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(71) Applicants *(for all designated States except US)*: **SCHERING CORPORATION** [US/US]; 2000 Galloping Hill Road, Kenilworth, New Jersey 07033 (US). **N.V. ORGANON** [NL/NL]; Kloosterstraat 6, NL-5349 AB Oss (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CARVEN, Gregory, John** [US/US]; 89 Brooks Street, Maynard, Massachusetts 01754 (US). **DULOS, Gradus, Johannes** [NL/NL]; Kloosterstraat 6, NL-5349 AB Oss (NL). **ELASSAISS-SCHAAP, Jeroen** [NL/NL]; Kloosterstraat 6, NL-5349 AB Oss (NL).

(74) Agent: **KLANN, Amy, G.**; Merck Sharp & Dohme Corp., 126 East Lincoln Avenue, Rahway, New Jersey 07065-0907 (US).

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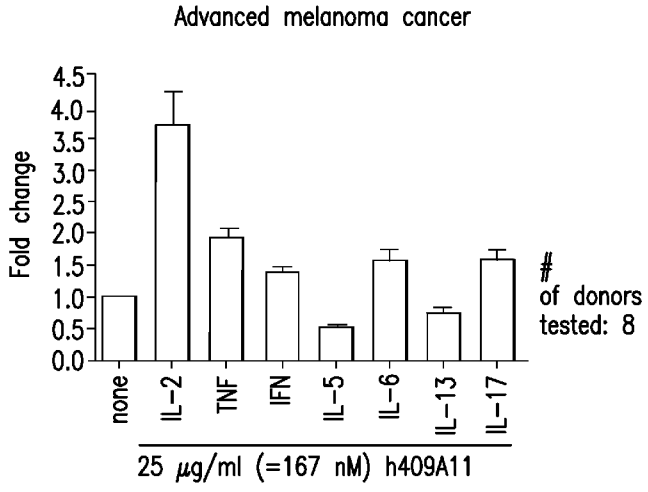


FIG.7E

(57) Abstract: The present invention relates to the identification and use of cytokine markers in bioassays for use in monitoring, diagnostic, patient selection, and treatment regimens involving blockade of the PD-1 pathway. In certain aspects, the invention provides methods and kits relating to these bioassays for selection and/or monitoring of treatment regimens involving PD-1 blockade. Biomarkers, kits, treatment regimens, therapeutic monitoring, and diagnostic methods related to anti-PD-1 therapies are provided.

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BIOASSAYS FOR DETERMINING PD-1 MODULATION

FIELD OF THE INVENTION

[0001] This application claims the benefit of U.S. provisional patent Application No. 61/367,657 filed July 26, 2010, which is herein incorporated by reference in its entirety.

[0002] The present invention relates to the identification and use of cytokine markers in bioassays for use in monitoring, diagnostic and treatment regimens involving PD-1 blockade. In certain aspects, the invention provides methods and kits relating to these bioassays for selection and/or monitoring of treatment regimens involving PD-1 blockade. Biomarkers, kits, treatment regimens, therapeutic monitoring, and diagnostic methods related to anti-PD-1 therapies are provided.

BACKGROUND OF THE INVENTION

[0003] Programmed Death 1 (PD-1), a member of the CD28 costimulatory gene family, is moderately expressed on naive T, B and NKT cells and up-regulated by T/B cell receptor signaling on lymphocytes, monocytes and myeloid cells (1). PD-1 has two known ligands with distinct expression profiles, PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-L2 expression is relatively restricted and is found on activated dendritic cells, macrophages and monocytes and on vascular endothelial cells (1-3). In contrast, PD-L1 is expressed more broadly including on naive lymphocytes and its expression is induced on activated B and T cells, monocytes and dendritic cells. Furthermore, by mRNA, it is expressed by non-lymphoid tissues including vascular endothelial cells, epithelial cells and muscle cells.

[0004] PD-1 is recognized as an important player in immune regulation and the maintenance of peripheral tolerance. In the mouse, this was shown to require PD-L1 expression on peripheral tissues and ligation of PD-1 on potentially autoreactive T cells to negatively modulate T cell activation involving an ITIM sequence in the PD-1 cytoplasmic domain (1, 4).

[0005] Depending on the specific genetic background, *pdcd1*$^{-/-}$mice spontaneously develop lupus-like phenomena or dilated cadiomyopathy (5, 6). Furthermore, antibody-

induced blockade of the PD-1 / PD-L1 pathway was demonstrated to accelerate the onset of autoimmune insulitis and diabetes in NOD mice (7).

[0006] Human cancers arising in various tissues were found to over-express PD-L1 or PD-L2. In large sample sets of e.g. ovarian, renal, colorectal, pancreatic, liver cancers and melanoma it was shown that PD-L1 expression correlated with poor prognosis and reduced overall survival irrespective of subsequent treatment (15-26). Similarly, PD-1 expression on tumor infiltrating lymphocytes was found to mark dysfunctional T cells in breast cancer and melanoma (27-28) and to correlate with poor prognosis in renal cancer (29). Using primary patient samples, it was shown that blockade of PD-1 or PD-L1 *in vitro* results in enhancement of human tumor-specific T cell activation and cytokine production (30). Consequently, in several murine syngeneic tumor models, blockade of either PD-1 or PD-L1 significantly inhibited tumor growth or induced complete regression.

[0007] A PD-1 blocking mAb (H409A11) was discovered and developed for use to treat human cancer patients and chronic virus-infected patients (described in co-pending application WO2008/156712).

[0008] Antigen-specific T cell dysfunction or tolerance is exemplified by the accumulated loss of the potential to produce Interleukin 2 (IL-2), Tumor Necrosis factor (TNF) α, perforin, interferon (IFN) γ (8) and inability to mount a proliferative response to T cell receptor triggering (1). The PD-1 pathway controls antigen-specific T cell tolerance and was found to be exploited in viral infection and tumor development to control and evade effective T cell immunity.

[0009] In chronic infection with LCMV (mouse), HIV, HBV or HCV (human), antigen-specific T cells were found to express aberrantly high levels of PD-1 correlating with their state of anergy or dysfunction (9). Blocking the PD-1 – PD-L1 interaction *in vivo* (LCMV) or *in vitro* (HIV, HCV, HBV) was shown to revive anti-viral T cell activity (10-12). PD-1 blockade in recently Simian Immunodeficiency Virus-infected macaques resulted in strong reduction of viral load and increased survival (13). Similarly, reduction in viral load was confirmed in second study using long-term SIV-infected rhesus macaques (14).

[0010] Overall, the PD-1/PD-L1 pathway is a well-validated target for the development of antibody therapeutics for cancer treatment. Anti-PD-1 antibodies may also be useful in chronic viral infection. Memory $CD8^+$T cells generated after an acute viral infection are highly functional and constitute an important component of protective immunity. In

contrast, chronic infections are often characterized by varying degrees of functional impairment (exhaustion) of virus-specific T-cell responses, and this defect is a principal reason for the inability of the host to eliminate the persisting pathogen. Although functional effector T cells are initially generated during the early stages of infection, they gradually lose function during the course of a chronic infection. Barber et al. (Barber et al., Nature 439: 682-687 (2006)) showed that mice infected with a laboratory strain of LCMV developed chronic infection resulting in high levels of virus in the blood and other tissues. These mice initially developed a robust T cell response, but eventually succumbed to the infection upon T cell exhaustion. The authors found that the decline in number and function of the effector T cells in chronically infected mice could be reversed by injecting an antibody that blocked the interaction between PD-I and PD-Ll.

[0011] Recently, it has been shown that PD-1 is highly expressed on T cells from HIV infected individuals and that receptor expression correlates with impaired T cell function and disease progression (Day et al., Nature 443:350-4 (2006).; Trautmann L. et al., Nat. Med. 12: 1198-202 (2006)). In both studies, blockade of the PD-1 pathway using antibodies against the ligand PD-Ll significantly increased the expansion of HIV-specific, IFN-gamma producing cells in vitro.

[0012] Other studies also implicate the importance of the PD-1 pathway in controlling viral infection. PD-1 knockout mice exhibit better control of adenovirus infection than wild-type mice (Iwai et al., Exp. Med. 198:39-50 (2003)). Also, adoptive transfer of HBV- specific T cells into HBV transgenic animals initiated hepatitis (Isogawa M. et al., Immunity 23:53-63 (2005)). The disease state of these animals oscillates as a consequence of antigen recognition in the liver and PD-1 upregulation by liver cells.

[0013] However, much of the information concerning the regulation and expression of PD-1 and its ligands PD-L1 and PD-L2 has been obtained using mouse cells, whereas characterization of their regulation and expression on human lymphocytes remains poorly defined, especially in response to modulation by anti-PD-1 treatment. Thus, there is a need for characterization of regulation and expression of PD-1 on human cells. Additionally, there is a need for developing methods to monitor effectiveness of treatments involving PD-1 blockade, such as those involved in treating cancer and infectious disease. In particular, a PD-1 translational biomarker assay to assess target engagement and modulation as well as PK/PD relations in patients dosed with one or more PD-1 blocking agents would be useful for monitoring and/or selection of patients.

SUMMARY OF THE INVENTION

[0014] In certain embodiments, the invention relates to a method for assessing PD-1 blockade in a mammalian subject treated with a PD-1-PD-L1 blocking agent comprising: a) measuring expression of at least one cytokine selected from the group consisting of IL-5, IL-2, IFNγ, IL-6, TNFα, and IL-17 in a blood sample from said subject; and b) comparing the expression of the at least one cytokine from step a) to a control, wherein higher expression of at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control and lower expression of IL-5 compared to a control is indicative of PD-1 blockade in the subject and suitability of said subject for treatment with at least one PD-1 pathway blocking agent; or wherein unchanged or lower expression at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control; and/or unchanged or elevated expression of IL-5 compared to a control is indicative of a lack of PD-1 blockade in response to at least one PD-1 pathway blocking agent in the subject and a lack of suitability of said subject for treatment with at least one PD-1 pathway blocking agent.

[0015] In certain embodiments, the invention relates to a method of treating cancer in a mammalian subject in need thereof, the method comprising the steps of: a) administering an effective amount of a PD-1-PD-L1 blocking agent; b) measuring expression of at least one cytokine selected from the group consisting of IL-5, IL-2, IFNγ, IL-6, TNFα, and IL-17 in a blood sample from said subject; and c) comparing the expression of the at least one cytokine of step b) to a control, wherein higher expression of at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control and lower expression of IL-5 compared to a control is indicative of PD-1 blockade in the subject and suitability of said subject for treatment with at least one PD-1 pathway blocking agent; or wherein unchanged or lower expression at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control; and/or unchanged or elevated expression of IL-5 compared to a control is indicative of a lack of PD-1 blockade in response to at least one PD-1 pathway blocking agent in the subject and a lack of suitability of said subject for treatment with at least one PD-1 pathway blocking agent.

[0016] In certain embodiments, the invention relates to a method of treating chronic infection in a mammalian subject in need thereof comprising: a) administering an effective amount of a PD-1-PD-L1 blocking agent; b) measuring expression of at least one cytokine

selected from the group consisting of IL-5, IL-2, IFNγ, IL-6, TNFα, and IL-17 in a blood sample from said subject; and c) comparing the expression of the at least one cytokine from step b) to a control, wherein higher expression of at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control and lower expression of IL-5 compared to a control is indicative of PD-1 blockade in the subject and suitability of said subject for treatment with at least one PD-1 pathway blocking agent; or wherein unchanged or lower expression at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control; and/or unchanged or elevated expression of IL-5 compared to a control is indicative of a lack of PD-1 blockade in response to at least one PD-1 pathway blocking agent in the subject and a lack of suitability of said subject for treatment with at least one PD-1 pathway blocking agent.

[0017] In certain embodiments, the mammalian subject is a human patient. In yet additional embodiments, the blood sample is a whole blood or PBMC sample.

[0018] In certain embodiments, expression is determined by gene expression analysis or immunoassay. In certain embodiments the gene expression analysis is selected from the group consisting of Northern blotting, PCR-based, SAGE, flow cytometry-based, and DNA microarray. In yet additional embodiments, the immunoassay is selected from the group consisting of ELISA, RIA, Western blot, luminescent immunoassay, fluorescent immunoassay.

[0019] In certain embodiments, the subject was treated with an effective amount of an antagonist PD-1 antibody.

[0020] In certain embodiments, the cancer is selected from the group consisting of brain, skin, colon, ovarian, renal, thyroid, lung, colorectal, pancreatic, liver, stomach, multiple myeloma, and melanoma.

[0021] In certain embodiments, the chronic infection is selected from viral infections consisting of human immunodeficiency virus (HIV), heptatitis B virus (HBV) or hepatitis C virus (HCV).

[0022] In certain embodiments, the invention relates to a kit for monitoring dosing levels in a mammalian subject dosed with one or more PD-1 blocking agents comprising at least one probe specific for a cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, IL-17, and IL-5.

[0023] In certain embodiments, the invention relates to a method of monitoring PD-1 blockade in a mammalian subject dosed with one or more PD-1 blocking agents comprising: a) obtaining a baseline biological sample from the subject prior to administering a dose of one or more PD-1 blocking agents; b) measuring the level of at least one biomarker selected from the group consisting of IL-5, IL-2, IFNγ, IL-6, TNFα, and IL-17 in the baseline biological sample by gene expression analysis or immunoassay, c) administering the one or more PD-1 blocking agents therapeutic agent to the subject; d) obtaining from the subject at least one subsequent biological sample; e) measuring the level of the at least one biomarker selected from the group consisting of IL-5, IL-2, IFNγ, IL-6, TNFα, and IL-17 in the subsequent sample by gene expression analysis or immunoassay; f) comparing the level of at least one biomarker in the subsequent biological sample with the level of at least one biomarker in the baseline biological sample, wherein a decrease in expression of IL-5 and an increase of at least one biomarker selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 in the subsequent biological sample indicates PD-1 blockade (i.e., target engagement).

[0024] In certain embodiments, at least one cytokine exhibits at least 2 fold higher expression than expression in a control sample.

BRIEF DESCRIPTION OF THE FIGURES

[0025] **Figures 1A-C** show PD-1, PD-L1 and PD-L2 surface expression on CD4+ and CD8+ T cells after SEB stimulation.

[0026] **Figures 2A-D** show that SEB-stimulated IL-2 production by healthy donor blood cells is enhanced by anti-PD-1 or anti-CTLA-4 and decreased by CTLA4Ig.

[0027] **Figures 3A-B** show that SEB-induced IL-2 production in whole blood cell cultures is enhanced by blockade of PD-1 or PD-L1 but not by PD-L2 blockade.

[0028] **Figures 4A-D** show that PD-1 blockade enhances Tetanus toxoid-antigen-specific T-cell IFNγ production.

[0029] **Figures 5A-D** show that PD-1 blockade enhances IL-2 production in blood cells from patient donors with prostate cancer (Figs. 5A-B) and advanced melanoma (Figs. 5C-D).

[0030] **Figure 6** shows that SEB-induced IL-2 production is enhanced by PD-1 blockade to a similar extent between healthy donors, melanoma cancer and prostate cancer patients.

[0031] **Figures 7A-E** show that PD-1 blockade enhances IL-2, IFNγ, IL-6, TNFα, IL-17 and decreases IL-5 cytokine production. These results represent the average response (±SEM) of several healthy donors, prostate cancer and advanced melanoma cancer donors.

[0032] **Figures 8 A-B** show that potentiation of SEB-induced IL-2 production in blood cells by PD-1 blockade is translatable to cynomolgus monkeys.

[0033] **Figures 9 A-B** show that further potentiation of IL-2 by exogenous addition of PD-1 blocking antibody was lost immediately following *in vivo* administration of h409A11. The duration of this pharmacodynamic effect occurred in a dose-dependent manner.

[0034] **Figures 10 A-B** show the representative fold change in SEB-induced IL-2 production in whole blood samples after single bolus intravenous dosing in Cynomolgus monkey and the pharmacodynamic model estimating dose response relationships.

DETAILED DESCRIPTION

[0035] A pharmacodynamic biomarker assay has been developed to quantify PD-1 blockade in blood samples from patients dosed with a PD-1-PD-L1 blocking agent. The assay utilized Staphylococcal enterotoxin B (SEB) to stimulate T cell receptor signaling in a subset of T cells bearing certain Vß chains, specifically Vß3, Vß12, Vß14, and Vß17 in human blood-derived T cells. SEB stimulation of whole blood induced IL-2 production that could be enhanced by PD-1 blockade in a dose dependent fashion.

[0036] Exemplary PD-1 blocking monoclonal antibodies are used to illustrate the effectiveness of the biomarker assays described herein. Three humanized PD-1 blocking monoclonal antibodies (*i.e.*, h409A11, h409A16, and h509A17) are described in WO2008/156712 for treating cancer as well as chronic infections such as chronic virus-infected patients.

[0037] The pharmacodynamic assays described herein can be used as a robust *in vitro* translational assay to assess the effect of PD-1 blockade in blood samples from individuals including from healthy donors (*e.g.*, serving as a baseline/control) and patients such as cancer patients, who have received treatment with an anti-PD-1 antibody such as h409A11. In two

examples, the PD-1 modulation bioassay (MBA) was used to determine the pharmacodynamic properties of humanized anti-PD-1 h409A11 following a single intravenous (IV) dose (0.3, 3.0 or 30 mg/kg) administration or multiple doses (6, 40 or 200 mg/kg) to cynomolgus macaques.

[0038] The PD-1 modulation bioassay is useful as a translational biomarker assay to assess target engagement and modulation as well as PK/PD relations in patients dosed with a PD-1 blocking mAb such as h409A11.

[0039] In certain embodiments, the invention provides a method for measuring the response to PD-1 modulating agents (*e.g.* h409A11 or other anti-PD-1 antibodies). In certain embodiments, the assay measures the response of whole blood cells or PBMC from individuals which were dosed with anti-PD-1 to a T cell stimulus (such as superantigen). When stimulated, T cells secrete cytokines including IL-2. In the presence of a PD-1 blocking agent, the level of cytokines such as IL-2 is increased.

[0040] In certain embodiments, laboratory test animals were dosed with anti-PD-1 antibody h409A11. Subsequently at various timepoints, whole blood was drawn and assayed for cytokine secretion (e.g. IL-2). Whole blood was diluted with cell culture medium (RPMI 1640 containing streptomycin and penicillin). Diluted whole blood was added to 96 well flat bottom plates containing additional h409A11or isotype control antibody at various concentrations and Staphylococcus Enterotoxin B (SEB). Final dilution of whole blood was 10 fold. Cultures were incubated for 3 days at 37C, 5% CO_2, 95% humidity after which supernatants were collected. Supernatants were frozen and assayed for IL-2 concentration using commercially available ELISA kits.

[0041] In certain embodiments, whole blood or PBMC derived from healthy human donors or from selected cancer patients were stimulated with a T cell stimulating agent such as SEB, in the presence or absence of a PD-1 blocking agent added in culture.

[0042] The PD-1 modulation bioassays described herein have been validated in laboratory test subjects, as well as samples from human cancer patients, and healthy volunteers.

[0043] In a particular embodiment, the activity of the h409A11 antibody was shown to correlate with the PK of the drug in cynomolgus macaques. Additionally, the activity of the h409A11 antibody in the MBA was determined to be dose dependant.

[0044] In accordance with the present invention there may be employed conventional molecular biology, microbiology, protein expression and purification, antibody, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. *See, e.g.,* Sambrook *et al.* (2001) *Molecular Cloning: A Laboratory Manual.* 3rd ed. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York; Ausubel *et al.* eds. (2005) *Current Protocols in Molecular Biology*. John Wiley and Sons, Inc.: Hoboken, NJ; Bonifacino *et al.* eds. (2005) *Current Protocols in Cell Biology*. John Wiley and Sons, Inc.: Hoboken, NJ; Coligan *et al.* eds. (2005) *Current Protocols in Immunology*, John Wiley and Sons, Inc.: Hoboken, NJ; Coico *et al.* eds. (2005) *Current Protocols in Microbiology*, John Wiley and Sons, Inc. : Hoboken, NJ; Coligan *et al.* eds. (2005) *Current Protocols in Protein Science*, John Wiley and Sons, Inc.: Hoboken, NJ; and Enna *et al.* eds. (2005) *Current Protocols in Pharmacology*, John Wiley and Sons, Inc.: Hoboken, NJ.; *Nucleic Acid Hybridization*, Hames & Higgins eds. (1985); *Transcription And Translation*, Hames & Higgins, eds. (1984); *Animal Cell Culture* Freshney, ed. (1986); *Immobilized Cells And Enzymes*, IRL Press (1986); Perbal, *A Practical Guide To Molecular Cloning* (1984); and Harlow and Lane. *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press: 1988).

