Abstract:
The described invention provides an immunopotent tumor-specific cancer cell product containing an immunogenic composition containing an immunostimulatory amount of an isolated population of dendritic cells contacted ex vivo with a cancer-specific cancer stem cell derived from a tumor cell, a tumor cell cluster, a tumor cell aggregate or a combination thereof expressing at least one cancer-specific antigen, the tumor cell, tumor cell cluster, tumor cell aggregate or the combination thereof being derived from peripheral blood; and an adjuvant; and a pharmaceutically acceptable carrier; as well as methods for preparing and using same.
IMMUNOGENIC COMPOSITIONS PREPARED FROM TUMOR CELLS DERIVED FROM PERIPHERAL BLOOD AND ORIGINATING FROM A SOLID TUMOR AND THEIR USE

CROSS-REFERENCE TO RELATED APPLICATION
[0001] This application claims the benefit of priority to U.S. Provisional Application No. 62/081,419, filed November 18, 2014, entitled "IMMUNOGENIC COMPOSITIONS PREPARED FROM TUMOR CELLS DERIVED FROM PERIPHERAL BLOOD AND ORIGINATING FROM A SOLID TUMOR AND THEIR USE", the content of which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION
[0002] The described invention generally relates to autologous cancer vaccines comprising dendritic cells contacted with at least one cancer-specific antigen.

BACKGROUND OF THE INVENTION

Immune response
[0003] Immune responses are initiated by an individual's encounter with a foreign antigenic substance/immunogen, for example, an infectious agent. The individual rapidly responds with the production of antibody molecules specific for epitopes of the immunogen and with the expansion and differentiation of antigen-specific regulatory and effector T-lymphocytes. The latter include cells that produce cytokines and killer T cells capable of lysing the infected cells. Generally this initial immune response is sufficient to control and eradicate the foreign substance.

generally consists of an enhanced antibody and T-cell response. This is the basis of vaccination.

**Cells of the immune system**

[0005] The immune system consists of lymphocytes, which are the cells that determine the specificity of immunity, and cells that interact with lymphocytes, which play roles in the presentation of antigen and in the mediation of immunologic functions. These cells include the monocyte/macrophages, dendritic cells and 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. Such cells 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).

[0006] Individual lymphocytes are specialized in that they are committed to respond to a limited set of structurally related antigens. This commitment, which exists before the first contact of the immune system with a given antigen, is expressed by the presence on the lymphocyte’s surface membrane of receptors specific for determinants (epitopes) on the antigen. Each lymphocyte possesses a 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).
[0007] 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**

[0008] 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. Naïve B cells are distinguished in that they do not express the marker CD27, while antigen-specific B cells do express CD27 (Perez-Andres et al. 2010) Cytometry Part B 78B (Suppl. 1) S47-S60). The 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. Antibody responses to most protein antigens are dependent on helper T cells. 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)).

[0009] 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)).

[0010] 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)).

[0011] 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 the 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., 97(9): 2063-2073 (1996)).

T-lymphocytes
[001.2] T-lymphocytes derive 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 their 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)).

[001.3] 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. Antibody and B-cell receptors evolved to bind to and to protect against microorganisms in extracellular fluids. In contrast, 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 antigen-presenting cells 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 antigen-presenting cell (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 antigen-presenting cell (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).

[0014] 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 sublineages: 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.

[0015] 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.

[0016] T cells also mediate 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.

[0017] 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)).

[0018] 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. The CD4+ T cells recognize only peptide/class II complexes

[0019] The TCR's ligand (i.e., the peptide/MHC protein complex) is created within antigen-presenting cells (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)).

[0020] 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 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)).

[0021] 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**

[0022] 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)).

[0023] 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

[0024] CD4+ T cells tend to differentiate into cells that principally secrete the cytokines IL-4, IL-5, IL-6, and IL-10 (TH2 cells) or into cells that mainly produce IL-2, IFN-γ, and lymphotoxin (TH1 cells). The TH2 cells are very effective in helping B-cells develop into antibody-producing cells, whereas the TH1 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 the CD4+ T cells with the phenotype of TH2 cells (i.e., IL-4, IL-5, IL-6 and IL-10) are efficient helper cells, TH1 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 cells involved in Induction of Cellular Immunity**

[0025] 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. The TH1 cells are effective in enhancing the microbicidal action because they produce IFN-γ. By contrast, two of the major cytokines produced by TH2 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)).

**Suppressor or Regulatory T (Treg) cells**
A controlled balance between initiation and downregulation of the immune response is important to maintain immune homeostasis. Mechanisms that contribute to the downregulation of the immune response include both apoptosis and T cell anergy (a tolerance mechanism in which the T cells are intrinsically functionally inactivated following an antigen encounter (Schwartz, R. H., "T cell anergy," Annu. Rev. Immunol., 21: 305-334 (2003)). 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 435: 598-604 (2005)). CD4+ Tregs that constitutively express the IL-2 receptor alpha (IL-2Ra) 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., 31: 1122-1131 (2001)). Depletion of CD4+CD25+ Tregs results in systemic autoimmune disease in mice; 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 (CD25high) and nonsuppressive (CD25low) 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., 177: 8338-8347 (200)).

Cytotoxic T Lymphocytes (CTL)

The 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 a series of enzymes produced by activated CTLs, referred to as granzymes. 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. The elements of the immune system include cellular immunity, humoral immunity, and the complement system.

**Natural Killer (NK cells)**

[0028] NK cells belong to the lymphoid lineage. Although NK cells are not configured to recognize specific target antigens, in the way that T cells are configured to recognize target antigens, the ability of NK cells to bind to the constant region of antibodies enables NK cells to specifically kill the cells that are tagged with antibodies. The NK cell’s recognition of the constant region of antibodies is mediated by the Fc receptor (of the NK cell) binding to the Fc portion of the antibody. This type of killing is called, antibody-dependent cell cytotoxicity (ADCC). NK cells can also kill cells independent of the mechanism of ADCC, where this killing requires expression of MHC class I to be lost or deficient in the target cell (see, e.g., Caligiuri (2008) Blood 112:461-469). NK cells have been reported to mediate cytotoxicity against cancer stem cells (see, e.g., Jewett and Tseng (2011) J. Cancer. 2:443-457).

**Antigen presenting cells**

[0029] Antigen presenting cells (APCs) are cells of the immune system used for presenting antigen to T cells. APCs include dendritic cells, monocytes, macrophages, marginal zone Kupffer cells, microglia, Langerhans cells, T cells, and B cells (see, e.g., Rodriguez-Pinto and Moreno (2005) Eur. J. Immunol. 35:1 097-1105). Antigen-presenting cells 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-

Dendritic cells

[0030] Dendritic cells are discrete leukocyte population(s) of antigen presenting cells that initiate specific T-lymphocyte activation and proliferation. Their key properties include (1) the ability to take up, process, and present antigen; (2) the ability to migrate selectively through tissues; and (3) the ability to interact with, stimulate and direct T-lymphocyte responses. Hart, DNJ, Blood 90(9): 3245-87, 3245 (1997). The encounter with an antigen induces the dendritic cell to mature from an antigen-capturing cell to an antigen-presenting cell that can activate T cells. Alberts, B. et al., Molecular Biology of the Cell, 4th Ed., Garland Science, NY (2002), p. 1394.


[0032] They efficiently capture and take up antigens in peripheral tissues and transport these antigens to the primary and secondary lymphoid organs where they express high levels of MHC class I and II molecules that present the processed peptides to T-cells for the priming of antigen-specific responses. Specifically, a DC acquires polypeptide antigens, where these antigens can be acquired from outside of the DC, or biosynthesized inside of the DC by an infecting organism. The DC processes the polypeptide, resulting in peptides of about ten amino acids in length, transfers the peptides to either MHC class I or MHC class II to form a complex, and
shuttles the complex to the surface of the DC. When a DC bearing a MHC class I/peptide complex contacts a CD8+ T-cell, the result is activation and proliferation of the CD8+ T-cell. Regarding the role of MHC class II, when a DC bearing a MHC class II/peptide complex contacts a CD4+ T-cell, the outcome is activation and proliferation of the CD4+ T-cell (Munz, et al. (2010) Curr. Opin. Immunol. 22:89-93; Monaco (1995) J. Leukocyte Biol. 57:543-547; Robinson, et al (2002) Immunology 105:252-262). Although dendritic cells presenting antigen to a T-cell can "activate" that T-cell, the activated T-cell might not be capable of mounting an effective immune response. Effective immune response by the CD8+ T-cell, for example, often requires prior stimulation of the DC by one or more of a number of interactions. These interactions include direct contact of a CD4+ T-cell to the DC (by way of contact of the CD4+ T-cell's CD40 ligand to the DCs CD40 receptor), or direct contact of a toll-like receptor (TLR) agonist to one of the dendritic cell's toll-like receptors (TLRs).

[0033] In exemplary implementations, the described invention provides agents that, alone or in combination with a dendritic cell vaccine, can stimulate an immune response against tumor antigens; agents that can deplete T regulatory cells (Tregs); agents that can enhance activity of CD8+ T cells or CD4+ T cells, and other agents that modulate the immune system. It encompasses reagents and methods for activating dendritic cells (DCs), with one or more immune adjuvants, such as a toll-like receptor (TLR) agonist, e.g., CpG-oligonucleotide (TLR9), imiquimod (TLR7), poly(I:C) (TLR3), glucopyranosyl lipid A (TLR4), murein (TLR2), flagellin (TLR5), as well as an adjuvant such as CD40 agonists, e.g., CD40-ligand, or the cytokine, interferon-gamma, prostaglandin E2, and the like. See, e.g., U.S. Pat. No. 7,993,659 issued to Noelle et al; US 7,993,648 issued to Kedl et al; US 7,935,804 issued to Dubensky et al, each of which is incorporated herein by reference in its entirety, in vitro treatment of DCs with one or more of the above adjuvant reagents, and/or administration of the adjuvant to a human subject, animal subject, or veterinary subject.
[0034] What is provided are, for example, toll-like receptor (TLR) agonists. For example, the imidazoquinolines imiquimod and resiquimod are TLR7 or TLR8 agonists. Stimulation of TLR7 of plasmacytoid dendritic cells results in expression of IFN-alpha. Stimulation of TLR7 of myeloid dendritic cells results in expression of interleukin-12, and a consequent Th1-type immune response. CpG oligonucleotides (CpG ODNs) are TLR9 agonists. CpG ODNs occur in three classes, CpG-A, CpG-B, and CpG-C. CpG-A stimulates NK cells, owing to its IFN-alpha producing effect on plasmacytoid DCs. CpG-B induces IFN-alpha, and upregulates co-stimulatory markers on pDCs and B cells. TLR3 agonists include polyriboinosinic-polyribocytidylic acid (poly I:C), which is an analogue of viral double stranded RNA (dsRNA). TLR4 agonists include monophosphoryl lipid A (MPL), which is a derivative of Salmonella minnesota lipopolysaccharide, and which is used as part of a vaccine against human papillomavirus. Attenuated bacteria are used in anti-cancer therapy. Mycobacterium bovis stimulates TLR2, and TLR4, and TLR9. Listeria monocytogenes stimulates various TLRs (US 2007/0207171 of Dubensky et al, which is incorporated herein by reference, in its entirety). See also, Galluzzi et al (2012) Oncoimmunology. 1:699-716; Adams (2009) Immunotherapy. 1:949-964.

[0035] Alpha-galactosylceramide (alpha-GalCer) is also provided (Schwaab and Ernstoff (2011) Therapy. 4:369-377). Alpha-GalCer provokes the activation of NKT cells that express certain T cell receptors (Lopez-Sagaseta et al (2012) PLoS Biol. 10:e1001412 (11 pages)). What is also provided are agonists of nucleotide-binding and oligomerization domain (NOD) receptors. NOD receptors include NOD1 and NOD2, which recognize distinct motifs of peptidoglycan (PGN). NOD agonists include N-acetylmuramyl-L-alanyl-D-isoglutamine (muramylidipeptide (MDP)), which binds to NOD2. NOD agonists include gamma-D-glutamyl-meso-diaminopimelic acid (iE-DAP), which binds to NOD1. NOD agonists include desmuramylpeptides (DMP), which binds to NOD1. See, e.g., Uehara et al (2006) J. Immunol. 177:1796-1804). NOD agonists are derived from fragments of peptidoglycan.

[0037] Lymphocyte activation gene-3 (LAG-3) blocking agents, such as anti-LAG-3 antibodies or soluble LAG-3 (e.g., LAG-3 Ig), can enhance immune response to cancers or infections. Anti-LAG-3 antibodies reduce the activity of Tregs (see, e.g., Huang, et al. (2004) Immunity 21:503-513; Triebel (2003) Trends Immunol. 24:61 9-622).


[0039] In addition, DCs express high levels of co-stimulatory molecules. The trafficking of immature DCs to sites of inflammation and or mature DCs to the T-cell area of secondary lymphoid organs is regulated by the expression of different
chemokines and chemokine receptors (Rubio M T, et al., International Immunology (2005) 17(1 2): 1561 ·1572; Sallusto F and Lanzavecchia A, Immunol. Rev. (2000) 177: 134-1 40; Dieu, M, et al., J. Ex. Med. (1998) 188: 373-386; Sallusto F, et al., Eur. J. Immunol. (1999) 29: 1617-1 625). Immature DCs express inflammatory chemokines (monocyte chemoattractant protein-1 (CCL2/MCP-1 ), macrophage inflammatory protein-1 a (CCL3/MIP-1 a), macrophage inflammatory protein-1 β (CCL4/MIP-1 β), regulated on activation, normal T-cell expressed and secreted (CCL5/RANTES), macrophage inflammatory protein-3a (CCL20/MIP-3a)) and chemokine receptors that bind to inflammatory chemokines (chemokine (C-C motif) receptor 1 (CCR1 ), chemokine (C-C motif) receptor 2 (CCR2), chemokine (C-C motif) receptor 5 (CCR5), chemokine (C-C motif) receptor 6 (CCR6) and chemokine (C-X-C motif) receptor 1 (CXCR1 )). Upon maturation, DCs down-regulate the inflammatory chemokines and their receptors and up-regulate constitutive chemokines such as interferon-y-inducible protein 10 (CXCL1 0/IP-1 0), thymus and activation regulated chemokine (CCL1 7/TARC), pulmonary and activation regulated chemokine (CCL1 8/PARC), macrophage inflammatory protein-3p (CCL1 9/MIP-3β), macrophage derived chemokine (CCL22/MDC), the chemokine receptor chemokine (C-C motif) receptor 7 (CCR7) and secondary lymphoid-tissue chemokine (CCL21/SLC) (Rubio M T, et al., International Immunology (2005) 17(1 2): 1561 ·1572).

[0040] The co-stimulatory chemokines expressed by mature DCs function to attract T-cells and to interact with receptors on T-cells to provide a second signal required to optimally activate antigen-specific T-cells (Dermime S, et al., British Medical Bulletin (2002) 62: 149-1 62; June C, et al., Immunol. Today (1994) 15:321 ·331 ). DCs have been shown to attract T helper 1 (Th1 ) and T helper 2 (Th2) cells, as well as naïve T-cells and memory T-cells. T helper cells express CCR4, a G-coupled protein that is the receptor for the chemokines MCP-1 , MIP-1 , RANTES, macrophage-derived chemokine and thymus and activation regulated chemokine (CCL1 7/TARC). DCs are known to express high levels of CCL1 7/TARC, which acts on the CCR4 receptor, and thus attracts and activates downstream immune cells (Vissers J L, et al., J. Leukoc. Biol. (2001 ) 69: 785-793). A particular advantage of
attracting or "recruiting" CD4+ T-cells is that they provide "help" for cross-priming naïve T-cells by expression of CD40L. The expression of CD40L permits DCs to activate CD8+ cytotoxic T-lymphocytes (Schoenberger S P, et al., Nature (1998) 393: 480-483). DCs also produce heterodimer IL-1.2p70, which is the major determinant of their ability to promote Th1 differentiation (Rubio M T, et al., International Immunology (2005) 17(1 2): 1561 -1572; Hilkens C M, et al., Blood (1997) 90: 1920-1 926).

[0041] DCs also express the CD45 antigen, including the CD45 RA, CD45RO and CD45 RC isoforms. Hart, DN, Blood 90(9): 3245-87 (1997), at 3250.


[0044] Dendritic cells differ functionally and phenotypically depending on their stage of activation. Hart, D.N.J. "Dendritic cells: unique leukocyte populations which control the primary immune response," Blood 90: 3245-87 (1997). Immature DCs are efficient at capturing and processing antigens, O'Doherty, U. et al., Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature," Immunology 82: 487-93 (1994). Maturation of DCs induced by inflammatory mediators results in reduced capacity to endocytose antigens and increased levels of adhesion and costimulatory molecules needed to optimal major histocompatibility complex (MHC)-dependent presentation of peptides to cytotoxic lymphocytes (CTLs). Banchereau, J. and Steinman, RM, "Dendritic cells and the control of immunity," Nature 392: 245-52 (1998); Hajek, R. and Butch, AW, "Dendritic cell biology and the application of dendritic cells to immunotherapy of multiple myeloma," Med. Oncol. 17: 215 (2000); Sallusto, F. et al., "Dendritic cells use micropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class I compartment: downregulation by cytokines and bacterial products," J. Exp. Med. 182: 389-400 (1995). Mature DCs express large amounts of MHC class I molecules and lack lineage markers, such as CD14 (monocytes/macrophages); CD3 (T-cells); CD19, CD20 and CD24 (B-cells); CD56 (NK cells); and CD66b (granulocytes). CD1a is preferentially expressed on human immature myeloid DC, whereas CD83 is found on mature DCs which also express high levels of costimulatory molecules (e.g., CD80, CD86, CD40) and adhesion molecules, including CD1a, CD1c, CD50, CD54, CD58 and CD102 Brossart, P. et al., "Dendritic cells in cancer vaccines," Exp. Hematol. 29: 1247-55 (2001).

Immunology of cancer

[0045] Cancer has 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. 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.

[0046] 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

[0047] 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.

**Circulating tumor cells**

[0048] Cancer stem cells (CSCs) circulating within the blood stream are known as circulating tumor cells (CTCs). CTCs express the markers of cancer stem cells (CSCs) that have adopted an epithelial to mesenchymal transition (EMT) for increased mobility and migration. CTCs express biomarkers including, for example, N-cadherin, vimentin, CD44 and fibronectin (Liu et al., Stem Cell Reports, 2014; 2: 78-91; Mani et al., Cell 2008; 4: 704-715).

[0049] Circulating tumor cells are shed by a primary tumor and ultimately cause distant metastases. CTC sampling is minimally invasive, repeatable, prognostic/predictive; and provides information about tumor biology and drug effects. Circulating tumor cells captured from peripheral blood have been shown to predict disease outcome and therapy response in cancer patients.

[0050] A variety of isolation methods have been developed to isolate CTC cells. For example, CTCs have been isolated from blood by methods that rely on immunomagnetic binding of cell surface epithelial cell adhesion molecules. This


[0052] Xu, et al. (Cancer Res. 70 (16): 6420-6 (2010)) reported experiments in which live PC3 and DU145 human prostate cancer cell lines were spiked into 1 mL human blood; the "CTCs" were captured on a 10 μm thick parylene-C slot microfilter and telomerase activity measured. The optimized slot size was 6 μm x 40 μm. Using a constant low-pressure delivery system, the microfilter was capable of cell capture from 1 mL of whole blood in less than 5 minutes, achieving 90% capture efficiency, 90% cell viability, and 200-fold sample enrichment. The captured live cells retained normal morphology by scanning electron microscopy and could be manipulated, further analyzed, or expanded on- or off-filter. Telomerase activity—a recognized cancer marker—was reliably detected by quantitative PCR from as few as 25 cancer cells added into 7.5 mL of whole blood and captured on the microfilter. Significant telomerase activity elevation was also measured from captured live cells processed from patients' blood samples for up to 24 hours from the time of blood draw and from single cancer cells lifted off of the microfilter.

[0053] In breast cancer, it has been reported that clusters of tumor cells that enter the bloodstream form metastases more often than single, circulating tumor cells (Bottos A and Hynes N E, Nature 2014; 514: 309-310).

