COMPOSITIONS AND METHODS FOR CANCER IMMUNOTHERAPY

[0001] The present application claims priority to US Provisional Patent Application 61/657,574 filed June 8, 2012, which is hereby incorporated in its entirety, including all tables, figures, and claims

BACKGROUND OF THE INVENTION

[0002] The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

[0003] The human immune system may generally be divided into two arms, referred to as "innate immunity" and "adaptive immunity." The innate arm of the immune system is predominantly responsible for an initial inflammatory response via a number of soluble factors, including the complement system and the chemokine/cytokine system; and a number of specialized cell types including mast cells, macrophages, dendritic cells (DCs), and natural killer cells. In contrast, the adaptive immune arm involves a delayed and a longer lasting antibody response together with CD8+ and CD4+ T cell responses that play a critical role in immunological memory against an antigen. A third arm of the immune system may be identified as involving γδ T cells and T cells with limited T cell receptor repertoires such as NKT cells and MAIT cells.

[0004] For an effective immune response to an antigen, antigen presenting cells (APCs) must process and display the antigen in a proper MHC context to a T cell, which then will result in either T cell stimulation of cytotoxic and helper T cells. Following antigen presentation successful interaction of co-stimulatory molecules on both APCs and T cells must occur or activation will be aborted. GM-CSF and IL-12 serve as effective pro-inflammatory molecules in many tumor models. For example, GM-CSF induces myeloid precursor cells to proliferate and differentiate into dendritic cells (DCs) although additional signals are necessary to activate their maturation to effective antigen-presenting cells necessary for activation of T cells. Barriers to effective immune therapies include tolerance to the targeted antigen that can limit induction of cytotoxic CD8 T cells of appropriate magnitude and function, poor trafficking of the generated T cells to sites of malignant cells, and poor persistence of the induced T cell response.

[0005] DCs that phagocytose tumor-cell debris process the material for major histocompatibility complex (MHC) presentation, upregulate expression of costimulatory molecules, and migrate to regional lymph nodes to stimulate tumor-specific lymphocytes. This pathway results in the proliferation and activation of CD4+ and CD8+ T cells that react to tumor-associated antigens. Indeed, such cells can be detected frequently in the blood, lymphoid tissues, and malignant lesions of patients.

[0006] New insights into the mechanisms underlying immune-evasion, together with combination treatment regimens that potentiate the potency of therapeutic vaccination—either directly or indirectly—through combination with immune checkpoint inhibitors or other therapies, have served as a basis for the development of vaccines that induce effective antitumor immunity.

[0007] Tumor cells genetically modified to secrete GM-CSF have been used in various strategies in an effort to generate an effective immune response to tumors, however systemic cytokine administration has not induced a direct anti-cancer response in randomized controlled trials. Irradiated GM-CSF-secreting tumor cells injected subcutaneously into patients have been shown to stimulate a local response comprising DCs, macrophages, and granulocytes. The accumulation of large numbers of APCs suggests that one function of GM-CSF in this model involved the augmentation of tumor antigen presentation. Moreover, tumor cell vaccines have shown to be safe in patients. However, the clinical efficacy of this approach has been yet to be proven.

[0008] In the context of infection, Toll-like receptor ("TLR") agonists have been shown to render dendritic cell activation immunogenic, whereas lack of TLR signaling can lead to tolerance. The implication from these studies is that localized TLR stimulation might enhance antitumor response when given as part of a combinatorial vaccine. WO2011139769 describes the formulation and use of a combined GM-CSF-secreting tumor cell (GVAX) vaccine, together with TLR4 stimulation, which reportedly provided anti-tumor efficacy in several murine models. Its efficacy in humans remains, however, to be proven.

[0009] There remains a need for improved compositions and methods for immunologic strategies to treating diseases such as cancer that can be refractory to traditional therapeutic approaches.

SUMMARY OF THE INVENTION

[0010] It is an object of the present invention to provide combination therapies for the treatment of cancer.

[0011] In a first aspect, the present invention provides compositions comprising:

one or more cyclic purine dinucleotides ("CDN") which binds to STimulator of INTerferon Gene ("STING") and induces STING-dependent TBK1 activation; and

an inactivated tumor cell which expresses and secretes one or more cytokines which stimulate dendritic cell induction, recruitment and/or maturation.

[0012] As described hereinafter, a number of CDNs find use in the present invention. Preferred cyclic purine dinuclotides include, but are not limited to, one or more of c-di-AMP, c-di-GMP, c-di-IMP, c-AMP-GMP, c-AMP-IMP, c-GMP-IMP, and analogs thereof. This list is not meant to be limiting.

[0013] Similarly, preferred costimulatory agent comprises one or more cytokines which stimulate dendritic cell induction, recruitment, and/or maturation include, but are not limited to, one or more of GM-CSF, CD40 ligand, IL-12, CCL3, CCL20, and CCL21. This list is not meant to be limiting.

[0014] The compositions of the present invention may be administered to individuals in need thereof by a variety of parenteral and nonparenteral routes in formulations containing pharmaceutically acceptable carriers, adjuvants and vehicles. Preferred routes are parenteral, and include but, are not limited to, one or more of subcutaneous, intravenous, intramuscular, intraarterial, intradermal, intrathecal and epidural administrations. Particularly preferred is administration by subcutaneous administration. Preferred pharmaceutical composition are formulated as aqueous or oil-in-water emulsions.

[0015] The compositions of the present invention may comprise, or be administered together with, one or more additional pharmaceutically active components such as adjuvants, lipids such as digitonin, liposomes, CTLA-4 and PD-1 pathway Antagonists, PD-1 pathway blocking agents, inactivated bacteria which induce innate immunity (e.g., inactivated or attenuated *Listeria monocytogenes*), compositions which mediate innate immune activation via Toll-like Receptors (TLRs), (NOD)-like receptors (NLRs), Retinoic

acid inducible gene-based (RIG)-I-like receptors (RLRs), C-type lectin receptors (CLRs), pathogen-associated molecular patterns ("PAMPs"), chemotherapeutic agents, etc.

[0016] As described hereinafter, cyclic purine dinuclotides formulated with one or more lipids can exhibit improved properties, including improved dendritic cell activation activity. Thus, the present invention also relates to a composition comprising one or more CDNs and one or more lipids. In certain preferred embodiments, one or more CDNs are formulated with digitonin, a liposomal formulation, and/or an oil-in-water emulsion. While these formulations of the invention may be administered without an inactivated tumor cell which expresses and secretes one or more cytokines which stimulate dendritic cell induction, recruitment and/or maturation, in certain embodiments, the formulation of CDNs with one or more lipids are provided together with one or more such cell lines.

[0017] In related aspects, the present invention relates to methods for inducing an immune response to a cancer in an individual. These methods comprise administering a composition according to the present invention to an individual in need thereof, wherein the inactivated tumor cell or a mixture of different tumor cells are type-matched to the individual's cancer.

[0018] In certain embodiments, the inactivated tumor cells or a mixture of different tumor cells are allogeneic tumor cells or autologous tumor cells or a mixture of the two.

[0019] The methods of the present invention may be directed to patients being treated for colorectal cancer, an aero-digestive squamous cancer, a lung cancer, a brain cancer, a liver cancer, a stomach cancer, a sarcoma, a leukemia, a lymphoma, a multiple myeloma, an ovarian cancer, a uterine cancer, a breast cancer, a melanoma, a prostate cancer, a pancreatic carcinoma, and a renal carcinoma.

BRIEF DESCRIPTION OF THE FIGURES

[0020] Fig. 1 depicts cyclic purine dinucleotide ("CDN")-mediated signaling. A CDN (e.g., c-di-AMP c-di-GMP, c-AMP-GMP), with the two purine nucleosides alternatively joined by a phosphate bridge with canonical bis-(3', 5') linkages, or non-canonical 2', 5' and 3',5' linkages, represented by c[G(2',5')pA(3',5')p]. The canonical or non-canonical CDNs induce production of both NF-kB dependent pro-inflammatory cytokines, and also IFN-β by binding to the cytosolic receptor STING (Stimulator of Interferon Genes),

activating signaling through the TBK-1/IRF-3 pathway, resulting in both autocrine and paracrine activation of DCs through binding to the IFN receptor and subsequent signaling..

[0021] Fig. 2A depicts CDN-adjuvinated T-cell responsiveness to HIV Gag.