Definitions

[0045] The following definitions are provided for clarity and illustrative purposes only, and are not intended to limit the scope of the invention.

[0046] As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the" include their corresponding plural references unless the context clearly dictates otherwise. All references cited herein are incorporated by reference to the same extent as if each individual publication, patent application, or patent, was specifically and individually indicated to be incorporated by reference.

Peripheral blood mononuclear cell

[0047] A peripheral blood mononuclear cell (PBMC) is any blood cell having a round nucleus, and includes for example: a lymphocyte, a monocyte or a macrophage. These blood cells are an important component in the immune system to fight infection and adapt to intruders. The lymphocyte population contains a mixture of T cells (CD4 and CD8 positive ~75%), B cells and NK cells (~25% combined).

[0048] PBMC cells are often extracted from whole blood using ficoll, a hydrophilic polysaccharide that separates layers of blood, with monocytes and lymphocytes forming a buffy coat under a layer of plasma. This buffy coat contains the PBMCs. PBMC's can be extracted from whole blood using a hypotonic lysis which will preferentially lyse red blood cells.

About or Approximately

[0049] The term "about" or "approximately" means within an acceptable range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *e.g.*, the limitations of the measurement system. For example, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Unless otherwise stated, the term "about" means within an acceptable error range for the particular value.

Administration

[0050] In the case of the present invention, parenteral and other routes of administration are possible. Such routes include intravenous, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, transmucosal, intranasal, rectal, vaginal, or transdermal routes. If desired, inactivated therapeutic formulations may be injected, e.g., intravascular, intratumor, subcutaneous, intraperitoneal, intramuscular, etc. In a preferred embodiment, the route of administration is oral. Although there are no physical limitations to delivery of the formulation, oral delivery is preferred because of its ease and convenience, and because oral formulations readily accommodate additional mixtures, such as milk and infant formula.

Adjuvant

[0051] As used herein, the term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically

enhances the immune response (Hood et al., *Immunology, Second Ed.*, 1984, Benjamin/Cummings: Menlo Park, California, p. 384). Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvants include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, and potentially useful human adjuvants such as N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine, N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine, BCG (*bacille Calmette-Guerin*) and *Corynebacterium parvum*. Preferably, the adjuvant is pharmaceutically acceptable.

Cytokine

[0052] The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, chemokines, and traditional polypeptide hormones. Examplary cytokines include: human IL-2, IFN-γ, IL-6, TNFα, IL-17, and IL-5.

Cytotoxic Agent

[0053] The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

Therapeutic Uses for the Assays and Methods of the Invention

[0054] The PD-1 blocking agents as described herein include those which specifically bind to human PD-1, can be used to increase, enhance, stimulate or up-regulate an immune response. The assays and methods of the invention are particularly suitable for screening, monitoring, or treating subjects having a disorder that can be treated by increasing the T-cell mediated immune response. Preferred subjects include human patients in need of enhancement of an immune response including patients with cancer and/or a chronic viral infection.

Cancer

[0055] The terms "cancer", "cancerous", or "malignant" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, leukemia, blastoma, and sarcoma. More particular examples of such cancers include squamous cell carcinoma, myeloma, small-cell lung cancer, non-small cell lung cancer, glioma, hodgkin's lymphoma, non-hodgkin's lymphoma, acute myeloid leukemia (AML), multiple myeloma, gastrointestinal (tract) cancer, renal cancer, ovarian cancer, liver cancer, lymphoblastic leukemia, lymphocytic leukemia, colorectal cancer, endometrial cancer, kidney cancer, prostate cancer, thyroid cancer, melanoma, chondrosarcoma, neuroblastoma, pancreatic cancer, glioblastoma multiforme, cervical cancer, brain cancer, stomach cancer, bladder cancer, hepatoma, breast cancer, colon carcinoma, and head and neck cancer.

[0056] PD-1 blocking agents such as those described herein include those used to treat cancer (*i.e.,* to inhibit the growth or survival of tumor cells). Preferred cancers whose growth may be inhibited using the antibodies or anti-PD-1 agents include cancers typically responsive to immunotherapy, but also cancers that have not hitherto been associated with immunotherapy. Non-limiting examples of preferred cancers for treatment include melanoma (e.g., metastatic malignant melanoma), renal cancer (e.g. clear cell carcinoma), prostate cancer (e.g. hormone refractory prostate adenocarcinoma), pancreatic adenocarcinoma, breast cancer, colon cancer, lung cancer (e.g. non-small cell lung cancer), esophageal cancer, squamous cell carcinoma of the head and neck, liver cancer, ovarian cancer, cervical cancer, thyroid cancer, glioblastoma, glioma, leukemia, lymphoma, and other neoplastic malignancies. The present invention relates to malignancies that demonstrate improved disease-free and overall survival in relation to the presence of tumor-infiltrating lymphocytes in biopsy or surgical material, *e.g.* melanoma, colorectal, liver, kidney, stomach/esophageal, breast, pancreas, and ovarian cancer. Such cancer subtypes are known to be susceptible to immune control by T lymphocytes. Additionally, the invention includes refractory or recurrent malignancies whose growth may be inhibited using the antibodies described herein. Particularly preferred cancers include those characterized by elevated expression of PD-1 and/or its ligands PD-L1 and/or PD-L2 in tested tissue samples, including: ovarian, renal, colorectal, pancreatic, breast, liver, glioblastoma, non-small cell lung cancer, gastric, esophageal cancers and melanoma. Preferred cancers also include those associated with persistent infection with viruses such as human immunodeficiency viruses, hepatitis viruses class A, B and C, Epstein Barr virus, human papilloma viruses that are known to be causally

related to for instance Kaposi's sarcoma, liver cancer, nasopharyngeal cancer, lymphoma, cervical, vulval, anal, penile and oral cancers.

Chemotherapeutic Agents and "Growth Inhibitory Agents"

[0057] A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. The methods described herein can be used in subjects that have had treatment with only PD-1-PD-L1 blocking agents (*i.e.*, no chemotherapeutic or "growth inhibitory agent" treatment). Alternatively, in certain instances, the methods described herein may be used in subjects that have had previous treatment with one or more chemotherapeutic agents, alone, or in combination with a "growth inhibitory agent". A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell. Thus, the growth inhibitory agent is one which significantly reduces the percentage of cells over-expressing such genes in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the vincas (vincristine and vinblastine) taxol, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information on such agents and chemotherapies can be found in *The Molecular Basis of Cancer*, Mendelsohn and Israel, eds., especially Chapter 1, entitled "Cell cycle regulation, oncogens, and antineoplastic drugs" by Murakami et al. (WB Saunders: Philadelphia, 1995).

Antibody or Antibody Fragments in Combination with Additional Agents

[0058] The antibody or antibody fragments can be used alone or in combination with: other anti-neoplastic agents or immunogenic agents (for example, attenuated cancerous cells, tumor antigens (including recombinant proteins, peptides, and carbohydrate molecules), antigen presenting cells such as dendritic cells pulsed with tumor derived antigen or nucleic acids, immune stimulating cytokines (for example, IL-2, IFNα2, GM-CSF), and cells transfected with genes encoding immune stimulating cytokines such as but not limited to GM-CSF); standard cancer treatments (for example, chemotherapy, radiotherapy or surgery); or other antibodies (including but not limited to antibodies to VEGF, EGFR, Her2/neu, VEGF receptors, other growth factor receptors, CD20, CD40, CD-40L, CTLA-4, OX-40, 4-1BB, and ICOS).

Infectious Diseases

[0059] The anti-PD-1 antibody or antibody fragments, or more generally, the PD-1-PD-L1 blocking agents can also be used to prevent or treat infections and infectious disease. These agents can be used alone, or in combination with vaccines, to stimulate the immune response to pathogens, toxins, and self-antigens. The antibodies or antigen-binding fragment thereof can be used to stimulate immune response to viruses infectious to humans, including but not limited to: human immunodeficiency viruses, hepatitis viruses class A, B and C, Epstein Barr virus, human cytomegalovirus, human papilloma viruses, and herpes viruses. The PD-1-PD-L1 blocking agents can be used to stimulate immune response to infection with bacterial or fungal parasites, and other pathogens. Viral infections with hepatitis B and C and HIV are among those considered to be chronic viral infections. Embodiments of the present invention include monitoring treatment of infectious diseases such as chronic viral diseases using the methods for assessing PD-1-PD-L1 blockade described herein. In addition, PD-1/PD-L1 blocking agents can be used to stimulate immunity by vaccination to prevent viral, bacterial and fungal infections. The present invention also relates to the use of PD-1 modulation bioassays to monitor PD-1 or PD-L1 blockade in the course of preventive vaccination.

DNA Amplification

[0060] "Amplification" of DNA as used herein denotes the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. For a description of PCR see Saiki *et al.*, Science 1988, 239:487.

Binding Composition

[0061] "Binding composition" refers to a molecule, small molecule, macromolecule, antibody, a fragment or analogue thereof, or soluble receptor, capable of binding to a target. "Binding composition" also may refer to a complex of molecules, e.g., a non-covalent complex, to an ionized molecule, and to a covalently or non-covalently modified molecule, e.g., modified by phosphorylation, acylation, cross-linking, cyclization, or limited cleavage, which is capable of binding to a target. "Binding composition" may also refer to a molecule in combination with a stabilizer, excipient, salt, buffer, solvent, or additive, capable of binding to a target. "Binding" may be defined as an association of the binding composition

with a target where the association results in reduction in the normal Brownian motion of the binding composition, in cases where the binding composition can be dissolved or suspended in solution.

Bispecific Antibody

[0062] "Bispecific antibody" generally refers to a covalent complex, but may refer to a stable non-covalent complex of binding fragments from two different antibodies, humanized binding fragments from two different antibodies, or peptide mimetics derived from binding fragments from two different antibodies. Each binding fragment recognizes a different target or epitope, e.g., a different receptor, e.g., an inhibiting receptor and an activating receptor. Bispecific antibodies normally exhibit specific binding to two different antigens.

Inhibition/Activation Assays

[0063] To examine the extent of inhibition, for example, samples or assays comprising a given, e.g., protein, gene, cell, or organism, are treated with a potential activator or inhibitor and are compared to control samples without the inhibitor. Control samples, i.e., not treated with antagonist, are assigned a relative activity value of 100%. Inhibition is achieved when the activity value relative to the control is about 90% or less, typically 85% or less, more typically 80% or less, most typically 75% or less, generally 70% or less, more generally 65% or less, most generally 60% or less, typically 55% or less, usually 50% or less, more usually 45% or less, most usually 40% or less, preferably 35% or less, more preferably 30% or less, still more preferably 25% or less, and most preferably less than 25%. Activation is achieved when the activity value relative to the control is about 110%, generally at least 120%, more generally at least 140%, more generally at least 160%, often at least 180%, more often at least 2-fold, most often at least 2.5-fold, usually at least 5-fold, more usually at least 10-fold, preferably at least 20-fold, more preferably at least 40-fold, and most preferably over 40-fold higher.

[0064] Endpoints in activation or inhibition can be monitored as follows. Activation, inhibition, and response to treatment, e.g., of a cell, skin tissue, keratinocyte, physiological fluid, tissue, organ, and animal or human subject, can be monitored by an endpoint. The endpoint may comprise a predetermined quantity or percentage of, *e.g.*, an indicia of inflammation, oncogenicity, or cell degranulation or secretion, such as the release of a

cytokine, toxic oxygen, or a protease. The endpoint may comprise, e.g., a predetermined quantity of ion flux or transport; cell migration; cell adhesion; cell proliferation; potential for metastasis; cell differentiation; and change in phenotype, e.g., change in expression of gene relating to inflammation, apoptosis, transformation, cell cycle, or metastasis (see, e.g., Knight (2000) Ann. Clin. Lab. Sci. 30:145-158; Hood and Cheresh (2002) Nature Rev. Cancer 2:91-100; Timme, *et al.* (2003) Curr. Drug Targets 4:251-261; Robbins and Itzkowitz (2002) Med. Clin. North Am. 86:1467-1495; Grady and Markowitz (2002) Annu. Rev. Genomics Hum. Genet. 3:101-128; Bauer, *et al.* (2001) Glia 36:235-243; Stanimirovic and Satoh (2000) Brain Pathol. 10:113-126).

Carrier

[0065] The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Alternatively, the carrier can be a solid dosage form carrier, including but not limited to one or more of a binder (for compressed pills), a glidant, an encapsulating agent, a flavorant, and a colorant. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

Coding Sequence or Sequence Encoding an Expression Product

[0066] A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, *i.e.,* the nucleotide sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

Control/Reference Expression Level

[0067] Various controls can be used in the present methods. A preferred control is unstimulated blood in the absence of a PD-1/PD-L1 blocking agent. Another preferred control is stimulated blood in the absence of a PD-1/PD-L1 blocking agent. Yet another

preferred control is unstimulated blood drawn from untreated control subjects. In certain methods an antibody control (e.g. an isotype control) may be used in comparative samples. In certain protocols, agents blocking PD-L1 or PD-L2 can be used to determine whether PD-1 preferentially exerts a measured biologic effect through either of these ligands. In other methods, agents modulating similar immune receptors can be used as controls, for instance antibodies blocking (or activating) CTLA-4, B7.1, B7.2, CD28, ICOS, and other members of the immune receptor protein families known to be expressed on blood cells.

Correlate

[0068] By "correlate" or "correlating" is meant comparing, in any way, the performance and/or results of a first analysis or protocol with the performance and/or results of a second analysis or protocol. For example, one may use the results of a first analysis or protocol in carrying out a second protocols and/or one may use the results of a first analysis or protocol to determine whether a second analysis or protocol should be performed. With respect to various embodiments herein, one may use the results of an analytical assay such as mRNA expression or an immunoassay to determine whether a dosage of an anti-PD-1 therapy engages the receptor and/ or is efficacious in reducing the size of a tumor or in treating a chronic viral infection.

Dosage

[0069] The dosage of a therapeutic formulation will vary widely, depending upon the nature of the disease, the patient's medical history, the frequency of administration, the manner of administration, the clearance of the agent from the host, and the like. The initial dose may be larger, followed by smaller maintenance doses. The dose may be administered as infrequently as weekly or biweekly, or fractionated into smaller doses and administered daily, semi-weekly, etc., to maintain an effective dosage level. In some cases, oral administration will require a higher dose than if administered intravenously.

Exogenous/Endogenous

[0070] "Exogenous" refers to substances that are produced or introduced outside an organism, cell, or human body, depending on the context. "Endogenous" refers to substances that are produced or introduced within a cell, organism, or human body, depending on the context.

Expression Construct

[0071] By "expression construct" is meant a nucleic acid sequence comprising a target nucleic acid sequence or sequences whose expression is desired, operatively associated with expression control sequence elements which provide for the proper transcription and translation of the target nucleic acid sequence(s) within the chosen host cells. Such sequence elements may include a promoter and a polyadenylation signal. The "expression construct" may further comprise "vector sequences." By "vector sequences" is meant any of several nucleic acid sequences established in the art which have utility in the recombinant DNA technologies of the invention to facilitate the cloning and propagation of the expression constructs including (but not limited to) plasmids, cosmids, phage vectors, viral vectors, and yeast artificial chromosomes.

[0072] Expression constructs may comprise vector sequences that facilitate the cloning and propagation of the expression constructs. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic host cells. Standard vectors useful in the current invention are well known in the art and include (but are not limited to) plasmids, cosmids, phage vectors, viral vectors, and yeast artificial chromosomes. The vector sequences may contain a replication origin for propagation in *E. coli*; the SV40 origin of replication; an ampicillin, neomycin, or puromycin resistance gene for selection in host cells; and/or genes (*e.g.*, dihydrofolate reductase gene) that amplify the dominant selectable marker plus the gene of interest.

Express and Expression

[0073] The terms "express" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form an "expression product" such as a protein. The expression product itself, *e.g.*. the resulting protein, may also be said to be "expressed" by the cell. An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means something that is inside a cell. The term "extracellular" means something that is outside a

cell. A substance is "secreted" by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell.

Transfection

[0074] The term "transfection" means the introduction of a foreign nucleic acid into a cell. The term "transformation" means the introduction of a "foreign" (*i.e.* extrinsic or extracellular) gene, DNA or RNA sequence to a cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. By "transfection" is meant the process of introducing one or more expression constructs into a host cell by any of the methods well established in the art, including (but not limited to) microinjection, electroporation, liposome-mediated transfection, calcium phosphate-mediated transfection, or virus-mediated transfection.

[0075] The introduced gene or sequence may also be called a "cloned" or "foreign" gene or sequence, may include regulatory or control sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cell's genetic machinery. The gene or sequence may include nonfunctional sequences or sequences with no known function. A host cell that receives and expresses introduced DNA or RNA has been "transformed" or "transfected" and is a "transformant", or a "transfectant", or a "clone". The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a different genus or species.

Expression System

[0076] The term "expression system" means a host cell and compatible vector under suitable conditions, *e.g.* for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell.

Gene or Structural Gene

[0077] The term "gene", also called a "structural gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine for example the conditions under which the

gene is expressed. Some genes, which are not structural genes, may be transcribed from DNA to RNA, but are not translated into an amino acid sequence. Other genes may function as regulators of structural genes or as regulators of DNA transcription.

[0078] A coding sequence is "under the control of" or "operatively associated with" expression control sequences in a cell when RNA polymerase transcribes the coding sequence into RNA, particularly mRNA, which is then trans-RNA spliced (if it contains introns) and translated into the protein encoded by the coding sequence.

[0079] The term "expression control sequence" refers to a promoter and any enhancer or suppression elements that combine to regulate the transcription of a coding sequence. In a preferred embodiment, the element is an origin of replication.

[0080] A "plurality of genes" as used herein refers to a group of identified or isolated genes whose levels of expression vary in different tissues, cells or under different conditions or biological states. The different conditions may be caused by exposure to certain agent(s)--whether exogenous or endogenous--which include hormones, receptor ligands, chemical compounds, etc. The expression of a plurality of genes demonstrates certain patterns. That is, each gene in the plurality is expressed differently in different conditions or with or without exposure to a certain endogenous or exogenous agents. The extent or level of differential expression of each gene may vary in the plurality and may be determined qualitatively and/or quantitatively according to this invention. A gene expression profile, as used herein, refers to a plurality of genes that are differentially expressed at different levels, which constitutes a "pattern" or a "profile." As used herein, the term "expression profile," "profile," "expression pattern," "pattern," "gene expression profile," and "gene expression pattern" are used interchangeably.

[0081] Gene expression profiles may be measured by using nucleotide or microarrays. These arrays allow tens of thousands of genes to be surveyed at the same time.

Arrays

[0082] As used herein, the term "microarray" refers to nucleotide arrays that can be used to detect biomolecules, for instance to measure gene expression. "Array," "slide," and "chip" are used interchangeably in this disclosure. Various kinds of arrays are made in research and manufacturing facilities worldwide, some of which are available commercially. There are, for example, two main kinds of nucleotide arrays that differ in the manner in

which the nucleic acid materials are placed onto the array substrate: spotted arrays and in situ synthesized arrays. One of the most widely used oligonucleotide arrays is GeneChip™ made by Affymetrix, Inc. The oligonucleotide probes that are 20- or 25-base long are synthesized in silico on the array substrate. These arrays tend to achieve high densities (*e.g.*, more than 40,000 genes per cm^2). The spotted arrays, on the other hand, tend to have lower densities, but the probes, typically partial cDNA molecules, usually are much longer than 20- or 25-mers. A representative type of spotted cDNA array is LifeArray made by Incyte Genomics. Pre-synthesized and amplified cDNA sequences are attached to the substrate of these kinds of arrays.

[0083] In one embodiment, the nucleotide is an array (*i.e.*, a matrix) in which each position represents a discrete binding site for a product encoded by a gene (e.g., a protein or RNA), and in which binding sites are present for products of most or almost all of the genes in the organism's genome. In another embodiment, the "binding site" (hereinafter, "site") is a nucleic acid or nucleic acid analogue to which a particular cognate cDNA can specifically hybridize. The nucleic acid or analogue of the binding site can be, *e.g.*, a synthetic oligomer, a full-length cDNA, a less-than full length cDNA, or a gene fragment.

[0084] Although the microarray may contain binding sites for products of all or almost all genes in the target organism's genome, such comprehensiveness is not necessarily required. Usually the microarray will have binding sites corresponding to at least about 50% of the genes in the genome, often at least about 75%, more often at least about 85%, even more often more than about 90%, and most often at least about 99%. Preferably, the microarray has binding sites for genes relevant to the action of the gene expression modulating agent of interest or in a biological pathway of interest.