**Rho Associated Coiled-Coil Kinase (ROCK)**

[0054] Rho associated coiled-coil kinase (ROCK) is a key regulator of actin organization and thus a regulator of cell migration. Rho kinase inhibitor Y-27632, in combination with fibroblast feeder cells, induces normal and tumor epithelial cells
from many tissues to proliferate indefinitely in vitro (Liu, Xuefeng et al. ROCK Inhibitor and Feeder Cells Induce the Conditional Reprogramming of Epithelial Cells The American Journal of Pathology, Volume 180, Issue 2, 599 - 607). ROCK inhibits the de-polymerization of actin filaments indirectly by phosphorylating and activating LIM kinase, which in turn phosphorylates ADF/cofilin, thereby inactivating its actin-de-polymerization activity. This results in the stabilization of actin filaments and an increase in their numbers. The increase in stable actin filaments and the loss of actin monomers contribute to a reduction of cell migration and cell shape definition. (Riento K, Ridley AJ (2003). "Rocks: multifunctional kinases in cell behaviours". Nat Rev Mol Cell Biol 4 (6): 446-56). It has been suggested that ROCK inhibitors, by preventing tumor spreading and blocking cell migration into neighboring tissue, may be useful for treating cancer disease (Hahmann C, Schroeter T (2010). "Rho-kinase inhibitors as therapeutics: from pan-inhibition to isoform selectivity". Cell Mol Life Sci 67 (2): 171-7). Although ROCK inhibitors may be effective in reducing adherent tumor cell behavior, other research indicates that these inhibitors could inadvertently increase metastatic potential of non-adherent CTCs by increasing their reattachment efficacy (Bhandary L, Whipple RA, Vitolo Ml, et al. ROCK inhibition promotes microtentacles that enhance reattachment of breast cancer cells. Oncotarget. 2015; 6(8):6251 - 6266).

**Phosphatase and Tensin Homolog (PTEN)**

[0055] Phosphatase and tensin homolog (PTEN) functions as a tumor suppressor. It acts as a dual-specificity protein phosphatase, dephosphorylating tyrosine-, serine- and threonine-phosphorylated proteins, and as a lipid phosphatase, removing the phosphate in the D3 position of the inositol ring from phosphatidylinositol 3,4,5-trisphosphate, phosphatidylinositol 3,4-diphosphate, phosphatidylinositol 3-phosphate and inositol 1,3,4,5-tetrakisphosphate. ROCK, which is a well-known effector of RhoA, upregulates the activity of PTEN. Activated PTEN downregulates Akt activity, which is essential for cell proliferation. PTEN regulates the phosphatidylinositol 3,4,5-trisphosphate and Akt signaling pathway and consequently modulates two critical cellular processes: cell cycle progression
and cell survival (Sun H et al. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway Proc Natl Acad Sci U S A. 1999 May 25; 96(11):6199-204.;; RhoA-ROCK-PTEN pathway acts as a molecular switch to control cell proliferation and determine anchorage dependence. In cells that are poorly attached to substrates, its inhibition is sufficient to restore cell proliferation without the need for physico-chemical modification of the material surface (Yang S, Kim HM. The RhoA-ROCK-PTEN pathway as a molecular switch for anchorage dependent cell behavior. Biomaterials. 2012 Apr; 33(10):2902-15).

[0056] The described invention provides CTCs isolated from blood for expansion and production of autologous circulating tumor cancer stem cell lines for producing autologous dendritic cell-circulating tumor cell (DC-CTC) vaccines for the treatment of solid tumors.

SUMMARY OF THE INVENTION

[0057] According to one aspect, the described invention provides an immunopotent tumor-specific cancer cell product comprising: an immunogenic composition comprising: an immunostimulatory amount of an activated population of dendritic cells contacted ex vivo with a cancer stem cell population expressing at least one cancer-specific antigen, the cancer stem cell population derived from a tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof derived from peripheral blood and originating from a solid tumor; an adjuvant; and a pharmaceutically acceptable carrier, wherein the immunopotent tumor-specific cancer cell product stimulates an effective immune response against one or more cancer-specific antigens.

[0058] According to another aspect, the described invention provides a method for preparing the tumor-specific cancer cell product according to claim 1, comprising: obtaining a blood sample from a patient comprising a tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof isolated from peripheral blood and originating from a tumor; expanding the tumor cell, tumor cell cluster,
tumor cell aggregate or a combination thereof by: transferring the tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof to an adherent substrate for about 2-4 weeks, wherein the adherent substrate comprises one or more of an Arginine-Glycine-Aspartic Acid (RGD)-rich compound and a serum-free medium; and feeding the cell population comprising the tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof every 2 days with fresh media, wherein the fresh media consists of a basal stem cell formulation supplemented with a protein/growth factor mixture, wherein the growth factor is a fibroblast growth factor (FGF), and epidermal growth factor (EGF), or a combination thereof; dissociating cells adherent to the adherent substrate enzymatically, transferring the dissociated adherent cells to a low adherent or ultra-low adherent surface to produce a cancer-specific stem cell culture comprising cancer stem cell spheroids; maintaining the cancer-specific stem cell culture comprising cancer stem cell spheroids for at least 14 days in a culture medium supplemented with growth factors that act through a MAPK pathway; sedimenting the spheroids to remove the single cells; transferring the spheroids to an adherent substrate; expanding the spheroids in vitro to establish a population of adherent cancer-specific stem cells; inactivating the population of adherent cancer-specific stem cells by irradiation, the irradiated population of adherent cancer-specific stem cells being characterized by an inability to proliferate; cryostoring the irradiated cancer-specific stem cells in vapor phase liquid nitrogen storage; preparing a population of dendritic cells by: obtaining peripheral blood mononuclear cells (PBMCs) by leukapheresis from the patient from whom peripheral blood sample comprising the tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof was obtained; purifying the collected PBMCs from other lymphocytes; incubating the purified PBMCs of step b. with GM-CSF and IL-4 for 6 days to generate the population of dendritic cells; thawing the irradiated cancer stem cells; contacting the population of dendritic cells with the thawed irradiated cancer stem cells for 18-24 hours to form a stimulated dendritic cell-tumor-derived cell antigen composition; and collecting and storing the dendritic cell-tumor-derived cell composition in vapor phase liquid nitrogen.
According to another aspect, the described invention provides a method for treating a cancer patient comprising: preparing for the cancer patient a patient-specific immunopotent tumor-specific cancer cell product comprising an immunostimulatory amount of an isolated population of dendritic cells contacted ex vivo with a population of cancer-specific cancer stem cells expressing a cancer-specific antigen by the method according to claim 1; administering the immunopotent tumor-specific cancer cell product to the cancer patient, wherein the immunogenic composition is effective to generate an immune response comprising activation and proliferation of CD4+ T cells, CD8+ T cells, B cells or a combination thereof; wherein the effective immune response is effective to improve a clinical parameter selected from progression-free survival, disease-free survival, time to progression, time to distant metastasis, overall survival of the subject when compared to a control.

According to some embodiments, the adjuvant is granulocyte-macrophage colony stimulating factor (GM-CSF).

According to some embodiments, the cancer stem cell population expresses at least one cancer-specific antigen with mesenchymal characteristics. According to some embodiments, the cancer stem cell population expresses at least one cancer-specific antigen with embryonic stem cell characteristics. According to some embodiments, the cancer stem cell population expresses at least one cancer-specific antigen with mixed stem cell characteristics.

According to some embodiments, the solid tumor is selected from the group consisting of a colon carcinoma, a renal carcinoma, a glioblastoma multiforme, a hepatocellular carcinoma, an ovarian carcinoma, a breast carcinoma and a prostate carcinoma.

According to some embodiments, the cancer stem cell population expressing at least one cancer-specific antigen is inactivated by irradiation.
According to some embodiments, the immunostimulatory amount comprises at least $1 \times 10^3$ cancer cell-specific antigen-stimulated dendritic cells.

According to some embodiments, the effective immune response comprises an effective T cell response, an effective B cell response, or a combination thereof. According to some embodiments, the effective immune response comprises activation and proliferation of CD4+ T cells, CD8+ T cells, B cells, or a combination thereof.

According to another aspect, the described invention provides a composition comprising (a) an inhibitor selected from the group consisting of a ROCK inhibitor, a PTEN inhibitor and a combination thereof; (b) a growth factor selected from the group consisting of FGF, EGF and a combination thereof; and (c) N-acetyl cysteine, wherein the composition is effective for isolating or purifying circulating tumor cells (CTCs) from a blood sample obtained from a cancer patient.

According to some embodiments, the ROCK inhibitor is selected from the group consisting of Y-27632, Thiazovivin, S1x-21 19, WF-536 [(+)-(R)-4-(1-aminoethyl)-N-(4-pyridyl) benzamide monohydrochloride] , RK1 -1447, 5-(1,4-Diazepane-1-sulfonyl)isoquinoline (Fasudil®), GSK429286A, 3-(4-Pyridyl)-1 H-indole (Rockout®), SR 3677 dihydrochloride, SB 772077B, AS 1892802, H 1152 dihydrochloride, GSK 269962, HA 1100 hydrochloride and Glycyl-H-1 152 dihydrochloride.

According to some embodiments, the PTEN inhibitor is selected from the group consisting of potassium bisperoxo(1,10-phenanthroline)oxovanadate (V) (bpV), SF 1670 and VO-OHpic.

According to some embodiments, the concentration of the ROCK inhibitor in the composition ranges from about 5 $\mu$M to about 20 $\mu$M, i.e., the concentration of the Rock inhibitor is about 5 $\mu$M, 6 $\mu$M, 7 $\mu$M, 8 $\mu$M, 9 $\mu$M, 10 $\mu$M, 11 $\mu$M, 12 $\mu$M, 13 $\mu$M, 14 $\mu$M, 15 $\mu$M, 16 $\mu$M, 17 $\mu$M, 18 $\mu$M, 19 $\mu$M, or 20 $\mu$M.
According to some embodiments, the concentration of the ROCK inhibitor in the composition is about 5 µM.

[0070] According to some embodiments, concentration of the PTEN inhibitor ranges from about 0.01 µM to about 10 µM, i.e., about 0.01 µM, 0.02 µM, 0.03 µM, 0.04 µM, 0.05 µM, 0.06 µM, 0.07 µM, 0.08 µM, 0.09 µM, 1.0 µM, 1.1 µM, 1.2 µM, 1.3 µM, 1.4 µM, 1.5 µM, 1.6 µM, 1.7 µM, 1.8 µM, 1.9 µM, 2 µM, 3 µM, 4 µM, 5 µM, 6 µM, 7 µM, 8 µM, 9 µM, or 10 µM. According to some embodiments, the concentration of the PTEN inhibitor in the composition ranges from about 0.5 µM to about 2 µM, i.e., the concentration of the PTEN inhibitor is about 0.5 µM, 0.6 µM, 0.7 µM, 0.8 µM, 0.9 µM, 1 µM, 1.1 µM, 1.2 µM, 1.3 µM, 1.4 µM, 1.5 µM, 1.6 µM, 1.7 µM, 1.8 µM, 1.9 µM or 2 µM. According to some embodiments, the concentration of the PTEN inhibitor is 1 µM.

[0071] According to some embodiments, the growth factor is epidermal growth factor (EGF) or fibroblast growth factor (FGF). According to some embodiments, the concentration of the growth factor in the composition is 10 ng/mL.

[0072] According to some embodiments, the concentration of N-acetyl cysteine in the composition is 1 mM.

[0073] According to some embodiments, the composition is formulated for deposition in a blood collection tube.

[0074] According to some embodiments, the described invention provides a method for isolating/purifying circulating tumor cells (CTCs) from a blood sample comprising: (a) obtaining a blood sample from a patient comprising a tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof originating from a tumor; (b) exposing the blood sample to the composition; (c) removing mononuclear cells from the blood sample, leaving the tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof;; (d) suspending the tumor cell, the tumor cell
cluster, the tumor cell aggregate or the combination thereof in a serum-free media comprising the composition; (e) exposing the suspended tumor cell, tumor cell cluster, tumor cell aggregate or combination thereof from step (d) to an adherent substrate; (f) dissociating cells adherent to the adherent substrate enzymatically; and (g) expanding the cells from step (f) in vitro to establish a population of adherent circulating tumor cells (CTCs). According to some embodiments, exposing step (b) is performed immediately after obtaining the blood sample. According to some embodiments, removing step (c) is performed by a method selected from the group consisting of density gradient and tangential centrifugation.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0075] Figure 1 shows a schematic of therapeutic targeting of cancer stem cells (CSCs).

[0076] Figure 2 shows a schematic of static electroporation.

[0077] Figure 3 shows the precision and consistency of flow electroporation. 8x1 0⁹ K582 cells were transfected with pGFP DNA using flow electroporation according to manufacturer's instructions. 28 fractions throughout the transfection process were collected and analyzed 24 hrs post transfection for cell viability (% cells excluding propidium iodide) and transfection efficiency (% GFP⁺ cells). A single, small-scale transfection of 4x10⁷ cells using static electroporation also was performed.

[0078] Figure 4 shows overall survival for 54 metastatic melanoma patients treated with patient-specific vaccine of dendritic cells loaded with autologous proliferating tumor cells.

[0079] Figure 5 shows the overall survival for metastatic melanoma patients treated with autologous dendritic cell-tumor cell (DC-TC) vaccine and autologous tumor cell (TC) vaccine.

[0080] Figure 6 shows survival differences between metastatic melanoma patients treated with DC-TC or TC who had no evidence of disease (NED) at the time of treatment.
Figure 7 shows survival differences between metastatic melanoma patients treated with DC-TC or TC who had detectable metastatic disease (Mets) at the time of treatment.

Figure 8 shows a non-adherent culture of melanoma cancer stem cell spheroids isolated using media formulated to select for cancer stem cells.

Figure § shows expression of CD148/CD271 during the production of melanoma tumor cells for use in an autologous DC-TC vaccine.

Figure 10 shows immunohistochemical staining for expression of vimentin and CD44 in melanoma cancer stem cell lines established from bulk tumor samples.

Figure 11 shows a microscopic image of a dendritic cell phagocytosing two melanoma cancer stem cells whose granules are stained for HMB-45 (gp100) melanoma antigens (Dillman R. et al., (2009) Cancer Biotherapy & Radiopharmaceuticals, 24: 311-319).

Figure 12 is a diagram summarizing the spike and retrieval experiment of Example 1.

Figure 13 is a schematic diagram for establishing the Spike/Retrieval (S/R) assay parameter of Example 1.

Figure 14 is a schematic diagram representing the Spike/Retrieval (S/R) procedure using FACS analysis described in Example 1.

Figure 15 is a schematic diagram representing the survival factor cocktail (SFC) optimization procedure described in Examples 2 and 3.

Figure 16 shows a Parylene-C slotted membrane filter used to filter circulating tumor cells (CTCs) by size from other blood cells.

Figure 17 shows clonally expanding CTCs captured on the Parylene-C slotted membrane filter.

Figure 18 shows that cells grown in serum containing media resulted in the expansion of monocyte/macrophage and dendritic cells on the Parylene-C slotted membrane filter.

Figure 19 shows that in serum free conditions, the myeloid lineage cleared, progressively leaving behind colonies of expanding tumor cells.
[0094] **Figure 20** shows tumor cells transferred to regular cell culture flasks separating from contaminant macrophages.

[0095] **Figure 21** shows the phenotypic change of cultured CTCs. Cells losing adherence (round cells) float, allowing for their removal by media exchange.

[0096] **Figure 22** is a schematic depicting the analysis of biological processes affected by exomic mutations in cultured tumor cells. The analysis revealed perturbations in o-glycosylation and in plasma membrane cell adhesion molecules.

[0097] **Figure 23** shows cells exposed to ROCK inhibitor after passage (A) and cells not exposed to ROCK inhibitor after passage (B). Cells exposed to ROCK inhibitor (A) displayed better adherence compared to cells that were not exposed to ROCK inhibitor (B).

[0098] **Figure 24** shows an established cell line from circulating tumor cells (CTCs).

[0099] **Figure 25** shows immunocytochemical characterization of expanded cell populations. Panels a,b: vimentin, Panel c: vimentin nuclear stain; Panels d,e: Magel, Panel f: Magel nuclear counterstain.

[0010] **Figure 26** shows immunocytochemical characterization of expanded cell populations. Panel a: EpCAM/NCAM; Panel b: EpCAM; Panel c: NCAM; Panel d: nuclear stain; Panel e: Vimentin/Snail; Panel f: Vimentin; Panel g: Snail; Panel h: nuclear counterstain.

**DETAILED DESCRIPTION OF THE INVENTION**

**Glossary**

[0011] Various terms used throughout this specification shall have the definitions set out herein.

[0012] 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 Cy1, 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 immunoglobulins (antibodies). The soluble product of an activated T lymphocyte is lymphokines.

[001 03] In exemplary implementations, the present disclosure encompasses reagents and methods for activating dendritic cells (DCs), with one or more immune adjuvants, such as a toll-like receptor (TLR) agonist, e.g., CpG-oligonucleotide (TLR9), imiquimod (TLR7), poly(I:C) (TLR3), glucopyranosyl lipid A (TLR4), murein (TLR2), flagellin (TLR5), as well as an adjuvant such as CD40 agonists, e.g., CD40-ligand, or the cytokine, interferon-gamma, prostaglandin E2, and the like. See, e.g., U.S. Pat. No. 7,993,659 issued to Noelle et al; US 7,993,648 issued to Kedl et al; US 7,935,804 issued to Dubensky et al, each of which is incorporated herein by reference in its entirety. The present disclosure encompasses in vitro treatment of DCs with one or more of the above adjuvant reagents, or in addition, or alternatively,
administration of the adjuvant to a human subject, animal subject, or veterinary subject.

[00104] As used herein, the term "administration" 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. Treatment of a cell encompasses contact of a reagent to the cell, as well as contact of a reagent to a fluid, where the fluid is in contact with the cell. "Administration" also encompasses in vitro and ex vivo treatments, e.g., of a cell, by a reagent, diagnostic, binding composition, or by another cell.

[00105] The term "biomarkers" (or "biosignatures") as used herein refers to peptides, proteins, nucleic acids, antibodies, genes, metabolites, or any other substances used as indicators of a biologic state. It is a characteristic that is measured objectively and evaluated as a cellular or molecular indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. The term "indicator" as used herein refers to any substance, number or ratio derived from a series of observed facts that may reveal relative changes as a function of time; or a signal, sign, mark, note or symptom that is visible or evidence of the existence or presence thereof. Once a proposed biomarker has been validated, it may be used to diagnose disease risk, presence of disease in an individual, or to tailor treatments for the disease in an individual (choices of drug treatment or administration regimes). In evaluating potential drug therapies, a biomarker may be used as a surrogate for a natural endpoint, such as survival or irreversible morbidity. If a treatment alters the biomarker, and that alteration has a direct connection to improved health, the biomarker may serve as a surrogate endpoint for evaluating clinical benefit. Clinical endpoints are variables that can be used to measure how patients feel, function or survive. Surrogate endpoints are
biomarkers that are intended to substitute for a clinical endpoint; these biomarkers are demonstrated to predict a clinical endpoint with a confidence level acceptable to regulators and the clinical community.

[001 06] 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).

[001 07] The terms "cancer stem cell (CSC)", "tumor initiating cell (TIC)", and "tumor propagating cell (TPC)" are used interchangeably to refer to a subpopulation of cells in a tumor that display characteristics similar to embryonic stem cells, e.g., the ability to undergo self-renewal, expression of specific surface markers, etc. (meaning they can give rise to cells of different lineages, i.e., they exhibit multipotency) (Robertson, F.M. et al., J. Biomolecular Screening 2010: 820-29).
[001 08] The term "chemokine" as used herein refers to a class of chemotactic cytokines that signal leukocytes to move in a specific direction. The terms "chemotaxis" or "chemotactic" refer to the directed motion of a motile cell or part along a chemical concentration gradient towards environmental conditions it deems attractive and/or away from surroundings it finds repellent.

[001 09] The term "cross-presentation" refers to a process whereby APCs acquire proteins from other tissue cells through endocytic mechanisms, especially phagocytosis or macropinocytosis. Rock, KL and Shen, L, Immunol. Rev. 207: 166-83 (2005). The internalized antigen can then be processed through at least two different mechanisms. In one pathway, the antigen is transferred from the phagosome into the cytosol, where it is hydrolyzed by proteasomes into oligopeptides that then are transported by the transporter associated with antigen processing to MHC class I molecules in the endoplasmic reticulum or phagosomes. Id. In a second pathway, the antigen is cleaved into peptides by endosomal proteases, particularly cathepsin S, and bound by class I molecules probably in the endocytic compartment itself. Id. Depending on the nature of the antigen, one or both of these pathways can contribute to cross-presentation in vivo. Id. The outcome of cross-presentation can be either tolerance or immunity. Id. Which of these outcomes occurs is thought to depend on whether antigens are acquired by themselves alone, leading to tolerance, or with immunostimulatory signals, leading to immunity. Id. One source of such signals is from dying cells that release immunostimulatory 'danger' signals that promote the generation of immunity to their cellular antigens. Id.

[001 10] 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. Generally, cytokines act locally. They include
type I cytokines, which encompass many of the interleukins including interleukin 2 (IL-2), as well as several hematopoietic growth factors; type II cytokines, including the interferons and interleukin-10; tumor necrosis factor ("TNF")-related molecules, including TNFa 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.

[001 11] As used herein, the term "contact" refers to the state or condition of touching or of being in immediate or local proximity. Contacting a composition to a target destination, such as, but not limited to, an organ, a tissue, a cell, or a tumor, may occur by any means of administration known to the skilled artisan.

[001 12] As used herein, the term "dendritic cell-tumor cell product (DC-TC)" refers to autologous activated dendritic cells isolated from peripheral blood and incubated with GM-CSF and IL-4 that have been loaded with antigens from inactivated circulating tumor cells by co-incubation or transfection.