[0022] Fig. 2B depicts The primary and secondary OVA-specific CD4 and CD8 T cell response in PBMC following immunization of mice with the vaccine compositions shown in the figure.

[0023] Fig. 2C depicts immune responses induced by CDN-adjuvanted vaccines using OVA as a model antigen.

[0024] Fig. 3A depicts growth inhibition of B16 melanoma in response to a GVAX/CDN combination vaccine (referred to as "Stingvax").

[0025] Fig. 3B depicts interferon-β induction in TRAMP-GM cells and bone marrow-derived macrophages in response to a GVAX/CDN combination vaccine.

[0026] Fig. 4 depicts concentration dependence of growth inhibition of B16 melanoma in response to a GVAX/CDN combination vaccine.

[0027] Fig. 5 depicts CD8+ T-cell infiltration of B16 melanoma tumors in response to a GVAX/CDN combination vaccine.

[0028] Fig. 6 depicts induction of mature interferon γ-producing splenic DC (CD11c+ cells) in response to a GVAX/CDN combination vaccine.

[0029] Fig. 7 depicts the induction of human dendritic cells upon CDN treatment, as assessed by expression of costimulatory molecules.

[0030] Fig. 8 depicts the expression of IFN-α in cultured human peripheral blood mononuclear cells isolated from 15 independent donors, following stimulation with various activators of innate immunity, including cyclic-di-GMP (CDG), Interferon Stimulating DNA (ISD), and poly inosine-cytosine (Poly I:C).

[0031] Fig. 9 depicts the synergistic mechanism of action of "STINGVAX."

DETAILED DESCRIPTION OF THE INVENTION

[0032] The FDA approval of Provenge® (Dendreon Corporation) for the treatment of castration-resistant metastatic prostate cancer (mCRPC) has validated active cancer

immunotherapy as a therapeutic area and reinvigorated the field. However, as an autologous dendritic cell (DC) based vaccine, Provenge® is impractical, complex and expensive, and provides only a modest—albeit significant—survival benefit. An improved cancer vaccine should be not only at least as effective as Provenge®, but also more practical, and preferably able to elicit objective responses. One step in this direction is ProstVac VF, a recombinant pox virus-based vaccine that encodes PSA and three co-stimulatory molecules (B7.1, ICAM-1, and Lfa-3). ProstVac VF was shown to increase overall survival in a randomized Phase 2 study conducted among men with mCRPC – though survival benefit did not reach $p<0.05$ statistical significance - and is currently being evaluated in a Phase 3 efficacy study. These vaccines use a single antigen expressed by both normal prostate tissue and prostate cancer. Targeting multiple cancer antigens with therapeutic vaccination strategies is desirable to reduce the potential negative impact of antigen-loss variants, patient-by-patient differences in tumor-associated antigen expression profiles, or MHC haplotype differences, all issues with single TAA vaccination strategies.

[0033] The present invention relates to a novel and highly active combination therapy which relies on a small molecule immune stimulator – cyclic-di-nucleotide (CDN) – that activates DCs via a recently discovered cytoplasmic receptor known as STING (Stimulator of Interferon Genes) formulated with irradiated allogeneic human tumor cell lines engineered to secrete high amounts of the DC growth factor, GM-CSF. This combination therapy can provide an ideal synergy of multiple tumor associated antigens, DC recruitment and proliferation (GM-CSF), coupled with a potent DC activation stimulus (CDN).

[0034] The CDNs cyclic-di-AMP (produced by Listeria monocytogenes) and its analog cyclic-di-GMP (produced by Legionella pneumophila) are recognized by the host cell as a PAMP (Pathogen Associated Molecular Pattern), which bind to the PRR (Pathogen Recognition Receptor) known as STING. STING is an adaptor protein in the cytoplasm of host mammalian cells which activates the TANK binding kinase (TBK1)—IRF3 signaling axis, resulting in the induction of IFN-β and other IRF-3 dependent gene products that strongly activate innate immunity. It is now recognized that STING is a component of the host cytosolic surveillance pathway, that senses infection with intracellular pathogens and in response induces the production of IFN-β, leading to the development of an adaptive protective pathogen-specific immune response consisting of both antigen-specific CD4 and CD8 T cells as well as pathogen-specific antibodies.

[0035] Definitions

[0036] "Administration" as it is used herein with regard to a human, mammal, mammalian subject, animal, veterinary subject, placebo subject, research subject, experimental subject, cell, tissue, organ, or biological fluid, refers without limitation to contact of an exogenous ligand, reagent, placebo, small molecule, pharmaceutical agent, therapeutic agent, diagnostic agent, or composition to the subject, cell, tissue, organ, or biological fluid, and the like. "Administration" can refer, *e.g.*, to therapeutic, pharmacokinetic, diagnostic, research, placebo, and experimental methods. Treatment of a cell encompasses contact of a reagent to the cell, as well as contact of a reagent to a fluid, where the fluid is in contact with the cell. "Administration" also encompasses in vitro and ex vivo treatments, *e.g.*, of a cell, by a reagent, diagnostic, binding composition, or by another cell. By "administered together" it is not meant to be implied that two or more agents be administered as a single composition. Although administration as a single composition is contemplated by the present invention, such agents may be delivered to a single subject as separate administrations, which may be at the same or different time, and which may be by the same route or different routes of administration.

[0037] By "purified" and "isolated" is meant that a specified species accounts for at least 50%, more often accounts for at least 60%, typically accounts for at least 70%, more typically accounts for at least 75%, most typically accounts for at least 80%, usually accounts for at least 85%, more usually accounts for at least 90%, most usually accounts for at least 95%, and conventionally accounts for at least 98% by weight, or greater, of the species present in a composition. The weights of water, buffers, salts, detergents, reductants, protease inhibitors, stabilizers (including an added protein such as albumin), and excipients are generally not used in the determination of purity.

[0038] "Specifically" or "selectively" binds, when referring to a ligand/receptor, nucleic acid/complementary nucleic acid, antibody/antigen, or other binding pair (e.g., a cytokine to a cytokine receptor) (each generally referred to herein as a "target biomolecule" or a "target") indicates a binding reaction which is related to the presence of the target in a heterogeneous population of proteins and other biologics. Specific binding can mean, e.g., that the binding compound, nucleic acid ligand, antibody, or binding composition derived from the antigen-binding site of an antibody, of the contemplated

method binds to its target with an affinity that is often at least 25% greater, more often at least 50% greater, most often at least 100% (2-fold) greater, normally at least ten times greater, more normally at least 20-times greater, and most normally at least 100-times greater than the affinity with a non-target molecule.

[0039] "Ligand" refers to a small molecule, nucleic acid, peptide, polypeptide, saccharide, polysaccharide, glycan, glycoprotein, glycolipid, or combinations thereof that binds to a target biomolecule. While such ligands may be agonists or antagonists of a receptor, a ligand also encompasses a binding agent that is not an agonist or antagonist, and has no agonist or antagonist properties. Specific binding of a ligand for its cognate target is often expressed in terms of an "Affinity." In preferred embodiments, the ligands of the present invention bind with affinities of between about 10^4 M^{-1} and about 10^8 M^{-1}. Affinity is calculated as $K_d = k_{off}/k_{on}$ (k_{off} is the dissociation rate constant, K_{on} is the association rate constant and K_d is the equilibrium constant).

[0040] Affinity can be determined at equilibrium by measuring the fraction bound (r) of labeled ligand at various concentrations (c). The data are graphed using the Scatchard equation: r/c = K(n-r): where r = moles of bound ligand/mole of receptor at equilibrium; c = free ligand concentration at equilibrium; K = equilibrium association constant; and n = number of ligand binding sites per receptor molecule. By graphical analysis, r/c is plotted on the Y-axis versus r on the X-axis, thus producing a Scatchard plot. Affinity measurement by Scatchard analysis is well known in the art. *See, e.g.*, van Erp *et al., J. Immunoassay* 12: 425-43, 1991; Nelson and Griswold, *Comput. Methods Programs Biomed.* 27: 65-8, 1988. In an alternative, affinity can be measured by isothermal titration calorimetry (ITC). In a typical ITC experiment, a solution of ligand is titrated into a solution of its cognate target. The heat released upon their interaction (ΔH) is monitored over time. As successive amounts of the ligand are titrated into the ITC cell, the quantity of heat absorbed or released is in direct proportion to the amount of binding. As the system reaches saturation, the heat signal diminishes until only heats of dilution are observed. A binding curve is then obtained from a plot of the heats from each injection against the ratio of ligand and binding partner in the cell. The binding curve is analyzed with the appropriate binding model to determine K_B, n and ΔH. Note that $K_B = 1/K_d$.