[0085] The nucleic acid or analogue is attached to a "solid support," which may be made from glass, plastic (*e.g.*, polypropylene, nylon), polyacrylamide, nitrocellulose, or other materials. A preferred method for attaching the nucleic acids to a surface is by printing on glass plates, as is described generally by Schena *et al.*, 1995, Quantitative monitoring of gene expression patterns with a complementary DNA microarray, Science 270:467-470. This method is especially useful for preparing microarrays of cDNA. See also DeRisi *et al.*, 1996, Use of a cDNA microarray to analyze gene expression patterns in human cancer, Nature Genetics 14:457-460; Shalon *et al.*, 1996, A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization, Genome Res. 6:639-645; and

Schena *et al.*, 1995, Parallel human genome analysis; microarray-based expression of 1000 genes, Proc. Natl. Acad. Sci. USA 93:10539-11286.

[0086] In a preferred embodiment, the microarray is a high-density oligonucleotide array, as described above. In a particularly preferred embodiment, the nucleotide arrays are the MG_U74 and MG_U74v2 arrays from Affymetrix.

Polymerase Chain Reaction

[0087] "Polymerase Chain Reaction" or "PCR" is an amplification-based assay used to measure the copy number of the gene. In such assays, the corresponding nucleic acid sequences act as a template in an amplification reaction. In a quantitative amplification, the amount of amplification product will be proportional to the amount of template in the original sample. Comparison to appropriate controls provides a measure of the copy number of the gene, corresponding to the specific probe used, according to the principle discussed above. Methods of "real-time quantitative PCR" using Taqman probes are well known in the art. Detailed protocols for real-time quantitative PCR are provided, for example, for RNA in: Gibson *et al.*, 1996, A novel method for real time quantitative RT-PCR. Genome Res. 10:995-1001; and for DNA in: Heid *et al.*, 1996, Real time quantitative PCR. Genome Res. 10:986-994.

[0088] A TaqMan-based assay can also be used to quantify polynucleotides. TaqMan based assays use a fluorogenic oligonucleotide probe that contains a 5' fluorescent dye and a 3' quenching agent. The probe hybridizes to a PCR product, but cannot itself be extended due to a blocking agent at the 3' end. When the PCR product is amplified in subsequent cycles, the 5' nuclease activity of the polymerase, for example, AmpliTaq, results in the cleavage of the TaqMan probe. This cleavage separates the 5' fluorescent dye and the 3' quenching agent, thereby resulting in an increase in fluorescence as a function of amplification.

[0089] Other suitable amplification methods include, but are not limited to, ligase chain reaction (LCR) (see, Wu and Wallace, 1989, Genomics 4: 560; Landegren et al., 1988 Science 241: 1077; and Barringer et al., 1990, Gene 89: 117), transcription amplification (Kwoh *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86: 1173), self-sustained sequence replication (Guatelli *et al.*, 1990, Proc. Nat. Acad. Sci. USA 87: 1874), dot PCR, and linker adapter PCR, etc.

Protein Arrays

[0090] Protein microarrays are also useful for certain assays. Examples of such protein arrays include the ProtoArray® Human Protein Microarray (from Invitrogen, Carlsbad, CA) which is a high-content, functional protein microarray that enables rapid profiling of thousands of biochemical interactions in as little as one day. Version 5.0 of the ProtoArray® Human Protein MoreMicroarray contains over 9,000 unique human proteins selected from multiple gene families. The proteins are arrayed in duplicate on a 1 inch x 3 inch glass slide along with controls for background, labeling, and detection.

Level of mRNA

[0091] The "level of mRNA" in a biological sample refers to the amount of mRNA transcribed from a given gene that is present in a cell or a biological sample. One aspect of the biological state of a biological sample (*e.g.*, a cell or cell culture) that can be measured is its transcriptional state. The transcriptional state of a biological sample includes the identities and abundances of the constituent RNA species, especially mRNAs, in the cell under a given set of conditions. Preferably, a substantial fraction of all constituent RNA species in the biological sample are measured, but at least a sufficient fraction is measured to characterize the action of an agent or gene modulator of interest. The level of mRNA may be quantified by methods described herein or may be simply detected, by visual detection by a human, with or without comparison to a level from a control sample or a level expected of a control sample.

Biological Sample

[0092] A "biological sample," as used herein refers to any sample taken from a biological subject, *in vivo* or *in situ*. A biological sample may be a sample of biological tissue, or cells or a biological fluid. Biological samples may be taken, according to this invention, from any kind of biological species, any types of tissues, and any types of cells, among other things. Cell samples may be isolated cells, primary cell cultures, or cultured cell lines according to this invention. Biological samples may be combined or pooled as needed in various embodiments. Preferred samples include whole blood or PBMC's.

Gene Expression

[0093] "Modulation of gene expression," as this term is used herein, refers to the induction or inhibition of expression of a gene. Such modulation may be assessed or measured by assays. Typically, modulation of gene expression may be caused by endogenous or exogenous factors or agents. The effect of a given compound can be measured by any means known to those skilled in the art. For example, expression levels may be measured by PCR, Northern blotting, Primer Extension, Differential Display techniques, etc.

[0094] "Induction of expression" as used herein refers to any observable or measurable increase in the levels of expression of a particular gene, either qualitatively or quantitatively. The measurement of levels of expression may be carried out according to this invention using any techniques that are capable of measuring RNA transcripts in a biological sample. Examples of these techniques include, as discussed above, PCR, TaqMan, Primer Extension, Differential display and nucleotide arrays, among other things.

Repression of expression

[0095] "Repression" or "inhibition" of expression, are used interchangeably according to this disclosure. It refers to any observable or measurable decrease in the levels of expression of a particular gene, either qualitatively or quantitatively. The measurement of levels of expression may be carried out using any techniques that are capable of measuring RNA transcripts in a biological sample. The examples of these techniques include, as discussed above, PCR, TaqMan, Primer Extension, Differential Display, and nucleotide arrays, among other things.

Gene chip or DNA chip

[0096] A "gene chip" or "DNA chip" is described, for instance, in U.S. Pat. Nos. 5,412,087, 5,445,934 and 5,744,305 and is useful for screening gene expression at the mRNA level. Gene chips are commercially available.

Kits

[0097] A "kit" is one or more of containers or packages, containing at least one "plurality of genes," as described above. In certain embodiments, any desired combination of the genes are provided on a solid support. Such kits also may contain various reagents or solutions, as well as instructions for use and labels.

Labels

[0098] A "detectable label" or a "detectable moiety" is a composition that when linked with a nucleic acid or a protein molecule of interest renders the latter detectable, via spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radioactive isotopes, magnetic beads, metallic beads, colloidal particles, fluorescent dyes, electron-dense reagents, enzymes, biotin, digoxigenenin or haptens. A "labeled nucleic acid or oligonucleotide probe" is one that is bound, either covalently, through a linker or a chemical bond, or noncovalently through ionic, vander Waals, electrostatic, hydrophobic interactions, or hydrogen bonds, to a label such that the presence of the nucleic acid or probe may be detected by detecting the presence of the label bound to the nucleic acid or probe.

Nucleic acid probe

[0099] A "nucleic acid probe" is a nucleic acid capable of binding to a target nucleic acid or complementary sequence through one or more types of chemical bond, usually through complementary base pairing usually through hydrogen bond formation. As used herein, a probe may include natural (i.e., A, G, C, or T or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in a probe may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. It will be understood by one of skill in the art that probes may bind target sequences that lack complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are preferably directly labeled with isotopes, for example, chromophores, luminphores, chromogens, or indirectly labeled with biotin to which a strepavidin complex may later bind. By assaying the presence or absence of the probe, one can detect the presence or absence of a target gene of interest.

***In situ* Hybridization**

[00100] "*In situ* hybridization" is a methodology for determining the presence of or the copy number of a gene in a sample, for example, fluorescence in situ hybridization (FISH) (see Angerer, 1987 Meth. Enzymol 152: 649). Generally, in situ hybridization comprises the following major steps: (1) fixation of tissue or biological structure to be analyzed; (2) prehybridization treatment of the biological structure to increase accessibility of target

nucleic acid, and to reduce nonspecific binding; (3) hybridization of the mixture of nucleic acids to the nucleic acid in the biological structure or tissue; (4) post-hybridization washes to remove nucleic acid fragments not bound in the hybridization, and (5) detection of the hybridized nucleic acid fragments. The probes used in such applications are typically labeled, for example, with radioisotopes or fluorescent reporters. Preferred probes are sufficiently long, for example, from about 50, 100, or 200 nucleotides to about 1000 or more nucleotides, to enable specific hybridization with the target nucleic acid(s) under stringent conditions.

[00101] Hybridization protocols suitable for use with the methods of the invention are described, for example, in Albertson (1984) EMBO J. 3:1227-1234; Pinkel (1988) Proc. Natl. Acad. Sci. USA 85:9138-9142; EPO Pub. No. 430:402; Methods in Molecular Biology, Vol. 33: In Situ Hybridization Protocols, Choo, ed., Humana Press, Totowa, N.J. (1994); etc.

Heterologous

[00102] The term "heterologous" refers to a combination of elements not naturally occurring. For example, heterologous DNA refers to DNA not naturally located in the cell, or in a chromosomal site of the cell. Preferably, the heterologous DNA includes a gene foreign to the cell. A heterologous expression regulatory element is such an element that is operatively associated with a different gene than the one it is operatively associated with in nature. Accordingly, a gene encoding a protein of interest is heterologous to the vector DNA in which it is inserted for cloning or expression, and it is heterologous to a host cell containing such a vector, in which it is expressed.

Homologous

[00103] The term "homologous" as used in the art commonly refers to the relationship between nucleic acid molecules or proteins that possess a "common evolutionary origin," including nucleic acid molecules or proteins within superfamilies (*e.g.*, the immunoglobulin superfamily) and nucleic acid molecules or proteins from different species (Reeck *et al.*, *Cell* 1987; 50: 667). Such nucleic acid molecules or proteins have sequence homology, as reflected by their sequence similarity, whether in terms of substantial percent similarity or the presence of specific residues or motifs at conserved positions.

Host Cell

[00104] The term "host cell" means any cell of any organism that is selected, modified, transformed, grown or used or manipulated in any way for the production of a substance by the cell. For example, a host cell may be one that is manipulated to express a particular gene, a DNA or RNA sequence, a protein or an enzyme. Host cells can further be used for screening or other assays that are described *infra*. Host cells may be cultured *in vitro* or one or more cells in a non-human animal (*e.g.*, a transgenic animal or a transiently transfected animal). Suitable host cells include but are not limited to *Streptomyces* species and *E. coli*.

Immune Response

[00105] An "immune response" refers to the development in the host of a cellular and/or antibody-mediated immune response to a composition or vaccine of interest. Such a response usually consists of the subject producing antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or antigens included in the composition or vaccine of interest.

Isolated

[00106] As used herein, the term "isolated" means that the referenced material is removed from the environment in which it is normally found. Thus, an isolated biological material can be free of cellular components, *i.e.*, components of the cells in which the material is found or produced. Isolated nucleic acid molecules include, for example, a PCR product, an isolated mRNA, a cDNA, or a restriction fragment. Isolated nucleic acid molecules also include, for example, sequences inserted into plasmids, cosmids, artificial chromosomes, and the like. An isolated nucleic acid molecule is preferably excised from the genome in which it may be found, and more preferably is no longer joined to non-regulatory sequences, non-coding sequences, or to other genes located upstream or downstream of the nucleic acid molecule when found within the genome. An isolated protein may be associated with other proteins or nucleic acids, or both, with which it associates in the cell, or with cellular membranes if it is a membrane-associated protein.

Mutant

[00107] As used herein, the terms "mutant" and "mutation" refer to any detectable change in genetic material (*e.g.*, DNA) or any process, mechanism, or result of such a change. This includes gene mutations, in which the structure (*e.g.*, DNA sequence) of a gene is altered, any gene or DNA arising from any mutation process, and any expression product (*e.g.*, protein or enzyme) expressed by a modified gene or DNA sequence. As used herein, the term "mutating" refers to a process of creating a mutant or mutation.

Nucleic Acid Hybridization

[00108] The term "nucleic acid hybridization" refers to anti-parallel hydrogen bonding between two single-stranded nucleic acids, in which A pairs with T (or U if an RNA nucleic acid) and C pairs with G. Nucleic acid molecules are "hybridizable" to each other when at least one strand of one nucleic acid molecule can form hydrogen bonds with the complementary bases of another nucleic acid molecule under defined stringency conditions. Stringency of hybridization is determined, *e.g.*, by (i) the temperature at which hybridization and/or washing is performed, and (ii) the ionic strength and (iii) concentration of denaturants such as formamide of the hybridization and washing solutions, as well as other parameters. Hybridization requires that the two strands contain substantially complementary sequences. Depending on the stringency of hybridization, however, some degree of mismatches may be tolerated. Under "low stringency" conditions, a greater percentage of mismatches are tolerable (*i.e.*, will not prevent formation of an anti-parallel hybrid). See *Molecular Biology of the Cell*, Alberts *et al.*, 3rd ed., New York and London: Garland Publ., 1994, Ch. 7.

[00109] Typically, hybridization of two strands at high stringency requires that the sequences exhibit a high degree of complementarity over an extended portion of their length. Examples of high stringency conditions include: hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% SDS, 1 mM EDTA at 65°C, followed by washing in 0.1x SSC/0.1% SDS at 68°C (where 1x SSC is 0.15M NaCl, 0.15M Na citrate) or for oligonucleotide molecules washing in 6x SSC/0.5% sodium pyrophosphate at about 37°C (for 14 nucleotide-long oligos), at about 48°C (for about 17 nucleotide-long oligos), at about 55°C (for 20 nucleotide-long oligos), and at about 60°C (for 23 nucleotide-long oligos)). Accordingly, the term "high stringency hybridization" refers to a combination of solvent and temperature where two strands will pair to form a "hybrid" helix only if their nucleotide sequences are almost perfectly complementary (see *Molecular Biology of the Cell*, Alberts *et al.*, 3rd ed., New York and London: Garland Publ., 1994, Ch. 7).

[00110] Conditions of intermediate or moderate stringency (such as, for example, an aqueous solution of 2×SSC at 65°C; alternatively, for example, hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% SDS, 1 mM EDTA at 65°C, and washing in 0.2 x SSC/0.1% SDS at 42°C) and low stringency (such as, for example, an aqueous solution of 2×SSC at 55°C), require correspondingly less overall complementarity for hybridization to occur between two sequences. Specific temperature and salt conditions for any given stringency hybridization reaction depend on the concentration of the target DNA and length and base composition of the probe, and are normally determined empirically in preliminary experiments, which are routine (see Southern, *J. Mol. Biol.* 1975; 98: 503; Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 2, ch. 9.50, CSH Laboratory Press, 1989; Ausubel *et al.* (eds.), 1989, *Current Protocols in Molecular Biology*, Vol. I, Green Publishing Associates, Inc., and John Wiley & Sons, Inc., New York, at p. 2.10.3).

[00111] As used herein, the term "standard hybridization conditions" refers to hybridization conditions that allow hybridization of sequences having at least 75% sequence identity. According to a specific embodiment, hybridization conditions of higher stringency may be used to allow hybridization of only sequences having at least 80% sequence identity, at least 90% sequence identity, at least 95% sequence identity, or at least 99% sequence identity.

[00112] Nucleic acid molecules that "hybridize" to any desired nucleic acids may be of any length. In one embodiment, such nucleic acid molecules are at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, and at least 70 nucleotides in length. In another embodiment, nucleic acid molecules that hybridize are of about the same length as the particular desired nucleic acid.

Nucleic Acid Molecule

[00113] A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of

the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear (*e.g.*, restriction fragments) or circular DNA molecules, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (*i.e.*, the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

Orthologs

[00114] As used herein, the term "orthologs" refers to genes in different species that apparently evolved from a common ancestral gene by speciation. Normally, orthologs retain the same function through the course of evolution. Identification of orthologs can provide reliable prediction of gene function in newly sequenced genomes. Sequence comparison algorithms that can be used to identify orthologs include without limitation BLAST, FASTA, DNA Strider, and the GCG pileup program. Orthologs often have high sequence similarity.

Operatively Associated

[00115] By "operatively associated with" is meant that a target nucleic acid sequence and one or more expression control sequences (*e.g.*, promoters) are physically linked so as to permit expression of the polypeptide encoded by the target nucleic acid sequence within a host cell.

Patient or Subject

[00116] "Patient" or "subject" refers to mammals and includes human and veterinary subjects.

Percent Sequence Similarity or Percent Sequence Identity

[00117] The terms "percent (%) sequence similarity", "percent (%) sequence identity", and the like, generally refer to the degree of identity or correspondence between different nucleotide sequences of nucleic acid molecules or amino acid sequences of proteins that may or may not share a common evolutionary origin (see Reeck *et al.*, *supra*). Sequence identity

can be determined using any of a number of publicly available sequence comparison algorithms, such as BLAST, FASTA, DNA Strider, GCG (Genetics Computer Group, Program Manual for the GCG Package, Version 7, Madison, Wisconsin), etc.

[00118] To determine the percent identity between two amino acid sequences or two nucleic acid molecules, the sequences are aligned for optimal comparison purposes. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, percent identity = number of identical positions/total number of positions (*e.g.*, overlapping positions) x 100). In one embodiment, the two sequences are, or are about, of the same length. The percent identity between two sequences can be determined using techniques similar to those described below, with or without allowing gaps. In calculating percent sequence identity, typically exact matches are counted.

[00119] The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 1990, 87:2264, modified as in Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 1993, 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.*, *J. Mol. Biol.* 1990; 215: 403. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to desired sequences. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to desired protein sequences. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, *Nucleic Acids Res.* 1997, 25:3389. Alternatively, PSI-Blast can be used to perform an iterated search that detects distant relationship between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. *See* ncbi.nlm.nih.gov/BLAST/ on the WorldWideWeb. Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, *CABIOS* 1988; 4: 11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0), which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

[00120] In a preferred embodiment, the percent identity between two amino acid sequences is determined using the algorithm of Needleman and Wunsch (*J. Mol. Biol.* 1970, 48:444-453), which has been incorporated into the GAP program in the GCG software package (Accelrys, Burlington, MA; available at accelrys.com on the WorldWideWeb), using either a Blossum 62 matrix or a PAM250 matrix, a gap weight of 16, 14, 12, 10, 8, 6, or 4, and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package using a NWSgapdna.CMP matrix, a gap weight of 40, 50, 60, 70, or 80, and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters is using a Blossum 62 scoring matrix with a gap open penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

[00121] In addition to the cDNA sequences encoding various desired proteins, polynucleotide molecules comprising nucleotide sequences having certain percentage sequence identities to any of the aforementioned sequences are also contemplated. Such sequences preferably hybridize under conditions of moderate or high stringency as described above, and may include species orthologs.

Pharmaceutically Acceptable

[00122] When formulated in a pharmaceutical composition, a therapeutic compound can be admixed with a pharmaceutically acceptable carrier or excipient. As used herein, the phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are generally believed to be physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

Pharmaceutically Acceptable Derivatives and Pharmaceutical Compositions

[00123] The term "pharmaceutically acceptable derivative" as used herein means any pharmaceutically acceptable salt, solvate or prodrug, e.g. ester, of the desired active agent, which upon administration to the recipient is capable of providing (directly or indirectly) the desired active agent, or an active metabolite or residue thereof. Such derivatives are recognizable to those skilled in the art, without undue experimentation. Nevertheless, reference is made to the teaching of Burger's Medicinal Chemistry and Drug Discovery, 5th Edition, Vol 1: Principles and Practice, which is incorporated herein by reference to the

extent of teaching such derivatives. Preferred pharmaceutically acceptable derivatives are salts, solvates, esters, carbamates, and phosphate esters. Particularly preferred pharmaceutically acceptable derivatives are salts, solvates, and esters. Most preferred pharmaceutically acceptable derivatives are salts and esters.

[00124] While it is possible to use a composition for therapy as is, it may be preferable to administer it in a pharmaceutical formulation, e.g., in admixture with a suitable pharmaceutical excipient, diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice. Accordingly, in one aspect, pharmaceutical composition or formulation comprises at least one active composition, or a pharmaceutically acceptable derivative thereof, in association with a pharmaceutically acceptable excipient, diluent and/or carrier. The excipient, diluent and/or carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

[00125] The pharmaceutical compositions can be formulated for administration in any convenient way for use in human or veterinary medicine.