[001 13] "Derived from," in the context of peptides derived from one or more cancer cells, encompasses the following. The cancer cell can be broken, for example, by a homogenizer or by osmotic bursting, resulting in a crude extract. Peptides, oligopeptides, and polypeptides of the crude extract can be exposed to dendritic cells, followed by processing of the peptides by the dendritic cells. Derived from also encompasses providing dendritic cells with intact cancer cells, where the cancer cells are living, or where the cancer cells have been treated with irradiation but are still metabolically active, or where the cancer cells have been treated with a nucleic acid cross-linking agent but are still metabolically active. "Derived from" includes mixtures of cancer cell debris, free cancer cell proteins, and irradiated cancer cells, that are taken up by dendritic cells, and therefore are derived from the cancer cells.
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.

The term "differentiation" as used herein refers to a property of cells to exhibit tissue-specific differentiated properties in culture.

The terms "disease" or "disorder" as used herein refer to an impairment of health or a condition of abnormal functioning.

The term "dose" as used herein refers to the quantity of a therapeutic substance prescribed to be taken at one time.

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. In other words, "increased expression" encompasses increased biosynthesis, or increased activity that is caused by phosphorylation, or an increased activity that is caused by migration from the cytosol...
to the nucleus.

[001 19] The term "fragment" or "peptide fragment" as used herein refers to a small part derived, cut off, or broken from a larger peptide, polypeptide or protein, which retains the desired biological activity of the larger peptide, polypeptide or protein.

[001 20] The term "growth" as used herein refers to a process of becoming larger, longer or more numerous, or an increase in size, number, or volume.

[001 21] 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.

[001 22] The term "immunomodulatory cell(s)" as used herein refer(s) to cell(s) that are capable of augmenting or diminishing immune responses by expressing chemokines, cytokines and other mediators of immune responses.

[001 23] The term "immunopotent" as used herein, refers to the ability to activate and guide a naïve immune system to mount a response toward a foreign protein.

[001 24] 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%, etc. 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).

[00125] The term "inflammatory cytokines" or "inflammatory mediators" as used herein refers to the molecular mediators of the inflammatory process, which may modulate the inflammation process being either pro- or anti-inflammatory in their effect. These soluble, diffusible molecules act both locally at the site of tissue damage and infection and at more distant sites. Some inflammatory mediators are activated by the inflammatory process, while others are synthesized and/or released from cellular sources in response to acute inflammation or by other soluble inflammatory mediators. Examples of inflammatory mediators of the inflammatory response include, but are not limited to, plasma proteases, complement, kinins, clotting and fibrinolytic proteins, lipid mediators, prostaglandins, leukotrienes, platelet-activating factor (PAF), peptides and amines, including, but not limited to, histamine, serotonin, and neuropeptides, and pro-inflammatory cytokines, including, but not limited to, interleukin-1 -beta (IL-1 β), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF-a), interferon-gamma (IF-γ), and interleukin-1 2 (IL-1 2).

[00126] The term "interleukin (IL)" as used herein refers to a cytokine secreted by, and acting on, leukocytes. Interleukins regulate cell growth, differentiation, and motility, and stimulates immune responses, such as inflammation. Examples of interleukins include interleukin-1 (IL-1), interleukin 2 (IL-2), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-1 2 (IL-1 2).

[00127] The term "interleukin-1 2" or "IL-1 2" as used herein refers to a cytokine that regulates the differentiation of naive T-cells into Th1 cells. It stimulates the growth and function of T-cells and alters the normal cycle of apoptotic cell death. IL-1 2 is one of a large group of cytokines that folds into a bundle of four alpha-helices. It is a heterodimer of 70kDa that is composed of two disulfide-linked
subunits, of mass 35kDa and 40kDa.

[001 28] The term "inhibit" and its various grammatical forms, including, but not limited to, "inhibiting" or "inhibition", are used herein to refer to reducing the amount or rate of a process, to stopping the process entirely, or to decreasing, limiting, or blocking the action or function thereof. Inhibition can include a reduction or decrease of the amount, rate, action function, or process of a substance by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99%.

[001 29] The term "isolated" is used herein to refer to material, such as, but not limited to, a nucleic acid, peptide, polypeptide, protein or cell, which is substantially or essentially free from components that normally accompany or interact with it as found in its naturally occurring environment, or, if the material is in its natural environment, the material has been non-naturally altered by deliberate human intervention. The alteration to yield the altered material may be performed on the material within, or removed from, its natural state.

[001 30] An "isolated molecule" is a molecule that is substantially pure and is free of other substances with which it is ordinarily found in nature or in vivo systems to an extent practical and appropriate for its intended use.

[001 31] The term "loaded with antigen(s)" as used herein refers to the ability of an antigen-presenting cell to capture whole cells, including live cells, necrotic cells, and dead cells, a cell lysate, acid elution cell extract, partially purified antigens, isolated antigens, partially purified peptides, purified peptides, isolated peptides, synthetic peptides, or any combination and then administered to a subject, including by cross-presentation.
The term "major histocompatibility complex" or "MHC" as used herein refers to a complex of vertebrate genes coding for a large family of cell-surface proteins that bind peptide fragments of foreign proteins and present them to T-lymphocytes to induce an immune response. The MHC also plays a role in resistance to infection and in susceptibility to a number of autoimmune diseases. The MHC complex is divided into three subgroups: MHC class I (MHC I); MHC class II (MHC II); and MHC class III (MHC III). MHC I molecules are present on nearly every nucleated cell of the body. MHC I presents peptides derived from cytosolic proteins and/or peptides from infectious agents. MHC II molecules are found only on specialized, antigen-presenting cell types such as macrophages, dendritic cells, activated T-cells and B-cells. MHC II presents peptides derived from extracellular proteins that are internalized by the cell from its environment, digested by lysosomes and bound by MHC II before its migration to the plasma membrane. MHC II interacts with helper (CD4+) T-cells to trigger an appropriate immune response. MHC III molecules include several secreted proteins comprising components of the complement system (e.g., C2, C and B factor), cytokines (e.g., TNF-a, LTA and LTB) and heat shock proteins (HSP).

The term "marker" is used herein to refer to a receptor, or a combination of receptors, found on the surface of a cell. These markers allow a cell type to be distinguishable from other kinds of cells. Specialized protein receptors (markers) that are capable of selectively binding or adhering to other signaling molecules coat the surface of every cell in the body. Cells use these receptors and the molecules that bind to them as a way of communicating with other cells and to carry out their proper function in the body.

The severity of a disease or disorder, as well as the ability of a treatment to prevent, treat, or mitigate, the disease or disorder can be measured, without implying any limitation, by a biomarker or by a clinical parameter. Biomarkers include blood counts, metabolite levels in serum, urine, or cerebrospinal fluid, tumor cell counts, cancer stem cell counts, tumor levels. Tumor levels can be determined by

Expression markers encompass genetic expression of mRNA or gene amplification, expression of an antigen, and expression of a polypeptide. Clinical parameters include progression-free survival (PFS), 6-month PFS, disease-free survival (DFS), time to progression (TTP), time to distant metastasis (TDM), and overall survival, without implying any limitation.

[00135] Expression of biomarkers by a single cell or by a population of cells can be determined by measuring expression of the polypeptide form of the biomarker or the mRNA form of the biomarker. Polypeptide expression can be measured using a labeled antibody, while nucleic acid expression can be measured by hybridization techniques.

[00136] 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.

[00137] 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.
[00138] The term "modify" as used herein means to change, vary, adjust, temper, alter, affect or regulate to a certain measure or proportion in one or more particulars.

[00139] The term "modulate" as used herein means to regulate, alter, adapt, or adjust to a certain measure or proportion.

[00140] The term "peptide" is used herein to refer to two or more amino acids joined by a peptide bond. The terms "polypeptide" and "protein" are used herein in their broadest sense to refer to a sequence of subunit amino acids, amino acid analogs, or peptidomimetics. The subunits generally are linked by peptide bonds. The polypeptides described herein may be isolated, chemically synthesized or recombinantly expressed. The term "peptidomimetic" as used herein refers to a small protein-like chain designed to mimic a peptide. A peptidomimetic typically arises from modification of an existing peptide in order to alter the molecule's properties. The term "protein" is used herein to refer to a large complex molecule or polypeptide composed of amino acids. The sequence of the amino acids in the protein is determined by the sequence of the bases in the nucleic acid sequence that encodes it.

[00141] 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.

[00142] 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.

[00143] 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.

[00144] 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 sulphonnic, 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, benzenesulphonate,
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.

[00145] The term "polypeptide" is used herein in its broadest sense to refer to a sequence of subunit amino acids, amino acid analogs or peptidomimetics, wherein the subunits are linked by peptide bonds.

[00146] The term "potency" as used herein refers to efficacy, effectiveness, strength or, typically, the dissociation constant, which indicates the concentration needed to inhibit binding.

[00147] The term "prevent" as used herein refers to effectual stoppage of action or progress.

[00148] 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.

**Priming**

[00149] 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.

[00150] 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. 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 in vivo appears to be a macrophage; and the critical second signal for helper T cell growth in vivo 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.

[00151] The term "PTEN inhibitor" as used herein refers to any molecule that inhibits a PTEN protein.

[00152] The term "purify" and its other grammatical forms as used herein refers to the process of isolating or freeing from foreign, extraneous, or objectionable elements.

[00153] The term "refractory" as used herein refers to the state of being unaffected, unresponsive, resistant or not fully responsive.

[00154] The term "ROCK inhibitor" as used herein refers to any molecule that inhibits a ROCK protein.

[00155] The term "spheroids" as used herein refers to spherical aggregates of cancer stem cells formed by culture of cancer cells in serum-free medium. The ability to form spheroids is a characteristic of cancer stem cells.

[00156] The term "stimulate" in any of its grammatical forms as used herein refers to inducing activation or increasing activity.
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.

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.

The term "substrate" as used herein refers to a surface upon which cells can grow. Exemplary substrates include, without limitation, adherent substrates and non-adherent substrates.

An adherent substrate can be one that is rich in RGD (Arg-Gly-Asp) tripeptide motifs (e.g., collagen, gelatin, MATRIGEL®). An "adherent substrate" is a surface that is configured to adhere to, and to collect, anchorage dependent cells. Moreover, the substrate can be an adherent substrate that is configured to adhere to and to collect anchorage dependent cells that are fibroblasts. RGD peptides also can be grafted on polymeric backbones such as polystyrene, hyaluronan, polylactic acid or combination thereof. The backbone can further carry proteoglycans. The proteoglycans can carry growth factors, e.g., FGF, epidermal growth factor (EGF), activin A or follistatin.

A non-adherent substrate can cause fast and efficient enrichment of the cultures with cancer stem cells and may be used when a large enough sample is provided, so that purification of tumor-CSC can begin immediately. If the sample is small (needle aspirate, peritoneal lavage) and non-adherent culture is not feasible, an adherent culture may be used for initial expansion, followed by a purification step on
a non-adherent substrate, then followed by another expansion under adherent conditions.

[00162] The term "target" as used herein refers to a biological entity, such as, for example, a protein, cell, organ, or nucleic acid, whose activity can be modified by an external stimulus. Depending upon the nature of the stimulus, there may be no direct change in the target, or a conformational change in the target may be induced.

[00163] 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.

[00164] 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.

[00165] 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.

[00166] 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.

[001 68] 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.

[001 69] 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.

[001 70] 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.

[00171] 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.

[00172] 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.

[00173] 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.

[001 74] The term "transplantation" as used herein, refers to removal and transfer of cells, a tissue or an organ from one part or individual to another.

[001 75] 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).

[001 76] The term "variant of a cancer-specific cancer stem cell antigen" is used herein to refer to a protein, peptide or polypeptide sequence in which at least one amino acid residue has been modified by deletion of an amino acid, insertion of an amino acid, or substitution of a second amino acid for a first amino acid at a specific position on the polypeptide. A skilled artisan can produce polypeptide variants having single or multiple amino acid substitutions, deletions, additions or replacements. These variants may include inter alia: (a) variants in which one or more amino acid residues are substituted with conservative or non-conservative amino acids; (b) variants in which one or more amino acids are added; (c) variants in which at least one amino acid includes a substituent group; (d) variants in which amino acid residues from one species are substituted for the corresponding residue in another species, either at conserved or non-conserved positions; and (d) variants in which a target protein is fused with another peptide or polypeptide such as a fusion partner, a protein tag or other chemical moiety, that may confer useful properties to the target protein, such as, for example, an epitope for an antibody. The techniques for obtaining such variants, including genetic
(suppressions, deletions, mutations, etc.), chemical, and enzymatic techniques are known to the skilled artisan. As used herein, the term "mutation" refers to a change of the DNA sequence within a gene or chromosome of an organism resulting in the creation of a new character or trait not found in the parental type, or the process by which such a change occurs in a chromosome, either through an alteration in the nucleotide sequence of the DNA coding for a gene or through a change in the physical arrangement of a chromosome. Three mechanisms of mutation include substitution (exchange of one base pair for another), addition (the insertion of one or more bases into a sequence), and deletion (loss of one or more base pairs).

[00177] According to one aspect, the described invention provides an immunopotent tumor-specific cancer cell product.

[00178] According to some embodiments, the tumor-specific cancer cell product is an immunogenic composition.

[00179] According to some embodiments, the immunogenic composition comprises a therapeutic amount of an isolated population of dendritic cells activated in vitro with a tumor-specific antigen derived from a population of purified cultivated tumor cells derived from circulating tumor cells (CTCs) obtained from a patient.

[00180] According to some embodiments, the therapeutic amount is effective to stimulate an immune response to one or more tumor specific antigens.

[00181] According to some embodiments, the composition comprises a pharmaceutically acceptable carrier.

[00182] According to some embodiments, the immunogenic composition further comprises an adjuvant. According to one such embodiment, the adjuvant is GM-CSF.
According to some embodiments, a source of the cancer cell expressing a cancer cell-specific antigen is a population of live tumor cell derived cancer stem cells found in peripheral blood.

**Tumor Antigens**

[001 84] Antigenic specificity can arise from the amino acid sequence of the antigen, from the degree of expression of that antigen by the tumor cell, from post-translational modification of the antigen, and the like.

[001 85] Specificity to a certain type of cancer cell can also arise from a particular fingerprint of a plurality of tumor antigens. Specificity can also arise, from the fact that a particular antigen, while expressed by a wide variety of tumor cells, has particular use in immunotherapy against a smaller number of tumor types. Specificity can also arise from the fact that a particular collection of MHC class I presentable and MHC class II presentable epitopes exist, on a particular polypeptide or polypeptide fragment. Also, specificity in an administered antigen can arise by omitting one or more peptides that can provoke immunotolerance. The skilled artisan can locate the relevant nucleic acid and polypeptide sequences, e.g., on the U.S. government's web site, at ncbi.nlm.nih.

[001 86] Exemplary glioblastoma-specific antigens include, but are not limited to, PTPRZ1; EGFR; SEC61 G; TNC; HER2; TRP-2; gp100; MAGE-1; IL13Ralpha2; AIM-2 (Phuphanich et al (2013) Cancer Immunol. Immunother. 62:125-135; Neidert et al (2012) J. Neurooncol.).

Exemplary renal cancer-specific antigens include, but are not limited to, carbonic anhydrase IX (CA-IX), MUC-1, and NYESO-1, and 5T4 (Tykodi et al (2012) J. Immunother. 35:523-533).


Exemplary melanoma-specific antigens include, but are not limited to, tyrosinase; gp75 (tyrosinase related protein-1 (TRP-1)); gp100 (Pmel17); Melan A/MART-1; TRP-2; MAGE family; BAGE family; GAGE family; NY-ESO-1; CDK4; β-catenin; mutated introns; N-acetylglucosaminyltransferase V gene product; MUM-1; p15; gangliosides (e.g., GM2, GD2, GM3, GD3); high molecular weight chondroitin sulfate proteoglycan; p97 melanotransferrin; and SEREX antigens (e.g., D-1, SSX-2) (Hodi FS, Clin Cancer Res, February 1, 2006; 12:673-678).

According to some embodiments, the population of live tumor cell derived cancer stem cells expressing a tumor-specific antigen is incapable of cell division but metabolically active.

According to one such embodiment, the population of live tumor cell derived cancer stem cells expressing a tumor-specific antigen is incapable of cell division after exposure to radiant energy, temperature (e.g., heat or cold), chemical methods (e.g., cytostatic, aldehyde, alcohol), or a combination thereof.

According to some embodiments, the population of live tumor cell derived cancer stem cells expressing a tumor-specific antigen is an irradiated cancer cell.
001.94] As used herein, the term "irradiated" refers to a process by which a subject is exposed to radiation, meaning the process in which energy is emitted by one body, transmitted through an intervening medium or space, and absorbed by another body. According to some embodiments, the radiation is selected from gamma-radiation, X-ray radiation, electron beam radiation, neutron beam radiation, proton beam radiation, electromagnetic radiation, visible light radiation, ultraviolet light radiation and the like. According to some embodiments, irradiation functions to prevent cell division of the cancer stem cells. According to some embodiments, irradiation prevents cell division, but also denatures cellular proteins.

001.95] According to some embodiments, the population of live tumor cell derived cancer stem cells expressing a tumor-specific antigen is a physically disrupted cancer cell. Examples of methods for physical disruption include, without limitation, sonication, cavitation, dehydration, ion depletion, or by toxicity from exposure to one or more salts.

001.96] According to some embodiments, the population of live tumor cell derived cancer stem cells expressing a tumor-specific antigen is a cancer cell in which nucleic acids are cross-linked with a nucleic acid cross-linking agent.

001.97] Examples of nucleic acid targeting agents include, without limitation, beta-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester; psoralens in combination with ultraviolet (UVA) irradiation; 4'-(4-amino-2-oxa)butyl-4',5',8-trimethylpsoralen ("S-59"); and 150 micromolar of psoralen S-59 and 3 J/cm2 UVA light (FX 1019 irradiation device, Baxter Fenwal, Round Lake, IL). See, U.S. Pat. Nos. 7,833,775 and 7,691,393.

001.98] According to some embodiments, the population of live tumor cell-derived cancer stem cells is derived from cells of a solid tumor.
Examples of solid tumors include, without limitation, a melanoma, a tissue of endodermal, mesodermal, or ectodermal origin (e.g., melanoma of neural crest origin, colon cancer of endoderm origin, renal cancer of mesoderm origin, glioblastoma of ectoderm origin, ovarian cancer of mixed mesoderm plus extra-embryonic origin) a hepatocellular carcinoma, colon carcinoma, ovarian carcinoma, glioblastoma multiforme, and tumor-cell derived vasculogenic-like cells, the source of which, for example is uveal melanoma, cutaneous melanoma, breast cancer, ovarian cancer, prostate cancer, and glioblastoma multiforme.

According to some embodiments, the cells of the solid tumor are a form of whole cells, including live cells, necrotic cells, dead cells, a cancer cell extract, a cancer cell lysate, or an acid eluate. According to some embodiments, the cells of the solid tumor comprise purified cancer cell peptides or cancer cell synthetic peptides.

According to some embodiments, the live cancer stem cell is a cancer progenitor cell characterized by continuous self-renewal and differentiation.

According to some embodiments, the live cells of the solid tumor are derived from peripheral blood of the subject. According to some embodiments, the live cells of the solid tumor in the peripheral blood of the subject are in cell clusters. According to some embodiments, the cell clusters are aggregates of cells. According to some embodiments, the aggregates of cells originate from the tumor and enter the vasculature.

According to some embodiments, the population of live tumor cell-derived cancer stem cells comprises a population of cancer stem cells expressing at least one tumor-specific antigen with mesenchymal characteristics.

According to some embodiments, the population of hepatocellular cancer stem cells expressing at least one tumor-specific antigen with mesenchymal
characteristics includes, but is not limited to, a loss of expression of at least one of the epithelial markers CK7, CK19, EpCAM and E-cadherin; and an increase in expression of at least one of the mesenchymal markers Slug/Snail, Twist, CD44, NCAM, N-cadherin, and vimentin by at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population.

[00205] According to some embodiments, the tumor antigen expressed by a population of vascular mimicry stem cells expressing at least one tumor-specific antigen with mesenchymal characteristics includes two or more of the biomarkers NCAM, Slug/Snail, CD24, and Twist by at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population express two or more of the biomarkers NCAM, Slug/Snail, CD24, Twist, N-cadherin, CD44, vimentin, CD133, Nanog and CD117. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population express two or more of the biomarkers NCAM, Slug/Snail, CD24, and Twist.