[0041] The term "subject" as used herein refers to a human or non-human organism. Thus, the methods and compositions described herein are applicable to both human and veterinary disease. In certain embodiments, subjects are "patients," i.e., living humans that are receiving medical care for a disease or condition. This includes persons with no defined illness who are being investigated for signs of pathology. Preferred are subjects who have an existing diagnosis of a particular cancer which is being targeted by the compositions and methods of the present invention. Preferred cancers for treatment with the compositions described herein include, but are not limited to prostate cancer, renal carcinoma, melanoma, pancreatic cancer, cervical cancer, ovarian cancer, colon cancer, head & neck cancer, lung cancer and breast cancer.

[0042] "Therapeutically effective amount" is defined as an amount of a reagent or pharmaceutical composition that is sufficient to show a patient benefit, i.e., to cause a decrease, prevention, or amelioration of the symptoms of the condition being treated. When the agent or pharmaceutical composition comprises a diagnostic agent, a "diagnostically effective amount" is defined as an amount that is sufficient to produce a signal, image, or other diagnostic parameter. Effective amounts of the pharmaceutical formulation will vary according to factors such as the degree of susceptibility of the individual, the age, gender, and weight of the individual, and idiosyncratic responses of the individual. "Effective amount" encompasses, without limitation, an amount that can ameliorate, reverse, mitigate, prevent, or diagnose a symptom or sign of a medical condition or disorder or a causative process thereof. Unless dictated otherwise, explicitly or by context, an "effective amount" is not limited to a minimal amount sufficient to ameliorate a condition.

[0043] "Treatment" or "treating" (with respect to a condition or a disease) is an approach for obtaining beneficial or desired results including and preferably clinical results. For purposes of this invention, beneficial or desired results with respect to a disease include, but are not limited to, one or more of the following: preventing a disease, improving a condition associated with a disease, curing a disease, lessening severity of a disease, delaying progression of a disease, alleviating one or more symptoms associated with a disease, increasing the quality of life of one suffering from a disease, and/or prolonging survival. Likewise, for purposes of this invention, beneficial or desired results with respect to a condition include, but are not limited to, one or more of the following:

preventing a condition, improving a condition, curing a condition, lessening severity of a condition, delaying progression of a condition, alleviating one or more symptoms associated with a condition, increasing the quality of life of one suffering from a condition, and/or prolonging survival. For instance, in embodiments where the compositions described herein are used for treatment of cancer, the beneficial or desired results include, but are not limited to, one or more of the following: reducing the proliferation of (or destroying) neoplastic or cancerous cells, reducing metastasis of neoplastic cells found in cancers, shrinking the size of a tumor, decreasing symptoms resulting from the cancer, increasing the quality of life of those suffering from the cancer, decreasing the dose of other medications required to treat the disease, delaying the progression of the cancer, and/or prolonging survival of patients having cancer. Depending on the context, "treatment" of a subject can imply that the subject is in need of treatment, e.g., in the situation where the subject comprises a disorder expected to be ameliorated by administration of a reagent.

[0044] The term "antibody" as used herein refers to a peptide or polypeptide derived from, modeled after or substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, capable of specifically binding an antigen or epitope. *See, e.g.* Fundamental Immunology, 3rd Edition, W.E. Paul, ed., Raven Press, N.Y. (1993); Wilson (1994; J. Immunol. Methods 175:267-273; Yarmush (1992) J. Biochem. Biophys. Methods 25:85-97. The term antibody includes antigen-binding portions, i.e., "antigen binding sites," (e.g., fragments, subsequences, complementarity determining regions (CDRs)) that retain capacity to bind antigen, including (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CHl domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CHl domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Single chain antibodies are also included by reference in the term "antibody."

[0045] Immunomodulatory Cell Lines

[0046] By “inactivated tumor cell” is meant a tumor cell (either "autologous" or "allogeneic" to the patient) which has which been treated to prevent division of the cells. For purposes of the present invention, such cells preserve their immunogenicity and their metabolic activity. Such tumor cells are genetically modified to express a transgene which is expressed within a patient as part of cancer therapy. Thus, a composition or vaccine of the invention comprises neoplastic (e.g., tumor) cells that are autologous or allogeneic to the patient undergoing treatment and is most preferably the same general type of tumor cell as is afflicting the patient. For example, a patient suffering from melanoma will typically be administered a genetically modified cell derived from a melanoma. Methods for inactivating tumor cells for use in the present invention, such as the use of irradiation, are well known in the art.

[0047] The inactivated tumor cells of the present invention are administered to the patient together with one or more costimulatory molecules or agents. A preferred costimulatory agent comprises one or more cytokines which stimulate dendritic cell induction, recruitment, and/or maturation. Methods for assessing such costimulatory agents are well known in the literature. Induction and maturation of DCs is typically assessed by increased expression of certain membrane molecules such as CD80 and CD86, and/or secretion of pro-inflammatory cytokines, such as IL-12 and type I interferons following stimulation.

[0048] In preferred embodiments, the inactivated tumor cells themselves are modified to express and secrete one or more cytokines which stimulate dendritic cell induction, recruitment, and/or maturation. The present invention is described in exemplary terms with regard to the use of GM-CSF. Thus, by way of example, the tumor cell may express a transgene encoding GM-CSF as described in U.S. Pat. Nos. 5,637,483, 5,904,920, 6,277,368 and 6,350,445, as well as in US Patent Publication No. 20100150946, each of which is expressly incorporated by reference herein. A form of GM-CSF-expressing genetically modified cancer cells or a "cytokine-expressing cellular vaccine" for the treatment of pancreatic cancer is described in U.S. Pat. Nos. 6,033,674 and 5,985,290, both of which are expressly incorporated by reference herein.

[0049] Other suitable cytokines which may be expressed by such inactivated tumor cells and/or bystander cells instead of, or together with, GM-CSF include, but are not

limited to, one or more of CD40 ligand, IL-12, CCL3, CCL20, and CCL21. This list is not meant to be limiting.

[0050] While it is preferred that the inactivated tumor cells administered to the subject express one or more cytokines of interest, the tumor cell line may be accompanied by an inactivated bystander cell line which expresses and secretes one or more cytokines which stimulate dendritic cell induction, recruitment, and/or maturation. The bystander cell line may provide all of the cytokines which stimulate dendritic cell induction, recruitment, and/or maturation, or may supplement cytokines which stimulate dendritic cell induction, recruitment, and/or maturation expressed and secreted by the inactivated tumor cells. By way of example, immunomodulatory cytokine-expressing bystander cell lines are disclosed in U.S. Pat. Nos. 6,464,973, and 8,012,469, Dessureault et al., Ann. Surg. Oncol. 14: 869-84, 2007, and Eager and Nemunaitis, Mol. Ther. 12: 18-27, 2005, each of which is expressly incorporated by reference herein.

[0051] By "Granulocyte-macrophage colony stimulating factor (GM-CSF) polypeptide" is meant a cytokine or fragment thereof having immunomodulatory activity and having at least about 85% amino acid sequence identity to GenBank Accession No. AAA52122.1.

[0052] Cyclic Purine Dinucleotides

[0053] As described herein, another of these costimulatory agents is a cyclic purine dinucleotide which binds to STING and induces STING-dependent TBK1 activation. Other costimulatory molecules which may be included are described hereinafter.

[0054] Prokaryotic as well as eukaryotic cells use various small molecules for cell signaling and intra- and intercellular communication. Cyclic nucleotides like cGMP, cAMP, etc. are known to have regulatory and initiating activity in pro- and eukaryotic cells. Unlike eukaryotic cells, prokaryotic cells also use cyclic purine dinucleotides as regulatory molecules. In prokaryotes, the condensation of two GTP molecules is catalyst by the enzyme diguanylate cyclase (DGC) to give c-diGMP, which represents an important regulator in bacteria.