[00126] Such pharmaceutical formulations may be presented for use in a conventional manner with the aid of one or more suitable excipients, diluents, and carriers. Pharmaceutically acceptable excipients assist or make possible the formation of a dosage form for a bioactive material and include diluents, binding agents, lubricants, glidants, disintegrants, coloring agents, and other ingredients. Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, ascorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used. An excipient is pharmaceutically acceptable if, in addition to performing its desired function, it is non-toxic, well tolerated upon ingestion, and does not interfere with absorption of bioactive materials.

[00127] Acceptable excipients, diluents, and carriers for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington: The Science and Practice of Pharmacy. Lippincott Williams & Wilkins (A.R. Gennaro edit. 2005). The choice of pharmaceutical excipient, diluent, and carrier can be selected with regard to the intended route of administration and standard pharmaceutical practice.

Therapeutically Effective Amount

[00128] A "therapeutically effective amount" means the amount of a compound that, when administered to a mammal for treating a state, disorder or condition, is sufficient to effect such state, disorder, or condition. The "therapeutically effective amount" will vary depending on the compound, the disease and its severity and the age, weight, physical condition and responsiveness of the mammal to be treated. In certain cases, "therapeutically effective amount" is used to mean an amount or dose sufficient to modulate, *e.g.*, increase or decrease a desired activity *e.g.*, by about 10 percent, preferably by about 50 percent, and more preferably by about 90 percent. Preferably, a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition in the host following a therapeutic regimen involving one or more therapeutic agents. The concentration or amount of the active ingredient depends on the desired dosage and administration regimen, as discussed below. Suitable dosages may range from about 0.01 mg/kg to about 100 mg/kg of body weight per day, week, or month. The pharmaceutical compositions may also include other biologically active compounds.

[00129] A therapeutically effective amount of the desired active agent can be formulated in a pharmaceutical composition to be introduced parenterally, transmucosally, *e.g.*, orally, nasally, or rectally, or transdermally. Preferably, administration is parenteral, *e.g.*, via intravenous injection, and also including, but is not limited to, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial administration.

[00130] In another embodiment, the active ingredient can be delivered in a vesicle, in particular a liposome (see Langer, Science, 1990;249:1527-1533; Treat *et al.*, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss: New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid.).

[00131] In yet another embodiment, the therapeutic compound(s) can be delivered in a controlled release system. For example, a polypeptide may be administered using intravenous infusion with a continuous pump, in a polymer matrix such as poly-lactic/glutamic acid (PLGA), a pellet containing a mixture of cholesterol and the active ingredient (Silastic^{RTM}; Dow Corning, Midland, MI; see U.S. Patent No. 5,554,601) implanted subcutaneously, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration.

[00132] The effective amounts of compounds containing active agents include doses that partially or completely achieve the desired therapeutic, prophylactic, and/or biological effect. The actual amount effective for a particular application depends on the condition being treated and the route of administration. The effective amount for use in humans can be determined from animal models. For example, a dose for humans can be formulated to achieve circulating and/or gastrointestinal concentrations that have been found to be effective in animals.

Polynucleotide or Nucleotide Sequence

[00133] A "polynucleotide" or "nucleotide sequence" is a series of nucleotide bases (also called "nucleotides") in a nucleic acid, such as DNA and RNA, and means any chain of two or more nucleotides. A nucleotide sequence typically carries genetic information, including the information used by cellular machinery to make proteins and enzymes. These terms include double or single stranded genomic and cDNA, RNA, any synthetic and genetically manipulated polynucleotide, and both sense and anti-sense polynucleotide (although only sense stands are being represented herein). This includes single- and double-stranded molecules, *i.e.*, DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid backbone. This also includes nucleic acids containing modified bases, for example thio-uracil, thio-guanine and fluoro-uracil.

[00134] The nucleic acids herein may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation sequences, introns, 5'- and 3'- non-coding regions, and the like. The nucleic acids may also be modified by many means known in the art. Non-limiting examples of such modifications include methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, and internucleotide modifications such as, for example, those with uncharged linkages (*e.g.*, methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (*e.g.*, phosphorothioates, phosphorodithioates, etc.). Polynucleotides may contain one or more additional covalently linked moieties, such as, for example, proteins (*e.g.*, nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (*e.g.*, acridine, psoralen, etc.), chelators (*e.g.*, metals, radioactive metals, iron, oxidative metals,

etc.), and alkylators. The polynucleotides may be derivatized by formation of a methyl or ethyl phosphotriester or an alkyl phosphoramidate linkage. Furthermore, the polynucleotides herein may also be modified with a label capable of providing a detectable signal, either directly or indirectly. Exemplary labels include radioisotopes, fluorescent molecules, biotin, and the like.

Promoter

[00135] The promoter sequences may be endogenous or heterologous to the host cell to be modified, and may provide ubiquitous (*i.e.*+, expression occurs in the absence of an apparent external stimulus) or inducible (*i.e.*, expression only occurs in presence of particular stimuli) expression. Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patents No. 5,385,839 and No. 5,168,062), the SV40 early promoter region (Benoist and Chambon, Nature 1981;290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, Cell 1980;22:787-797), the herpes thymidine kinase promoter (Wagner *et al.*, Proc. Natl. Acad. Sci. USA 1981; 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, Nature 1982; 296:39-42); prokaryotic promoters such as the alkaline phosphatase promoter, the trp-lac promoter, the bacteriophage lambda P_L promoter, the T7 promoter, the beta-lactamase promoter (Villa-Komaroff, *et al.*, Proc. Natl. Acad. Sci. USA 1978;75:3727-3731), or the tac promoter (DeBoer, *et al.*, Proc. Natl. Acad. Sci. USA 1983;80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American 1980;242:74-94; promoter elements from yeast or other fungi such as the Gal4 promoter, the ADC (alcohol dehydrogenase) promoter, and the PGK (phosphoglycerol kinase) promoter.

Small Molecule

[00136] The term "small molecule" refers to a compound that has a molecular weight of less than about 2000 Daltons, less than about 1000 Daltons, or less than about 500 Daltons. Small molecules, without limitation, may be, for example, nucleic acids, peptides, polypeptides, peptide nucleic acids, peptidomimetics, carbohydrates, lipids, or other organic (carbon containing) or inorganic molecules and may be synthetic or naturally occurring or optionally derivatized. Such small molecules may be a therapeutically deliverable substance or may be further derivatized to facilitate delivery or targeting.

Substantially Homologous or Substantially Similar

[00137] In a specific embodiment, two DNA sequences are "substantially homologous" or "substantially similar" when at least about 80%, and most preferably at least about 90% or 95% of the nucleotides match over the defined length of the DNA sequences, as determined by sequence comparison algorithms, such as BLAST, FASTA, DNA Strider, etc. An example of such a sequence is an allelic or species variant of the specific genes of the invention. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system.

[00138] Similarly, in a particular embodiment, two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 80% of the amino acids are identical, or greater than about 90% are similar. Preferably, the amino acids are functionally identical. Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, *Version 10*, Madison, Wisconsin) pileup program, or any of the programs described above (BLAST, FASTA, etc.).

Substantially Identical

[00139] By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 80%, more preferably at least 90%, and most preferably at least 95% identity in comparison to a reference amino acid or nucleic acid sequence. For polypeptides, the length of sequence comparison will generally be at least 20 amino acids, preferably at least 30 amino acids, more preferably at least 40 amino acids, and most preferably at least 50 amino acids. For nucleic acid molecules, the length of sequence comparison will generally be at least 60 nucleotides, preferably at least 90 nucleotides, and more preferably at least 120 nucleotides.

[00140] The degree of sequence identity between any two nucleic acid molecules or two polypeptides may be determined by sequence comparison and alignment algorithms known in the art, including but not limited to BLAST, FASTA, DNA Strider, and the GCG Package (Madison, Wisconsin) pileup program (see, for example, Gribskov and Devereux Sequence *Analysis Primer* (Stockton Press: 1991) and references cited therein). The percent

similarity between two nucleotide sequences may be determined, for example, using the Smith-Waterman algorithm as implemented in the BESTFIT software program using default parameters.

Therapeutically or Prophylactically Effective Amount of an Antibody

[00141] The compositions used in any of the methods described herein may include a "therapeutically effective amount" or a "prophylactically effective amount" of one or more antibody or antigen-binding portion of an antibody. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the antibody or antibody portion may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody or antibody portion to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody or antibody portion are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Treating or Treatment

[00142] "Treating" or "treatment" of a state, disorder or condition includes:

[00143] (1) preventing or delaying the appearance of clinical or sub-clinical symptoms of the state, disorder or condition developing in a mammal that may be afflicted with or predisposed to the state, disorder or condition but does not yet experience or display clinical or subclinical symptoms of the state, disorder or condition; or

[00144] (2) inhibiting the state, disorder or condition, i.e., arresting, reducing or delaying the development of the disease or a relapse thereof (in case of maintenance treatment) or at least one clinical or sub-clinical symptom thereof; or

[00145] (3) relieving the disease, i.e., causing regression of the state, disorder or condition or at least one of its clinical or sub-clinical symptoms.

[00146] The benefit to a subject to be treated is either statistically significant or at least perceptible to the patient or to the physician.

Vaccine

[00147] As used herein, the term "vaccine" refers to a composition comprising a cell or a cellular antigen, and optionally other pharmaceutically acceptable carriers, administered to stimulate an immune response in an animal, preferably a mammal, most preferably a human, specifically against the antigen and preferably to engender immunological memory that leads to mounting of a protective immune response should the subject encounter that antigen at some future time. Vaccines often comprise an adjuvant.

Variant

[00148] The term "variant" may also be used to indicate a modified or altered gene, DNA sequence, enzyme, cell, etc., *i.e.*, any kind of mutant.

Vector, Cloning Vector and Expression Vector

[00149] The terms "vector", "cloning vector" and "expression vector" refer to the vehicle by which DNA can be introduced into a host cell, resulting in expression of the introduced sequence. In one embodiment, vectors comprise a promoter and one or more control elements (*e.g.*, enhancer elements) that are heterologous to the introduced DNA but are recognized and used by the host cell. In another embodiment, the sequence that is introduced into the vector retains its natural promoter that may be recognized and expressed by the host cell (Bormann *et al.*, J. Bacteriol. 1996;178:1216-1218).

[00150] Vectors typically comprise the DNA of a transmissible agent, into which foreign DNA is inserted. A common way to insert one segment of DNA into another segment of DNA involves the use of enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites. A "cassette" refers to a DNA coding sequence or segment of DNA that codes for an expression product that can be inserted into a vector at defined restriction sites. The cassette restriction sites are designed to ensure insertion of the cassette in the proper reading frame. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA

having inserted or added DNA, such as an expression vector, can also be called a "DNA construct". A common type of vector is a "plasmid", which generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and which can readily be introduced into a suitable host cell. A plasmid vector often contains coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. Coding DNA is a DNA sequence that encodes a particular amino acid sequence for a particular protein or enzyme. Promoter DNA is a DNA sequence which initiates, regulates, or otherwise mediates or controls the expression of the coding DNA. Promoter DNA and coding DNA may be from the same gene or from different genes, and may be from the same or different organisms. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, *e.g.* antibiotic resistance, and one or more expression cassettes. Vector constructs may be produced using conventional molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.*, 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); F.M. Ausubel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

General

[00151] The present invention provides methods of monitoring target engagement and efficacy of PD-1 blockade, and in particular for monitoring an anti-PD-1 therapy in the treatment of cancer or chronic viral infections as described herein. In particular, increased serum levels of any one of, or all of IL-2, IFNγ, IL-6, TNFα, and IL-17 alone or in combination with the observation of decreased IL-5 expression, provide biomarker signatures indicating PD-1-PD-L1 target engagement as well as efficacy.

Sample Collection and Preparation

[00152] The biomarkers can be measured in biological samples from subjects before and after exposure of the subject to one or more therapeutic agents. Accordingly, samples may be collected from a subject over a period of time. Furthermore, obtaining numerous samples from a subject over a period of time can be used to verify results from earlier detections and/or identify a differential expression as a result of exposure to a therapeutic

drug. Generally, biological samples can be collected from a subject via biopsy, but may be collected using other known clinical methods, such as the collection of peripheral blood, including serum, or bone marrow for hematological malignancies, or the collection of peripheral blood mononuclear cells for surrogate assays of target inhibition/biomarker measurement.

[00153] Samples can be analyzed without additional preparation and/or separation procedures. In alternative embodiments, sample preparation and/or separation can involve, without limitation, any of the following procedures, depending on the type of sample collected and/or types of biomarkers searched: removal of high abundance polypeptides; addition of preservatives and calibrants, desalting of samples; concentration of sample substances; protein digestions; and fraction collection. In yet further embodiments, sample preparation techniques concentrate information-rich biomarkers and deplete polypeptides or other substances that would carry little or no information such as those that are highly abundant in or native to the tumor. Examples of methods for further processing samples include: electrophoretic separation, size exclusion chromatography, proteolytic digestion, and ultracentrifugation.

Detection and Quantitation of Biomarkers

[00154] Any suitable method can be used to detect (a differential presence of) one or more of the biomarkers described herein. Successful practice of the invention can be achieved with one or a combination of methods that can detect and, preferably, quantify the biomarkers. These methods include, without limitation, hybridization-based methods including those employed in biochip arrays, mass spectrometry (e.g., laser desorption/ionization mass spectrometry), fluorescence (e.g. sandwich immunoassay), surface plasmon resonance, ellipsometry and atomic force microscopy. For nucleic acid biomarkers, methods for detection and quantitation include PCR, quantitative PCR, northern blot analysis, southern blot analysis, mass spectrometry and the like.

[00155] Methods may further include, by one or more of electrospray ionization mass spectrometry (ESI-MS), ESI-MS/MS, ESI-MS/$(MS)^n$, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), desorption/ionization on silicon (DIOS), secondary ion mass spectrometry (SIMS), quadrupole time-of-flight (Q-TOF), atmospheric pressure chemical ionization mass spectrometry (APCI-MS), APCI-MS/MS, APCI-$(MS)^n$, atmospheric pressure photoionization mass spectrometry (APPI-MS), APPI-MS/MS, and APPI-$(MS)_n$, quadrupole

mass spectrometry, fourier transform mass spectrometry (FTMS), and ion trap mass spectrometry, where n is an integer greater than zero. Spectroscopic methods for detecting and quantifying protein biomarkers are known in the art and are described, for example in U.S. Pat. Nos. 5,719,060; 6,225,047; 5,719,060; 6,124,137 and PCT International Publication No. WO 03/64594.

[00156] In another embodiment of the invention, the biomarkers of the invention are measured by a method other than mass spectrometry or other than methods that rely on a measurement of the mass of the biomarker. In one such embodiment that does not rely on mass, the biomarkers of this invention are measured by immunoassay. Immunoassay requires biospecific capture reagents, such as antibodies, to capture the biomarkers. Antibodies can be produced by methods well known in the art, e.g., by immunizing animals with the biomarkers. Biomarkers can be isolated from samples based on their binding characteristics. Alternatively, if the amino acid sequence of a polypeptide biomarker is known, the polypeptide can be synthesized and used to generate antibodies by methods well known in the art.

[00157] This invention contemplates traditional immunoassays including, for example, sandwich immunoassays including ELISA or fluorescence-based immunoassays, as well as other enzyme immunoassays. Nephelometry is an assay done in liquid phase, in which antibodies are in solution. Binding of the antigen to the antibody results in changes in absorbance, which is measured. In the SELDI-based immunoassay, a biospecific capture reagent for the biomarker is attached to the surface of an MS probe, such as a pre-activated ProteinChip array. The biomarker is then specifically captured on the biochip through this reagent, and the captured biomarker is detected by mass spectrometry.

[00158] In one embodiment, the biomarker can be a nucleic acid, wherein the nucleic acid can be detected and/or quantified using methods known in the art. For example, nucleic acid biomarkers may be detected using PCR (disclosed in U.S. Pat. No. 4,683,195, U.S. Pat. No. 4,683,202 and U.S. Pat. No. 4,965,188 and others in detail). In one embodiment, a real time PCR method is used to enable a convenient and reliable quantitative measurement of biomarker nucleic acids having a wide dynamic range. The real time PCR technique includes the method by using a TaqMan probe using ABIPRISM7700.™. (Applied Biosystems) and the method by using LightCycler.™. (Roche Diagnostics). Particularly in the latter case, in a high rate reaction cycle in which a temperature cycle of PCR is completed for some 10 minutes, a change of an amplified amount of a DNA synthesized for every cycle can be detected in a real time. DNA detection method of the real time PCR method includes 4

methods using a DNA-binding pigment (intercalator), a hybridization probe (kissing probe), TaqMan probe, or Sunrise Uniprimer (molecular beacon). On the other hand, the expression level of a biomarker gene can be analyzed by using a DNA-binding pigment such as SYBR GreenI. SYBR GreenI is a binding pigment specific to a double strand of the DNA and, when bound to a double strand, an inherent fluorescence intensity is reinforced. By adding SYBR GreenI at the PCR reaction and measuring the fluorescence intensity at the end of each cycle of an elongation reaction, the increase in a PCR product can be detected. For detection of a biomarker gene, similar to normal PCR, a primer is designed by using a commercialized gene analysis software on the basis of a sequence of the biomarker gene. SYBR GreenI detects a nonspecific product and, thus, requires designing an optimal primer. Required designing standards are a length of an oligomer, a base composition of the sequence, a GC content, and a Tm value.

[00159] Detection methods may include use of a microarray/biochip array. Biochip arrays useful in the invention include protein and nucleic acid arrays. One or more biomarkers are captured on the biochip array and subjected to laser ionization to detect the molecular weight of the products. Analysis of the products is, for example, by molecular weight of the one or more biomarkers against a threshold intensity that is normalized against total ion current.

[00160] The biochip surfaces may, for example, be ionic, anionic, hydrophobic; comprised of immobilized nickel or copper ions, comprised of a mixture of positive and negative ions; and/or comprised of one or more antibodies, single or double stranded nucleic acids, proteins, peptides or fragments thereof, amino acid probes, or phage display libraries. Many protein biochips are described in the art. These include, for example, protein biochips produced by Ciphergen Biosystems (Fremont, Calif.), Packard BioScience Company (Meriden Conn.), Zyomyx (Hayward, Calif.) and Phylos (Lexington, Mass.). Examples of such protein biochips are described in the following patents or patent applications: U.S. Pat. No. 6,225,047 (Hutchens and Yip, "Use of retentate chromatography to generate difference maps," May 1, 2001); International publication WO 99/51773 (Kuimelis and Wagner, "Addressable protein arrays," Oct. 14, 1999); U.S. Pat. No. 6,329,209 (Wagner et al., "Arrays of protein-capture agents and methods of use thereof," Dec. 11, 2001) and International publication WO 00/56934 (Englert et al., "Continuous porous matrix arrays," Sep. 28, 2000).

[00161] Biomarkers may be captured with capture reagents immobilized to a solid support, such as a biochip, a multiwell microtiter plate, a resin, or nitrocellulose membranes

that are subsequently probed for the presence of proteins. Capture can be on a chromatographic surface or a biospecific surface. For example, a tumor sample containing the biomarkers may be placed on the active surface of a biochip for a sufficient time to allow binding. Then, unbound molecules are washed from the surface using a suitable eluant, such as phosphate buffered saline. In general, the more stringent the eluant, the more tightly the proteins must be bound to be retained after the wash.

[00162] Upon capture on a biochip, analytes can be detected by a variety of detection methods selected from, for example, a gas phase ion spectrometry method, an optical method, an electrochemical method, atomic force microscopy and a radio frequency method. Also of interest is the use of mass spectrometry, for example, SELDI. Optical methods include, for example, detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, birefringence or refractive index (e.g., surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry). Optical methods include microscopy (both confocal and non-confocal), imaging methods and non-imaging methods. Immunoassays in various formats (e.g., ELISA) are popular methods for detection of analytes captured on a solid phase. Electrochemical methods include voltametry and amperometry methods. Radio frequency methods include multipolar resonance spectroscopy.

Qualification of Disease Status

[00163] The biomarkers of the present invention have a number of uses. For example, determining efficacy and/or receptor engagement following therapy with an anti-PD-1 therapeutic agent. The biomarkers can be used alone or in combination with other products. The biomarkers are differentially present in blood samples of a patient before and after exposure to a therapeutic drug. For example, some of the markers are expressed at an elevated level and/or are present at a higher frequency after treatment, while some of the products are expressed at a decreased level and/or are present at a lower frequency after treatment. Therefore, generating a biomarker profile for a subject would provide useful information regarding treatment status (e.g., indicating efficacy of cancer or chronic viral infection treatment).