[00206] According to some embodiments, the population of ovarian cancer stem cells expressing at least one tumor-specific antigen with mesenchymal characteristics includes, but is not limited to, loss of expression of at least one of epithelial biomarkers CK8, CK18 and EpCAM, wherein loss of expression of a biomarker refers to undetectable expression or expression in 40% or less, 35% or less, 30% or less, 25% or less, 20% or less, 15% or less, 10% or less, 9% or less, 8% or less, 7% or less, 6% or less, 5% or less, 4% or less, 3% or less, 2% or less, 1% or less, 0.5% or less, 0.1% or less of the cells, and an increase in expression of at least one of the mesenchymal biomarkers Slug/Snail, Twist, CD44, NCAM, N-cadherin, and vimentin in at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population.
According to some embodiments, the population of colon cancer stem cells expressing at least one tumor-specific antigen with mesenchymal characteristics includes, but is not limited to, an increase in the expression of at least one of the mesenchymal markers Slug/Snail, CD44, Twist, N-cadherin, and vimentin in at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population. According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population express two or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist and CD1 17. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population express two or more of the biomarkers N-cadherin, Slug/Snail vimentin, CD1 17, CD44, CD24, γ-synuclein, FMNL2, b-catenin, Nanog, CD1 47, p3GHT8, LGR5, CD29, CXCFr, CD1 33, and DCiK1. According to some embodiments at least 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population express two or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist and CD1 17.

According to some embodiments, the population of glioblastoma multiforme stem cells expressing at least one tumor-specific antigen with mesenchymal characteristics includes, but is not limited to, expression of two or more of the biomarkers Slug/Snail, Sox10, Twist, vimentin and N-cadherin by at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population. According to some embodiments, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population express two or more of the biomarkers Slug/Snail, Sox10, Twist, vimentin, N-cadherin, nestin, S100, P53 and Ki-67. According to some embodiments, at least 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population express two or more of the biomarkers Slug/Snail, Sox10, Twist, vimentin and N-cadherin.

According to some embodiments, the population of live tumor cell-derived cancer stem cells comprises a population of “early” cancer stem cells with characteristics of embryonic stem cells.
[0021 0] According to some embodiments, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" hepatocellular cancer stem cells express two or more of biomarkers EpCAM, E-cadherin, Nanog, Sox2, Sox7, Sox17, Oct3/4, Fox2A, Ov1, OV6, c-kit, CD133, and CD90. According to some embodiments, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" hepatocellular cancer stem cells express two or more of biomarkers EpCAM, E-cadherin, Nanog, Sox2, Sox7, Sox17, Oct3/4, Fox2A, Ov1, OV6, c-kit, CD133 and CD90. According to some embodiments, at least 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" hepatocellular cancer stem cells express two or more of biomarkers Nanog, Sox2, Oct3/4, and c-kit.

[0021 1] According to some embodiments, at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" vascular mimicry stem cells express two or more of biomarkers EpCAM, CD133, CD44, Nanog, Sox2, Oct3/4, CD117, and Ki-67. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" vascular mimicry stem cells express two or more of biomarkers EpCAM, CD133, CD44, Nanog, Sox2, Oct3/4, CD117, Ki-67, TGFβR, and CD24. According to some embodiments, at least 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" vascular mimicry stem cells express two or more of biomarkers EpCAM, CD133, CD44, Nanog, Sox2, Oct3/4, CD117, Ki-67.

[0021 2] According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" ovarian cancer stem cells express two or more of biomarkers EpCAM, CD133, CD44, Nanog, Sox2, Oct3/4, CD117 and Ki-67. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" ovarian cancer stem cells express two or more of biomarkers EpCAM, CD133, CD44, Nanog, Sox2, Oct3/4, CD117, Ki-67, CA-125,
MUC-1, TGFβR, and CD24. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" ovarian cancer stem cells express two or more of biomarkers EPCAM; CD133, CD44, Nanog, Sox2, Oct3/4, CD17, and Ki-67.

[0021] According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" colon cancer stem cells express two or more of biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2 and CD133. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" colon cancer stem cells express two or more of biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2, CD133, EpCAM, E-cadherin, Sox7, Sox17, ALDH1A1, LGR5, Hes1, DPPA2, CD9, KRAS, ESA, BMI1, CD166, CD24, CD29, CD44, CD166 and CDCP1. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" colon cancer stem cells express two or more of biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2 and CD133.

[0021] According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" glioblastoma multiforme cancer stem cells express two or more of biomarkers CD133, Oct3/4, Sox2, CD271, nestin, Nanog, and CD15. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" glioblastoma multiforme cancer stem cells express two or more of biomarkers CD133, Oct3/4, Sox2, CD271, nestin, Nanog, CD15, Sox3, EGFR, vimentin, S100 and CD44. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" glioblastoma multiforme cancer stem cells express two or more of biomarkers CD133, Oct3/4, Sox2, CD271, nestin, Nanog and CD15.
According to some embodiments, the population of live tumor cell-derived cancer stem cells comprises a population of cancer stem cells with mixed stem cell characteristics having a mixed differentiation profile.

According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of hepatocellular cancer stem cells with mixed stem cell characteristics express two or more of biomarkers AFP, CK7, CK19, EpCAM, E-cadherin, Nanog, FoxA2, HNF4a, and ABCG2. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of hepatocellular cancer stem cells with mixed stem cell characteristics express two or more of biomarkers AFP, CK7, CK19, EpCAM, E-cadherin, Nanog, FoxA2, HNF4a and ABCG2.

According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of vascular mimicry cancer stem cells with mixed stem cell characteristics express two or more of biomarkers ABCG2, CD133, CD24, CD44, CD34, CD117, CK8, EpCAM, Ki-67, Nanog, N-cadherin, NCAM, Oct3/4, Slug/Snail, Twist, vimentin, ALDH, TGFβR, Sox2, EGFR, nestin, TP53, VEGF-R1, VEGF-R2, VE-Cadherin, VEGF-A, vWF, PECAM, and UEA-1. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of vascular mimicry cancer stem cells with mixed stem cell characteristics express two or more of biomarkers ABCG2, CD133, CD24, CD44, CD34, CD117, CK8, EpCAM, Ki-67, Nanog, N-cadherin, NCAM, Oct3/4, Slug/Snail, Twist, vimentin, ALDH, TGFβR, Sox2, EGFR, nestin, TP53, VEGF-R1, VEGF-R2, VE-cadherin, VEGF-A, vWF, PECAM and UEA-1.

According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of ovarian cancer stem cells with mixed characteristics express two or more of biomarkers EpCAM, CA-125, MUC-1, CD117, CK8, CK18 and Ki-67. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%,
98%, 99% of the cells in the population of ovarian cancer stem cells with mixed characteristics express two or more of biomarkers EpCAM, CA-1 25, MUC-1, CD1 17, CK8, CK1 8, Ki-67, CA 19-9, Her2/neu, NCAM, ganglioside CD2, esterogen receptor alpha, testosterone TGFβR, EGFR, TAG-72, CD46, He-4, ALDH, CD1 33, CD44, ABCG2, nestin and TP53. According to some embodiments, at least about 90%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of ovarian cancer stem cells with mixed stem cell characteristics express two or more of biomarkers EpCAM, CA-1 25, MUC-1, CD1 17, CK8, CK1 8 and Ki-67.

[00219] According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of colon cancer stem cells with mixed characteristics express two or more of biomarkers Hes1, MSI1, ALDH1 B1, ALDH1 A1, EpCAM, -CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki67, AFP and DClkl. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of colon cancer stem cells with mixed characteristics express two or more of biomarkers HES1, MSI1, ALDH1 B1, ALDH1 A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP and DClkl.

[00220] According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of glioblastoma multiforme cancer stem cells with mixed characteristics express two or more of biomarkers CD1 33, nestin, beta-tubulin III, GFAP, O1, EphA2, ABCG2, EGFRvIII, survivin, vimentin, S100, PDGF-Ra, NSE, nestin, MAGE1, CD271, TRP2, NG2, CD44, ALDH and P53. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of glioblastoma multiforme cancer stem cells with mixed characteristics express two or more of CD1 33, nestin, beta-tubulin III, GFAP, O1, EphA2, ABCG2, EGFRvIII, survivin, vimentin, S100, PDGF-Ra, NSE, nestin, MAGE1, CD271, TRP2, NG2, CD44, ALDH, and P53.
According to some embodiments, the isolated population of dendritic cells comprises at least one tumor-specific cancer cell antigen.

According to some embodiments, CD83 is expressed by at least some of the isolated population of dendritic cells comprising at least one tumor-specific cancer cell antigen.

According to some embodiments, the therapeutic amount of the isolated population of dendritic cells comprises $1 \times 10^3$ or more dendritic cells contacted and activated ex vivo with a cancer cell-specific antigen derived from a population of patient-specific isolated cancer cells sourced from circulating tumor cells (CTCs).

According to some embodiments, the dendritic cells are immune adjuvant-activated dendritic cells.

According to some embodiments, the immune adjuvant for activating dendritic cells comprises a TLR agonist or an adjuvant.

According to some embodiments, the TLR agonist is selected from CpG-oligonucleotide (TLR9), imiquimod (TLR7), poly(I:C) (TLR3), glucopyranosyl lipid A (TLR4), murein (TLR2), flagellin (TLR5).

According to some embodiments, the adjuvant is a CD40 agonist.

According to some embodiments, the CD40 agonist is selected from a CD40-ligand, interferon-gamma, or prostaglandin E2.

According to some embodiments, the stimulated effective immune response comprises an effective T cell response, an effective B cell response, or
both.

[00230] According to some embodiments, the therapeutic amount is immunostimulatory and is effective (1) to stimulate an effective immune response; and (2) to improve a clinical parameter selected from progression-free survival, disease-free survival, time to progression, time to distant metastasis, overall survival of the subject when compared to dendritic cells not displaying the tumor-specific stem cell antigen or a suitable control.

[00231] According to some embodiments, a stimulated effective immune response comprises a population of cancer-antigen stimulated dendritic cells interacting with a T cell population, resulting in activation and proliferation of antigen-specific T cells.

[00232] According to some embodiments, the T cells are CD4+ T cells, CD8+ T cells, or a combination thereof.

[00233] According to some embodiments, a stimulated effective immune response comprises a population of cancer-antigen stimulated dendritic cells interacting with a B cell population, resulting in activation and proliferation of antigen-specific B cells.

[00234] According to some embodiments, an effective B cell response comprises production of antibodies that specifically recognize a cancer stem cell antigen that mediates antibody dependent cell cytotoxicity (ADCC).

[00235] According to some embodiments, the antibody-dependent cell cytotoxicity is mediated by NK cells.

[00236] According to another aspect, the described invention provides a method for preparing an immunopotent tumor-specific cancer stem cell product.
comprising an immunopotent immunogenic composition comprising a therapeutic amount of an isolated population of dendritic cells activated in vitro with a tumor-specific antigen derived from a population of purified circulating tumor cells (CTCs) derived from a patient.

**Part A: Preparing a tumor-specific population of cancer stem cells expressing a tumor-specific antigen.**

[00237] **STEP 1:** Obtaining a tumor sample comprising a tumor cell expressing a tumor specific antigen.

[00238] According to some embodiments, the tumor sample expressing a tumor specific antigen is derived from a solid tumor.

[00239] Examples of solid tumors include, without limitation, a melanoma, a tissue of endodermal, mesodermal, or ectodermal origin (e.g., melanoma of neural crest origin, colon cancer of endoderm origin, renal cancer of mesoderm origin, glioblastoma of ectoderm origin, ovarian cancer of mixed mesoderm plus extra-embryonic origin) a hepatocellular carcinoma, colon carcinoma, ovarian carcinoma, glioblastoma multiforme, and tumor-cell derived vasculogenic-like cells, the source of which, for example is uveal melanoma, cutaneous melanoma, breast cancer, ovarian cancer, prostate cancer, and glioblastoma multiforme.

[00240] According to some embodiments, the tumor sample expressing a tumor specific antigen derived from a solid tumor expressing a tumor specific antigen is a tumor cell circulating in peripheral blood. According to some embodiments, the live tumor cells in the peripheral blood of the subject are in cell clusters. According to some embodiments, the cell clusters are aggregates of cells. According to some embodiments, the aggregates of cells originate from the tumor and enter the vasculature.
STEP 2: Expanding the number of tumor cells expressing a tumor specific antigen in vitro.

According to some embodiments, the method comprises expanding the number of tumor cells expressing a tumor specific antigen in vitro by
(a) Isolating a tumor cell, tumor cell cluster, tumor cell aggregate, or a combination thereof derived from in peripheral blood;
(b) Optionally dissociating the tumor cell cluster, tumor cell aggregate or a combination thereof into a single cell suspension;
(c) Transferring the cells of the single cell suspension to an adherent substrate for about 2-4 weeks;
(d) Feeding cultures containing the adherent cells of step (c) every 2 days with fresh medium;
(e) Optionally dissociating the adherent cultures to a single cell suspension;
(f) Transferring the cell suspension to a low adherent surface or an ultra-low-adherent surface in serum-free medium to remove anchorage-dependent differentiated cells to form a culture comprising cancer cell spheroids, and maintaining the spheroid culture in a culture medium supplemented with growth factors to produce a cancer cell culture comprising cancer cell spheroids comprising tumor specific cancer stem cells;
(g) Sedimenting the spheroids to collect the spheroids;
(h) Dissociating cells from the spheroids mechanically and enzymatically to yield a single-cell suspension;
(i) Transferring the cell suspension to an adherent substrate; and
(j) Expanding cell number in vitro to establish a population of adherent tumor-specific cancer stem cells derived from a tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof derived from in peripheral blood.

According to some embodiments, the adherent substrate contains one or more Arginine-Glycine-Aspartic Acid (RGD)-rich compound and a serum-free medium. According to some embodiments, the fresh medium consists of a basal
stem cell formulation supplemented with a protein/growth factor mixture. According to some embodiments, the growth factor is a fibroblast growth factor (FGF), epidermal growth factor (EGF), or both.

[00244] According to some embodiments, spheroids of cancer stem cells are characterized by expression of biomarkers.

[00245] For example, according to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the hepatocellular cancer stem cell spheroid population express two or more of biomarkers AFP, CK7, CK19, EpCAM, E-cadherin, Ov1 and OV6. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the hepatocellular cancer stem cell spheroid population express two or more of biomarkers AFP, EpCAM, Ov1 and Ov6. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in a purified the hepatocellular cancer stem cell spheroid population express one or more of biomarkers ABCG2, AFP, CD133, CD44, CD90, CK19, CK7; c-KIT, E-cadherin, EpCAM, FoxA2, HNF4a, Ki67, Nanog, N-cadherin, NCAM (CD56), Oct3/4, Ov1, OV6, Slug/Snail, Sox17, Sox2, Sox7, Twist and vimentin.

[00246] For example, according to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the vascular mimicry cancer stem cell spheroid population express two or more biomarkers EpCAM, CD117, ALDH, CD133, CD24, Ki-67, NCAM, vimentin, CK8, TGFβR, EGFR, CD44, ABCG2, Slug/Snail, nestin and TP53. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the vascular mimicry cancer stem cell spheroid population express two or more of biomarkers EpCAM, CD117, ALDH, CD133, CD24 and Ki-67. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the vascular mimicry spheroid population express two or more of biomarkers EpCAM, CD117, ALDH, CD133, CD24, and Ki-67.
[00247] For example, according to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the colon cancer stem cell spheroid population express two or more biomarkers CD133, Hes1, CD44, CD24, CD1 66 and CD29. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the colon cancer stem cell spheroid population express two or more biomarkers CD133, Hes1, CD44, CD24, CD1 66, CD29, CK7, CK1 9, E-cadherin, CK20, ESA, ALDH, CDX1, LGR5, and DCikl. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the colon cancer stem cell spheroid population express two or more biomarkers CD133, Hes1, CD44, CD24, CD1 66 and CD29.

[00248] For example, according to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the ovarian cancer stem cell spheroid population express two or more biomarkers EpCAM, CA-1 25, MUC-1, CD1 17, He-4, ALDH, CD133, CD24, Ki-67, CA1 9-9, HER/neu, NCAM, galglioside CD2, estrogen receptor alpha, vimentin, CK8, CK1 8, AFP, testosterone, TGFβR, EGFR, TAG-72, CD46, CD44, ABCG2, Slug/Snail, nestin and TP53. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the ovarian cancer stem cell spheroid population express two or more biomarkers AFP, CK7, CK1 9, EpCAM, E-cadherin, Ov1 and OV6. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the colon cancer stem cell spheroid population express two or more biomarkers EpCAM, CA-1 25, MUC-1, CD1 17, He-4, ALDH, CD133, CD24 and Ki-67.

[00249] For example, according to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the glioblastoma multiforme (GBM) cancer stem cell spheroid population express two or more biomarkers CD133, nestin, Sox2 and CD271. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the GBM cancer stem cell spheroid population express two or more of biomarkers
According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the colon cancer stem cell spheroid population express two or more of biomarkers CD133, nestin, Sox2 and CD271.

[00250] According to some embodiments, in step (h) the spheroids are gently dissociated by exposure to collagenase IV and mechanical pipetting.

[00251] According to some embodiments, in step (h) the spheroids are gently dissociated by exposure to dispase 2U/ml for 24-48 hours in the culture media.

[00252] According to some embodiments, in step (e), for those CSCs sensitive to physical and enzymatic manipulation during passaging, the method comprises:

(i) seeding cells at a low density 5,000, 10,000 and 15,0000 cells/cm2, on final adherent surface in media containing 15% FBS
(ii) allowing the cells to expand for 4 days; and
(iii) Feeding cultures on MWF feeding schedule with media that contained only 5% FBS.

[00253] **STEP 3:** Differentiating cancer stem cells

[00254] For cancer stem cells with an early cancer stem cell differentiation profile, expanding the adherent cultures for 2-4 weeks on the adherent substrate in a serum-free culture medium containing FGF and activin A.

[00255] According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" hepatocellular cancer stem cells express two or more of biomarkers EpCAM, E-cadherin, Nanog, Sox2, Sox7, Sox17, Oct3/4, Fox2A, Ov1, OV6, c-kit, CD133, and CD90. According
to some embodiments, at least at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" hepatocellular cancer stem cells express two or more of biomarkers EpCAM, E-cadherin, Nanog, Sox2, Sox7, Sox17, Oct3/4, Fox2A, Ov1, OV6, c-kit, CD133 and CD90. According to some embodiments, at least at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" hepatocellular cancer stem cells express two or more of biomarkers Nanog, Sox2, Oct3/4, and c-kit.

[00256] According to some embodiments, at least at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" vascular mimicry stem cells express two or more of biomarkers EpCAM, CD133, CD44, Nanog, Sox2, Oct3/4, CD17, and Ki-67. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" vascular mimicry cells express two or more of biomarkers EpCAM, CD133, CD44, Nanog, Sox2, Oct3/4, CD17, Ki-67, TGFβR, and CD24. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" vascular mimicry cells express two or more of biomarkers EpCAM, CD133, CD44, Nanog, Sox2, Oct3/4, CD17, and Ki-67.

[00257] According to some embodiments, at least at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" ovarian cancer stem cells express two or more of biomarkers EpCAM, CD133, CD44, Nanog, Sox2, Oct3/4, CD17 and Ki-67. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" ovarian cancer stem cells express two or more of biomarkers EpCAM, CD133, CD44, Nanog, Sox2, Oct3/4, CD17, Ki-67, CA-1 25, MUC-1, TGFβR, and CD24. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" ovarian cancer stem cells express two or more of biomarkers EPCAM; CD133, CD44, Nanog, Sox2, Oct3/4, CD17, and Ki-67.
According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" colon cancer stem cells express two or more of biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2 and CD133. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" colon cancer stem cells express two or more of biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2, CD133, EpCAM, E-cadherin, Sox7, Sox17, ALDH1A1, LGR5, Hes1, DPPA2, CD9, KRAS, ESA, BMI1, CD166, CD24, CD29, CD44, CD166 and CDCP1. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" colon cancer stem cells express two or more of biomarkers Nanog, Sox2, Oct3/4, c-kit, FoxA2 and CD133.

According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" glioblastoma multiforme cancer stem cells express two or more of biomarkers CD133, Oct3/4, Sox2, CD271, nestin, Nanog, and CD15. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" of the cells in the population of "early" GBM cancer stem cells express two or more of biomarkers CD133, Oct3/4, Sox2, CD271, nestin, Nanog, CD15, Sox3, EGFR, vimentin, S100 and CD44. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of "early" GBM cancer stem cells express two or more of biomarkers CD133, Oct3/4, Sox2, CD271, nestin, Nanog and CD15.

(ii) For cancer stem cells with a mixed differentiation profile; expanding the adherent cultures for 2-4 weeks on the adherent substrate in a serum-containing medium containing growth factors FGF and EGF.

According to some embodiments, about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mixed hepatocellular cancer stem cells express two or more of biomarkers AFP, CK7, CK19, EpCAM, E-cadherin,
Nanog, FoxA2, HNF4a, and ABCG2. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mixed hepatocellular cancer stem cells express two or more of biomarkers AFP, CK7, CK19, EpCAM, E-cadherin, Nanog, FoxA2, HNF4a and ABCG2.