[0055] Recent work suggests that cyclic diGMP or analogs thereof can also stimulate or enhance immune or inflammatory response in a patient or can enhance the immune

response to a vaccine by serving as an adjuvant in mammals. Cytosolic detection of pathogen-derived DNA requires signaling through TANK binding kinase 1 (TBK1) and its downstream transcription factor, IFN-regulatory factor 3 (IRF3). A transmembrane protein called STING (stimulator of IFN genes; also known as MITA, ERIS, MPYS and TMEM173) functions as the signaling receptor for these cyclic purine dinucleotides, causing stimulation of the TBK1-IRF3 signalling axis and a STING-dependent type I interferon response. See, e.g., Fig. 1. Burdette et al., Nature 478: 515-18, 2011 demonstrated that STING binds directly to cyclic diguanylate monophosphate, but not to other unrelated nucleotides or nucleic acids.

[0056] Suitable cyclic purine dinucleotides for use in the present invention are described in some detail in, e.g., U.S. Patent Nos. 7,709458 and 7,592,326; WO2007/054279; and Yan et al., Bioorg. Med. Chem Lett. 18: 5631 (2008), each of which is hereby incorporated by reference. Preferred cyclic purine dinuclotides include, but are not limited to, c-di-AMP, c-di-GMP, c-di-IMP, c-AMP-GMP, c-AMP-IMP, and c-GMP-IMP, and analogs thereof including, but not limited to, phosphorothioate analogues.

[0057] Adjuvants

[0058] In addition to the inactivated tumor cell(s) and cyclic purine dinuclotide(s) described above, the compositions of the present invention may further comprise one or more additional substances which, because of their adjuvant nature, can act to stimulate the immune system to respond to the cancer antigens present on the inactivated tumor cell(s). Such adjuvants include, but are not limited to, lipids, liposomes, inactivated bacteria which induce innate immunity (e.g., inactivated or attenuated *Listeria monocytogenes*), compositions which mediate innate immune activation via Toll-like Receptors (TLRs), (NOD)-like receptors (NLRs), Retinoic acid inducible gene-based (RIG)-I-like receptors (RLRs), and/or C-type lectin receptors (CLRs). Examples of PAMPs include lipoproteins, lipopolypeptides, peptidoglycans, zymosan, lipopolysaccharide, neisserial porins, flagellin, profillin, galactoceramide, muramyl dipeptide. Peptidoglycans, lipoproteins, and lipoteichoic acids are cell wall components of Gram-positive. Lipopolysaccharides are expressed by most bacteria, with MPL being one example. Flagellin refers to the structural component of bacterial flagella that is

secreted by pathogenic and commensal bacterial. α-Galactosylceramide (α-GalCer) is an activator of natural killer T (NKT) cells. Muramyl dipeptide is a bioactive peptidoglycan motif common to all bacteria. This list is not meant to be limiting. Preferred adjuvant compositions are described below.

[0059] CTLA-4 and PD-1 pathway Antagonists

[0060] CTLA-4 is thought to be an important negative regulator of the adaptive immune response. Activated T cells upregulate CTLA-4, which binds CD80 and CD86 on antigen-presenting cells with higher affinity than CD28, thus inhibiting T-cell stimulation, IL-2 gene expression and T-cell proliferation. Anti-tumor effects of CTLA4 blockade have been observed in murine models of colon carcinoma, metastatic prostate cancer, and metastatic melanoma.

[0061] Ipilimumab (Yervoy™) and tremelimumab are humanized monoclonal antibodies that bind to human CTLA4 and prevent its interaction with CD80 and CD86. Phase I and II studies using ipilimumab and tremelimumab have demonstrated clinical activity in cancer patients. Other negative immune regulators which may be targeted by a similar strategy include programmed cell death 1, B and T lymphocyte attenuator, transforming growth factor beta β, interleukin-10, and vascular endothelial growth factor.

[0062] PD-1 is another negative regulator of adaptive immune response that is expressed on activated T-cells. PD-1 binds to B7-H1 and B7-DC, and the engagement of PD-1 suppresses T-cell activation. Anti-tumor effects have been demonstrated with PD-1 pathway blockade. BMS-936558, MK3475, CT-011, AMP-224 and MDX-1106 have been reported iin the literature to be examples of PD-1 pathway blockers which may find use in the present invention.

[0063] TLR Agonists

[0064] The term "Toll like receptor" (or "TLR") as used herein refers to a member of the Toll-like receptor family of proteins or a fragment thereof that senses a microbial product and/or initiates an adaptive immune response. In one embodiment, a TLR activates a dendritic cell (DC). Toll like receptors (TLRs) are a family of pattern recognition receptors that were initially identified as sensors of the innate immune system that recognize microbial pathogens. TLRs comprise a family of conserved membrane

spanning molecules containing an ectodomain of leucine-rich repeats, a transmembrane domain and an intracellular TIR (Toll/IL-1R) domain. TLRs recognize distinct structures in microbes, often referred to as "PAMPs" (pathogen associated molecular patterns). Ligand binding to TLRs invokes a cascade of intra-cellular signaling pathways that induce the production of factors involved in inflammation and immunity.

[0065] In humans, ten TLR have been identified. TLRs that are expressed on the surface of cells include TLR-1,-2,-4,-5, and -6, while TLR-3, -7/8, and -9 are expressed with the ER compartment. Human dendritic cell subsets can be identified on the basis of distinct TLR expression patterns. By way of example, the myeloid or "conventional" subset of DC (mDC) expresses TLRs 1–8 when stimulated, and a cascade of activation markers (e.g. CD80, CD86, MHC class I and II, CCR7), pro-inflammatory cytokines, and chemokines are produced. A result of this stimulation and resulting expression is antigen-specific CD4+ and CD8+ T cell priming. These DCs acquire an enhanced capacity to take up antigens and present them in an appropriate form to T cells. In contrast, the plasmacytoid subset of DC (pDC) expresses only TLR7 and TLR9 upon activation, with a resulting activation of NK cells as well as T-cells. As dying tumor cells may adversely affect DC function, it has been suggested that activating DC with TLR agonists may be beneficial for priming anti-tumor immunity in an immunotherapy approach to the treatment of cancer. It has also been suggested that successful treatment of breast cancer using radiation and chemotherapy requires TLR4 activation.

[0066] TLR agonists known in the art and finding use in the present invention include, but are not limited to, the following:

Pam3Cys, a TLR-1/2 agonist;
CFA, a TLR-2 agonist;
MALP2, a TLR-2 agonist;
Pam2Cys, a TLR-2 agonist;
FSL-1, a TLR-2 agonist;
Hib-OMPC, a TLR-2 agonist;
polyribosinic:polyribocytidic acid (Poly I:C), a TLR-3 agonist;
polyadenosine-polyuridylic acid (poly AU), a TLR-3 agonist;

Polyinosinic-Polycytidylic acid stabilized with poly-L-lysine and carboxymethylcellulose (Hiltonol®), a TLR-3 agonist;
monophosphoryl lipid A (MPL), a TLR-4 agonist;
LPS, a TLR-4 agonist;
bacterial flagellin, a TLR-5 agonist;
sialyl-Tn (STn), a carbohydrate associated with the MUC1 mucin on a number of human cancer cells and a TLR-4 agonist;
imiquimod, a TLR-7 agonist;
resiquimod, a TLR-7/8 agonist;
loxoribine, a TLR-7/8 agonist; and
unmethylated CpG dinucleotide (CpG-ODN), a TLR-9 agonist.

[0067] Because of their adjuvant qualities, TLR agonists are preferably used in combinations with other vaccines, adjuvants and/or immune modulators, and may be combined in various combinations. Thus, in certain embodiments, the cyclic purine dinucleotides that bind to STING and induces STING-dependent TBK1 activation and an inactivated tumor cell which expresses and secretes one or more cytokines which stimulate dendritic cell induction, recruitment and/or maturation, as described herein can be administered together with one or more TLR agonists for therapeutic purposes.

[0068] Lipids and Liposomes

[0069] Liposomes are vesicles formed from one ("unilamellar") or more ("multilamellar") layers of phospholipid. Because of the amphipathic character of the phospholipid building blocks, liposomes typically comprise a hydrophilic layer presenting a hydrophilic external face and enclosing a hydrophilic core. The versatility of liposomes in the incorporation of hydrophilic/hydrophobic components, their non-toxic nature, biodegradability, biocompatibility, adjuvanticity, induction of cellular immunity, property of sustained release and prompt uptake by macrophages, makes them attractive candidates for the delivery of antigens.