[00164] The detection of a differential presence of a plurality of biomarkers in a blood or tumor sample may improve the indication of therapeutic efficacy of a therapeutic drug in the treatment of a cancer or chronic viral infection.

[00165] The biomarkers of the present invention can be used to adjust the dosage of therapeutic agents provided to a subject. For example, to achieve maximal target

engagement of an anti-PD-1 agent or blockade, the responsible treating physician can adjust the dosage such that the expression of any one or more of the cyokines IL-2, IFNγ, IL-6, TNFα, IL-17, and IL-5 is optimized. Similarly, increased the expression of IL-2, IFNγ, IL-6, TNFα, and IL-17, and decreased expression of IL-5 is indicative of tumor inhibition efficacy. Again, by optimizing the expression of any of IL-2, IFNγ, IL-6, TNFα, IL-17, and IL-5 by varying the PD-1-PD-L1 blocking agent (*e.g.*, anti-PD1 agent) dosage, the treating physician can achieve maximal tumor inhibition. Thus, by changing the dosage level in a systematic manner, the optimal level of therapeutic PD-1-PD-L1 blocking agent is determined for a subject. Amelioration of symptoms may be monitored concurrently. This method can be used to find an optimal dosage level at which to treat a subject.

[00166] The biomarkers of the present invention may also be used for identification of cancer patients or patients diagnosed with a chronic viral infection that are most likely to benefit from treatment with an agent that blocks the PD-1/PD-L1 pathway. The present methods may be used to identify patients lacking a response to T cell stimulation in the blood in the presence or absence of a PD-1/PD-L1 modulating agent, who may ultimately be deselected for subsequent treatment with such agents.

[00167] The biomarkers of the present invention may also be used for identification of an anti-tumor response or monitoring the efficacy of a therapeutic drug in a cancer subject taking into account the amount of the biomarker(s) in a tumor sample before and after exposure of the subject to a therapeutic drug (up or down regulation of the biomarker(s)). The amounts are measured under the same or substantially similar experimental conditions but at different time periods preceding and following treatment. The biomarkers of the invention can also be used to identify an agent useful in the treatment of cancer.

[00168] In certain embodiments of the methods of qualifying treatment status, the methods further comprise managing subject treatment based on the status. The invention also provides for such methods where the biomarkers (or specific combination of biomarkers) are measured again after such subject management. In these cases, the methods are used to monitor the status of the cancer or chronic viral infection, *e.g.*, candidacy for treatment with anti-PD1 or other PD-1-PD-L1 blocking agent, alone or in combination with another therapeutic agent, response to such treatment, remission of the disease or progression of the disease.

[00169] In another embodiment of the invention, the output from a detection device can subsequently be processed, stored, and further analyzed or assayed using a bio-informatics system. A bio-informatics system may include one or more of the following,

without limitation: a computer; a plurality of computers connected to a network; a signal processing tool(s); a pattern recognition tool(s); a tool(s) to control flow rate for sample preparation, separation, and detection.

[00170] The data processing utilizes mathematical foundations. In another embodiment of the invention, dynamic programming is used to align a separation axis with a standard separation profile. Intensities may be normalized, for example, by fitting roughly 90% of the intensity values into a standard spectrum. The data sets can then be fitted using wavelets designed for separation and mass spectrometer data. In yet another embodiment of the invention, data processing filters out some of the noise and reduces spectrum dimensionality, potentially allowing for pattern recognition.

[00171] Following data processing, pattern recognition tools can be utilized to identify subtle differences between phenotypic states. Pattern recognition tools are based on a combination of statistical and computer scientific approaches, which provide dimensionality reduction. Such tools are scalable. Data so obtained may be stored on a computer readable medium.

Kits

[00172] In one aspect, the invention provides kits for qualifying cancer or infection status in a subject, wherein the kits can be used to detect the differential presence of the biomarkers described herein. For example, the kits can be used to detect a differential presence of any combination of the biomarkers in tumor samples of cancer subjects or in subjects with a chronic viral infection, before and after exposure to a therapeutic drug. The kits of the invention have many applications. For example, the kits can be used to monitor efficacy of a therapeutic drug in a subject with cancer and/or a chronic viral infection. The kits can also be used to identify agents useful in the treatment of cancer and/or a chronic viral infection.

[00173] In specific embodiments, kits of the invention contain a biomarker, which is optionally isotopically or fluorescently labeled.

[00174] The kits of the invention may include instructions, reagents, testing equipment (test tubes, reaction vessels, needles, syringes, etc.), standards for calibration, and/or equipment. Reagents may include acids, bases, oxidizing agents, and marker species. The instructions provided in a kit according to the invention may be directed to suitable operational parameters in the form of a label or a separate insert.

[00175] The kits may also include an adsorbent, wherein the adsorbent retains one or more biomarkers described herein (polynucleotide or polypeptide), and written instructions

for use of the kit for qualification of cancer and/or a chronic viral infection status in a subject. Such a kit could, for example, comprise: (a) a substrate comprising an adsorbent thereon, wherein the adsorbent is suitable for binding a biomarker, and (b) instructions to detect the biomarker(s) by contacting a sample (e.g.blood) with the adsorbent and detecting the product(s) retained by the adsorbent. Accordingly, the kit could comprise (a) a DNA probe that specifically binds to a biomarker; and (b) a detection reagent. Such a kit could further comprise an eluant (as an alternative or in combination with instructions) or instructions for making an eluant, wherein the combination of the adsorbent and the eluant allows detection of the biomarker using, for example, gas phase ion spectrometry.

[00176] The abbreviations in the specification correspond to units of measure, techniques, properties or compounds as follows: "min" means minutes, "h" means hour(s), "µl" or "µL" means microliter(s), "ml" or "mL" means milliliter(s), "mM" means millimolar, "M" means molar, "mmole" means millimole(s), "kb" means kilobase, "mAb" means monoclonal antibody "bp" means base pair(s), and "IU" means International Units. "Polymerase chain reaction" is abbreviated PCR; "Reverse transcriptase polymerase chain reaction" is abbreviated RT-PCR; "Estrogen receptor" is abbreviated ER; "DNA binding domain" is abbreviated DBD; "Untranslated region" is abbreviated UTR; "Sodium dodecyl sulfate" is abbreviated SDS; and "High Pressure Liquid Chromatography" is abbreviated HPLC.

GENERAL METHODS

Antibodies

[00177] Functional grade purified antibodies used were anti-PD-1 (including mouse-anti-human hPD-1.09A as described in WO2008/156712, or humanized anti-PD-1:h409A11 corresponding to the humanized antibody according to the light and heavy chains deposited as ATCC reference No. 0801470_SPD-L-11 and No. 081469_SPD-H as described in WO2008/156712), anti-PD-L1 and anti-PD-L2 (eBioscience), anti-CTLA-4 (mouse anti human CTLA4 clone 14D3; eBioscience or Innogenetics [Zwijnaarde, Belgium]), and CTLA-4Ig. Isotype controls used were mouse-IgG1 (R&D systems), mouse-IgG2a (eBiosciences), human-IgG (purchased Sigma and further purified) and human-IgG4 (Sigma).

[00178] To study PD-1 blockade, hPD-1.09A and its corresponding humanized antibody h409A11 were used (as described in WO2008/156712). Monoclonal antibody

h409A11 is a humanized anti-PD-1 monoclonal antibody of the IgG4/kappa isotype as described in WO2008/156712. The antibody hPD-1.09A is a mouse anti-human monoclonal antibody of the mouse IgG1 isotype also described in WO2008/156712. The binding affinity of h409A11 and hPD-1.09A for PD-1 is in the low pM range and their binding of both PD-1 ligands is blocked with an IC50 of < 0.1 nM.

Results

Superantigen-induced PD-1, PD-L1, and PD-L2 Expression on Human T cells

[00179] The following directly conjugated mouse anti-human antibodies were used for flow cytometry: anti-CD3 PerCP-Cy5.5 (clone SK7), anti-CD4 APC (clone RPA-T4), anti-CD4 PE-Cy7 (clone SK3), anti-CD8 FITC (clone SK1), anti-CD8 APC (clone RPA-T8), anti-CD8 APC-H7 (clone SK1), anti-PD-1 FITC (clone MIH4), anti-PD-1 APC (clone MIH4), anti-PD-L1 PE (clone MIH1), anti-PD-L2 PE (clone MIH18) and isotype controls IgG1κ FITC (clone MOPC-21), IgG2aκ FITC (clone G155-178), IgG1κ PE (clone MOPC-21), IgG2aκ PE (clone G155-178), IgG1κ APC (clone MOPC-21) and IgG2aκ APC (clone G155-178) (all from BD Biosciences, Breda, the Netherlands).

[00180] The expression of human PD-1 and its ligands on T cells was analyzed following Staphylococcus Enterotoxin B (SEB) stimulation. Cryopreserved PBMC (5E4 cells/well) in medium (RPMI1640 supplemented with penicillin, streptomycin and 10% FBS [Gibco, Invitrogen Corporation]) was added to 96-well NUNCLON delta surface plates (NUNC™). Cells were cultured in the presence or absence of 0.1 µg/ml SEB (Sigma) for 0 to 4 days at 37°C, 5% CO_2 and 97% humidity. PBMCs from two identical wells were pooled and resuspended in 150 µl sampling buffer (PBS + 20% HI FBS + 0.02% sodium azide). Cells were incubated in the dark on ice with 10 µl of anti-PD-1, anti-PD-L1, anti-PD-L2 PE, anti-CD3, anti-CD4 or anti- CD8 (all from Becton & Dickinson). After 30 minutes, PBMC were washed twice with washing solution (PBS + 2% HI FBS + 0.02% sodium azide). Pooled, unstained PBMC, either SEB stimulated or not, were used as negative control. All samples were analyzed with using a FACS Canto II cytometer and FACS Diva software 6.1 (both Becton Dickinson, Breda, the Netherlands). Unlabelled cells were used to set forward scatter (FSC) and sideward scatter (SSC) voltages appropriately. The FSC threshold was set to 15,000 events. Then, fluorescence intensities of approximately 20,000-50,000 cells per sample were measured.

[00181] Levels of PD-1, PD-L1 and PD-L2 expression were monitored on gated CD4+ and CD8+ T cells. Data was analyzed by setting a live gate around the lymphocytes (based on FSC and SSC) after exclusion of the dead cell population (stained with cell viability solution). Thereafter, CD3+CD4+ and CD3+CD8+ cells in the live gate were distinguished. Within the CD3+CD4+ and CD3+CD8+ T cell subpopulations, median fluorescence intensities (MFI) of the various B7 family members were calculated. Histogram overlays and average of the MFI from 2-4 donors over 2 experiments were made with WinMDI 2.8 software to visualize expression levels of B7 family members and their respective isotype controls.

[00182] Low level baseline expression of PD-1 on the surface of both CD4+ and CD8+ T cells was enhanced from day 2-3 onwards after SEB stimulation. At day 4, most of the T cells in culture expressed PD-1 on their surface with the highest expression observed on CD4+ cells. In addition, SEB induced PD-L1 expression became readily detectable around day 1-2 and still increased at day 4 after stimulation. SEB-induced PD-1 and PD-L1 expression was consistently observed in blood samples from 2-4 individuals as shown in Figure 1A-B. In contrast, PD-L2 was not expressed on naïve (non-stimulated: NS) or SEB-stimulated T cells (*See* Fig. 1C).

SEB-induced human T-cell IL-2 production is enhanced by PD-1 blockade

[00183] Superantigens, such as SEB, activate T-cells by linking MHC class II molecules on antigen presenting cells to the Vß element of the TCR resulting in the production of cytokines including the autocrine growth factor Interleukin-2 (IL-2). Compared to a typical recall antigen-induced T-cell response where 0.1-0.001% of the T cells might be activated, SEB is capable of activating up to 10-20% of the T-cells in human blood depending on the fraction of T cells bearing the Vβ3, Vβ12, Vβ14, and Vβ17 found in each particular blood donor. Therefore, SEB was used for a T-cell based IL-2 secretion assay to determine the level of target modulation by h409A11 in human whole blood cells (WBC).

[00184] The results in Figures 2A-D illustrate SEB-stimulated IL-2 production by healthy donor blood cells is enhanced by anti-PD-1 or anti-CTLA-4 and decreased by CTLA4Ig.

[00185] In these experiments, heparinized whole blood was obtained from healthy donors (Sanquin blood bank Nijmegen, The Netherlands), Blood was diluted in medium

(RPMI supplemented with 80 U/ml penicillin, 80 μg/ml streptomycin) and added to 96-well NUNCLON delta surface plates (NUNC™). Multiple dilutions of heparinized blood were tested with 1:10 determined to be the most optimal. Next, diluted blood was pre-incubated for 30-60 minutes with mAb or isotype control (25 to 0.00025 and 0 μg/ml).

[00186] Stimulation was performed with 0.1 μg/ml SEB for 0-4 days. Supernatant was collected for 0 to 4 days at 37°C, 5% CO_2 and 97% humidity. IL-2 was measured with ELISA (eBioscience or Biosource) as main read-out for T cell activation. For simultaneous detection of 27 cytokines/chemokines in culture supernatant samples, a Bio-Plex/Luminex human cytokine 27-plex panel kit from Bio-Rad was used. The 27-plex kit included antibody-coated beads, detection antibody and standards for detection of eotaxin, FGF basic, IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF bb, RANTES, TNF-α and VEGF using a 96 well filter plate as described by the manufacturer (Bio-Rad, Hercules, CA). To determine IL-2 fold change, the cytokine level in the presence of antibody (plus SEB) is divided by the cytokine level in the absence of antibody. A 2-fold change (e.g. detected in the presence of anti-PD-1) thus means that the absolute concentration of cytokines measured in the experiment is twice the amount found in the SEB-stimulated control experiment.

[00187] In Fig. 2 A the bars show the standard error of the mean (±SEM) IL-2 production in pg/mL following incubation with 25 μg/mL (167 nM) anti-PD-1, anti-CTLA4 or CTLA4Ig. In Fig. 2B the mean (±SEM) fold change in IL-2 across donors (±SEM) is depicted. Each IL-2 value is compared to its own isotype control to determine the fold change or activity ratio (fold change IL-2 of 4 means 4-fold increase in IL-2 production when compared to SEB alone). Numbers inside each bar indicate the number of donors represented. In Figs. 2C-D blood from healthy donors (n=3) was pre-incubated for 30-60 minutes with mAb or isotype control at multiple concentrations (25-0.00025 and 0 μg/ml) and the IL-2 production (Fig. 2C) and IL-2 fold change (Fig. 2D) was calculated. For Figs. 2A-D, Mouse (m)IgG1 is the isotype control for anti-PD-1.09A (09A). Human (h) IgG4 is the isotype control for h409A11, Mouse (m) IgG2a is the isotype control for anti-CTLA4 and hIgG is the isotype for CTLA4Ig.

[00188] In particular, SEB-stimulated IL-2 production by healthy donor blood cells was enhanced in the presence of 25 μg/mL (167 nM) anti-PD-1 or anti-CTLA-4 antibodies (Figure 2A). Similar enhancement of IL-2 production was observed in the presence of either the mouse anti-human PD-1.09A (09A) parental mAb or its humanized derivative h409A11

(Figure 2A). In contrast, CTLA-4/Ig decreased the IL-2 production (consistent with its reported mechanism of action). Figure 2B depicts the stimulation index relative to control SEB-induced IL-2 production. Under these conditions, both anti-PD-1 and anti-CTLA-4 enhanced IL-2 production up to ~3 to 4-fold (Figure 2B), in a dose-dependent fashion (Figure 2C and 2D). Under these conditions, SEB-induced IL-2 production by whole-blood cells and its modulation by anti-PD-1, in the form of either the mouse anti-human PD-1.09A (09A) parental mAb or its humanized derivative h409A11, is a robust and quantifiable read-out of target modulation.

Anti-PD-1 and Anti-PD-L1 but not Anti-PD-L2 Modulate the SEB-induced IL-2 production by Human WBC

[00189] To determine the involvement of PD-1 ligands, PD-L1 and PD-L2 blocking mAbs were used in the SEB-induced IL-2 assay. Anti-PD-1.09A enhanced the production of IL-2 and fold change compared to SEB-stimulation, which was reproduced using an anti-PD-L1 antibody (Figure 3 A and B).

[00190] The results shown in Figures 3A-B illustrate that SEB-induced IL-2 production is enhanced by blockade of PD-1 or PD-L1 but not by PD-L2 blockade. In these experiments, fresh blood was diluted 1:10 and pre-incubated for 30-60 minutes with antibodies to PD-1, PD-L1 and PD-L2 and thereafter stimulated with 0.1μg/mL SEB. In Fig 3A the bars show the mean (± SEM) IL-2 production in pg/mL following incubation with 25 μg/mL (167 nM) of the indicated antibody. In Fig. 3B the mean (± SEM) fold change in IL-2 across donors (± SEM) is depicted. Each IL-2 value is compared to its own control to determine the fold change (fold change IL-2 of 4 means 400% IL-2 production when compared to SEB alone (set at 100%)). Numbers inside each bar indicate the number of donors represented. Mouse (m) IgG1 is the isotype control for mouse anti human PD-1.09A (PD-1.09A), anti-PD-L1 and anti-PD-L2.

[00191] In contrast to the results with anti-PD-1 blockade, IL-2 production could not be modulated using anti-PD-L2. This result can be explained at least in part by the lack of PD-L2 expression observed upon SEB stimulation of human PBMC (*See*, Figure 1C). No IL-2 production was observed in response to anti-PD-1 in the absence of SEB stimulation, a finding that suggests that PD-1 controls T cell activity only in the context of antigen receptor stimulation. In conclusion, modulation of the PD-1 pathway in SEB-stimulated whole blood

can be achieved by PD-1 or PD-L1 blockade (but not PD-L2) and suggests that in the blood compartment the PD-1 – PD-L1 interaction is a relevant co-inhibitory pathway.

Human T-cell Response to Tetanus Toxoid is Enhanced by PD-1 blockade

[00192] To determine that antigen-specific T cell receptor triggering was modulated by anti-PD-1, a human T-cell recall assay was developed using tetanus toxoid (TT) antigen to stimulate pre-existing memory T cells in healthy donor blood. Here, cryopreserved PBMC from recently [<1 year] TT revaccinated donors (Sanquin Blood Bank Nijmegen, The Netherlands) were thawed at 37°C. Cells (1.5E5/well) in medium DMEMF12 supplemented with 80 U/ml penicillin, 80 µg/ml streptomycin and 10% FBS (Gibco, Invitrogen Corporation) were added to 96-well NUNCLON delta surface plates (NUNC™). Antibody or isotype control was pre-incubated with mouse anti-PD-1.09A, anti-CTLA4 or their respective controls mIgG1, mIgG2a at 25 µg/mL (167 nM) (Figs. 4A-B) or with a dose range of h409A11, anti-PD-1.09A or their respective controls hIgG4 or mIgG1 (Figs. 4C-D) after which TT was added. Multiple TT concentrations were tested, with 1 µg/ml found to be optimal. Cells were incubated for 7 days and supernatant was analyzed for IFNγ content using ELISA (eBioscience). To determine the fold change in IFNγ levels, the cytokine level in the presence of antibody is compared to the cytokine level in the absence of antibody. A 2-fold change thus means 200% cytokine production (*e.g.* by anti-PD-1) when compared to TT alone (set at 100%).

[00193] As shown in Figure 4A, production of IFNγ was enhanced by PD-1 blockade using h409A11, or its parental mouse anti-PD-1 mAb hPD-1.09A in PBMC cultures in a dose-dependent manner. In contrast, anti-CTLA-4 did not modulate IFNγ production in parallel cultures (Figure 4 A and B).

[00194] The number of independent healthy donors used to determine the average production of IFNγ +/- SEM is indicated for each condition in the bar graphs (Figs. 4A-B). Dose-response curve in Figure 4C represents a typical example of one donor.

Dose-Dependent Potentiation of IL-2 Production By PD-1 Blockade In Blood Cells From Prostate Cancer And Melanoma Cancer Patients

[00195] Binding of PD-1 to its ligand receptors down-regulates immune responses, and it is this immune modulation that is likely to be exploited to evade immune surveillance in

cases of tumor progression. The SEB assay was used to determine responsiveness of blood T-cells from cancer patients to anti-PD-1 and h409A11.

