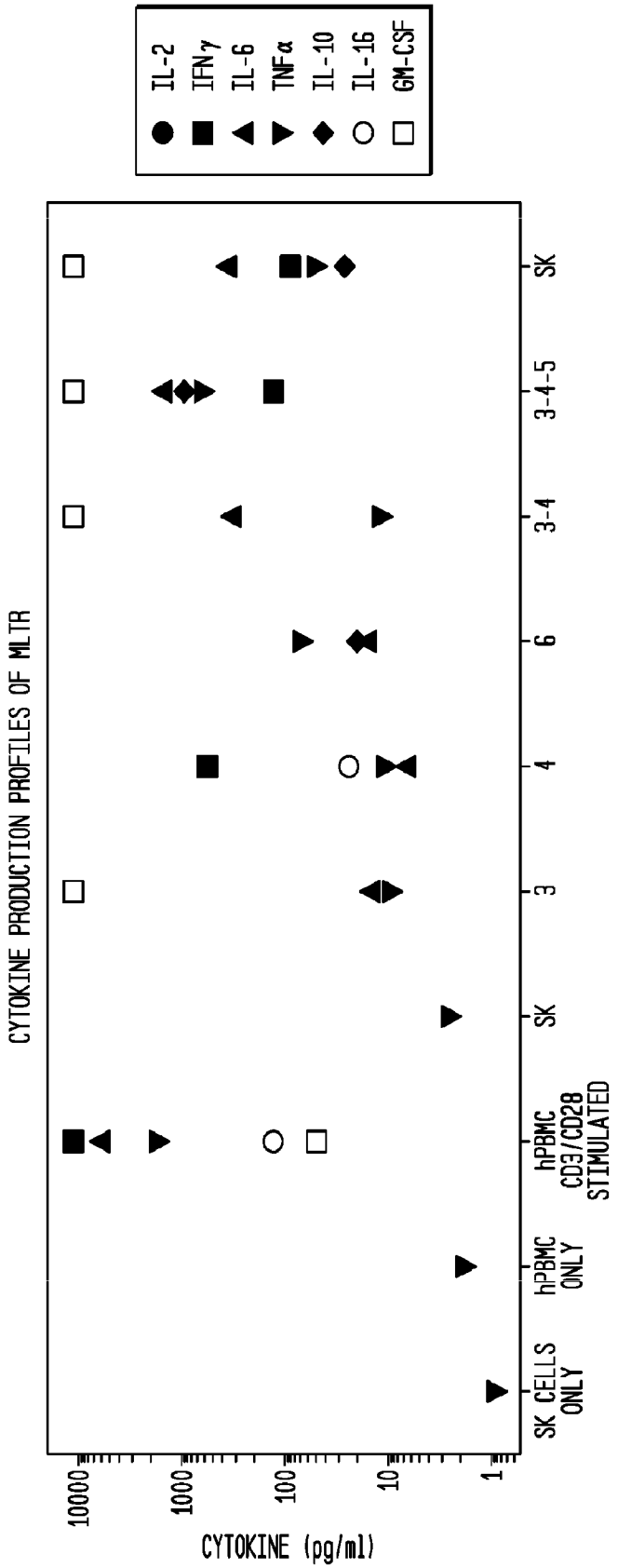

CD5 T CELL MARKERS
MEI
15
10
5
0
CD69
CD25
3-4-6
3-4-5
3-4
6
4
3
SK
hPBMC

FIG. 16


CYTOKINE PRODUCTION PROFILES OF MLTR
IL-2
IFNγ
IL-6
TNFα
IL-10
IL-16
GM-CSF
CYTOKINE (pg/ml)
10000
1000
100
10
1
SK CELLS ONLY
hPBMC ONLY
hPBMC CD3/CD28 STIMULATED
SK
3
4
6
3-4
3-4-5
SK