[0070] WO2010/104833, which is incorporated by reference herein in its entirety, describes liposomal preparations which comprise:

a) an aqueous vehicle;

b) liposomes comprising

(i) dimyristoylphosphatidylcholine ("DMPC"),

(ii) dimyristoylphosphatidylglycerol ("DMPG"), dimyristoyltrimethylammonium propane ("DMTAP"), or both DMPG and DMTAP,

and

(iii) at least one sterol derivative; and

c) one or more immunogenic polypeptide(s) or carbohydrate(s) covalently linked to between 1% and 100% of said at least one sterol derivative.

[0071] Such liposomal formulations, referred to herein as VesiVax® (Molecular Express, Inc.), with our without the "immunogenic polypeptide(s) or carbohydrate(s)" referred to above, can contain one or more additional components such as peptidoglycan, lipopeptide, lipopolysaccharide, monophosphoryl lipid A, lipoteichoic acid, resiquimod, imiquimod, flagellin, oligonucleotides containing unmethylated CpG motifs, beta-galactosylceramide, muramyl dipeptide, all-trans retinoic acid, double-stranded viral RNA, heat shock proteins, dioctadecyldimethylammonium bromide, cationic surfactants, toll-like receptor agonists, dimyristoyltrimethylammoniumpropane, and nod-like receptor agonists. Advantageously, these liposomal formulations can be used to deliver one or more cyclic purine dinucleotides in accordance with the present invention.

[0072] Moreover, while the liposomal formulations discussed above employ a" steroid derivative" as an anchor for attaching an immunogenic polypeptide or carbohydrate to a liposome, the steroid may simply be provided as an unconjugated steroid such as cholesterol.

[0073] Suitable methods for preparing liposomes from lipid mixtures are well known in the art. *See, e.g.*, Basu & Basu, Liposome Methods and Protocols (Methods in Molecular Biology), Humana Press, 2002; Gregoriadis, Liposome Technology, 3rd Edition, Informa HealthCare, 2006. Preferred methods include extrusion, homogenization, and sonication methods described therein. An exemplary method for preparing liposomes for use in the present invention, which comprises drying a lipid

mixture, followed by hydration in an aqueous vehicle and sonication to form liposomes, is described in WO2010/104833.

[0074] In certain embodiments, the liposomes are provided within a particular average size range. Liposome size can be selected, for example, by extrusion of an aqueous vehicle comprising liposomes through membranes having a preselected pore size and collecting the material flowing through the membrane. In preferred embodiments, the liposomes are selected to be substantially between 50 and 500 nm in diameter, more preferably substantially between 50 and 200 nm in diameter, and most preferably substantially between 50 and 150 nm in diameter. The term "substantially" as used herein in this context means that at least 75%, more preferably 80%, and most preferably at least 90% of the liposomes are within the designated range.

[0075] Other lipid and lipid-like adjuvants which may find use in the present invention include oil-in-water (o/w) emulsions (see, e.g., Muderhwa et al., J. Pharmaceut. Sci. 88: 1332–9, 1999)), VesiVax® TLR (Molecular Express, Inc.), digitonin (see, e.g., U.S. Patent 5,698,432), and glucopyranosyl lipids (see, e.g., United States Patent Application 20100310602).

[0076] Chemotherapeutic Agents

[0077] In additional embodiments the methods further involve administering to the subject an effective amount of one or more chemotherapeutics as an additional treatment for the patient's tumor. In certain embodiments the one or more chemo therapeutics is selected from abiraterone acetate, altretamine, anhydrovinblastine, auristatin, bexarotene, bicalutamide, BMS 184476, 2,3,4,5,6-pentafluoro-N-(3-fluoro-4-methoxyphenyl)benzene sulfonamide, bleomycin, N,N-dimethyl-L-valyl-L-valyl-N-methyl-L-valyl-L-proly- 1-Lproline-t-butylamide, cachectin, cemadotin, chlorambucil, cyclophosphamide, 3',4'-didehydro-4'-deoxy-8'-norvin-caleukoblastine, docetaxol, doxetaxel, cyclophosphamide, carboplatin, carmustine, cisplatin, cryptophycin, cyclophosphamide, cytarabine, dacarbazine (DTIC), dactinomycin, daunorubicin, decitabine dolastatin, doxorubicin (adriamycin), etoposide, 5-fluorouracil, finasteride, flutamide, hydroxyurea and hydroxyureataxanes, ifosfamide, liarozole, lonidamine, lomustine (CCNU), MDV3100, mechlorethamine (nitrogen mustard), melphalan, mivobulin isethionate, rhizoxin, sertenef, streptozocin, mitomycin, methotrexate, taxanes, nilutamide, onapristone,

paclitaxel, prednimustine, procarbazine, RPR109881, stramustine phosphate, tamoxifen, tasonermin, taxol, tretinoin, vinblastine, vincristine, vindesine sulfate, and vinflunine.

[0078] Pharmaceutical Compositions

[0079] The term "pharmaceutical" as used herein refers to a chemical substance intended for use in the cure, treatment, or prevention of disease and which is subject to an approval process by the U.S. Food and Drug Administration (or a non-U.S. equivalent thereof) as a prescription or over-the-counter drug product. Details on techniques for formulation and administration of such compositions may be found in Remington, The Science and Practice of Pharmacy 21st Edition (Mack Publishing Co., Easton, PA) and Nielloud and Marti-Mestres, Pharmaceutical Emulsions and Suspensions: 2nd Edition (Marcel Dekker, Inc, New York).

[0080] For the purposes of this disclosure, the pharmaceutical compositions may be administered by a variety of means including orally, parenterally, by inhalation spray, topically, or rectally in formulations containing pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used here includes but is not limited to subcutaneous, intravenous, intramuscular, intraarterial, intradermal, intrathecal and epidural injections with a variety of infusion techniques. Intraarterial and intravenous injection as used herein includes administration through catheters. Administration via intracoronary stents and intracoronary reservoirs is also contemplated. The term oral as used herein includes, but is not limited to oral ingestion, or delivery by a sublingual or buccal route. Oral administration includes fluid drinks, energy bars, as well as pill formulations.

[0081] Pharmaceutical compositions may be in any form suitable for the intended method of administration. When used for oral use for example, tablets, troches, lozenges, aqueous or oil suspensions, dispersible powders or granules, emulsions, hard or soft capsules, syrups or elixirs may be prepared. Compositions intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions may contain one or more agents including sweetening agents, flavoring agents, coloring agents and preserving agents, in order to provide a palatable preparation. Tablets containing a drug compound in admixture with non-toxic pharmaceutically acceptable excipient which are suitable for manufacture of

tablets are acceptable. These excipients may be, for example, inert diluents, such as calcium or sodium carbonate, lactose, calcium or sodium phosphate; granulating and disintegrating agents, such as maize starch, or alginic acid; binding agents, such as starch, gelatin or acacia; and lubricating agents; such as magnesium stearate, stearic acid or talc. Tablets may be uncoated, or may be coated by known techniques including enteric coating, colonic coating, or microencapsulation to delay disintegration and adsorption in the gastrointestinal tract and/or provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate alone or with a wax may be employed.

[0082] Formulations for oral use may be also presented as hard gelatin capsules where the drug compound is mixed with an inert solid diluent, for example calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, such as peanut oil, liquid paraffin or olive oil.

[0083] Pharmaceutical compositions may be formulated as aqueous suspensions in admixture with excipients suitable for the manufacture of aqueous-suspensions. Such excipients include a suspending agent, such as sodium carboxymethylcellulose, methylcellulose, hydroxypropyl methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia, and dispersing or wetting agents such as a naturally occurring phosphatide (e.g., lecithin), a condensation product of an alkylene oxide with a fatty acid (e.g., polyoxyethylene stearate), a condensation product of ethylene oxide with a long chain aliphatic alcohol (e.g., heptadecaethyleneoxycetanol), a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol anhydride (e.g., polyoxyethylene sorbitan monooleate). The aqueous suspension may also contain one or more preservatives such as ethyl or n-propyl p-hydroxy-benzoate, one or more coloring agents, one or more flavoring agents and one or more sweetening agents, such as sucrose or saccharin.

[0084] Oil suspensions may be formulated by suspending the active ingredient in a vegetable oil, such as arachis oil, olive oil, sesame oil or coconut oil, or a mineral oil such as liquid paraffin. The oral suspensions may contain a thickening agent, such as beeswax, hard paraffin or cetyl alcohol. Sweetening agents, such as those set forth above, and

flavoring agents may be added to provide a palatable oral preparation. These compositions may be preserved by the addition of an antioxidant such as ascorbic acid.