[00196] Fresh blood was obtained from 15 prostate cancer patients (Department of Urology, Radboud University Nijmegen Medical Centre, Nijmegen, the Netherlands) including 5 hormone treatment resistant (castration resistant), 5 responders to hormone treatment and 5 with enhancing PSA-levels after prostatectomy. Fresh blood was also obtained from 10 advanced melanoma cancer patients with stage IV disease (Department of Medical Oncology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands).

[00197] Anti-PD-1 or control monoclonal antibody was added to SEB-stimulated whole blood cultures from prostate cancer (Figures 5A-B) or melanoma patients (Figures 5C-D) and mAb-dependent modulation of the IL-2 response was determined (as described above for healthy human donor blood samples). Briefly, fresh blood from prostate cancer (Figure 5 A and B) or advanced melanoma (Figure 5 C and D) patients was diluted and added to 96-well culture plates. Following 30-60 min. pre-incubation with mouse anti-PD-1.09A, humanized anti-PD-1 H409A11, anti-CTLA4, CTLA4Ig or isotype controls, SEB was added to a final concentration of 0.1 μg/mL. Culture supernatants were collected after 4 days and analyzed for IL-2 production (Figure 5 A and C) by ELISA. To compare values across multiple donors, IL-2 levels were normalized and a fold change (or activity ratio) relative to SEB-stimulation alone was calculated (Figure 5B and D). Mouse (m)IgG1 is the isotype control for anti-PD-1.09A (09A), human (h) IgG4 is the isotype control for H409A11, Mouse (m)IgG2a is the isotype control for anti-CTLA4 and hIgG is the control for CTLA4Ig.

[00198] PD-1 blockade by h409A11 or its parental mouse mAb hPD-1.09A at 25 μg/mL (167 nM) potentiated the SEB induced IL-2 response ~3 to 4-fold (*See*, Figures 5 A and C).

[00199] Additionally, modulation by anti-PD-1 was found to occur in a dose-dependent fashion in blood cell cultures established from both prostate cancer and melanoma patient populations (*See,* Figures 5B and D). Among patient donors with prostate cancer, potentiation of SEB-induced IL-2 production by h409A11 or hPD-1.09A was found to be similar between hormone treatment-resistant, hormone treatment–responders and patients with rising PSA-levels after prostatectomy. Anti-CTLA also enhanced IL-2 production in a dose-dependent manner, albeit with a reduced potency when compared to h409A11. The

costimulatory inhibitor CTLA4Ig down-regulated IL-2 production in healthy donor blood. These experiments illustrate that evaluating the modulation of SEB-induced IL-2 production is a robust bioassay to determine PD-1 target modulation in the blood compartment of cancer patients and provides an assay to determine target modulation (*i.e.*, responsiveness) in mammals treated with a PD-1 blockade (e.g., h409A11) including primates and human patients.

Potency of Anti-PD-1 Modulated SEB-Induced IL-2 Production is Similar Between Healthy Donors and Cancer Patients

[00200] The IL-2 dose response curve for each subject was fit using a four parameter logistic equation producing the minimum, maximum, pEC50 (−log EC50) and hill slope. The pEC50 was statistically analyzed using a one-way ANOVA on treatment group. No statistical significance (P= 0.066) was observed between the healthy donor groups, H409A11 treated, and those treated with the parental mouse mAb hPD-1.09A.

[00201] These results are shown in Figure 6. Fresh blood was diluted 1:10 and pre-incubated for 30-60 minutes in the presence or absence of a dose response (0.00025 μg/mL – 25 μg/mL) PD-1.09A or h409A11. Thereafter, blood cells were stimulated with 0.1μg/mL SEB. Each IL-2 value is compared to its own control and the mean pEC50 and EC50 after PD-1 blockade is calculated. Bars show the mean (± SEM) IL-2 pEC50 in μg/mL. Numbers inside each bar indicate the number of donors represented. The mean EC50 in μg/mL for healthy, melanoma and prostate cancer after PD-1 h409A11 blockade is 0.053 μg/mL (= 0.354 nM), 0.086 μg/mL(= 0.575 nM), 0.052 μg/mL (= 0.347 nM) and after PD-1.09A blockade 0.020 μg/mL (= 0.134 nM), 0.017 μg/mL (= 0.114 nM)and 0.035 μg/mL (= 0.234 nM) respectively. These experiments demonstrate that h409A11 enhanced IL-2 production upon SEB stimulation of whole blood-derived cells from prostate and melanoma cancer patients to a similar potency and with a similar dose-response relationship as observed for healthy donors. Overall, SEB-induced IL-2 production is enhanced by PD-1 blockade to a similar extent between healthy donors, melanoma cancer and prostate cancer patients.

PD-1 blockade enhances IL-2, IFNγ, TNFα, IL-6 and IL-17 and decreases IL-5 cytokine production in healthy donors and cancer patients blood cells

[00202] Using the above described assay, the ability of PD-1 blockade to modulate additional cytokines was determined by multiplex analysis. For simultaneous detection of 27

cytokines/chemokines in culture supernatant samples, a Bio-Plex/Luminex human cytokine 27-plex panel kit from Bio-Rad was used. The 27-plex kit included antibody coated beads, detection antibody and standards for detection of eotaxin, FGF basic, IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF bb, RANTES, TNF-α and VEGF using a 96-well filter plate as described by the manufacturer (Bio-Rad, Hercules, CA). To determine fold change, the cytokine level in the presence of antibody (plus SEB) is divided by the cytokine level in the absence of antibody. A 2-fold change thus means 200% cytokine production (*e.g.* by anti-PD-1) when compared to SEB alone (set at 100%).

[00203] In addition to IL-2, TNFα, IFNγ, IL-6 and IL-17 were found to be increased by hPD-1.09A (Figure 7A, B C) and h409A11 (Figure 7 D and E) in SEB-stimulated blood cultures from healthy donors, prostate cancer patients and advanced melanoma cancer patients following a similar pattern. In contrast, h409A11 and hPD-1.09A decreased IL-5 levels. Under these conditions, no modulation of IL-13, eotaxin, FGF, IL-1β, IL-1Ra, IL-4, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-15, G-CSF, GM-CSF, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, RANTES, TNF-α, or VEGF was observed. These experiments suggest that PD-1 blocking agents in the context of antigen-receptor stimulation favour a potentiation or skewing towards a TH1-like T cell response.

[00204] Diluted whole blood was pre-incubated for 30-60 minutes with 25 μg/ml (=167 nM) h409A11, PD-1.09A or isotype control antibody and cultured following 0.1 μg/mL SEB incubation. Supernatants were collected after 4 days of culture and analyzed for cytokine production by multiplex analysis. To compare values across multiple donors, cytokine levels were normalized and a fold change (or activity ratio) was calculated as the level of cytokine produced in the presence of SEB plus antibody divided by the level of IL-2 produced in the absence of antibody (SEB only). Figures 7A-E represent the average response (± SEM) of several healthy donors (Fig. 7A), prostate cancer (Figs. 7B and D) and advanced melanoma cancer donors (Figs. 7C and E). None = SEB alone.

[00205] **TABLE 1 Cytokine Reference Numbers**

Cytokine	**NCBI/GenBank Reference Number**
IL-2 (Human) also known as: IL2, TCGF, lymphokine	Protein: NP_000577 (SEQ ID NO:2) mRNA: NM_000586 (SEQ ID NO:1)
IFN-γ (Human)	Protein: NP_000610 (SEQ ID NO:4)

also known as: IFNγ, IFNG; IFG; IFI, type II interferon	mRNA: NM_000619 (SEQ ID NO:3)
IL-6 (Human) also known as: IL6; HGF; BSF2; HSF; IFNB2	Protein: NP_000591 (SEQ ID NO:6) mRNA: NM_000600 (SEQ ID NO:5)
TNFα (Human) also known as: TNF; DIF; TNF-alpha; TNFA; TNFSF2	Protein: NP_000585 (SEQ ID NO:8) mRNA: NM_000594 (SEQ ID NO:7)
IL-17(Human) also known as: IL-17A; IL17; CTLA8	Protein: NP_002181 (SEQ ID NO:10) mRNA: NM_002190 (SEQ ID NO:9)
IL-5 (Human) also known as: IL5; EDF; TRF	Protein: NP_000870 (SEQ ID NO:12) mRNA: NM_000879 (SEQ ID NO:11)

Potentiation of IL-2 production in blood cells by PD-1 blockade in healthy donor and cancer patient blood is translatable to cynomolgus monkey blood

[00206] Humanized antibody h409A11 and its parental mouse mAb hPD-1.09A have been shown to bind and block cynomolgus PD-1 with similar affinity and potency as determined for human PD-1 (See WO2008/156712). Before *in vivo* dosing of H409A11 in cynomolgus monkey, its activity was tested *in vitro* in cynomolgus monkey donor blood (n=12) using the SEB whole blood assay. Figures 8A-B illustrate potentiation of IL-2 production in blood cells by PD-1 blockade is translatable to cynomolgus monkeys. For these experiments, diluted fresh blood from 12 cynomolgus monkey donors was pre-incubated for 30-60 minutes with H409A11 or its hIgG4 isotype control after which SEB at 1 μg/mL (most optimal for cyno) was added. Supernatants were collected after 3 days and analyzed for IL-2 production by ELISA. To compare values across multiple donors, IL-2 levels were normalized and a fold change (or activity ratio) was calculated as described above in the methods section.

[00207] The results shown in Figures 8A-B illustrate that h409A11 enhanced IL-2 production in the SEB assay to a similar extent as observed in healthy donors and cancer patient blood cells. These data confirm cross-reactivity of these antibodies with cynomolgus PD-1 and indicate similar PD-1 biology across these two species. Furthermore, these results indicate that the SEB whole blood assay is translatable to the cynomolgus primate and

supports the selection of the cynomolgus macaque as a relevant species for toxicological studies of h409A11.

Dose and time-dependent modulation of SEB-induced IL-2 production by cynomolgus monkey blood cells following administration of H409A11

[00208] Two non-human primate studies were designed to characterize the pharmacokinetics (PK), pharmacodynamics (PD) and tolerability of h409A11. The PD-1 modulation assay was used to follow target modulation following a single intravenous (IV) dose or multiple IV doses of h409A11 to normal cynomolgus macaques.

[00209] In the single-dose study, four dose groups consisted of 3 monkeys including vehicle, 0.3 mg/kg, 3 mg/kg or 30 mg/kg h409A11. Whole blood was collected at predose day -7, day 0 and after h409A11 or vehicle administration at day 1, day 7, day 28, day 56 and day 84 for *ex-vivo* analysis using the PD-1 bioassay.

[00210] In a second, multiple dose study, dose groups of 12 cynomolgus macaques were dosed weekly for one month with vehicle, 6 mg/kg, 40 mg/kg, or 200 mg/kg h409A11. Blood was collected twice pretest (Days -11 and -5) and 24 hours after dosing on Days 0 and 28 from all animals. Blood was also obtained on Days 56, 98, 126 and 154 from animals assigned to the recovery period.

***Ex vivo* Analysis of PD-1 Blockade in Cynomolgus Monkeys following a single dose of anti-PD-1 h409A11**

[00211] Female cynomolgus monkeys (*Macaca fascicularis*) were administered a single dose of vehicle, 0.3 mg/kg, 3 mg/kg or 30 mg/kg h409A11 by IV injections. Each dose group consisted of 3 monkeys. The study was performed in a blinded fashion. Cynomolgus monkey blood was obtained at predose day -7, day 0 and after h409A11 administration at day 1, day 7, day 28, day 56 and day 84 after dosing. Within 4-6 hours after collection, blood was diluted 1:10 with culture medium (Gibco; RPMI 1640 with penicillin, streptomycin and L-glutamine). Diluted cynomolgus blood was added to 96-wells flat bottom plates ((NUNC™) and pre-incubated for 30-60 minutes with several (25 μg/mL – 0.00025 μg/mL) concentrations of h409A11, isotype control, or medium control alone. Thereafter, 1 μg/mL SEB was added. Supernatants were collected after 3 days of culture (found to be an optimal incubation time for cynomolgus monkey blood) at 37°C, 5% CO_2 and 97% humidity

and transferred to flat bottom plates or micronic tubes. Production of IL-2 was quantified with ELISA as indicated by the manufacturer (human IL-2 kit, Ready-SET-Go, eBiosciences). IL-2 levels (pg/mL) were calculated using the 4-parameter formula in GraphPad Prism. To compare values between different time points/assays, levels were normalized and a fold change was calculated. For normalization, IL-2 production (in pg/ml) after h409A11 incubation and SEB stimulation is divided by IL-2 production after SEB stimulation (in pg/mL).

Potentiation of IL-2 was dose-dependently lost after h409A11 dosing

[00212] As shown in Figures 8A-B, *ex vivo,* exogenous addition of h409A11 and SEB to whole blood collected from control animals potentiated IL-2 production ~2-fold. The results in Figure 9A illustrate that following administration of h409A11, potentiation of IL-2 production was not observed in whole blood collected from dosed animals, indicating maximal target modulation as a result of h409A11 infusion. For these experiments, diluted fresh blood from 12 cynomolgus monkey donors was pre-incubated for 30-60 minutes with h409A11 or its hIgG4 isotype control after which SEB at 1 μg/mL was added. Supernatants were collected after 3 days and analyzed for IL-2 production by ELISA. IL-2 levels were normalized and a fold change (or activity ratio) was calculated as indicated in the Materials and Methods. ANOVA for repeated measures was used for statistical analysis.

[00213] Immediately after administration, the lack of *ex vivo* potentiation of IL-2 response was similar at all dose levels, suggesting that even at the lowest infused dose maximal target engagement and modulation was achieved in the blood compartment. *In vivo* target modulation was gradually lost over time, signified by the fact that *ex vivo* addition of h409A11 could again modulate SEB-induced IL-2 production. As shown in Figure 9, prolongation of target engagement and modulation occurred in a dose-dependent manner. This finding is in agreement with the clearance of drug substance (h409A11) from the circulation

PK-PD Relationship

[00214] Models were developed to describe the PK and PK-PD of h409A11 on IL-2 fold changes after single bolus intravenous dosing in the aforementioned cynomolgus monkey study. The basic PK-PD relationship consists of a turnover model with the drug effect on degradation of IL-2 fold change, where the drug effect is defined as an Emax

equation (also known as hyperbolic). An example of curve fit with individual posthoc fits is shown in Figure 10A, for the 30 mg/kg dose group. Model predictions of indirect drug effects on IL-2 fold change as a function of serum concentrations are shown in Figure 10B, and plotted as a straight line. Numbers indicate the dose levels at which observations underlying these drug effects were made. The serum concentration of h409A11 that is associated with 50% reduction of IL-2 fold change (IC_{50}) is 15.6 mg/l (approximate 95%-confidence interval: 6.69 – 36.6 mg/l). This *in vivo* potency was confirmed in a subsequent analysis on the cynomolgus monkey study following multiple doses of anti-PD-1 h409A11. The model results allow inter- and extrapolation of PD effects with respect to dose and time. Moreover, these results allow comparison of the PK-PD relationship between species in a translational fashion thereby adding to the power of the biomarker measurements.

***Ex vivo* Analysis of PD-1 Blockade in Cynomolgus Monkeys following a multiple doses of anti-PD-1 h409A11**

[00215] To assess the usefulness of the PD-1 MBA as a biomarker for target engagement following multiple doses of anti-PD-1, four dose groups of 12 cynomolgus macaques were dosed weekly for one month with vehicle, 6 mg/kg, 40 mg/kg, or 200 mg/kg h409A11 and followed using the assay for four months after the end of the dosing period. Prior to dosing, blood was collected from all animals on Days -11 and -5 and tested using the SEB whole blood assay. In these cultures, *ex vivo* addition of h409A11 potentiated IL-2 production (1.6 to 4.5 fold; mean 2.3 fold), which was consistent with previous studies using human and cynomolgus monkey whole blood. After administration of h409A11 on Day 0, the ability of *ex vivo* addition of drug to increase IL-2 levels was lost (presumably due to existing target engagement by h409A11). This is shown by an activity ratio ~1 for all animals which received anti-PD-1.

[00216] For most animals, the observed PD effect was also observed on Day 28 (on which each animal had received its fifth antibody dose) as indicated by an activity ratio ~1 (*i.e.* no change in SEB-stimulated IL-2 production after *ex vivo* addition of h409.A11). This observed pharmacodynamic effect was sustained into the recovery period for all h409A11 dose groups but returned to (pre-study) baseline levels in a dose-dependent manner in animals from the 6 and 40 mg/kg dose groups, in other words, the duration or prolongation of IL-2 production enhanced following administration of anti-PD-1 was found to be dose-dependent (Figure 9B).

[00217] The observed pharmacodynamic effect (Fold change IL-2 ~1) was sustained post dosing and returned to baseline (pre-study) levels over time in a dose-dependent manner. During the recovery period, gradual increase in the ability to potentiate IL-2 production was observed in the 6 mg/kg and 40 mg/kg dose groups with mean IL-2 values returning to control levels by Day 98 and Day 154, respectively. IL-2 production was inhibited throughout the recovery period, and to an extent similar to that observed during the dosing period in animals administered 200 mg/kg h409A11.

[00218] The PD-1 bioassay is useful as a translational biomarker assay to assess target engagement and modulation as well as PK/PD relations in patients dosed with h409A11 and is expected to be similarly useful for monitoring patients dosed with any agent that blocks the PD-1 pathway by inhibiting the PD-1-PD-L1 interaction.

CONCLUSIONS

[00219] Humanized h409A11 and its parental mouse mAb PD-1.09A enhance T cell activation in the context of T cell receptor stimulation, using either SEB or TT. The *ex vivo* SEB assay demonstrates that inhibition of the PD-1 pathway, *i.e.*, target modulation of the PD-1-PD-L1 interaction can be determined *ex vivo* in a dose-dependent fashion, irrespective of the blood donor. PD-1 blockade *in vivo* was shown to enhance IL-2, IFNγ, IL-6, TNFα, IL-17 and decreases IL-5 cytokine production. This cytokine/biomarker profile indicates that h409A11 and PD-1.09A may induce a skewed TH1-like T cell response. Importantly, the observed cytokine/biomarker profile after PD-1 blockade is highly similar between healthy donors, advanced melanoma cancer and prostate cancer patients.

[00220] IL-2 production could not be modulated using anti-PD-L2. This could be explained by the lack of PD-L2 expression observed upon SEB stimulation of human PBMC. This suggests that at least in the blood compartment, the PD-1 – PD-L1 interaction is a relevant co-inhibitory pathway. The observed potency for h409A11 modulation of blood cell IL-2 production is strikingly similar between different donors. In these experiments, the IL-2 EC50 varies from ~0.1-0.6 nM between healthy donors and cancer patients, rendering this assay to be robust and useful in a clinical setting. This is further underscored by the data obtained in blood cultures from cynomolgus monkeys dosed with h409A11 in the non-GLP PK study. Further evidence in multiple dose primate GLP-toxicity study with h409A11 has confirmed the use of the SEB assay to determine pharmacodynamic effects of anti-PD-1. The PD-1 MBA will be useful for monitoring, measuring or predicting *in vivo* responses for

patients dosed with anti-PD-1 agents that interfere with the PD-1-PD-L1 pathway for treatment of cancer or chronic diseases such as hepatitis (including hepatitis B or C).

[00221] The *ex vivo* pharmacodynamic assay as described herein is useful for measuring the response to T cell modulating agents in animals or humans dosed with such an agent. Preferred embodiments measure the response to PD-1-PD-L1 modulating agents (e.g. h409A11 or other anti-PD-1 antibodies). These assays measure the response of whole blood cells or PBMC from dosed individuals in response to a T cell stimulus (such as superantigen). When stimulated, T cells secrete cytokines including IL-2. In the presence of a PD-1 blocking agent, the level of certain cytokines including: IL-2, IFNγ, IL-6, TNFα, and IL-17 are increased, while the expression of IL-5 is decreased.

[00222] Based on the PK/PD model derived using this biomarker assay, it was surprisingly observed that the *ex vivo* IC50 value of the pharmacodynamic effect after a single dose of h409A11 (15.6 mg/L) is orders of magnitude higher than the *in vitro* Kd of the antibody, (approximately 4.0 μg/L), as well as the IC50 in functional assays, approximately 0.10-0.12 mg/L. This difference between *in vitro* and *in vivo* potency was unexpected, and underscores the importance of using such an *ex vivo* approach to monitor or predict *in vivo* responses for patients dosed with anti-PD-1.

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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

While the compositions and methods of this invention have been described in terms of specific embodiments, it will be apparent to those of skill in the art that variations may be

applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the scope of the invention as defined by the appended claims. It is further to be understood that all values are approximate, and are provided for description.