[00261] According to some embodiments, 1. According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mixed vascular mimicry cancer stem cells express two or more of biomarkers ABCG2, CD1 33, CD24, CD44, CD34, CD1 17, CK8, EpCAM, Ki-67, Nanog, N-cadherin, NCAM, Oct 3/4, Slug/Snail, Twist, vimentin, ALDH, TGFβR, Sox2, EGFR, nestin, TP53, VEGF-R1, VEGF-R2, VE-Cadherin, VEGF-A, vWF, PECAM, and UEA-1. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mixed vascular mimicry cancer stem cells express two or more of biomarkers ABCG2, CD1 33, CD24, CD44, CD34, CD1 17, CK8, EpCAM, Ki-67, Nanog, N-cadherin, NCAM, Oct 3/4, Slug/Snail, Twist, vimentin, ALDH, TGFβR, Sox2, EGFR, nestin, TP53, VEGF-R1, VEGF-R2, VE-cadherin, VEGF-A, vWF, PECAM and UEA-1.

[00262] According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mixed ovarian cancer stem cells express two or more of biomarkers EpCAM, CA-1 25, MUC-1, CD1 17, CK8, CK1 8 and Ki-67. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mixed ovarian cancer stem cells express two or more of biomarkers EpCAM, CA-1 25, MUC-1, CD1 17, CK8, CK1 8, Ki-67, CA 19-9, Her2/neu, NCAM, ganglioside CD2, esterogen receptor alpha, testosterone TGFβR, EGFR, TAG-72, CD46, He-4, ALDH, CD1 33, CD44, ABCG2, nestin and TP53. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of the mixed ovarian cancer stem cell population express two or more of biomarkers EpCAM, CA-1 25, MUC-1, CD1 17, CK8, CK1 8 and Ki-67.
According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mixed colon cancer stem cells express two or more of biomarkers Hes1, MSI1, ALDH1 A1, EpCAM, -CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki67, AFP and DCIkl. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mixed colon cancer stem cells express two or more of biomarkers HES1, MSI1, ALDH1 B1, ALDH1 A1, EpCAM, G-CSF, Hiwi, CD44, CD49f, ESA, EphBR, ABCG2, NCAM, Ki-67, AFP and DCIkl.

According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mixed glioblastoma multiforme cancer stem cells express two or more of biomarkers CD133, nestin, beta-tubulin III, GFAP, O1, EphA2, ABCG2, EGFRvIII, survivin, vimentin, S100, PDGF-Ra, NSE, nestin, MAGE1, CD271, TRP2, NG2, CD44, ALDH and P53. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mixed GBM cancer stem cells express two or more of CD133, nestin, beta-tubulin III, GFAP, O1, EphA2, ABCG2, EGFRvIII, survivin, vimentin, S100, PDGF-Ra, NSE, nestin, MAGE1, CD271, TRP2, NG2, CD44, ALDH, and P53.

(iii) For epithelial to mesenchymal transition (EMT) cancer stem cells, expanding the adherent cultures for 2-4 weeks on the adherent substrate in a serum containing medium comprising FGF.

According to some embodiments, the term "mesenchymal-like tumor-specific CSC" or E MT-HCC-CSC (epithelial to mesenchymal transitioned (EMT) cancer stem cells)" is used to refer to a population in which the spheroids have undergone a process of EMT characterized by the loss of the expression of at least one epithelial marker, e.g., CK7, CK19, EpCAM and E-cadherin, and an increase in
the expression of at least one mesenchymal markers, e.g., Slug/Snail (Slg/Snl), Twist, CD44, NCAM, N-cadherin, and vimentin.

[00266] According to some embodiments, the population of hepatocellular cancer stem cells expressing at least one tumor-specific antigen with mesenchymal characteristics includes, but is not limited to, a loss of the expression of at least one of the epithelial markers CK7, CK19, EpCAM and E-cadherin; and an increase in the expression of at least one of the mesenchymal markers Slug/Snail, Twist, CD44, NCAM, N-cadherin, and vimentin to at least 30% of the cells in the population.

[00267] According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mesenchymal vascular mimicry cancer stem cells express two or more of the biomarkers NCAM, Slug/Snail, CD44, and Twist. According to some embodiments, at least about at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mesenchymal vascular mimicry cancer stem cells express two or more of the biomarkers NCAM, Slug/Snail, CD44, Twist, N-cadherin, CD44, vimentin, CD133, Nanog and CD17. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mesenchymal vascular mimicry cancer stem cells express two or more of the biomarkers NCAM, Slug/Snail, CD44, and Twist.

[00268] According to some embodiments, at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mesenchymal ovarian cancer stem cells are characterized by a loss of expression of at least one of epithelial biomarkers CK8, CK18 and EpCAM, where loss of expression of a biomarker refers to undetectable expression or expression in 40% or less of the cells, and an increase in expression of at least one of mesenchymal biomarkers Slug/Snail, Twist, CD44, NCAM, N-cadherin, and vimentin.
According to some embodiments, at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mesenchymal colon cancer stem cells are characterized by an increase in the expression of at least one of the mesenchymal markers Slug/Snail, CD44, Twist, N-cadherin, and vimentin. According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mesenchymal colon cancer stem cells express two or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist and CD1 17. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mesenchymal colon cancer stem cells express two or more of the biomarkers N-cadherin, Slug/Snail vimentin, CD1 17, CD44, CD24, γ-synuclein, FMNL2, b-catenin, Nanog, CD1 47, p3GhT8, LGR5, CD29, CXCFr, CD1 33, and DCIkl. According to some embodiments at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mesenchymal colon cancer stem cells express two or more of the biomarkers N-cadherin, Slug/Snail, vimentin, Twist and CD1 17.

According to some embodiments, at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mesenchymal glioblastoma multiforme cancer stem cells express two or more of the biomarkers Slug/Snail, Sox1 0, Twist, vimentin and N-cadherin. According to some embodiments, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mesenchymal GBM cancer stem cells express two or more of the biomarkers Slug/Snail, Sox1 0, Twist, vimentin, N-cadherin, nestin, S100, P53 and Ki-67. According to some embodiments, at least about 90%, 95%, 96%, 97%, 98%, 99% of the cells in the population of mesenchymal GBM cancer stem cells express two or more of the biomarkers Slug/Snail, Sox1 0, Twist, vimentin and N-cadherin.

STEP 4: Passaging and expanding the number of tumor-derived cancer stem cells in the cultures.
[00272] **STEP 5**: Inactivating the tumor-derived population of cancer stem cells expressing a tumor-specific antigen.

[00273] According to some embodiments, the inactivated population of live tumor cell derived cancer stem cells expressing a tumor-specific antigen is incapable of cell division but metabolically active.

[00274] According to one such embodiment, the inactivated population of live tumor cell derived cancer stem cells expressing a tumor-specific antigen is incapable of cell division after exposure to radiant energy, temperature (e.g., heat or cold), chemical methods (e.g., cytostatic, aldehyde, alcohol), or a combination thereof.

[00275] According to some embodiments, the inactivated population of live tumor cell derived cancer stem cells expressing a tumor-specific antigen is an irradiated cancer cell.

[00276] As used herein, the term "irradiated" refers to a process by which a subject is exposed to radiation, meaning the process in which energy is emitted by one body, transmitted through an intervening medium or space, and absorbed by another body. According to some embodiments, the radiation is selected from gamma-radiation, X-ray radiation, electron beam radiation, neutron beam radiation, proton beam radiation, electromagnetic radiation, visible light radiation, ultraviolet light radiation and the like. According to some embodiments, irradiation functions to prevent cell division of the cancer stem cells. According to some embodiments, irradiation prevents cell division, but also denatures cellular proteins.

[00277] According to some embodiments, the population of live tumor cell derived cancer stem cells expressing a tumor-specific antigen is a physically disrupted cancer cell. Examples of methods for physical disruption include, without
limitation, sonication, cavitation, dehydration, ion depletion, or by toxicity from exposure to one or more salts.

[00278] According to some embodiments, the inactivated population of live tumor cell derived cancer stem cells expressing a tumor-specific antigen is a cancer cell in which nucleic acids are cross-linked with a nucleic acid cross-linking agent.


[00280] **STEP 6:** cryostoring the irradiated tumor-derived cells in a vapor phase liquid nitrogen storage at -86° C.

**PART B:** preparing a population of immunopotent dendritic cells activated in vitro with a tumor-specific antigen derived from the population of purified cultivated tumor cells derived from the subject.

[00281] A method to prepare a population of immunopotent dendritic cells activated in vitro with a tumor-specific antigen derived from the population of purified cultivated tumor cells derived from the subject comprises:

[00282] a Obtaining peripheral blood mononuclear cells (PBMCs) by leukapheresis from the patient from whom the tumor cell, tumor cell cluster, tumor cell aggregate, or a combination thereof was derived from peripheral blood in (a);
[00283] 2’ Optionally shipping the collected PBMCs in (a) to a manufacturing facility;

[00284] 3’ Purifying the collected PBMCs from other lymphocytes;

[00285] 4’ incubating the purified PBMCs with GM-CSF and IL-4 for 6 days to generate the activated dendritic cells.

**Dendritic Cell Preparation**

[00286] Peripheral blood mononuclear cells (PBMCs) are obtained by leukapheresis from a patient from whom a tumor cell, tumor cell cluster, tumor cell aggregate or combination thereof was derived from the patient's peripheral blood. Optionally, the collected PBMCs are shipped to a manufacturing facility. The collected PBMCs are purified from other lymphocytes. According to some embodiments, DCs are purified from PBMCs by counter flow density centrifugation (elutriation), meaning a process by which monocytes are purified from other lymphocytes in order to enrich for cells that can be turned into APCs or DCs). According to some embodiments, low density DCs can be prepared by gradient separation over a density gradient to obtain PBMCs, T-lymphocyte depletion (optional), overnight incubation for 16 hours in autologous serum/medium (± cytokines), gradient separation, and positive selection (e.g., flow cytometry, immunomagnetic beads) for activation antigens, e.g., CD80, CD83, CD86, and CMRF-44 (Fearnley, DB et al, Blood 89: 3708 (1997); Caux, C. et al., J. Exp. Med. 180 (5): 1841-47 (1994)),CD80. Hart, DNJ, Blood 90(9): 3245-87 (1997);Hock, B.D. et al., Immunol. 83: 573-81 (1994). According to some embodiments, low density DCs can be prepared by gradient separation over a density gradient to obtain PBMCs, T-lymphocyte depletion (optional), overnight incubation for 16 hours in autologous serum/medium (± cytokines), metrizamide gradient separation, and positive selection (e.g., flow cytometry, immunomagnetic beads) to obtain a lineage-negative, CD83+ cell population. Zhou, L and Tedder, TF, J. Immunol. 154: 3821-35
According to some embodiments, purified PBMCs are incubated with GM-CSF and IL-4 for 6 days to generate DCs.

**PART C: preparing the immunopotent immunogenic composition**

According to some embodiments, the method for preparing the immunopotent immunogenic composition comprises:

- **Step 1**: Thawing the purified tumor cell product of Step 6; and

- **Step 2**: activating the population of dendritic cells in vitro by contacting the population of dendritic cells with a form of the population of tumor-specific cancer stem cells expressing a tumor-specific antigen by: Contacting the thawed irradiated tumor-derived cell antigen product with the activated dendritic cells for 18-24 hours to form an activated dendritic cell/tumor-derived cell antigen composition; and collecting and storing the dendritic cell/tumor-derived cell composition in vapor phase liquid nitrogen.

According to some embodiments, the dendritic cell presents a processed tumor-specific peptide within the dendritic cell. According to some embodiments, the dendritic cell presents a tumor-specific processed peptide on a dendritic cell surface. According to some embodiments, the tumor antigen derives from a tumor cell, tumor cell cluster, tumor cell aggregate, or a combination thereof derived from peripheral blood. According to some embodiments, the source of the tumor cell, tumor cell cluster, tumor cell aggregate or combination thereof derived from peripheral blood is a solid tumor.

According to some embodiments, the immunopotent immunogenic composition can be prepared by transfection. Exemplary transfection methods include, but are not limited to, chemical-based methods and non-chemical based
methods. Chemical-based methods include, without limitation, calcium phosphate, highly branched organic compounds (e.g., dendrimers), liposomes, and cationic polymers (e.g., DEAD-dextran, polyethylenimine). Non-chemical based methods include, but are not limited to, cell squeezing, sonoporation, optical transfection, impalefection, hydrodynamic delivery, magnetofection, viral transduction, electroporation and the like.

[00293] MaxCyte® transfection technology (MaxCyte, Inc., Gaithersburg, MD) is based on the general principles of electroporation, which involves the application of an electric field to a cell suspension, causing the cell membrane to become transiently permeable and encouraging external material to enter the cell. By safely and repeatedly inserting nearly any molecule, including genes, proteins, DNA or RNA, into any target cell, without the use of added biological or chemical agents, MaxCyte® electroporation enables transfection of a wide range of cells with loading efficiencies exceeding 90%. The technology is highly scalable from, for example, 5x10^5 cells in seconds using small-scale, static electroporation to 1x10^10 cells in less than 30 minutes using flow electroporation. MaxCyte® scalable electroporation has been successfully applied in \textit{ex vivo} cell therapy, protein production and drug discovery pipelines where reproducibility, efficiency and the need for increased cell numbers are critical.

[00294] The MaxCyte® system can perform small and large-scale electroporation using a single instrument and a single electroporation protocol. The term "static electroporation" refers to small-scale electroporation in which cells and the material(s) to be transfected are mixed and placed into a small processing chamber that is then loaded into the instrument for electroporation (Figure 2).

[00295] The term "flow electroporation" refers to large-scale electroporation in which cells and the material(s) to be transfected are mixed and placed into the source bag of a large-scale processing assembly. The sterile processing assembly is then loaded onto the MaxCyte® instrument. For example, when cell processing
begins, 3 ml fractions flow in succession through the electroporation chamber and continue into the cell collection bag. Up to $1 \times 10^{10}$ cells can be transfected in less than 30 minutes using flow electroporation. Transfected cells can be used immediately following a 20 minute recovery period or cryopreserved for later use.

[00296] According to some embodiments, the form of the population of tumor-specific cancer stem cells expressing a tumor-specific antigen is as a population of whole cells, a population of live cells, a population of dead cells, a population of necrotic cells, a cell lysate, a cell extract, a purified tumor-specific antigen, fragment or variant thereof.

[00297] According to another aspect, the described invention provides a method for treating a subject suffering from a cancer, comprising:

[00298] (1) Preparing for a cancer patient a patient-specific immunogenic composition comprising a therapeutic amount of an isolated population of dendritic cells contacted ex vivo with a population of cancer stem cells expressing a cancer-specific antigen derived from a tumor cell, a tumor cell cluster, a tumor cell aggregate or a combination thereof derived from peripheral blood;

[00299] (2) administering the immunogenic composition to the cancer patient; and

[00300] (3) eliciting an immunoeffective immune response comprising activation and proliferation of CD4+ T cells, CD8+ T cells, B cells or a combination thereof;

[00301] wherein the effective immune response is effective to improve a clinical parameter selected from progression-free survival (PFS), disease-free survival, time to progression, time to distant metastasis and overall survival (OS) of the subject when compared to a control.

[00302] According to some embodiments, the tumor sample expressing a tumor specific antigen is derived from a tumor cell, a tumor cell cluster, a tumor cell aggregate or a combination thereof derived from peripheral blood. According to some
embodiments, the live tumor cells in the peripheral blood of the subject are in cell clusters. According to some embodiments, the cell clusters are aggregates of cells. According to some embodiments, the aggregates of cells originate from the tumor and enter the vasculature.

[00303] According to another aspect, the described invention provides a composition for isolating/purifying CTCs from a blood sample.


[00305] According to some embodiments, concentration of the ROCK inhibitor in the composition ranges from about 1 µM to about 20 µM. According to some embodiments, concentration of the ROCK inhibitor ranges from about 5 µM to about 10 µM. According to some embodiments, the concentration of the ROCK inhibitor is 1 µM. According to some embodiments, the concentration of the ROCK inhibitor is 2 µM. According to some embodiments, the concentration of the ROCK inhibitor is 3 µM.
According to some embodiments, the concentration of the ROCK inhibitor is 4 µM. According to some embodiments, the concentration of the ROCK inhibitor is 5 µM. According to some embodiments, the concentration of the ROCK inhibitor is 6 µM. According to some embodiments, the concentration of the ROCK inhibitor is 7 µM. According to some embodiments, the concentration of the ROCK inhibitor is 8 µM. According to some embodiments, the concentration of the ROCK inhibitor is 9 µM. According to some embodiments, the concentration of the ROCK inhibitor is 10 µM. According to some embodiments, the concentration of the ROCK inhibitor is 11 µM. According to some embodiments, the concentration of the ROCK inhibitor is 12 µM. According to some embodiments, the concentration of the ROCK inhibitor is 13 µM. According to some embodiments, the concentration of the ROCK inhibitor is 14 µM. According to some embodiments, the concentration of the ROCK inhibitor is 15 µM. According to some embodiments, the concentration of the ROCK inhibitor is 16 µM. According to some embodiments, the concentration of the ROCK inhibitor is 17 µM. According to some embodiments, the concentration of the ROCK inhibitor is 18 µM. According to some embodiments, the concentration of the ROCK inhibitor is 19 µM. According to some embodiments, the concentration of the ROCK inhibitor is 20 µM.

[00306] According to some embodiments, the composition comprises a PTEN inhibitor. Examples of PTEN inhibitors include, but are not limited to, potassium bisperoxo(1,10-phenanthroline)oxovanadate (V) (bpV) (Sigma-Aldrich), SF 1670 (Tocris/R&D Systems), VO-OHpic (Tocris/R&D Systems) and the like.

[00307] According to some embodiments, concentration of the PTEN inhibitor ranges from about 0.01 µM to about 10 µM. According to some embodiments, the concentration of PTEN inhibitor ranges from about 0.5 µM to about 2 µM. According to some embodiments, the concentration of the PTEN inhibitor is 0.01 µM. According to some embodiments, the concentration of the PTEN inhibitor is 0.025 µM. According to some embodiments, the concentration of the PTEN inhibitor is 0.05 µM. According to some embodiments, the concentration of the PTEN inhibitor is 0.075 µM. According to some embodiments, the concentration of the PTEN inhibitor is 0.1 µM. According to some embodiments, the concentration of the PTEN inhibitor is 0.25 µM. According to some embodiments, the concentration of the PTEN inhibitor is 0.5 µM. According to some embodiments, the concentration of the PTEN inhibitor is 1 µM. According to some embodiments, the concentration of the PTEN inhibitor is 2 µM. According to some embodiments, the concentration of the PTEN inhibitor is 5 µM.
some embodiments, the concentration of the PTEN inhibitor is 0.5 µM. According to some embodiments, the concentration of the PTEN inhibitor is 0.75 µM. According to some embodiments, the concentration of the PTEN inhibitor is 1 µM. According to some embodiments, the concentration of the PTEN inhibitor is 2 µM. According to some embodiments, the concentration of the PTEN inhibitor is 3 µM. According to some embodiments, the concentration of the PTEN inhibitor is 4 µM. According to some embodiments, the concentration of the PTEN inhibitor is 5 µM. According to some embodiments, the concentration of the PTEN inhibitor is 6 µM. According to some embodiments, the concentration of the PTEN inhibitor is 7 µM. According to some embodiments, the concentration of the PTEN inhibitor is 8 µM. According to some embodiments, the concentration of the PTEN inhibitor is 9 µM. According to some embodiments, the concentration of the PTEN inhibitor is 10 µM.

[00308] According to some embodiments, the tumor includes, but is not limited to, a melanoma, a tissue of endodermal, mesodermal, or ectodermal origin (e.g., melanoma of neural crest origin, colon cancer of endoderm origin, renal cancer of mesoderm origin, glioblastoma of ectoderm origin, ovarian cancer of mixed mesoderm plus extra-embryonic origin) a hepatocellular carcinoma, colon carcinoma, ovarian carcinoma, glioblastoma multiforme, and tumor-cell derived vasculogenic-like cells, the source of which, for example is uveal melanoma, cutaneous melanoma, breast cancer, ovarian cancer, prostate cancer, and glioblastoma multiforme.

[00309] 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.
[0031 0] 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.

[0031 1] 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.

[0031 2] 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

[0031 3] 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.

**Materials and Methods**

**Generation of Autologous Dendritic Cells**

[00314] Dendritic cells were generated by a plastic adherence method as described by Choi and Luft (Choi (1998) Clin. Cancer Res. 4:2709-2716; Luft (1998) Exp. Hematol. 26:489-500). Briefly, patients underwent leukapheresis (i.e., the filtering of blood to collect peripheral blood mononuclear cells (PBMCs) (about 6 liters). The autologous leukapheresis product was subjected to ficoll-hypaque (GE Healthcare, Buckinghamshire, United Kingdom) density gradient separation. The resulting PBMCs were placed in antibiotic-free AIMV medium (Invitrogen, Grand Island, NY) supplemented with 1,000 IU/mL each of IL-4 (CellGenix, Freisberg, Germany) and GM-CSF (Berlex, Seattle, WA) (DC medium) at $15 \times 10^8$ cells/mL in cell cultivation flasks (Corning-Costar, Corning, NY). After one hour incubation, the non-adherent population was discarded and fresh DC medium was added to the flasks. The following morning, the non-adherent cells were discarded, the flasks were washed once with ambient temperature PBS, and fresh DC medium was added. The flasks were then cultivated for 6 days at which time flow cytometry evaluation was performed to determine the percentage and phenotype of DC generated by this approach (pre-load DC).