[0085] Dispersible powders and granules of the disclosure suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, a suspending agent, and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those disclosed above. Additional excipients, for example sweetening, flavoring and coloring agents, may also be present.

[0086] The pharmaceutical compositions of the disclosure may also be in the form of oil-in-water emulsions. The oily phase may be a vegetable oil, such as olive oil or arachis oil, a mineral oil, such as liquid paraffin, or a mixture of these. Suitable emulsifying agents include naturally-occurring gums, such as gum acacia and gum tragacanth, naturally occurring phosphatides, such as soybean lecithin, esters or partial esters derived from fatty acids and hexitol anhydrides, such as sorbitan monooleate, and condensation products of these partial esters with ethylene oxide, such as polyoxyethylene sorbitan monooleate. The emulsion may also contain sweetening and flavoring agents.

[0087] Syrups and elixirs may be formulated with sweetening agents, such as glycerol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative, a flavoring or a coloring agent.

[0088] The pharmaceutical compositions of the disclosure may be in the form of a sterile injectable preparation, such as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent such as a solution in 1,3-butane-diol or prepared as a lyophilized powder. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile fixed oils may conventionally be employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid may likewise be used in the preparation of injectables.

[0089] The amount of active ingredient that may be combined with the carrier material to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a time-release formulation intended for oral administration to humans may contain approximately 20 to 500 mg of active material compounded with an appropriate and convenient amount of carrier material which may vary from about 5 to about 95% of the total compositions. It is preferred that the pharmaceutical composition be prepared which provides easily measurable amounts for administration. Typically, an effective amount to be administered systemically is about 0.1 mg/kg to about 100 mg/kg and depends upon a number of factors including, for example, the age and weight of the subject (e.g., a mammal such as a human), the precise condition requiring treatment and its severity, the route of administration, and will ultimately be at the discretion of the attendant physician or veterinarian. It will be understood, however, that the specific dose level for any particular patient will depend on a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex and diet of the individual being treated; the time and route of administration; the rate of excretion; other drugs which have previously been administered; and the severity of the particular condition undergoing therapy, as is well understood by those skilled in the art.

[0090] As noted above, formulations of the disclosure suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active ingredient, as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The pharmaceutical compositions may also be administered as a bolus, electuary or paste.

[0091] A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free flowing form such as a powder or granules, optionally mixed with a binder (e.g., povidone, gelatin, hydroxypropyl ethyl cellulose), lubricant, inert diluent, preservative, disintegrant (e.g., sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface active or dispersing agent. Molded tablets may be made in a suitable machine using a mixture of the powdered compound moistened with an inert liquid diluent. The tablets

may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropyl methylcellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric or colonic coating to provide release in parts of the gut other than the stomach. This is particularly advantageous with the compounds of formula 1 when such compounds are susceptible to acid hydrolysis.

[0092] Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavored base, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert base such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

[0093] Formulations for rectal administration may be presented as a suppository with a suitable base comprising for example cocoa butter or a salicylate.

[0094] Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient such carriers as are known in the art to be appropriate.

[0095] Formulations suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain antioxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

[0096] As used herein, pharmaceutically acceptable salts include, but are not limited to: acetate, pyridine, ammonium, piperazine, diethylamine, nicotinamide, formic, urea, sodium, potassium, calcium, magnesium, zinc, lithium, cinnamic, methylamino, methanesulfonic, picric, tartaric, triethylamino, dimethylamino, and

tris(hydoxymethyl)aminomethane. Additional pharmaceutically acceptable salts are known to those skilled in the art.

[0097] An effective amount for a particular patient may vary depending on factors such as the condition being treated, the overall health of the patient, the route and dose of administration and the severity of side effects. Guidance for methods of treatment and diagnosis is available (see, e.g., Maynard, et al. (1996) A Handbook of SOPs for Good Clinical Practice, Interpharm Press, Boca Raton, FL; Dent (2001) Good Laboratory and Good Clinical Practice, Urch Publ., London, UK).

[0098] An effective amount may be given in one dose, but is not restricted to one dose. Thus, the administration can be two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, or more, administrations of pharmaceutical composition. Where there is more than one administration of a pharmaceutical composition in the present methods, the administrations can be spaced by time intervals of one minute, two minutes, three, four, five, six, seven, eight, nine, ten, or more minutes, by intervals of about one hour, two hours, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 hours, and so on. In the context of hours, the term "about" means plus or minus any time interval within 30 minutes. The administrations can also be spaced by time intervals of one day, two days, three days, four days, five days, six days, seven days, eight days, nine days, ten days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, and combinations thereof. The invention is not limited to dosing intervals that are spaced equally in time, but encompass doses at non-equal intervals.

[0099] A dosing schedule of, for example, once/week, twice/week, three times/week, four times/week, five times/week, six times/week, seven times/week, once every two weeks, once every three weeks, once every four weeks, once every five weeks, and the like, is available for the invention. The dosing schedules encompass dosing for a total period of time of, for example, one week, two weeks, three weeks, four weeks, five weeks, six weeks, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, and twelve months.

[00100] Provided are cycles of the above dosing schedules. The cycle can be repeated about, e.g., every seven days; every 14 days; every 21 days; every 28 days; every 35 days; 42 days; every 49 days; every 56 days; every 63 days; every 70 days; and the like. An interval of non dosing can occur between a cycle, where the interval can be about, e.g., seven days; 14 days; 21 days; 28 days; 35 days; 42 days; 49 days; 56 days; 63 days; 70 days; and the like. In this context, the term "about" means plus or minus one day, plus or minus two days, plus or minus three days, plus or minus four days, plus or minus five days, plus or minus six days, or plus or minus seven days.

[00101] Methods for co-administration with an additional therapeutic agent are well known in the art (Hardman, et al. (eds.) (2001) Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10th ed., McGraw-Hill, New York, NY; Poole and Peterson (eds.) (2001) Pharmacotherapeutics for Advanced Practice:A Practical Approach, Lippincott, Williams & Wilkins, Phila., PA; Chabner and Longo (eds.) (2001) Cancer Chemotherapy and Biotherapy, Lippincott, Williams & Wilkins, Phila., PA).

[00102] As noted, the compositions of the present invention are preferably formulated as pharmaceutical compositions for parenteral or enteral delivery. A typical pharmaceutical composition for administration to an animal comprises a pharmaceutically acceptable vehicle such as aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like. See, e.g., Remington's Pharmaceutical Sciences, 15th Ed., Easton ed. , Mack Publishing Co., pp 1405-1412 and 1461- 1487 (1975); The National Formulary XIV, 14th Ed., American Pharmaceutical Association, Washington, DC (1975) . Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc*. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to routine skills in the art.

[00103] Repeated administrations of a particular vaccine (homologous boosting) have proven effective for boosting humoral responses. Such an approach may not be effective at boosting cellular immunity because prior immunity to the vector tends to impair robust

antigen presentation and the generation of appropriate inflammatory signals. One approach to circumvent this problem has been the sequential administration of vaccines that use different antigen-delivery systems (heterologous boosting). In a heterologous boosting regimen, at least one prime or boost delivery comprises delivery of the inactivated tumor cell/cyclic purine dinucleotide compositions described herein. The heterologous arm of the regimen may comprise delivery of antigen using one or more of the following strategies:

inactivated bacteria or viruses comprising the antigen of interest, which are particles that have been treated with some denaturing condition to render them ineffective or inefficient in mounting a pathogenic invasion;

purified antigens, which are typically naturally-produced antigens purified from a cell culture of the pathogen or a tissue sample containing the pathogen, or a recombinant version thereof;

live viral or bacterial delivery vectors recombinantly engineered to express and/or secrete antigens in the host cells of the subject. These strategies rely on attenuating (e.g., via genetic engineering) the viral or bacterial vectors to be non-pathogenic and non-toxic;

antigen presenting cell (APC) vectors, such as a dendritic cell (DC) vector, which comprise cells that are loaded with an antigen, or transfected with a composition comprising a nucleic acid encoding the antigen (e.g., Provenge® (Dendreon Corporation) for the treatment of castration-resistant metastatic prostate cancer);

liposomal antigen delivery vehicles; and

naked DNA vectors and naked RNA vectors which may be administered by a gene gun, electroporation, bacterial ghosts, microspheres, microparticles, liposomes, polycationic nanoparticles, and the like.