All references cited herein are incorporated by reference to the same extent as if each individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, was specifically and individually indicated to be incorporated by reference. This statement of incorporation by reference is intended by Applicants, pursuant to 37 C.F.R. §1.57(b)(1), to relate to each and every individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, each of which is clearly identified in compliance with 37 C.F.R. §1.57(b)(2), even if such citation is not immediately adjacent to a dedicated statement of incorporation by reference. The inclusion of dedicated statements of incorporation by reference, if any, within the specification does not in any way weaken this general statement of incorporation by reference. Citation of the references herein is not intended as an admission that the reference is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

WHAT IS CLAIMED IS:

1. A method for assessing PD-1 blockade in a mammalian subject treated with a PD-1-PD-L1 blocking agent comprising:

a) measuring expression of at least one cytokine selected from the group consisting of IL-5, IL-2, IFNγ, IL-6, TNFα, and IL-17 in a blood sample from said subject; and

b) comparing the expression of the at least one cytokine from step a) to a control,

wherein higher expression of at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control and lower expression of IL-5 compared to a control is indicative of PD-1 blockade in the subject and suitability of said subject for treatment with at least one PD-1 pathway blocking agent; or

wherein unchanged or lower expression at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control; and/or unchanged or elevated expression of IL-5 compared to a control is indicative of a lack of PD-1 blockade in response to at least one PD-1 pathway blocking agent in the subject and a lack of suitability of said subject for treatment with at least one PD-1 pathway blocking agent.

2. The method of claim 1, wherein said mammalian subject is a human patient.

3. The method of claim 1, wherein said blood sample is a whole blood or PBMC sample.

4. The method of claim 1, wherein said expression is determined by gene expression analysis or immunoassay.

5. The method of claim 4, wherein said gene expression analysis is selected from the group consisting of is selected from the group consisting of Northern blotting, PCR-based, SAGE, flow cytometry-based, and DNA microarray.

6. The method of claim 4, wherein the immunoassay is selected from the group consisting of ELISA, RIA, Western blot, luminescent immunoassay, fluorescent immunoassay..

7. The method of claim 1, wherein the subject was treated with an effective amount of an antagonist PD-1 antibody.

8. A method of treating cancer in a mammalian subject in need thereof, the method comprising the steps of:

a) administering an effective amount of a PD-1-PD-L1 blocking agent;

b) measuring expression of at least one cytokine selected from the group consisting of IL-5, IL-2, IFNγ, IL-6, TNFα, and IL-17 in a blood sample from said subject; and

c) comparing the expression of the at least one cytokine of step b) to a control,

wherein higher expression of at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control and lower expression of IL-5 compared to a control is indicative of PD-1 blockade in the subject and suitability of said subject for treatment with at least one PD-1 pathway blocking agent; or

wherein unchanged or lower expression at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control; and/or unchanged or elevated expression of IL-5 compared to a control is indicative of a lack of PD-1 blockade in response to at least one PD-1 pathway blocking agent in the subject and a lack of suitability of said subject for treatment with at least one PD-1 pathway blocking agent.

9. A method of treating chronic infection in a mammalian subject in need thereof comprising:

a) administering an effective amount of a PD-1-PD-L1 blocking agent;

b) measuring expression of at least one cytokine selected from the group consisting of IL-5, IL-2, IFNγ, IL-6, TNFα, and IL-17 in a blood sample from said subject; and

c) comparing the expression of the at least one cytokine from step b) to a control,

wherein higher expression of at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control and lower expression of

IL-5 compared to a control is indicative of PD-1 blockade in the subject and suitability of said subject for treatment with at least one PD-1 pathway blocking agent; or

wherein unchanged or lower expression at least one cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 when compared to a control; and/or unchanged or elevated expression of IL-5 compared to a control is indicative of a lack of PD-1 blockade in response to at least one PD-1 pathway blocking agent in the subject and a lack of suitability of said subject for treatment with at least one PD-1 pathway blocking agent.

10. The method of claim 8 or 9, wherein said mammalian subject is a human patient.

11. The method of claim 8 or 9, wherein said test sample is a whole blood or PBMC sample.

12. The method of claim 8 or 9, wherein said expression is determined by gene expression analysis or immunoassay.

13. The method of claim 12, wherein said gene expression analysis is selected from the group consisting of is selected from the group consisting of Northern blotting, PCR-based, SAGE, flow cytometry-based, and DNA microarray.

14. The method of claim 12, wherein the immunoassay is selected from the group consisting of ELISA, RIA, Western blot, luminescent immunoassay, fluorescent immunoassay.

15. The method of claim 8 or 9, wherein said anti-PD1 therapeutic agent comprises an antagonist PD-1 antibody.

16. The method of claim 8, wherein the cancer is selected from the group consisting of ovarian, renal, colorectal, pancreatic, breast, liver, gastric, esophageal cancers and melanoma.

17. The method of claim 9, wherein the chronic infection is selected from viral infections consisting of human immunodeficiency virus (HIV), hepatitis B virus (HBV) or hepatitis C virus (HCV).

18. A kit for monitoring dosing levels in a mammalian subject dosed with one or more PD-1 blocking agents comprising at least one probe specific for a cytokine selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, IL-17, and IL-5.

19. A method of monitoring PD-1 blockade in a mammalian subject dosed with one or more PD-1 blocking agents comprising:

a) obtaining a baseline biological sample from the subject prior to administering a dose of one or more PD-1 blocking agents;

b) measuring the level of at least one biomarker selected from the group consisting of IL-5, IL-2, IFNγ, IL-6, TNFα, and IL-17 in the baseline biological sample by gene expression analysis or immunoassay,

c) administering the one or more PD-1 blocking agents to the subject;

d) obtaining from the subject at least one subsequent biological sample;

e) measuring the level of the at least one biomarker selected from the group consisting of IL-5, IL-2, IFNγ, IL-6, TNFα, and IL-17 in the subsequent sample by gene expression analysis or immunoassay;

f) comparing the level of at least one biomarker in the subsequent biological sample with the level of at least one biomarker in the baseline biological sample, wherein a decrease in expression of IL-5 and an increase of at least one biomarker selected from the group consisting of IL-2, IFNγ, IL-6, TNFα, and IL-17 in the subsequent biological sample indicates PD-1 blockade (i.e., target engagement).

20. The method of claim 19 wherein the subject is a candidate for anti-cancer or anti-viral treatment.

21. The method of any one of claims 1, 8, 9, or 19 wherein the at least one cytokine or biomarker exhibits at least 2 fold higher expression than expression in a control sample.

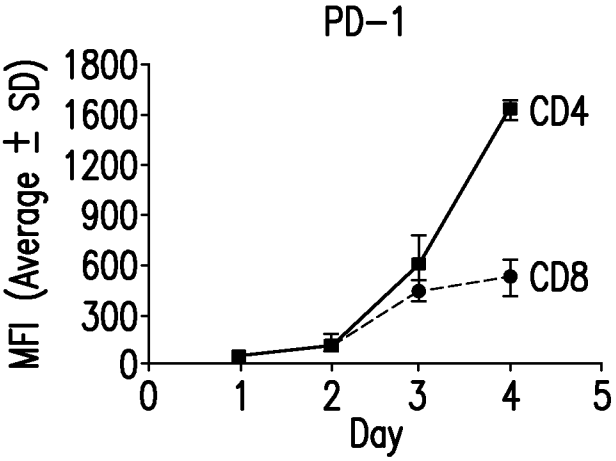


FIG.1A

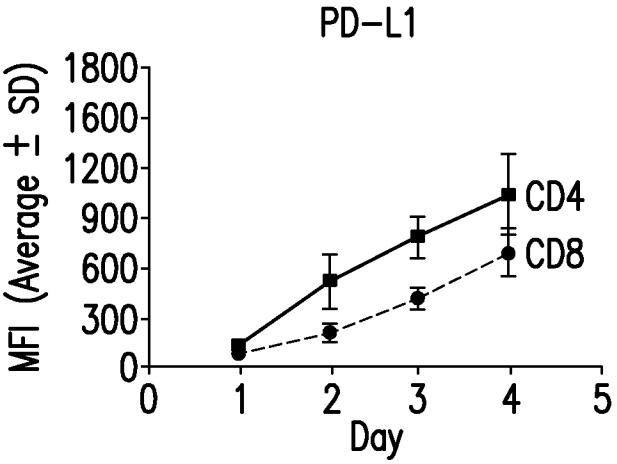


FIG.1B

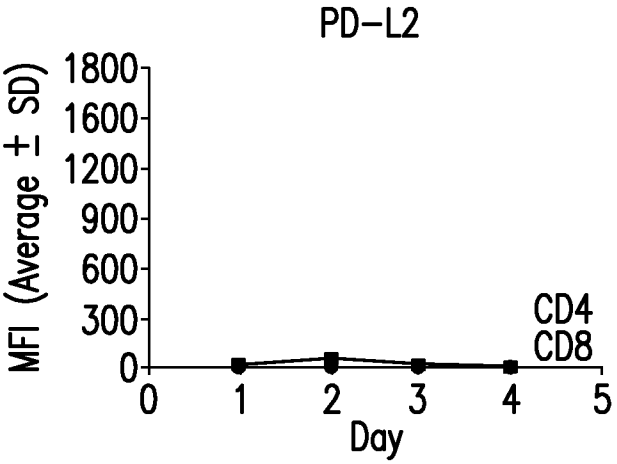


FIG.1C

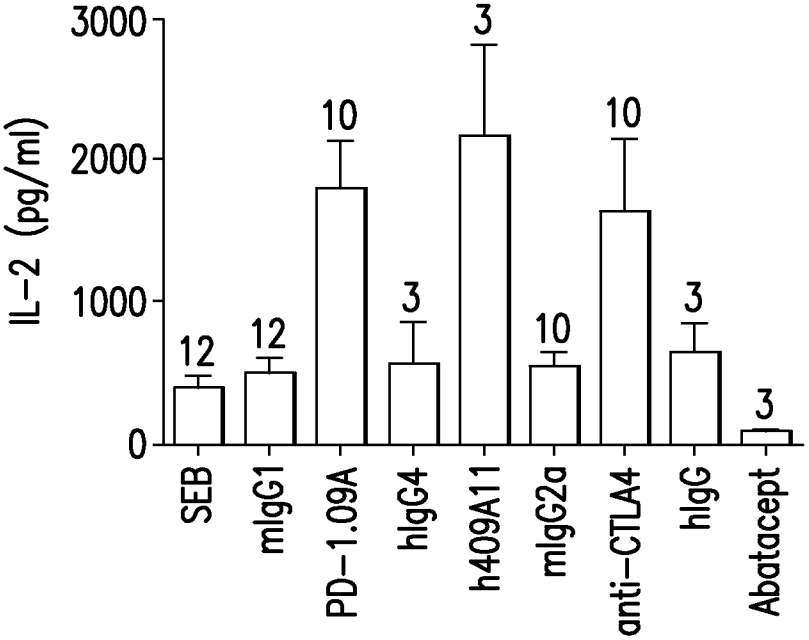

IL-2 (pg/ml)
3000
2000
1000
0
12
12
10
3
3
10
10
3
3
SEB
mIgG1
PD-1.09A
hIgG4
h409A11
mIgG2a
anti-CTLA4
hIgG
Abatacept

FIG.2A

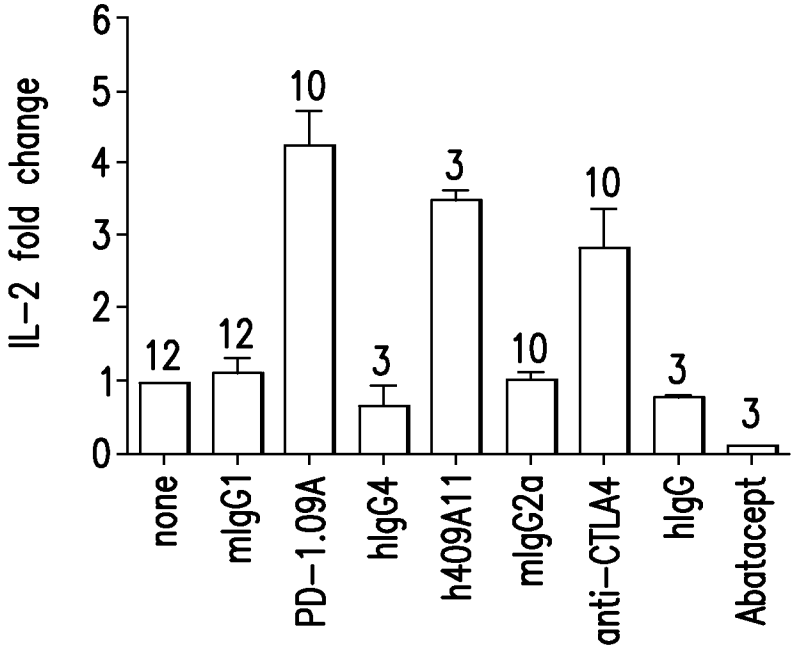

IL-2 fold change
6
5
4
3
2
1
0
12
12
10
3
3
10
10
3
3
none
mIgG1
PD-1.09A
hIgG4
h409A11
mIgG2a
anti-CTLA4
hIgG
Abatacept

FIG.2B

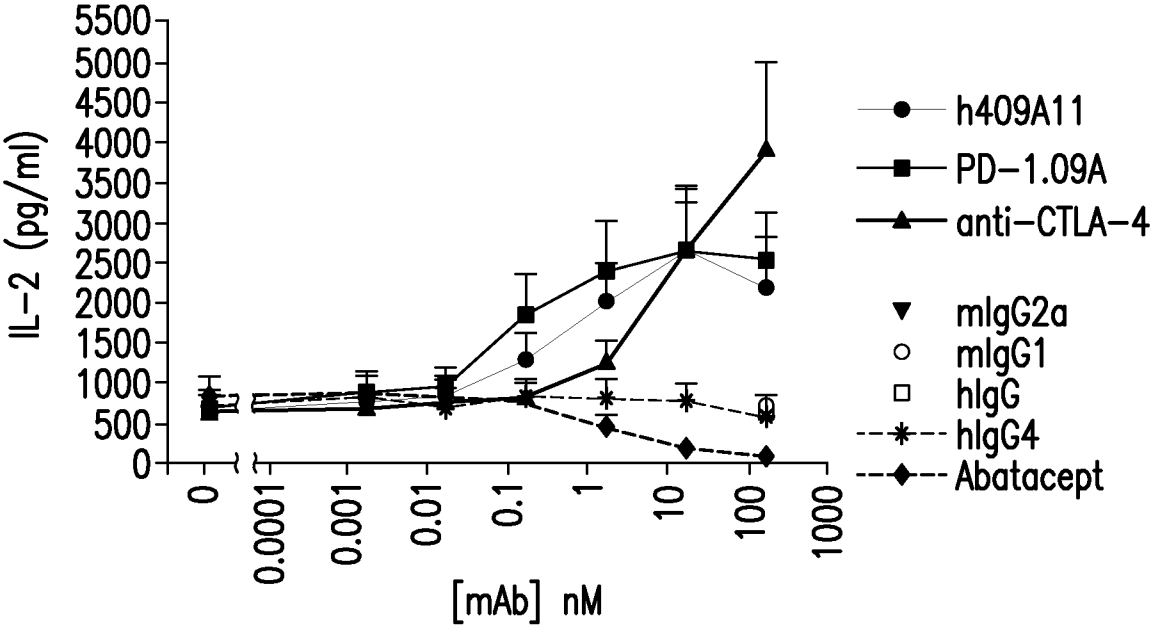

IL-2 (pg/ml)
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
0.0001
0.001
0.01
0.1
1
10
100
1000
[mAb] nM
h409A11
PD-1.09A
anti-CTLA-4
mIgG2a
mIgG1
hIgG
hIgG4
Abatacept

FIG.2C

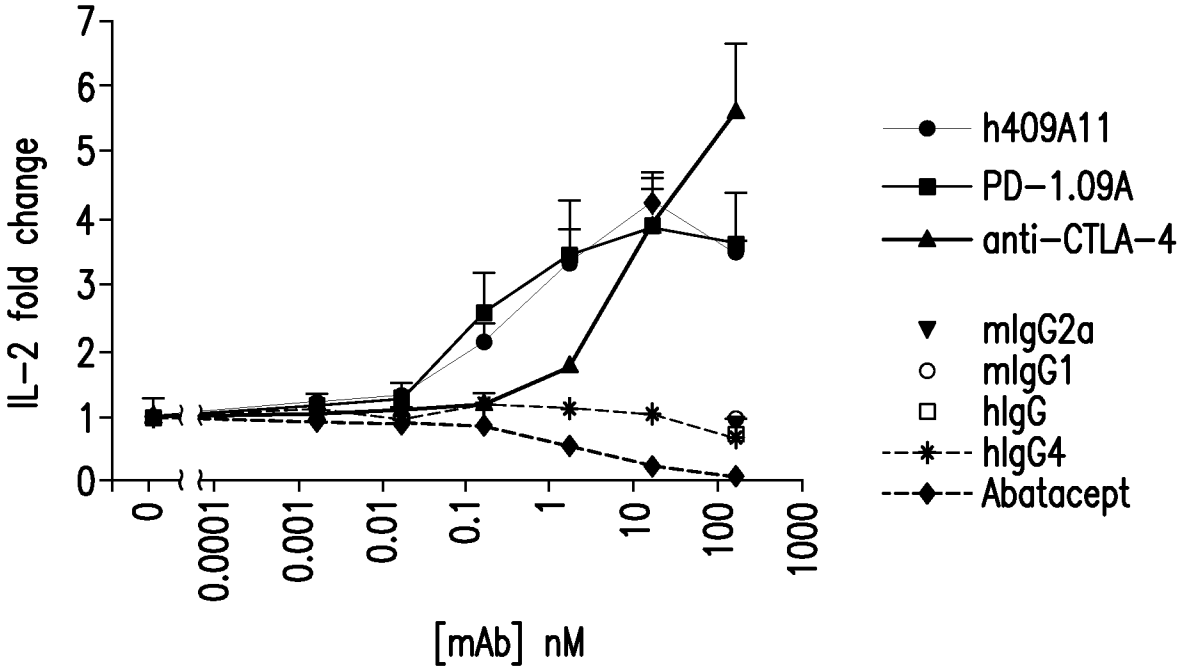

IL-2 fold change
7
6
5
4
3
2
1
0
0
0.0001
0.001
0.01
0.1
1
10
100
1000
[mAb] nM
h409A11
PD-1.09A
anti-CTLA-4
mIgG2a
mIgG1
hIgG
hIgG4
Abatacept

FIG.2D

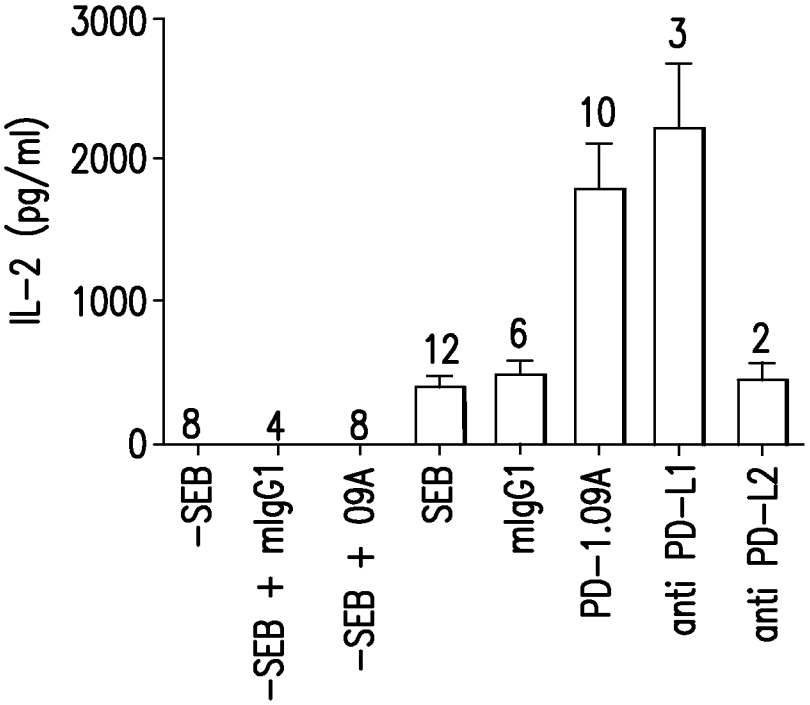

IL-2 (pg/ml)
3000
2000
1000
0
8
4
8
12
6
10
3
2
-SEB
-SEB + mIgG1
-SEB + 09A
SEB
mIgG1
PD-1.09A
anti PD-L1
anti PD-L2