[00315] Phenotypic characterization of the dendritic cell populations was performed using monoclonal antibodies against the following surface markers obtained from BD Pharmingen San Diego, CA: anti-MHC class II conjugated to PerCp, anti CD11c conjugated to APC, anti-CD80, anti-CD83, anti-CD86 conjugated to PE. Isotype controls were used to determine percent positive cells.

**Isolation and Enrichment of CTCs from Blood**
CTCs can be isolated by targeting their physical properties, such as size, density, electric charges, deformability, and their biological properties, such as cell surface protein expression, and viability (Alix-Panabieres and Pantel, 2013). A Parylene-C slot microfilter can be used to capture and enrich live CTCs from whole blood rapidly and efficiently based on differential size and deformability (Xu et al, 2010). Captured cells can be immunofluorescently stained for cancer-specific protein markers and imaged on the filter using a fully automated, multi-spectral scanning and imaging platform (Zeiss) specially configured for this purpose. Alternatively, captured cells can be lysed for qPCR analysis, or picked cell-by-cell using a motorized micropipette apparatus (Eppendorf) specially configured for this application. A LiquidBiopsy EpCAM-based microfluidic CTC capture platform (Cynvenio, Inc., Westlake Village, CA), a ClearCell FX size-based microfluidic CTC platform (Clearbridge Biomedics, Inc.), Stem Cell Technologies immunomagnetic WBC depletion platform, and the DepArray platform (Silicon Biosystems), which uses dielectrophoretic "cages" to trap and manipulate single cells, allowing rapid and robust recovery of rare ultra-pure CTCs can also be used.

Autologous CTCs can be characterized by the expression of cytokeratins, tumor specific markers, adhesion molecules, proliferation markers and/or EMT markers and can be detected by flow cytometry or immunohistochemistry.

Autologous CTCs can be exposed to 100 Gy radiation in a cobalt 60 source irradiator. Irradiation efficiency can be confirmed by nonproliferative assay. Irradiated CTCs can either be used immediately to create autologous DC-TC vaccine or placed in vapor phase liquid nitrogen storage prior to use.

**Preparation of Autologous Dendritic Cell-Circulating Tumor Cell (DC-CTC) Vaccine Composition**

Patient-specific vaccine compositions are prepared by co-incubating autologous circulating tumor cells with IFN-γ. After incubation with IFN-γ, the
autologous circulating tumor cells are co-incubated with the activated autologous dendritic cells for 18 to 24 hrs.

[00320] Alternatively, patient-specific vaccine compositions are prepared by electroporation of autologous dendritic cells with a cell lysate prepared from cultures of circulating tumor cells incubated with IFN-γ or from immunogenic proteins isolated from proliferating tumor cells or circulating tumor cells incubated with IFN-γ.

[00321] The DC-CTC mixtures are collected and cryopreserved in vapor phase liquid nitrogen to retain viability of the DC-CTC population.

[00322] The DC-CTC mixtures are tested for activating dendritic cells using one or more immune adjuvants, for example, toll-like receptor (TLR) agonist (e.g., CpG-oligonucleotide (TLR9), imiquimod (TLR7), pol I:C (TLR3), glucopyranosyl lipid A (TLR4), murein (TLR2), flagellin (TLR5)), CD40 agonists (e.g., CD40-ligand), interferon-gamma, prostaglandin E1, etc. (US Patent Nos. 7,993,659; 7,993,648, 7,935,804). Dendritic activation is determined, for example, by ELISpot assay, ICS assay and/or tetramer assay which determine an immune stimulatory amount of the composition compared to control.

**Example 1: Use of Established Tumor Cell Lines to Determine the Viability and Proliferative Capacity of Circulating Tumor Cells (CTCs) in Blood**

[00323] Viable CTCs that have maintained their proliferative capacity are necessary for many non-clinical and clinical applications. Despite advancing technologies for isolation and enrichment of CTCs, technology for stabilizing CTCs in blood after collection but before processing, is lacking. Much of the current technology is based on fixation of the CTCs, rendering them non-viable and incapable of proliferating (Qin et al., Cancer Cell International 2014; 14:23). Accordingly, there is an unmed need for the development of collection systems that can maintain the viability and proliferation of CTCs under shipping conditions for approximated worst-case shipping duration.
As summarized in Figure 12, an established tumor cell line can be used as a proxy for CTCs in order to determine conditions that will make whole blood more conducive to CTC survival. A spike/retrieval (S/R) system can be used to (i) introduce a pre-determined number of live tumor cells to whole blood collected in K3EDTA vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ) or to Hanks Balanced Salt Solution (HBSS) (Sigma-Aldrich, St. Louis, MO); (ii) retrieve the cells from the whole blood or HBSS; and (iii) determine the viability and other functional properties of the tumor cells.

**Biological Material Procurement**

A tumor cell line representative of a highly metastatic cancer, for example, melanoma (Sk-Mel-28), breast (BT20) or non-small lung cancer (NCI-H460) can be obtained. The acquired line can be expanded and then frozen in a small working bank containing approximate 50 vials of $1 \times 10^6$ cells/vial. The viability of the cells in the frozen bank can be tested by thawing one random vial and performing a standard viability test (e.g., trypan blue exclusion).

Whole blood can be freshly obtained from consented healthy donors. The blood samples can be stored refrigerated during transportation (4°C) and used immediately (within 2-3 hours from collection).

**Lacing Whole Blood with Tumor Cells**

A determined amount of whole blood collected in K3EDTA vacutubes can be mixed with various numbers of cells from a cryopreserved stock. Two days prior to mixing, the tumor cells can be thawed and maintained in culture conditions to restore metabolic and proliferative activity. The culture can be harvested by enzymatic dissociation and the cell count and viability can be recorded. The cell pellet can be re-suspended in a minimum amount of saline and used for five dilutions in 1 mL of blood each (Table 2 and Figure 13). Another set of mixtures with identical concentrations of tumor cells can be made using Hanks Balanced Salt Solution.
(HBSS) instead of blood. The mixtures can be analyzed immediately for tumor cell retrieval.

Table 2. Exemplary experimental set up for spiking 1 ml of anticoagulated whole blood or HBSS with various concentrations of tumor cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Known concentration</th>
<th>Retrieval 1</th>
<th>Retrieval 2</th>
<th>Retrieval 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Conc.</td>
<td>Viability</td>
<td>Conc.</td>
</tr>
<tr>
<td>1</td>
<td>0 cells/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10 cells/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>100 cells/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1,000 cells/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>10,000 cells/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Retrieval and Viability

The laced blood and HBSS mixtures can be subjected to a flow-cytometric analysis. Identical procedures of preparation and labeling can be performed on each sample. The blood sample can be washed with saline solution and the red blood cells can be lysed with FACS Lysing Solution (BD Biosciences, San Jose, CA). The mixture can be labeled for CD3 (T cell specific antigen) and CD45 (leucocyte common antigen). 7-AAD (7-Aminoactinomycin D) can be used to label non-viable cells. As an alternative method for RBC separation, Ficoll density gradient (GE Healthcare Bio-Sciences, Pittsburgh, PA) can be applied and the buffy coat can be subjected to FACS analysis (Figure 14).

The viable tumor cells are CD3/CD45/7ADD negative. The test can be repeated 3 times as in Table 2 and the results from blood and HBSS can be statistically analyzed. By performing a paired parametric test between the known concentration series and retrieved concentrations, the accuracy of the retrieval system can be validated and correction factors can be introduced if necessary. Additionally, this experiment can be used to establish a reliable threshold of detection, by setting an acceptable variance between determinations. Using power analyses, the minimum number of cells necessary for a reliable detection can be
established. The detection threshold is necessary to establish a spiking concentration for the subsequent experiments that minimizes the detection system noise.

[00331] To remove leukocytes from the buffycoat, Dynabeads® CD45, super paramagnetic beads covalently coupled to anti-human CD45 antibody (Life technologies, Grand Island, NY) can be used. Dynabeads® enable isolation or depletion of CD45+ leukocytes. The remaining cells unbound to the beads can be collected from supernatant and analyzed for total number and viability. In addition, this method can be used to analyze the proliferative and spherogenic properties of the retrieved circulating tumor cells (Figure 15).

[00332] These experiments can be used to establish an S/R system that is reliable with low noise, establish the minimum spiking threshold with tumor cells, and provide a method to retrieve purified tumor cells for further investigations.

Establishment of a Baseline Survival Curve

[00333] Using the validated S/R system, a baseline survival curve of the tumor cells can be established anticipating standard transportation conditions (refrigerated packaging, 4°C). For each time point, a minimum volume of spiked blood sample and a reliable concentration of tumor cells (as determined during the S/R system validation experiments) can be used. The samples can be exposed for 1 hour to room temperature, simulating blood collection and preparation for transportation) followed by refrigeration at 4°C for: 12, 24, 36, 48, 60, 72, 84, 96 and 108 hours.

[00334] The results of the retrieval can be included in a table and the survival plotted on a Kaplan-Meier (K-M) graph. This experiment can be used to establish a baseline survival of tumor cells exposed to blood.

Identification of Factors that Enhance Tumor Cell Survival in Collected Blood
The following experiments can be used to improve the survival of tumor cells in whole blood, with the goal of achieving viability greater than 50% at 96 hours. With the intent of manufacturing scalability for clinical applications, and based on a quality-by-design concept, factors that are suitable for viable and scaled commercial manufacturing were selected. Only components that have a predicted individual stability on the order of 3 months are considered for testing. In addition, only calcium independent factors are considered, as the preferred anticoagulant is a chelator (K3EDTA).

Whole blood, a highly complex tissue, poses unique challenges that threaten CTC viability. Briefly, blood is a composite of erythrocytes (red blood cells), leukocytes (white blood cells), platelets, protein rich plasma (e.g., albumin, coagulation proteins), nutrients/metabolites (glucose) and dissolved gases (i.e., oxygen and carbon dioxide). In order to effectively sustain CTCs in this environment, the potential implications posed by each of these components must be anticipated and preventative solutions must be developed.

These challenges and the potential solutions are summarized in Table 3.

Table 3. List of experimental challenges and solutions

<table>
<thead>
<tr>
<th>Factors that could impact cell survival</th>
<th>Parameter</th>
<th>Specific approach</th>
<th>General approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulation</td>
<td>Ca depletion by K3EDTA</td>
<td>To be investigated</td>
<td>- Temperature decrease, - Selective erythrolysis - Dilution</td>
</tr>
<tr>
<td>Metabolic depletion</td>
<td>Hypoxia/acidosis</td>
<td>Buffering, NaHCO3,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Energetic substrate depletion</td>
<td>Addition of glucose and L-glutamine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactic acid accumulation and free radicals</td>
<td>Scavengers: N-acetyl-cysteine, glutathione</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitric oxide</td>
<td>Scavengers: N-Acetyl-</td>
<td></td>
</tr>
</tbody>
</table>
Coagulation. Coagulation is a major concern for CTC viability in whole blood. Blood contains platelets and serum proteins (i.e., coagulation factors) that induce clotting as a mechanism to prevent blood loss. If drawn blood is not properly treated with an anti-coagulant, the blood will clot and trap/compromise the viability of the CTCs. Coagulation is a complex cascade process with many steps and layers of regulation (reviewed Furie et al., Cell 1988, 53:505-18). Despite this complexity, calcium (Ca\(^{2+}\)) is absolutely required for coagulation. As a means of preventing coagulation, anti-coagulation agents (e.g., heparin, sodium citrate, EDTA) are added to whole blood to chelate Ca\(^{2+}\) ions. For example, K3 EDTA can be incorporated into these experiments using the recommended concentration of 1.5 mg/mL of blood (NCCLS, 1996). It is unknown whether K3EDTA is cytotoxic to CTCs. Therefore, initial experiments can be performed to test the in vitro toxicity of K3EDTA. For example, a 96 well plate containing 10,000 cells/well in off-the-clot human serum (Valley Biomedical, Winchester, VA) or culture media, can be subjected to various K3EDTA concentrations (0.0; 0.5, 1.0; 1.5 mg/mL) and analyzed for morphology and survival at 12, 24, and 48 hours at 37°C (Table 4).
[00340] **Metabolism.** One milliliter (1 ml) of blood contains 4 - 8 million erythrocytes, which accounts for 40-45% of the blood volume. An erythrocyte uses glucose and glycolysis as its sole source of energy. Pyruvate is the end product of glycolysis. In cells with mitochondria and oxidative metabolism, pyruvate is converted completely into CO₂ and H₂O, termed aerobic glycolysis (Feig et al., Journal of clinical Investigation 1972, 51:1547-54). In erythrocytes, which lack mitochondria and oxidative metabolism, pyruvate is reduced to lactic acid (the product of anaerobic glycolysis). As peripheral blood is collected from a venous source, the oxygen load is already diminished. It is expected that blood kept at body temperature will rapidly consume the glucose from the serum, decrease O₂ levels, and increase the concentration of CO₂ and lactic acid. As a consequence, hydrogen ions accumulate and can decrease the pH of the blood (acidosis). Oxygenation of the blood sample is not practical after collection in vacutainer tubes. Measures that reduce the basal metabolism and consequently the oxygen and energetic substrate consumption should be in place for any transportation system. A "hibernation" status could be accomplished by simple reduction in the storage temperature during transportation and manipulations. The effect of temperature induced hibernation can be tested by subjecting whole blood samples spiked with tumor cells to ambient temperature (18°C) and 4°C (Table 4).

[00341] Without being bound by theory, it is hypothesized that supplementing whole blood with cell culture medium can improve survival by increasing metabolic substrate (e.g., glucose, L-glutamine) levels, adding antioxidants (e.g., glutathione) and providing additional pH buffering capacity (e.g., HEPES, sodium bicarbonate and phosphate buffer systems). The addition of cell culture medium can also produce a dilution of the whole blood that may be beneficial for survival by reducing cell density. For example, the culture medium can contain: a basal formulation such as DMEM:F12 supplemented with insulin, glutathione, putrescine, and HEPES buffer. To test this hypothesis, whole blood can be diluted with cell culture medium or a specialized commercial hibernation media (e.g.,
HypoThermosol®, Sigma-Aldrich, St. Louis, MO; or Prime-XV®, Irvine Scientific, Santa Ana, CA) and tested with the lacing/retrieval system (Table 4).

[00342] Hemolysis of erythrocytes during storage and shipment could reduce O₂ levels in the blood due to released hemoglobin. Without being bound by theory, the addition of the hemoglobin-binding glycoprotein heptaglobin can be used to scavenge free hemoglobin and prevent oxygen reduction (Ascenzi et al., IUBMB Life 2005, 57:749-59). The inclusion of heptaglobin has the added benefit of acting as an anti-inflammatory agent when complexed with hemoglobin. More specifically, during inflammation, macrophages express the cell surface receptor CD1 63 which binds heptaglobin-hemoglobin complexes and mediates its endocytosis. Following exposure to heptaglobin-hemoglobin complexes, macrophages release the anti-inflammatory cytokine interleukin 10 (IL-10) (Ascenzi et al., 2005). Three different phenotypic variants of the heptaglobin gene exist in human: heptaglobin 1-1, 2-1 and 2-2 (Langlois et al., Clinical Chemistry 1996, 42:589-600). These heptaglobin polymorphisms have different abilities to interact with CD1 63 when bound to hemoglobin. For example, heptaglobin 1-1 interacts weakly with CD1 63, heptaglobin 2-1 moderately binds to CD1 63 and heptaglobin strongly interacts with CD1 63. The cytoprotective effect of heptaglobin 2-2 on CTCs viability at concentrations of 10 µg/mL and 100 µg/mL can be determined (Table 4).

[00343] **Nitric Oxide and free radicals.** The effect of the NO scavenger carboxy-PTIO at concentrations of 10 and 100 µM on CTC viability in whole blood can be tested (Table 4). Cells in the blood rapidly consume dissolved oxygen and release nitric oxide (NO) as a specific response to hypoxia. High levels of NO may be cytostatic or cytotoxic for tumor cells, whereas low level activity of NO can have the opposite effect and promote tumor growth. Therefore, one challenge in keeping CTCs viable in blood up to 96 hours is the potential cytotoxic effect of NO.

[00344] **Immune activation.** In addition to the NO scavenger carboxy-PTIO, the NO-synthase inhibitor L-NG-methylarginine can be tested as means to suppress inflammatory responses at concentration of 10 and 100 µM (Table 4)
One percent of all blood cells (4,000 - 10,000 cells per ml) are white blood cells (neutrophils, basophils, eosinophils, monocytes, and lymphocytes). Commonly referred to as leukocytes, white blood cells are principally responsible for both the innate and adaptive immune systems. Biochemical and physiological perturbations during whole blood storage can decrease the pH and alter metabolite levels triggering immune responses (Yazdanbakhsh et al., Hematology 2011, 2011:46-9). NO production by hypoxic erythrocytes was found to be responsible for macrophage activation. Without being bound by theory, it is hypothesized that suppressing immune cell activation will improve CTC survival.

[00345] **Specific survival pathways.** A cocktail of cytokines that enhance the survival pathways of the cells can be used to extend the viability of CTCs. These factors include, but are not limited to, ligands for receptor tyrosine kinases (e.g., FGF, EGF, PDGF, NGF and HGF), TGF beta proteins, Rho kinase (ROCK) inhibitors and PTEN inhibitors. These factors are known for their specific effects on normal and tumor cells in cell culture media or in vivo. Each of these factors can be tested at two concentrations (high and low) for their ability to extend CTC viability in the presence of blood and K3DETA (Table 4).

[00346] CTCs enter the blood stream via an epithelial-to-mesenchymal (EMT) transition. EMT, driven by the interplay between growth factor signaling activities, induces epithelial cells to lose their polarity and adhesion properties and adopt a migratory mesenchymal fate. During carcinogenesis, EMT supports metastasis through tumor-initiating cell dissociation from the primary tumor and spread to other sites in the body. Growth factor signaling pathways with known roles in oncogenic EMT include, but are not limited to, epidermal growth factor (EGF) pathway, the WNT signaling pathway, the transforming growth factor beta (TGFβ) pathway, the Notch pathway, the Hedgehog pathway and integral signaling (Mimeault et al., Annals of Oncology 2007, 18:1 605-19). These growth factors also play key
roles in cancer stem cell growth and survival. Therefore, these growth factors (either alone or in combination) can be integrated to stabilize CTC viability, recovery and function.

[00347] Rho-associated protein kinases (ROCK) mediate various cellular functions such as cell shape, motility, secretion, proliferation, and gene expression. ROCK expression is increased in metastatic tumors and is believed to contribute to invasive properties (Liu et al., Cancer Research 2009, 69:8742-51). In other tumors, increased ROCK expression causes tumor inhibition (Castro et al. Stem Cells 2013, 31:12-22). The use of ROCK inhibitors (e.g., Y27632) in embryonic stem cell culture systems permits survival of isolated cells at clonal dilutions. Therefore, CTC survival in the presence of blood and Y27632 can be tested (Table 4).

[00348] CTC survival in the presence of blood and PTEN Inhibitor SF1670 can be tested (Table 4). It has been shown that the phosphoinositide 3-kinase (PI3K) signaling pathway is required for maintenance of embryonic stem cells (ESC) pluripotency by regulating Nanog and Sox2, both of which are transcription factors involved in self-renewal of ESCs. In addition, the PI3K pathway is known to be negatively regulated by phosphatase and tensin homolog deleted on chromosome ten (PTEN), a well-known tumor suppressor that is deleted or mutated in various types of cancer. Inhibition of PTEN tumor suppressor promotes the generation of induced pluripotent stem cells (Liao et al., Molecular Therapy 2013, 21:1242-50).