[00104] A prime vaccine and a boost vaccine can be administered by any one or combination of the following routes. In one aspect, the prime vaccine and boost vaccine are administered by the same route. In another aspect, the prime vaccine and boost vaccine are administered by different routes. The term “different routes” encompasses, but is not limited to, different sites on the body, for example, a site that is oral, non-oral,

enteral, parenteral, rectal, intranode (lymph node), intravenous, arterial, subcutaneous, intramuscular, intratumor, peritumor, intratumor, infusion, mucosal, nasal, in the cerebrospinal space or cerebrospinal fluid, and so on, as well as by different modes, for example, oral, intravenous, and intramuscular.

[00105] An effective amount of a prime or boost vaccine may be given in one dose, but is not restricted to one dose. Thus, the administration can be two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, or more, administrations of the vaccine. Where there is more than one administration of a vaccine the administrations can be spaced by time intervals of one minute, two minutes, three, four, five, six, seven, eight, nine, ten, or more minutes, by intervals of about one hour, two hours, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 hours, and so on. In the context of hours, the term "about" means plus or minus any time interval within 30 minutes. The administrations can also be spaced by time intervals of one day, two days, three days, four days, five days, six days, seven days, eight days, nine days, ten days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, and combinations thereof. The invention is not limited to dosing intervals that are spaced equally in time, but encompass doses at non-equal intervals, such as a priming schedule consisting of administration at 1 day, 4 days, 7 days, and 25 days, just to provide a non-limiting example.

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EXAMPLES

[00155] The following examples serve to illustrate the present invention. These examples are in no way intended to limit the scope of the invention.

[00156] Example 1

[00157] One approach to stimulate immunity against a broad repertoire of TAAs is "GVAX," which are vaccines based on allogeneic human tumor cell lines that are engineered to secrete GM-CSF, the primary cytokine that stimulates DC recruitment, differentiation and maturation. GVAX vaccines have formed the basis of multiple clinical trials in several cancer indications, and have been shown to be safe, well-tolerated, immunogenic and shown to provide some clinical benefit. A Phase 3 clinical study comparing prostate GVAX immunotherapy (G) to docetaxel plus prednisone (D + P) in men with mCRPC was prematurely terminated by the sponsor when an early unscheduled futility analysis revealed that the trial had < 30% chance of meeting its predefined primary endpoint of improvement in overall survival. Continued follow-up and analysis of the >600 patients on study, however, revealed that the Kaplan-Meier survival curve for the G arm crossed above the D + P arm at ~ 21 months. Furthermore, patients with a predicted survival time of $\geq$18 months at baseline, based on the Halabi nomogram, had a 2.5 month survival benefit when treated with GVAX, with a 30% "tail" of long-term survivors, as compared to chemotherapy These results showed

that GVAX prostate immunotherapy provided a survival benefit over chemotherapy, which correspondingly shows ~2 month survival benefit as compared to placebo.

[00158] Since TLR-targeted adjuvants used individually that signal through MyD88- and TRIF-dependent pathways are typically poor inducers of CD8 T cell immunity, we assessed whether CDN, which signals through the cytoplasmic STING receptor, could facilitate priming of both MHC class I- and class II-restricted immunity. CDN induced priming of both Th1 CD4 and CD8 T cells specific for the vaccine recombinant protein Ag, HIV Gag or OVA. Balb/c mice were vaccinated twice subcutaneously in the base of the tail (s.c.) 3 wks apart with 5 μg of HIV Gag protein formulated with 2% oil-in-water adjuvant (Addavax, Invivogen) and CDN at the dose level indicated in Fig. 2.

[00159] As shown in Fig. 2A, CDN-adjuvanted HIV Gag vaccines induce a polyfunctional Ag-specific Th1 CD4 T cell response. The secondary CD4 T cell response was measured at 5 days post boost by intracellular cytokine staining of IFN-γ, IL-2 and TNF-α positive splenocytes following stimulation with the I-A^d restricted HIV Gag epitope. Bars represent individual mice. As shown in Fig. 4B, the magnitude of the CDN-dependent vaccine induced T cell response is enhanced by formulation of CDN with VesiVax® liposomes.

[00160] The primary (1) and secondary (2) OVA-specific CD4 and CD8 T cell response in PBMC following s.c. immunization of groups of 5 C57BL/6 mice with the vaccine compositions shown in Figure 2 was measured by IFN-γ ELISPOT, and the results depicted in Fig. 2C. Groups of 5 C57BL/6 mice were immunized s.c. twice at a 3-wk interval with the vaccines indicated in the Figure, or with $5x10^6$ CFU of Lm-OVA, given i.v. At 4 weeks post boost, mice were challenged with $5x10^5$ PFU of VV-OVA, and 5 days later the ovaries were harvested, processed and VV-OVA was quantitated by plaque assay.

[00161] As shown in the figure, vaccine potency was dependent on formulation, and our initial results indicate that formulation with VesiVax® liposomes was optimal, due likely to efficient vaccine delivery into the cytosol. Notably, mice vaccinated with CDN-adjuvanted OVA were completely protected by challenge with vaccinia virus. By comparison with the negative control group given HBSS, this level of protection was >4 logs. The level of protection afforded by the CDN-adjuvanted vaccine was better than either MPL-adjuvanted OVA (using the human MPL dose of 50 μg), or Listeria-OVA vaccines.

[00162] Fig. 7 depicts the induction of human dendritic cells upon CDN treatment, as assessed by expression of costimulatory molecules such as CD80 and CD86. In this experiment, CD14+ monocytes were isolated from PBMC and cultured in the presence of GM-CSF and IL-4. On day 6, 10^5 DCs were treated with 100 ng/ML LPS, 20 μM CDN (c-di-AMP); 369μg liposomes (VesiVax®), or liposomes plus CDN, as indicated in the figure. The indicated costimulatory molecules were detected 48 hrs later by flow cytometry. As noted, liposomes substantially improve the ability of CDN to induce dendritic cell maturation.

[00163] Example 2.

[00164] The B16 melanoma tumor model is aggressive and poorly immunogenic, and therapeutic vaccination with irradiated GM-CSF secreting B16 melanoma tumor cells (B16-GM) has not been effective unless combined with blockade of immune checkpoints, such as CTLA-4 or PD-1. In this example, we show that a single injection of STINGVAX (CDN formulated with digitonin and incubated with irradiated B16-GM) significantly inhibited the growth of established palpable B16 tumors, when administered at 7 days post B16 tumor cell implantation when the tumor was palpable and established.

[00165] $5x10^4$ B16 melanoma cells were inoculated in the footpad of C57BL/6 mice and when tumors were palpable at 7 days, mice were injected once s.c. in the contralateral thigh with the vaccines indicated in Fig. 3. The following amounts of vaccine components were used for each injection: irradiated B16 GM-CSF (GVAX), $1.5x10^6$ cells; CDN only, 20 ng; digitonin, 10 μg/mL. STINGVAX was prepared by incubation of irradiated B16 GM-CSF with CDN and digitonin at 20 °C for 30 min, washing 3x with PBS, and the resulting composition injected after resuspension in 200 μL of PBS. See also Woodward et al., Supporting online material 27 May 2010 on Science Express DOI: 10.1126/science.1189801, for formulation information. Tumor growth was measured daily. Growth was significantly inhibited by STINGVAX vs No Rx Group ($P<0.01$). As shown in Fig. 3A, tumor inhibition was dependent on combination of B16-GM with CDN, as treatment with either component alone had no impact on tumor growth as compared to untreated control mice. Additionally, the CD8 T cell response specific for p15E, an endogenous retrovirus-specific Ag expressed on the B16 tumor, was enhanced in STINGVAX-treated tumor-bearing mice, compared to B16-GM treated mice (data not shown).

[00166] From these data, it is believed that the the synergy between STING-targeted CDN and irradiated GM-secreting tumor cell vaccines requires that the CDN-dependent induction of IFN-β results in the activation of GM-CSF-differentiated DCs, thus providing a strong "Signal 3," resulting in a more-effective antitumor T cell response. For this reason, we determined whether CDN could directly induce the production of IFN-β in TRAMP-GM cells. As shown in Fig 3B, when formulated with digitonin, CDN efficiently induced the expression of IFN-β in both TRAMP-GM and primary macrophages, but not in macrophages from goldenticket (*gt*) mice which lack a functional STING protein.

[00167] Fig. 4 demonstrates the dose-dependency of the therapeutic benefit exhibited by the STING-targeted CDN / GVAX combination. In this experiment, $5x10^4$ B16 melanoma cells were inoculated in the footpad of C57BL/6 mice. Once tumor is palpable at day 6, GVAX (B16 GM-CSF (GVAX), $1.5x10^6$ cells) or the CDN/GVAX combination at 2, 20, or 200ng CDN/animal was injected subcutaneously into the contralateral thigh. In the case of the combination treatment, the CDN was formulated with digitonin at 10 μg/mL for 30min at 20°C and subsequently washed 4x with PBS to remove non-incorporated CDN prior to injection as described above. Tumor volume was measured daily in a total of 20 animals/treatment group. As shown in the figure, the antitumor response was increased as the concentration of CDN increased from 2 ng/animal to 20 ng/animal, but no further increase was observed by increasing the dosage to 200 ng/mL.

[00168] Fig. 5 provides further evidence of a synergistic antitumor response to the CDN/GVAX combination. In this figure, B16 melanoma cells were harvested from the animals and the cells fixed onto slides for immunohistochemistry. Fluorescein-labeled anti-CD8 antibodies were ised to visualize CR8+ T-cell infiltration into the tumor. DAPI was used to counterstain the nucleus of the tumor cells. The four panels shown in the figure are untreated B16 melanoma cells (A), and cells treated with CDN (B), GVAX (C) and CDN /GVAX (D). As shown, a substantial improvement in CD*+ T-cell tumor infiltration is observed with CDN /GVAX in comparison to either of CDN or GVAX alone.

[00169] Similarly, Fig. 6 demonstrates an improved induction of mature interferon γ-producing splenic DC (CD11c+ cells) by CDN /GVAX in comparison to either of CDN or GVAX alone. In this experiment, mice were treated as described above, and the spleen from 2 mice/group were harvested. Total splenocytes were harvested and stained with anti-CD11c

and anti-IFNa conjugates. The treatment (untreated B16, CDN, CDN + digitonin, GVAX, and CDN/GVAX + digitonin) are indicated in the figure.

[00170] Example 3.

[00171] Figure 9 depicts the synergistic mechanism of action of "STINGVAX," which is STING-activating cyclic purine dinucleotides co-formulated with irradiated GM-CSF expressing allogeneic tumor cells (GVAX; STING + GVAX = STINGVAX). The GVAX tumor cell vaccines provides an un-biased presentation of multiple tumor associated antigens to the immune system. GM-CSF produced by GVAX recruits dendritic cells (DCs) to the injection site. CDNs activate the recruited DCs, which in turn activate or prime potent antigen-specific CD4 and CD8 T cells that traffic to and kill the tumor, resulting in a clinical benefit. STINGVAX can enhance the tumor response either by serving as a depot for the GM-CSF recruited DCs, or through autocrine signaling express both TBK-1/IRF-3 dependent IFN-β and NF-κB pro-inflammatory cytokines that in combination activate the GM-CSF recruited DCs.

[00172] The mechanism for the increased anti-tumor efficacy of STINGVAX is believed due to the activation of innate immunity that is mediated by direct binding of CDNs to STING, triggering a conformational change in this receptor, resulting in signaling through the TBK-1/IRF-3 axis and activation of type 1 interferons (IFN), including IFN-α and IFN-β. GM-CSF produced by the GVAX tumor cell vaccine recruits dendritic cells (DCs) to the injection site. CDNs activate the recruited DCs, which in turn activate or prime potent antigen-specific CD4 and CD8 T cells that traffic to and kill the tumor, resulting in a clinical benefit.

[00173] To determine the levels of IFN-α as a signature of CDN potency to activate innate immunity, 1x 10^6 primary human PBMCs isolated from fifteen independent human donors were incubated in a 96 well U bottom plate for 30 min at 37°C, 5% CO2 with 50 μM of c-di-GMP (CDG), 1 μg/mL of Interferon Stimulatory DNA (ISD), or 4 μg/mL of Poly (I:C) utilizing Effectene transfection reagent (Qiagen) to transfer the molecules into the PBMC. ISD (Interferon Stimulating DNA) is TLR independent (Stetston, D.B. et. al. Immunity 24, 93–103, January 2006) and signals through cGAS, and is thus STING-dependent, while Poly (I:C) can signal through both TLR3 and RIG-I pathways, and are thus STING-independent. After 30 minutes, the cells were washed and replaced with

RPMI media containing 10% FBS and incubated at 37°C, 5% CO2. After 24 hours incubation IFN-α levels were determined by Cytometric Bead Array (CBA, BD Biosciences) (Fig. 8). These results demonstrate that cyclic-di-GMP activates innate immunity in human leukocytes prepared from multiple independent donors, thus supporting the mechanism of action of STINGVAX.

[00174] One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The examples provided herein are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention.

[00175] It is to be understood that the invention is not limited in its application to the details of construction and to the arrangements of the components set forth in the following description or illustrated in the drawings. The invention is capable of embodiments in addition to those described and of being practiced and carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein, as well as the abstract, are for the purpose of description and should not be regarded as limiting.

[00176] As such, those skilled in the art will appreciate that the conception upon which this disclosure is based may readily be utilized as a basis for the designing of other structures, methods and systems for carrying out the several purposes of the present invention. It is important, therefore, that the claims be regarded as including such equivalent constructions insofar as they do not depart from the spirit and scope of the present invention.

[00177] While the invention has been described and exemplified in sufficient detail for those skilled in this art to make and use it, various alternatives, modifications, and aimprovements should be apparent without departing from the spirit and scope of the invention. The examples provided herein are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims.

[00178] It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

[00179] All patents and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

[00180] The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

[00181] Other embodiments are set forth within the following claims.

We claim:

1. A composition comprising:

a cyclic purine dinucleotide which binds to STING and induces STING-dependent TBK1 activation; and

an inactivated tumor cell which expresses and secretes one or more cytokines which stimulate dendritic cell induction, recruitment and/or maturation.

2. A composition according to claim 1, further comprising a pharmaceutically acceptable excipient.

3. A composition according to claim 1 or 2, wherein the inactivated tumor cell expresses and secretes GM-CSF.

4. A composition according to claim 1 or 2, wherein the inactivated tumor cell expresses and secretes CCL20.

5. A composition according to claim 1 or 2, wherein the inactivated tumor cell expresses and secretes CCL3.

6. A composition according to claim 1 or 2, wherein the inactivated tumor cell expresses and secretes IL-12p70.

7. A composition according to claim 1 or 2, wherein the inactivated tumor cell expresses and secretes FLT-3 ligand.

8. A composition according to one of claims 1-7, further comprising one or more of a CTLA-4 antagonist and a TLR-4 agonist.

9. A composition according to one of claims 1-8, wherein the tumor cell is inactivated by treatment with radiation.

10. A composition according to one of claims 1-9, wherein the cyclic purine dinuclotide is selected from the group consisting of c-di-AMP, c-di-GMP, c-di-IMP, c-AMP-GMP, c-AMP-IMP, and c-GMP-IMP, or combinations thereof.

11. A composition according to one of claims 1-10, wherein the cyclic purine dinuclotide is formulated with one or more lipids.

12. A composition according to claim 11, wherein the one or more lipids comprise digitonin.

13. A composition according to claim 11, wherein the one or more lipids form a liposome.

14. A composition according to one of claims 1-13, further comprising one or more adjuvants.

15. A composition according to claim 14, wherein the one or more adjuvants comprise CpG and/or monophosphoryl lipid A.

16. A method of inducing an immune response to a cancer in an individual, comprising:

administering a composition according to one of claims 1-15 to the individual, wherein the inactivated tumor cell or a mixture of different tumor cells are type-matched to the individual's cancer.

17. A method according to claim 16, wherein the inactivated tumor cell or a mixture of different tumor cells is an allogeneic tumor cell line or lines.

18. A method according to claim 16, wherein the inactivated tumor cell is an autologous tumor cell.

19. A method according to one of claims 16-18, wherein the tumor cell is selected from the group consisting of a colorectal cancer cell, an aero-digestive squamous cancer cell, a lung cancer cell, a brain cancer cell, a liver cancer cell, a stomach cancer cell, a sarcoma cell, a leukemia cell, a lymphoma cell, a multiple myeloma cell, an ovarian cancer cell, a uterine cancer cell, a breast cancer cell, a melanoma cell, a prostate cancer cell, a pancreatic carcinoma cell, and a renal carcinoma cell.