[00349] **Table 4.** Experimental matrix of factors that will be tested for tumor cells survival in blood samples

<table>
<thead>
<tr>
<th>Variable</th>
<th>Test groups</th>
<th>Active ingredient concentration</th>
<th>Duration (hours)</th>
<th>Total data points</th>
</tr>
</thead>
<tbody>
<tr>
<td>K3EDTA toxicity</td>
<td>Culture media</td>
<td>0.0; 0.5; 1.0; 1.5</td>
<td>12; 24; 48</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Human serum</td>
<td>0.0; 0.5; 1.0; 1.5</td>
<td>12; 24; 48</td>
<td>12</td>
</tr>
<tr>
<td>Temperature</td>
<td>Whole blood</td>
<td>18 °C, 4 °C</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>18</td>
</tr>
<tr>
<td>Variable</td>
<td>Test groups</td>
<td>Active ingredient concentration</td>
<td>Duration (hours)</td>
<td>Total data points</td>
</tr>
<tr>
<td>---------------------------</td>
<td>---------------------</td>
<td>---------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Dilution</td>
<td>Culture media</td>
<td>0%; 20%; 30%; 40% 50%</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>HypoTherosol</td>
<td>10%; 20%; 30%; 40% 50%</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Prime-XV</td>
<td>10%; 20%; 30%; 40% 50%</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>10%; 20%; 30%; 40% 50%</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>45</td>
</tr>
<tr>
<td>Hemoglobin scavenger</td>
<td>Heptaglobin 2-2</td>
<td>10-1 00 μg/mL</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>18</td>
</tr>
<tr>
<td>NO scavenger</td>
<td>carboxy-PTIO</td>
<td>10-1 00 μM</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>18</td>
</tr>
<tr>
<td>NO-synthase inhibitor</td>
<td>L-NG-methylarginine</td>
<td>10-1 00 μM</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>18</td>
</tr>
<tr>
<td>RTK</td>
<td>FGF</td>
<td>10-1 00 ng/mL</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>EGF</td>
<td>10-1 00 ng/mL</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>18</td>
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<tr>
<td></td>
<td>IGF</td>
<td>10-1 00 ng/mL</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
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<tr>
<td></td>
<td>PDGF</td>
<td>1-10 ng/mL</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
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</tr>
<tr>
<td></td>
<td>NGF</td>
<td>10-1 00 ng/mL</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>HGF</td>
<td>10-50 ng/mL</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>18</td>
</tr>
<tr>
<td>TGFb-R</td>
<td>Activin A</td>
<td>5-10 ng</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>TGFb</td>
<td>1-20 ng/mL</td>
<td>12; 24; 36;</td>
<td>18</td>
</tr>
<tr>
<td>Variable</td>
<td>Test groups</td>
<td>Active ingredient concentration</td>
<td>Duration (hours)</td>
<td>Total data points</td>
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<tr>
<td>----------------</td>
<td>-------------</td>
<td>---------------------------------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Wnt</td>
<td>WNT3A</td>
<td>1 - 20 ng/mL</td>
<td>48; 60; 72; 84; 96; 108</td>
<td>18</td>
</tr>
<tr>
<td>Rock Inhibitor</td>
<td>Y27632</td>
<td>5-10 μM</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>18</td>
</tr>
<tr>
<td>PTEN Inhibitor</td>
<td>SF1 670</td>
<td>1-10 μM</td>
<td>12; 24; 36; 48; 60; 72; 84; 96; 108</td>
<td>18</td>
</tr>
</tbody>
</table>

**Experimental design**

[00350] The factors listed in Table 4 can be tested in 96 well plate format. Tumor cells can be seeded at a density of 10,000 cells/well on cell culture treated plastic in a media that contains RPMI (Sigma-Aldrich, St. Louis, MO) and 5% FBS (Life Technologies, Grand Island, NY). The cells can be allowed to recover from cryogenic "stasis" for 48 hours before experimentation. Active ingredients can be reconstituted at the listed concentrations in freshly collected blood (within 1-3 hours from collection in K3EDTA).

[00351] Strategically, 96-well plates offer the advantage that multiple growth factor combinations can be tested in parallel and provide a convenient platform for cell proliferation assays (e.g., MTT, EMD Millipore, Billerica, MA). For each time point, a plate containing a group of factors in 3 replicates, and one control line with no factors added can be prepared. After exposure to blood for the tested time, at the temperature that was determined to be optimal in the previous experiment, the blood will be removed by aspiration, cells rinsed with HBSS and cell culture media added. Next, a second control line on each plate can be seeded containing a serial dilution of increasing cell densities from 1000 to 50,000 cells per well in culture media. The plate can be incubated for 24 hours in a 37°C/CO₂ incubator. After incubation, an MTT assay can be performed to assess cell viability. The replicates can be averaged and the readings can be normalized to the serial diluted line to determine the viable cell number. Each test factor condition can be compared to a control (no factor condition).
These experiments can be used to determine the factors that are significant for cell survival by analyzing the survival curves at 12; 24; 36; 48; 60; 72; 84; 96; 108 hours.

**Example 2: Use of Established Tumor Cell Lines to Determine the Effect of a Survival Factors Cocktail (SFC) on Survival of Tumor Cells in Collected Blood Samples**

[00352] In this experiment, the factors determined in the previous experiments can be mixed with blood collected on K3EDTA laced with tumor cells. The mixture can be incubated at the optimal storage temperature in parallel with a control mixture not containing survival factors. The mixture can be analyzed with the validated S/R method and compared to baseline survival curve. This experiment can be used to confirm that the composition of the survival factor cocktail (SFC), when added to whole blood, contributes to the extended viability of CTCs.

[00353] Cells retrieved by Ficoll gradient and Dynabead separation can be used to demonstrate the proliferative and spherogenic ability of tumor cells in collected blood. Retrieved cells can be plated in a cell culture system consisting of polystyrene cell culture vessels (flasks, 6-well plate) and media that is formulated for cell expansion. In parallel, cells not exposed to blood samples can be plated at the same density and used as a control. The expansion rate can be compared to the control cells, and phenotypical analyses with specific markers (e.g., CD146, CD271) for the cell line can be used to confirm the preservation of phenotype and proliferative capability.

[00354] A spherogenic assay can be initiated by dissociating the adherent cultures and transferring the cells in ultra-low adherent cell culture vessels (Corning, Tewksbury, MA) in serum-free media. The spherogenic assay has been routinely used with cancer stem cells to definitively demonstrate the intrinsic properties of 'stem cells' of self-renewal in an isolated sub-population of tumor cells (Yuan et al, Oncogene 2004, 23: 9392-9400).
Example 3: Testing of Survival Factors Cocktail (SFC) on Additional Solid Carcinomas

[00355] The survival factor cocktail (SFC) can be tested on a second solid carcinoma type tumor (e.g., melanoma, hepatocellular carcinoma, ovarian carcinoma, colon carcinoma, GBM, breast or non-small lung).

[00356] Recovered cells after Ficoll and CD45-Dynabeads separation can be subjected to cell proliferation assays and spherogenic assays. It has been observed that the majority of carcinomas that are successfully cultivated towards isolation of tumor initiating cells are spherogenic in appropriate conditions. In some cases, tumor initiating cell phenotype can be demonstrated by the presence of markers such as CD133 in ovarian cancer side population or CD20 in some cases of non-spherogenic melanoma cell lines. The proliferative potential can be assessed by cultivation for 3 passages and establishment of growth curves in comparison to equivalent starting cell numbers that were not exposed to the blood storage/transportation conditions.

[00357] The results can be used to demonstrate that (i) the SFC can be applied to other tumor types; (ii) these tumor cells maintain viability greater than 50%; and (iii) the tumor cells retain their proliferative and spherogenic/phenotypic characteristics.

Example 4: Double-blind Trial of Autologous Vaccines Consisting of Adjuvant GM-CSF + dendritic cells loaded with irradiated circulating tumor cells (DC-CTC) versus GM-CSF + PBMC (MC) in Patients with Ovarian Cancer

[00358] Cancer stem cells (CSCs) circulating within the blood stream are known as circulating tumor cells (CTCs). CTCs express the markers of cancer stem cells (CSCs) that have adopted an epithelial to mesenchymal transition (EMT) for increased motility and migration. CTCs express biomarkers including, for example, N-cadherin, vimentin, CD44, and fibronectin (Liu et al., (2014) Stem Cell Reports
CTCs isolated from blood for expansion and production of autologous circulating tumor cancer stem cell lines can be used to produce autologous dendritic cell-circulating tumor cell (DC-CTC) vaccines for the treatment of solid tumors.

For example, a double-blind study can be performed in which study patients can be randomized in a 2:1 ratio to receive either DC-CTC (autologous dendritic cells loaded with irradiated autologous circulating tumor cells in GM-CSF) or control (MC) (autologous PBMC in GM-CSF). Patients eligible for treatment can be those (1) who have undergone debulking surgery, (2) for whom a cell line has been established, (3) who have undergone leukapheresis, (4) are scheduled for primary adjuvant chemotherapy, and (5) who have an ECOG performance grade of 0 or 1.

The primary endpoint of this trial can be death from any cause with the primary endpoint of overall survival (OS) from the date of randomization. Progression-free survival (PFS) can be a secondary endpoint and can be calculated as the time from the date of randomization for treatment to subjective tumor progression or death. Progression can be subjectively defined by the treating physician, and can be based on tumor marker levels (e.g. CA-125) and/or imaging. Secondarily, PFS and OS can also be defined from the date of debulking surgery.

Patients can be stratified into two groups: (1) platinum-resistant, based on progression during adjuvant chemotherapy or detectable disease at the conclusion of adjuvant therapy or (2) platinum-sensitive, with no evidence of disease (NED) at the conclusion of adjuvant therapy (per elevated blood CA-125 and/or tumor markers and/or detection of disease by physical examination or imaging) as summarized in the Table 5.

<table>
<thead>
<tr>
<th>Table 5. Summary of Stratification and Treatment Plan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platinum Sensitive</td>
</tr>
</tbody>
</table>

2:78-91 ; Mani et al., Cell 2008, 4:704-715.
Details regarding the trial design are as follows:

**Primary Objective:**
- Compare overall survival (OS) from the date of randomization for patients treated with DC-CTC (autologous dendritic cells pulsed with autologous irradiated circulating tumor cells in GM-CSF) to patients treated with autologous blood mononuclear cells in GM-CSF (MC) control.

**Secondary Objectives:**
- Establish safety of repeated administration of DC-CTC.
- Compare duration of progression-free survival (PFS) from the date of randomization for patients treated with DC-CTC to patients treated with MC.
- In the subset of platinum-sensitive patients with no evidence of disease (NED) after post-debulking chemotherapy, compare OS and PFS from date of randomization.
- In the subset of platinum-resistant patients, compare OS and PFS from date of randomization.
- Compare OS and PFS from the date of randomization in patients who have achieved optimal tumor debulking to patients whose tumor debulking was suboptimal. Optimal tumor debulking is defined as a debulking procedure that leaves patients with residual tumor nodules less than or equal to 1 cm in diameter.
- Compare OS and PFS from date of diagnosis and date of debulking surgery in patients treated with NBS-23 to patients treated with MC.
- Determine the percentage of patients who discontinue treatment due to reactions attributable to the treatment to GM-CSF.

**Study Treatment Arm:**

- **DC-CTC:** Autologous dendritic cells loaded with antigens from irradiated autologous circulating tumor cells and suspended in GM-CSF (treatment arm).
MC: Autologous peripheral blood mononuclear cells suspended in GM-CSF will serve as the control arm.

Patient Population: Female subjects 18 years or older with newly diagnosed stage III or IV ovarian cancer, who are candidates for receipt of chemotherapy. Performance status must be an ECOG score of 0 or 1 at the time of enrollment for treatment.

Inclusion Criteria:

Pre-Treatment Phase: Tissue Procurement and Establishment of Tumor Cell Line

Histologic diagnosis of epithelial ovarian, fallopian tube or primary peritoneal carcinoma.

Advanced (metastatic, stage III or IV) epithelial ovarian, fallopian tube or primary peritoneal carcinoma and enough CTCs obtained to establish a short-term circulating tumor cell line.

Age ≥ 18 years

Each patient must be aware of the neoplastic nature of her disease process and must willingly consent to the manipulation of tumor tissue for efforts to establish a tumor cell line. Patients will sign an Informed Consent to allow their blood/tissue to be given to NeoStem and for efforts to establish an autologous circulating tumor cell line

Patients must have the ability and willingness to travel to Newport Beach, California for administration of treatment.

Treatment Phase

Successful establishment of an autologous epithelial ovarian, fallopian tube, or primary peritoneal cancer cell line by NeoStem.

Patients must previously have been staged as having stage III (intraperitoneal) or Stage IV (distant metastatic) ovarian, fallopian tube, or primary peritoneal cancer and have initiated or completed standard adjuvant chemotherapy, which may include intravenous and/or intraperitoneal chemotherapy using standard regimens. Patients will be characterized as being in complete remission per physical exam, CT scans, and CA-125, or not in complete remission.
[00387] Medical fitness to undergo a leukapheresis, including peripheral venous access or access by central vein if necessary.

[00388] Negative pregnancy test for women of childbearing potential and use of effective contraception (hormonal or barrier method of birth control) for women of childbearing potential prior to therapy, during therapy, and 4 months after completing therapy.

[00389] Patients with one or a few brain metastases that have been treated with stereotactic radiotherapy consisting of a single dose, such as Gamma Knife or Cyberknife, are allowed to be included in the study, but need wait one week after such treatment.

[00390] Additional written informed consent for treatment including explanation of the procedures to be followed, the experimental nature of the therapy, alternatives, potential benefits, side effects, risks, and discomforts associated with this investigational therapy, will be obtained.

[00391] Exclusion Criteria:

[00392] Pre-Treatment Phase: Blood/Tissue Procurement and Establishment of Tumor Cell Line

[00393] ECOG performance status greater than 2.

[00394] Treatment Phase

[00395] Known positive for hepatitis B or C or HIV.

[00396] Pregnant or lactating women.

[00397] Underlying cardiac disease associated with myocardial dysfunction that requires active medical treatment, or unstable angina related to atherosclerotic cardiovascular disease, or under treatment for arterial or venous peripheral vascular disease

[00398] Diagnosis of any other invasive cancer which is considered to be life-threatening within the next five years, and/or taking anti-cancer therapy for cancer other than ovarian (such as continuation of hormonal therapy for breast cancer diagnosed more than five years earlier).

[00399] Active infection or other active medical condition that could be eminently life-threatening, including active blood clotting or bleeding diathesis.
Active central nervous system metastases at the time of treatment.

Known autoimmune disease, immunodeficiency, or disease process that involves the use of immunosuppressive therapy.

A low malignancy potential tumor.

Received another investigational drug within 28 days of the first dose.

Treatment Duration: The study product will be administered subcutaneously weekly for three consecutive weeks and then approximately every three to four weeks (at the time subsequent maintenance or secondary therapy is given), for up to a total of eight doses of treatment given over four to six months.

Dosage: Each dose of DC-CTC or MC contains approximately 5-10 million cells. Each dose is suspended in 500 μg GM-CSF prior to administration.

Mode of Administration: Subcutaneous (SC) injections.

Study Endpoints:

Primary Efficacy Endpoint:

Overall Survival (OS): time to death from date of randomization.

Secondary Efficacy Endpoints:

The success rate for establishing a tumor cell line.

Progression Free Survival (PFS): time to disease progression or death from date of randomization.

OS and PFS for the subset of patients who are platinum-sensitive after adjuvant therapy.

OS and PFS for the subset of patients who are platinum-resistant after/during primary adjuvant chemotherapy.

OS and PFS from dates of initial diagnosis and/or debulking surgery.

Safety Endpoints:

Adverse events attributed to the cell therapy (including GM-CSF) and not attributed to chemotherapy to assess safety and tolerability.

Abnormal findings by history and physical examination, vital signs, clinical laboratory tests (safety), and other tests as clinically indicated, that might be attributed to the cell therapy.
Study Duration and Follow-Up: All patients who qualify for participation in the study will be followed for up to 5 years from date of randomization or until death, whichever occurs first.

Sources of Materials

Blood will be obtained and CTCs can be isolated. The antigen source is autologous circulating tumor cells (CTCs) from the patient's blood sample. Blood will be handled with strict adherence to sterility protocols to ensure that samples are sterile. Specimens are shipped by overnight courier to the manufacturing facility (NeoStem) within 48 hours of procurement.

Since patients will be undergoing concurrent chemotherapy in all treatment groups, our therapy will be an adjunctive therapy, which is consistent with the general consensus that vaccine therapies are most efficacious as adjunctive (rather than front line) therapy.

Confidentiality and Informed Consent

Confidentiality and privacy of all participants will be maintained in accordance with the Health Insurance Portability and Accountability Act (HIPAA) of 1996. All reports and communications relating to the patients in the study will identify each patient only by the patient's initials and study number. Any images (e.g., scans) or laboratory reports will be de-identified before leaving, or in anyway being transmitted from, the clinical site. Patient names will be available to Sponsor representatives (e.g., study monitor) during review of medical records. This will be treated with strict adherence to professional standards of confidentiality.

Written informed consent for treatment will include explanation of the procedures to be followed, the experimental nature of the therapy, alternatives, potential benefits, side effects, risks, and discomforts associated with this investigational therapy, will be obtained.

Statistical Methods:
Null-Hypotheses: [1] For all patients, OS is no different for patients who receive DC-CTC compared to patients who receive MC; [2] For platinum-sensitive patients, OS is no different for patients who receive DC-CTC compared to patients who receive MC, [3] For patients who are platinum-resistant, OS is no different for patients who receive DC-CTC compared to patients who receive MC.

Analyses: Kaplan-Meier curves will display survival times for each treatment group. The log rank test will be used to analyze OS to test the null hypothesis of no treatment difference. The Cox regression model and the Wald test will be used to estimate the hazard ratio associated with treatment and to identify the significance of potential prognostic factors and their impact, if any on the treatment differences. Analyses based on the intention-to-treat population will be considered as the primary analysis, analyses based on the Per-Protocol population will be considered as a sensitivity analysis. Subgroup analyses of the OS endpoint will mimic the plan laid out for the primary endpoint for platinum-resistant patients and platinum-sensitive patients separately.

Example 5: Isolation and Expansion of Circulating Tumor Cells (CTCs) from Cancer Patient Blood Samples

Blood samples from patients diagnosed with various cancers (melanoma, prostate, renal and bladder) were collected in 10 mL vacutainer tubes containing K₂EDTA. After collection, the tubes were immediately transported to the processing laboratory and maintained at 4°C until use. Mononucleated cells were separated from components of whole blood by a Ficoll-Paque (GE Healthcare) gradient process. The separation layer (buffy coat) was further re-suspended in Hanks Balanced Salt Solution and filtered through a parylene-C slotted filtering membrane using a constant low-pressure delivery apparatus (figure 16). Bo Lu; Tong Xu; Zheng, S.; Goldkorn, A.; Yu-Chong Tai, "Parylene membrane slot filter for the capture, analysis and culture of viable circulating tumor cells," in Micro Electro Mechanical Systems (MEMS), 2010 IEEE 23rd International Conference on, vol., no., pp.935-938, 24-28 Jan. 2010. The filter that retained the cells with sizes above
the slot dimension was immediately transferred in a special formulated cell culture media to sustain cell viability and proliferation. This media is composed of basal media and lineage supplement formulation (Tables 6 and 7).

Table 6. Basal media formulation

<table>
<thead>
<tr>
<th>Component Description</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>CALCIUM CHLORIDE (ANHY)</td>
<td>116.61</td>
</tr>
<tr>
<td>COPPER SULFATE-5H2O</td>
<td>0.0013</td>
</tr>
<tr>
<td>POTASSIUM CHLORIDE</td>
<td>312</td>
</tr>
<tr>
<td>MAGNESIUM CHLORIDE (ANHY)</td>
<td>28</td>
</tr>
<tr>
<td>MAGNESIUM SULFATE (ANHY)</td>
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</tr>
<tr>
<td>SODIUM CHLORIDE</td>
<td>6250</td>
</tr>
<tr>
<td>SODIUM PHOSPHATE DIBASIC, ANHY</td>
<td>71</td>
</tr>
<tr>
<td>SODIUM PHOSPHATE MONOBASIC H2O</td>
<td>62</td>
</tr>
<tr>
<td>ZINC SULFATE-7H2O</td>
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</tr>
<tr>
<td>L-ALANINE</td>
<td>9</td>
</tr>
<tr>
<td>L-ARGININE-HCL</td>
<td>148</td>
</tr>
<tr>
<td>L-ASPARAGINE-H2O</td>
<td>16</td>
</tr>
<tr>
<td>L-ASPARTICACID</td>
<td>20</td>
</tr>
<tr>
<td>L-CYSTEINE-HCL-H2O</td>
<td>18</td>
</tr>
<tr>
<td>L-CYSTINE-2HCL</td>
<td>30</td>
</tr>
<tr>
<td>L-GLUTAMIC ACID</td>
<td>15</td>
</tr>
<tr>
<td>GLYCINE</td>
<td>20</td>
</tr>
<tr>
<td>L-HISTIDINE-HCL-H2O</td>
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<tr>
<td>L-ISOLEUCINE</td>
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<td>L-LEUCINE</td>
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<tr>
<td>L-LYSINE-HCL</td>
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<tr>
<td>L-METHIONINE</td>
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<tr>
<td>L-PHENYLALANINE</td>
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<tr>
<td>L-PROLINE</td>
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<tr>
<td>L-SERINE</td>
<td>30</td>
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<tr>
<td>L-THREONINE</td>
<td>53</td>
</tr>
<tr>
<td>L-TRYPTOPHAN</td>
<td>9</td>
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<tr>
<td>L-TYROSINE-2NA-2H2O</td>
<td>56</td>
</tr>
<tr>
<td>L-VALINE</td>
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<tr>
<td>CALCIUM D-PANTOTHENATE</td>
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<td>FOLIC ACID</td>
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<tr>
<td>Niacinamide</td>
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<tr>
<td>PYRIDOXAL HYDROCHLORIDE</td>
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<td>PYRIDOXINE-HCL</td>
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</tr>
<tr>
<td>RIBOFLAVIN</td>
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<tr>
<td>THIAMINE-HCL</td>
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<tr>
<td>VITAMIN B12</td>
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</tr>
<tr>
<td>D-GLUCOSE</td>
<td>2000</td>
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<tr>
<td>HEPES</td>
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<tr>
<td>Component Description</td>
<td>mg/L</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------</td>
</tr>
<tr>
<td>HYPOXANTHINE-2NA</td>
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<tr>
<td>LINOLEIC ACID</td>
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</tr>
<tr>
<td>DL-ALPHA-LIPOIC ACID</td>
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</tr>
<tr>
<td>SODIUM PYRUVATE</td>
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</tr>
<tr>
<td>THYMIDINE</td>
<td>0.365</td>
</tr>
<tr>
<td>SODIUM BICARBONATE</td>
<td>2100</td>
</tr>
</tbody>
</table>

**Table 7. Lineage supplement formulation**

<table>
<thead>
<tr>
<th>Components</th>
<th>Formulation (per unit, for 1 L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>value</td>
</tr>
<tr>
<td>WATER</td>
<td>QS to 50</td>
</tr>
<tr>
<td>HUMAN SERUM ALBUMIN</td>
<td>2.5</td>
</tr>
<tr>
<td>TRANSFERRIN PARTIALLY SATURATED</td>
<td>20</td>
</tr>
<tr>
<td>INSULIN</td>
<td>20</td>
</tr>
<tr>
<td>T3</td>
<td>0.002</td>
</tr>
<tr>
<td>SELENITE</td>
<td>0.01</td>
</tr>
<tr>
<td>PROGESTERONE</td>
<td>0.005</td>
</tr>
<tr>
<td>PUTRESCINE</td>
<td>10</td>
</tr>
<tr>
<td>GLUTATHIONE</td>
<td>1</td>
</tr>
<tr>
<td>CARNITINE</td>
<td>2</td>
</tr>
<tr>
<td>BIOTIN</td>
<td>0.05</td>
</tr>
<tr>
<td>L-GLUTAMINE</td>
<td>365</td>
</tr>
<tr>
<td>ETHANOLAMINE</td>
<td>15</td>
</tr>
<tr>
<td>HEPES</td>
<td>1</td>
</tr>
</tbody>
</table>

[00430] In addition, the media was supplemented with growth factors (EGF 10 ng/mL, and FGF 10 ng/mL). For first day only, the media was supplemented with N-Acetyl-Cysteine 1 mM (163.19 μg/mL); 1 μM PTEN inhibitor bpV(phen) (Potassium Bisperoxo(1,10-phenanthroline)oxovanadate(V)trihydrate, Sigma-Aldrich) and with 5 μM Rho-Associated-Kinase (ROCK) inhibitor (Y-27632, Stem Cell Technologies). The media was partially replaced Monday-Wednesday-Friday and supplemented with FGF/EGF combination, 10 ng/mL of each growth factor.

[00431] After 2 weeks from isolation, clonally expanding cells were observed on the surface of the filter device (Figure 17). In some cultures, fetal bovine serum was used to supplement the media. Other conditions maintained serum-free media for the duration of cell expansion. When cultures reached confluence, the cells were
dissociated using TrypLE, re-plated in regular cell culture flasks at a lower cell density, and maintained by replacing media every other day.

[00432] Removal of Contaminant Blood Cells from the Filtering Device:

[00433] The majority of monocytes that were captured on the filter along with CTCs differentiated into macrophages, and in lesser number, into dendritic cells (Figure 18). The differentiation into macrophages was accentuated by the presence of FBS in the media. Conversely, serum-free media progressively cleared macrophages (Figure 19).

[00434] Removal of the live macrophages from the filter device was attempted by using 1 µL/mL chlodronate liposomes (FormuMax®), from manufacturer's original solution. Although the macrophages visibly underwent apoptosis, microbiological contamination in some cultures lead to discontinuation of this liposome system because this system cannot be sterilized.

[00435] When the cultures were dissociated with TrypLE, only the tumor cells were lifted from the filter device, while the macrophages remained attached. This method efficiently separated the macrophages from the tumor cells without introducing potential microbial contamination (Figure 20).

**Example 6: Phenotypic Transformation of Cultured CTCs**

[00436] After 3-4 weeks of expansion, adherent tumor cells began to lose their ability to attach to substrate as evidenced by cells floating in the media (Figure 21). Under continuous culture, the floating cells formed spheroid structures that progressively expanded. A mutational analysis of the non-adherent cultured tumor cells was performed. The results of the mutational analysis revealed common mutations that affected the hemophilic integrin pathway, calcium metabolism and o-linked glycosylation processes (Figure 22). Without being limited by theory, these results could explain the mechanism of metastasis by activation of mutated pathways in certain conditions such as epithelial to mesenchymal transition.
[00437] Research indicates that, although ROCK inhibitors may be effective in reducing adherent tumor cell behavior, these inhibitors could inadvertently increase metastatic potential of non-adherent CTCs by increasing their reattachment efficacy (Bhandary L, Whipple RA, Vitolo MI, et al. ROCK inhibition promotes microtentacles that enhance reattachment of breast cancer cells. Oncotarget. 2015;6(8):6251 - 6266). Therefore, the ability of ROCK inhibitor Y-27632 to reverse the loss of adherence by tumor cells was investigated. 5 μM of ROCK inhibitor Y-27632 was added to culture media of non-adherent tumor cells. The tumor cells exposed to 5 μM of ROCK inhibitor re-gained adherence after overnight exposure, thus indicating that the loss of adherence by tumor cells can be reversed by the addition of ROCK inhibitor Y-27632 into the culture media (Figure 23).

Example 7: Enhanced Survival of CTCs by Combined Effect of Rock Inhibitor and PTEN Inhibitor

[00438] One disadvantage of a fixed slot size filter is that it may not capture CTCs smaller than the slot size. In this study, ROCK and PTEN inhibitors were used to isolate CTCs by a differential adherence method. The method was used to remove lymphocytes in a neutral media in order to avoid non-specific activation of cytotoxic effectors, followed by enzymatic dissociation of tumor cells and establishment of tumor cell lines without the use of a fixed slot size filter.

[00439] The successful expansion of single circulating tumor cells in the absence of feeder cells has been attributed to the combination of ROCK inhibitor and phosphatase and tensin homolog (PTEN) inhibitor in the initial cultures. The high RhoA activity in cells that are poorly attached to substrates can cause an increase in the activity of ROCK, a well-known effector of RhoA, that upregulates the activity of phosphatase and tensin homolog (PTEN).
Without being limited by theory, we believe that the poorly adherent phenotype of CTCs is predominant and that attempts to isolate CTCs by an adherent method in the absence of ROCK inhibitors could fail. Therefore, non-adherent CTCs were exposed to ROCK inhibitor Y-27632 as described in Example 6 in order to restore adherence and facilitate isolation. Adherent tumor cells were separated from adherent macrophages by exposing the cell cultures to a gentle enzymatic digestion using TrypLE (Life Technologies). This gentle enzymatic digestion lifts only tumor cells from the culture surface; leaving macrophages attached.

The administration of PTEN inhibitors activates the phosphoinositide 3-kinase (PI3K) pathway, which is important for self-renewal, proliferation, and maintenance of self-renewing cells. Without being limited by theory, the use of a PTEN inhibitor alone or combination with a ROCK inhibitor can influence survival and proliferation pathways, thereby establishing a tumor cell line from isolated CTCs. Non-adherent cells were exposed to 1 μM PTEN inhibitor potassium bisperoxo(1,10-phenanthroline)oxovanadate (V) (Sigma-Aldrich) and 5 μM ROCK inhibitor Y-27632. As shown in Figure 24, the addition of the inhibitors in combination resulted in a potent anti-apoptotic and proliferative effect that rescued captured CTCs. As shown in Figure 25 and Figure 26, immune-histological characterization of expanded tumor cells revealed a typical tumor phenotype (Vimentin, Magel, Snail, CD44, EpCAM and NCAM).

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. An immunopotent tumor-specific cancer cell product comprising:
   a) an immunogenic composition comprising:
      i) an immunostimulatory amount of an activated population of dendritic
         cells contacted ex vivo with a cancer stem cell population expressing
         at least one cancer-specific antigen, the cancer stem cell population
         derived from a tumor cell, tumor cell cluster, tumor cell aggregate or a
         combination thereof derived from peripheral blood and originating from
         a solid tumor;
      ii) an adjuvant; and
   b) a pharmaceutically acceptable carrier,

   wherein the immunopotent tumor-specific cancer cell product stimulates an
   effective immune response against one or more cancer-specific antigens.

2. The immunopotent tumor-specific cancer cell product according to claim 1,
   wherein the adjuvant is granulocyte-macrophage colony stimulating factor (GM-
   CSF).

3. The immunopotent tumor-specific cancer cell product according to claim 1,
   wherein the cancer stem cell population expresses at least one cancer-specific
   antigen with mesenchymal characteristics.

4. The immunopotent tumor-specific cancer cell product according to claim 1,
   wherein the cancer stem cell population expresses at least one cancer-specific
   antigen with embryonic stem cell characteristics.

5. The immunopotent tumor-specific cancer cell product according to claim 1,
   wherein the cancer stem cell population expresses at least one cancer-specific
   antigen with mixed stem cell characteristics.

6. The immunopotent tumor-specific cancer cell product according to claim 1,
   wherein the solid tumor is selected from the group consisting of a colon
carcinoma, a renal carcinoma, a glioblastoma multiforme, a hepatocellular carcinoma, an ovarian carcinoma, a breast carcinoma and a prostate carcinoma.

7. The immunopotent tumor-specific cancer cell product according to claim 1, wherein the cancer stem cell population expressing at least one cancer-specific antigen is inactivated by irradiation.

8. The immunopotent tumor-specific cancer cell product according to claim 1, wherein the immunostimulatory amount comprises at least $1 \times 10^3$ cancer cell-specific antigen-stimulated dendritic cells.

9. The immunopotent tumor-specific cancer cell product according to claim 1, wherein the effective immune response comprises an effective T cell response, an effective B cell response, or a combination thereof.

10. The immunopotent tumor-specific cancer cell product according to claim 9, wherein the effective immune response comprises activation and proliferation of CD4+ T cells, CD8+ T cells, B cells, or a combination thereof.

11. A method for preparing the tumor-specific cancer cell product according to claim 1, comprising:

1) obtaining a blood sample from a patient comprising a tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof isolated from peripheral blood and originating from a tumor;

2) incubating the blood sample for about 30 minutes to 108 hours at 2-8°C in the presence of a survival factor cocktail,

wherein the survival factor cocktail comprises an antioxidant, a ligand for receptor tyrosine kinase and a ligand for G protein-coupled receptors, and wherein the survival factor cocktail dilutes the whole blood between 10% to 200%;

3) expanding the tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof by:
a. transferring the tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof to an adherent substrate for about 2-4 weeks, wherein the adherent substrate comprises one or more of an Arginine-Glycine-Aspartic Acid (RGD)-rich compound and a serum-free medium; and

b. feeding the cell population comprising the tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof every 2 days with fresh media, wherein the fresh media consists of a basal stem cell formulation supplemented with a protein/growth factor mixture, wherein the growth factor is a fibroblast growth factor (FGF), and epidermal growth factor (EGF), or a combination thereof;

(4) dissociating cells adherent to the adherent substrate enzymatically,

(5) transferring the dissociated adherent cells to a low adherent or ultra-low adherent surface to produce a cancer-specific stem cell culture comprising cancer stem cell spheroids;

(6) maintaining the cancer-specific stem cell culture comprising cancer stem cell spheroids for at least 14 days in a culture medium supplemented with growth factors that act through a MAPK pathway;

(7) sedimenting the spheroids to separate from single cells;

(8) transferring the spheroids to an adherent substrate;

(9) expanding the spheroids in vitro to establish a population of adherent cancer-specific stem cells;

(10) inactivating the population of adherent cancer-specific stem cells by irradiation, the irradiated population of adherent cancer-specific stem cells being characterized by an inability to proliferate;

(11) cryostoring the irradiated cancer-specific stem cells in vapor phase liquid nitrogen storage;
preparing a population of dendritic cells by:

a. obtaining peripheral blood mononuclear cells (PBMCs) by leukapheresis from the patient from whom peripheral blood sample comprising the tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof was obtained in step (1);

b. purifying the collected PBMCs from other lymphocytes;

c. incubating the purified PBMCs of step b. with GM-CSF and IL-4 for 6 days to generate the population of dendritic cells;

(13) thawing the irradiated cancer stem cells of step (10);

(14) contacting the population of dendritic cells of step (11) with the thawed irradiated cancer stem cells of step (12) for 18-24 hours to form a stimulated dendritic cell-tumor-derived cell antigen composition; and

(15) collecting and storing the dendritic cell-tumor-derived cell composition in vapor phase liquid nitrogen.

12. A method for treating a cancer patient comprising:

a. preparing for the cancer patient a patient-specific immunopotent tumor-specific cancer cell product comprising an immunostimulatory amount of an isolated population of dendritic cells contacted ex vivo with a population of cancer-specific cancer stem cells expressing a cancer-specific antigen by the method according to claim 1;

b. administering the immunopotent tumor-specific cancer cell product to the cancer patient, wherein the immunogenic composition is effective to generate an immune response comprising activation and proliferation of CD4+ T cells, CD8+ T cells, B cells or a combination thereof; wherein the effective immune response is effective to improve a clinical parameter selected from progression-free survival, disease-free survival, time to progression, time to distant metastasis, overall survival of the subject when compared to a control.

13. A composition comprising:
a. an inhibitor selected from the group consisting of a ROCK inhibitor, a PTEN inhibitor and a combination thereof;

b. a growth factor selected from the group consisting of FGF, EGF and a combination thereof; and

c. N-acetyl cysteine,

wherein the composition is effective to isolate circulating tumor cells (CTC) from a blood sample obtained from a cancer patient.

14. The composition according to claim 13, wherein the ROCK inhibitor is selected from the group consisting of Y-27632, Thiazovivin, Slx-21 19, WF-536 [(+)-(R)-4-(1-aminoethyl)-N-(4-pyridyl) benzamide monohydrochloride], RK1-1447, 5-(1,4-Diazepane-1-sulfonyl)isoquinoline (Fasudil®), GSK429286A, 3-(4-Pyridyl)-1H-indole (Rockout®), SR 3677 dihydrochloride, SB 772077B, AS 1892802, H 1152 dihydrochloride, GSK 269962, HA 1100 hydrochloride and Glycyl-H-1 152 dihydrochloride.

15. The composition according to claim 13, wherein the PTEN inhibitor is selected from the group consisting of potassium bisperoxo(1,10-phenanthroline)oxovanadate (V) (bpV), SF 1670 and VO-OHpic.

16. The composition according to claim 13, wherein concentration of the ROCK inhibitor ranges from about 5 µM to about 10 µM.

17. The composition according to claim 16, wherein the concentration of the ROCK inhibitor is 5 µM.

18. The composition according to claim 13, wherein concentration of the PTEN inhibitor ranges from about 0.5 µM to about 2 µM.

19. The composition according to claim 18, wherein the concentration of the PTEN inhibitor is 1 µM.

20. The composition according to claim 13, wherein concentration of the growth factor is 10 ng/mL.
21. The composition according to claim 13, wherein concentration of the N-acetyl cysteine is 1 mM.

22. The composition according to claim 13, wherein the composition is formulated for deposition in a blood collection tube.

23. A method for purifying circulating tumor cells (CTCs) from a blood sample comprising, in order:

   a. obtaining a blood sample from a patient comprising a tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof originating from a tumor;

   b. exposing the blood sample to the composition according to claim 13;

   c. removing mononuclear cells from the blood sample, leaving the tumor cell, tumor cell cluster, tumor cell aggregate or a combination thereof;

   d. suspending the tumor cell, the tumor cell cluster, the tumor cell aggregate or the combination thereof in a serum-free media comprising the composition according to claim 13;

   e. exposing the suspended tumor cell, tumor cell cluster, tumor cell aggregate or combination thereof from (d) to an adherent substrate;

   f. dissociating cells adherent to the adherent substrate enzymatically; and

   g. expanding the cells from (f) in vitro to establish a population of adherent circulating tumor cells (CTCs).
Tumor stem cells can move through the blood stream to form new metastasis and grow to form new tumors.

Therapies that fail to target tumor stem cells are not curative.
Figure 3

Cell Viability (%)
eGFP+ Cells (%)

Collected Fractions

Large scale (6x10^9 cells)
Small scale (40x10^6 cells)
Overall survival for 54 metastatic melanoma patients treated with patient-specific vaccine of dendritic cells loaded with autologous proliferating tumor cells.

Update September 2011: All survivors followed at least 5 years, none lost to F/U. Median overall survival (OS) = 5 yrs & 5-year OS = 50%.
Figure 5

Randomized Phase II DC-TC vs TC in metastatic melanoma

Median survival = NR vs 15.9 mos & 2-year OS =72% vs 31% (p=0.007)
HR=0.27 (95% CI 0.098 to 0.729); 21 deaths (5 DC-TC vs 16 TC)
Median time F/U survivors: 23.8 vs 15.4 mos (Sept 2011)
Figure 6

DC-TC vs TC if NED at Randomization

--- NED TC (n=11) --- NED DC-TC (n=8)

P=0.005  NED=no evidence of disease
Figure 7

DC-TC vs TC if detectable metastases at the time of Randomization

P = 0.11

- Mets TC (n=13)
- Mets DC-TC (n=10)

7/13 TC were M1c, 4/7 had ↑LDH; 6/10 DC were M1c, 5/6 had ↑LDH
Figure 8
Figure 10

Vimentin

CD44
Figure 12

Cell line procurement and working cell bank establishment

Group matched healthy donor blood collection IRB

Test individual survival factors

Test survival factors cocktail

Test survival cocktail: viability/MTT

Test proliferative ability, spherogenicity and phenotype

Provide NCI 100 aliquots of cocktail

Provide NCI final report

Develop spike/retrieval (S/R) system

Establish/verify optimal storage temperature

Generate SOP for use of cocktail and S/R system

Provide NCI SOP for use of cocktail and S/R system
Figure 15

- Optimize survival factors cocktail
  - Test on additional tumor line
    - Adapt cocktail to vacutube
      - POC for lyophilized factors in vacutube
        - Small scale use
          - Technology transfer and optimize manufacturing process
            - Develop specifications
              - Stability validation for 3 months
                - Validate label free retrieving system (USC) with laced samples
                  - Patient blood collection and CTC isolation
                    - Test proliferative ability and phenotype
                      - Deliver 1000 vacutubes to NCI
Figure 18
20/26

Figure 20
Figure 21
Figure 23

A

B
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
C12N 15/09(2006.01)i, C12N 13/00(2006.01)i, C07K 14/485(2006.01)i, C07K 14/50(2006.01)i, C07K 14/535(2006.01)i, C07K 14/82(2006.01)i, A61K 35/13(2014.01)i, A61K 39/00(2006.01)i, A61P 35/00(2006.01)i

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

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12N 15/09; C12Q 1/02; C12N 5/00; A61K 39/39; A61N 5/00; C07K 14/08; A61K 31/704; A61K 38/18; A61P 37/04; G01N 33/50; C12N 13/00; C07K 14/485; C07K 14/50; C07K 14/535; C07K 14/82; A61K 35/13; A61K 39/00; A61P 35/00

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: immunopotent tumor-specific cancer cell, dendritic cell, circulating tumor cell

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td>A</td>
<td>wo 2014-164464 A1 (CALIFORNIA STEM CELL, INC.) 09 Oct ober 2014 See claims 1, 4-6, 8-9 and 16-17; paragraphs [0015], [0017], [0059H0061], [0067], [0073], [0078], [0131], [0139], [0149], [0194H0195] and [0202].</td>
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Date of the actual completion of the international search
29 February 2016 (29.02.2016)

Date of mailing of the international search report
29 February 2016 (29.02.2016)

Name and mailing address of the ISA/KR
International Application Division
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Form PCT/ISA/210 (second sheet) (January 2015)
Box No. II  Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

1. [X] Claims Nos.: 12
   because they relate to subject matter not required to be searched by this Authority, namely:
   Claim 12 pertains to a method for treatment of the human body by therapy, and thus relate to a subject matter which this
   International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.

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

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

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

This International Searching Authority found multiple inventions in this international application, as follows:
- Invention 1 (claims 1-11) relating to an immunopotent tumor-specific cancer cell product.
- Invention 2 (claims 13-23) relating to circulating tumor cells.

Since the above mentioned inventions do not share any of the technical features identified, a "special technical relationship" between the inventions, as defined in PCT Rule 13.2, does not exist. Accordingly, the international application does not relate to a
one invention or to a single inventive concept.

[X] Unsearchable claim: 12

Claim 12 pertains to method for treatment of the human body by therapy.

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

2. [X] As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment
   of any additional fees.

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

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

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the
   payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest
   fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.
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Form PCT/ISA/2 10 (patent family annex) (January 2015)