FIG.3A

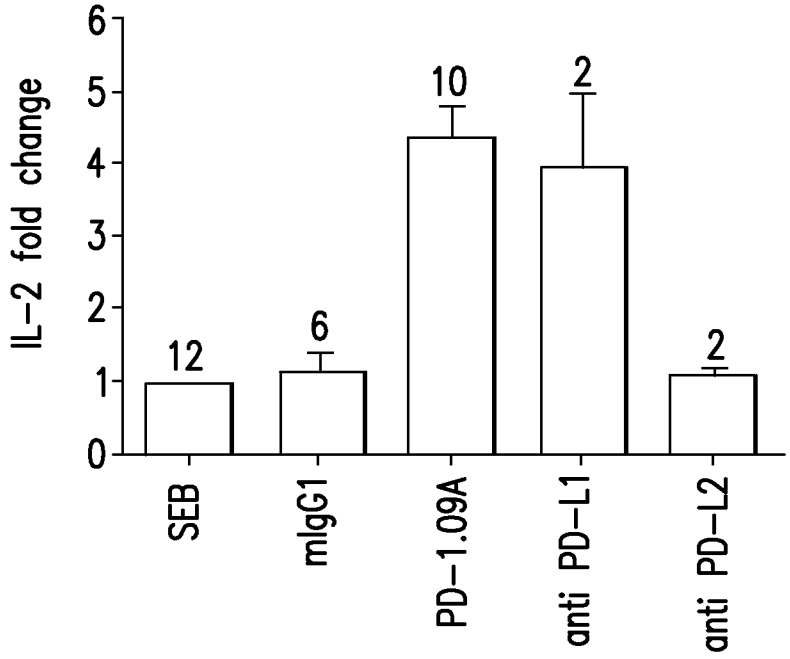

IL-2 fold change
6
5
4
3
2
1
0
12
6
10
2
2
SEB
mIgG1
PD-1.09A
anti PD-L1
anti PD-L2

FIG.3B

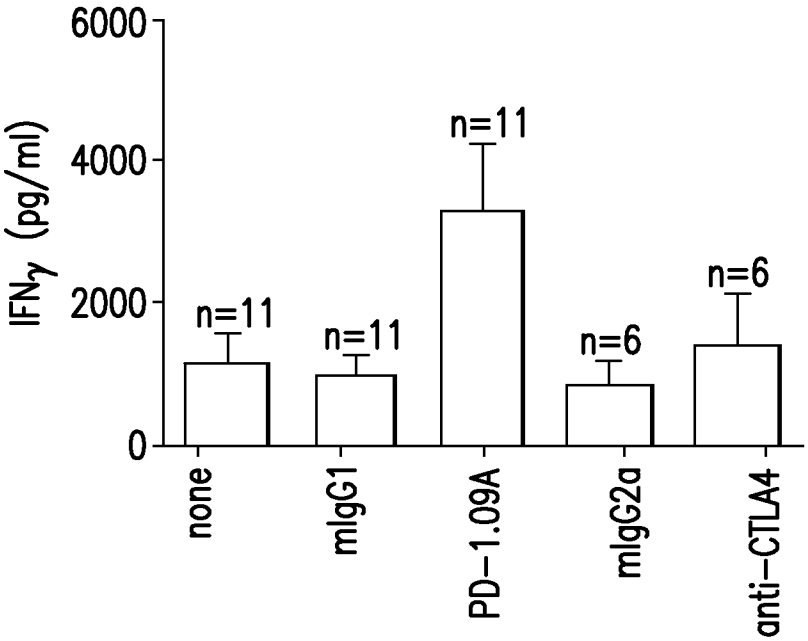

6000
4000
2000
0
IFNγ (pg/ml)
n=11
n=11
n=11
n=6
n=6
none
mIgG1
PD-1.09A
mIgG2a
anti-CTLA4

FIG.4A

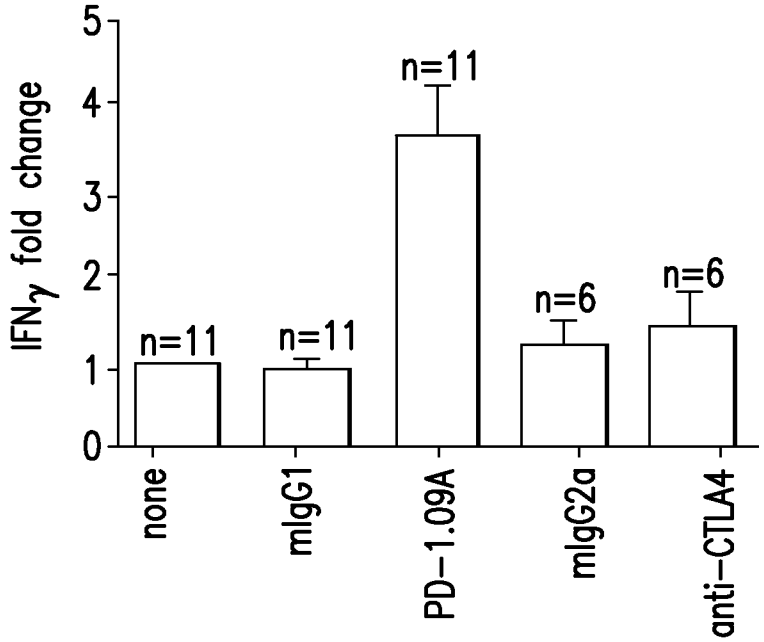

5
4
3
2
1
0
IFNγ fold change
n=11
n=11
n=11
n=6
n=6
none
mIgG1
PD-1.09A
mIgG2a
anti-CTLA4

FIG.4B

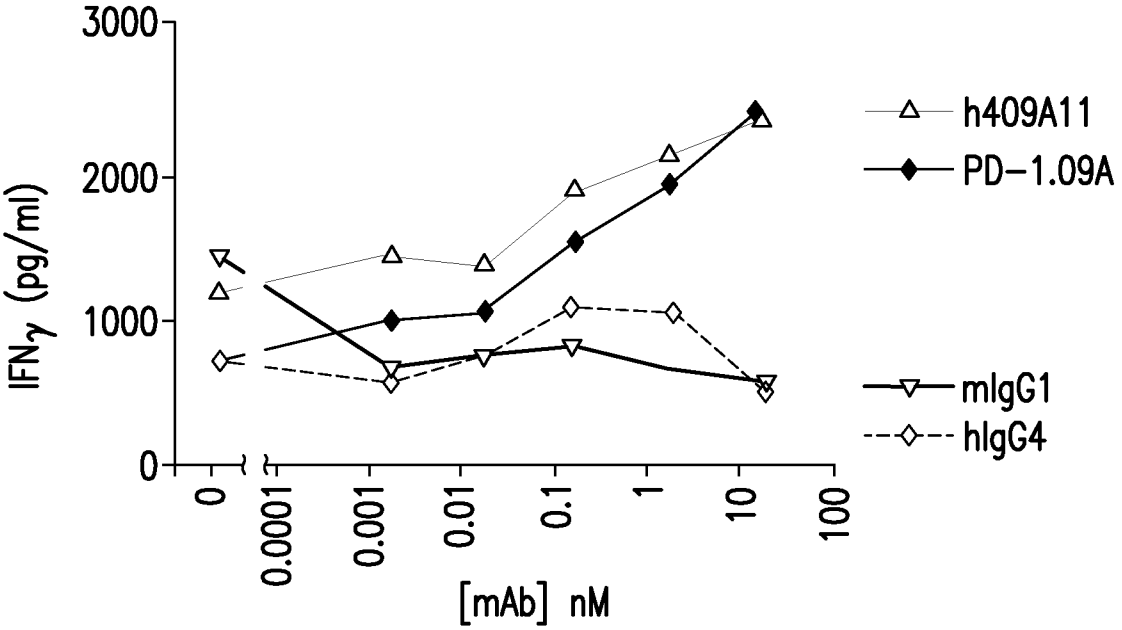

3000
2000
1000
0
IFNγ (pg/ml)
h409A11
PD-1.09A
mIgG1
hIgG4
0
0.0001
0.001
0.01
0.1
1
10
100
[mAb] nM

FIG.4C

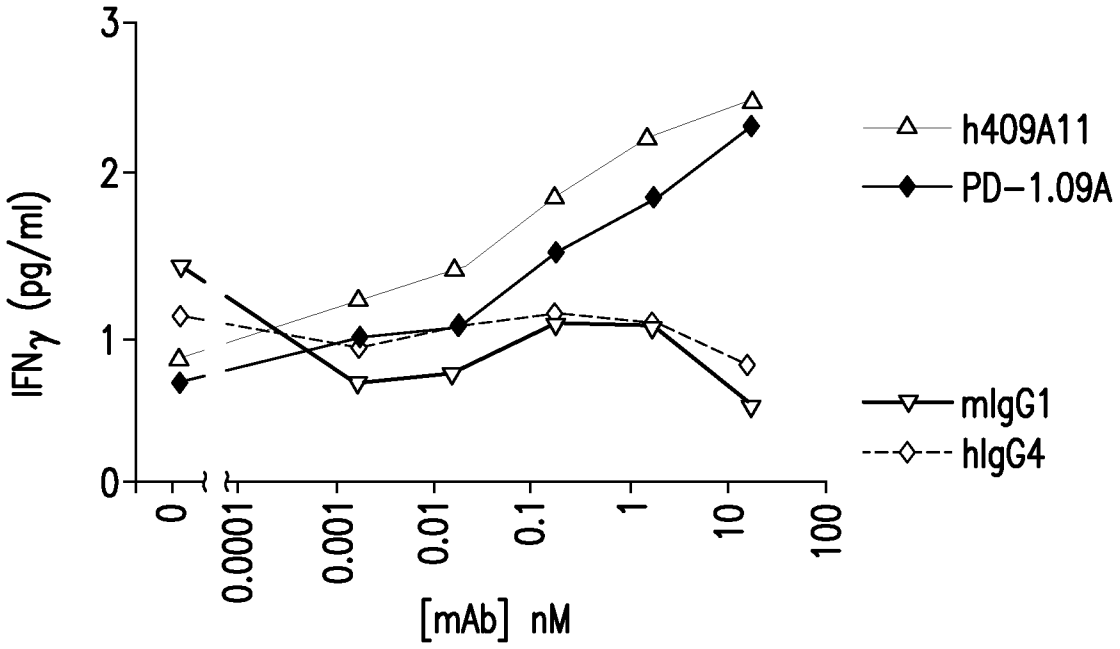

3
2
1
0
IFNγ (pg/ml)
h409A11
PD-1.09A
mIgG1
hIgG4
0
0.0001
0.001
0.01
0.1
1
10
100
[mAb] nM

FIG.4D

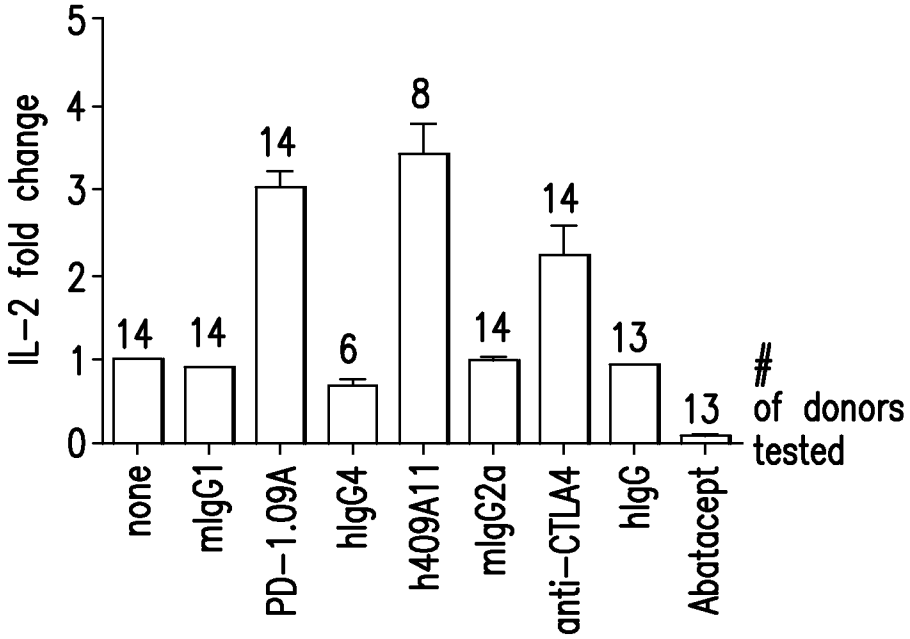

IL-2 fold change
5
4
3
2
1
0
14
14
14
6
8
14
14
13
13
# of donors tested
none
mIgG1
PD-1.09A
hIgG4
h409A11
mIgG2a
anti-CTLA4
hIgG
Abatacept

FIG.5A

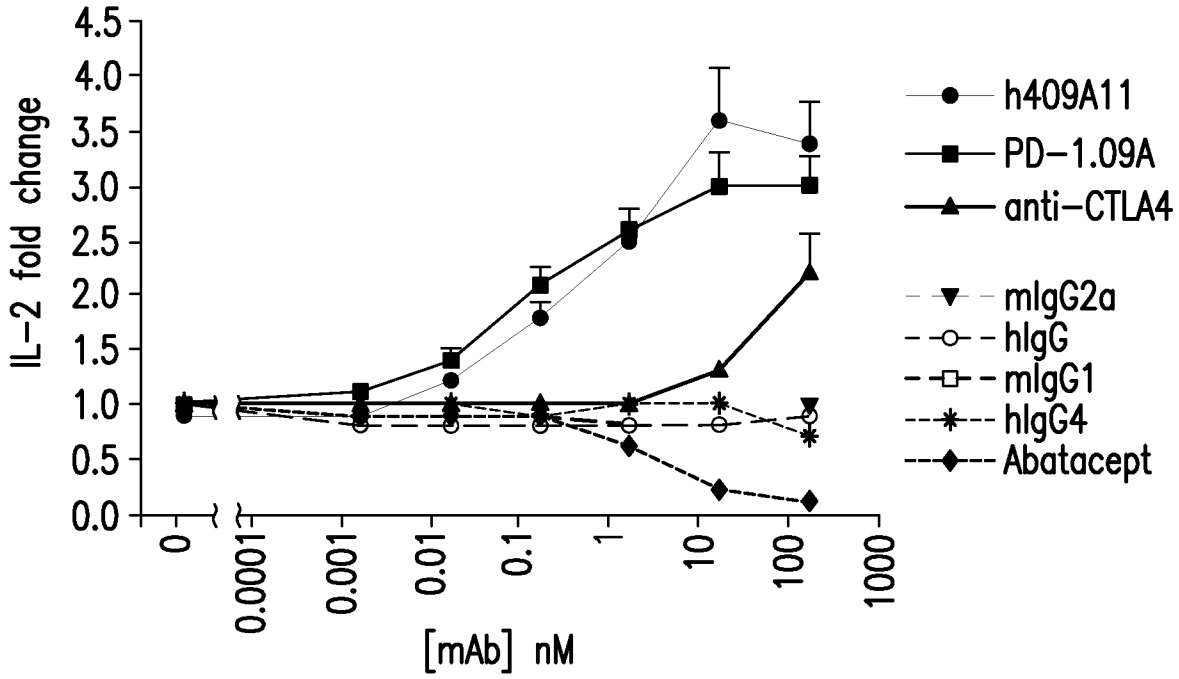

IL-2 fold change
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.0001
0.001
0.01
0.1
1
10
100
1000
[mAb] nM
h409A11
PD-1.09A
anti-CTLA4
mIgG2a
hIgG
mIgG1
hIgG4
Abatacept

FIG.5B

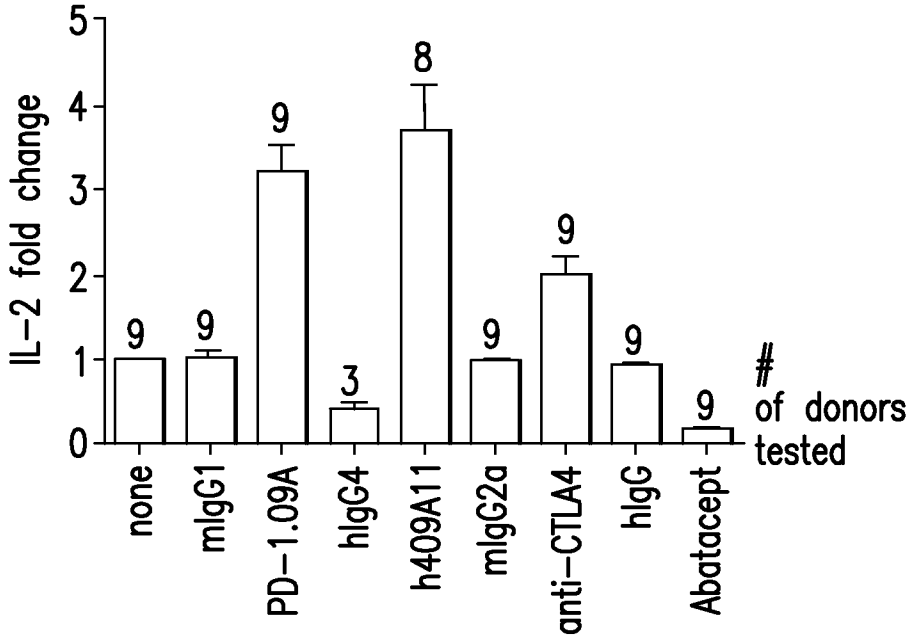

IL-2 fold change
5
4
3
2
1
0
9
9
9
3
8
9
9
9
9
# of donors tested
none
mIgG1
PD-1.09A
hIgG4
h409A11
mIgG2a
anti-CTLA4
hIgG
Abatacept

FIG.5C

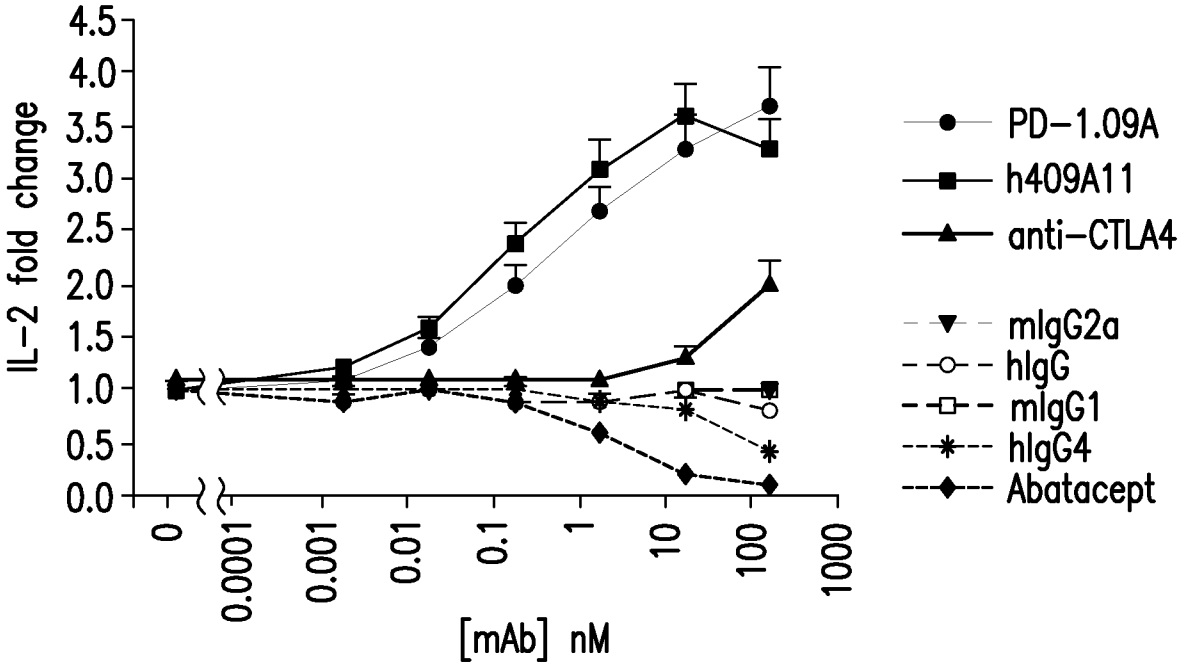

IL-2 fold change
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
0.0001
0.001
0.01
0.1
1
10
100
1000
[mAb] nM
PD-1.09A
h409A11
anti-CTLA4
mIgG2a
hIgG
mIgG1
hIgG4
Abatacept

FIG.5D

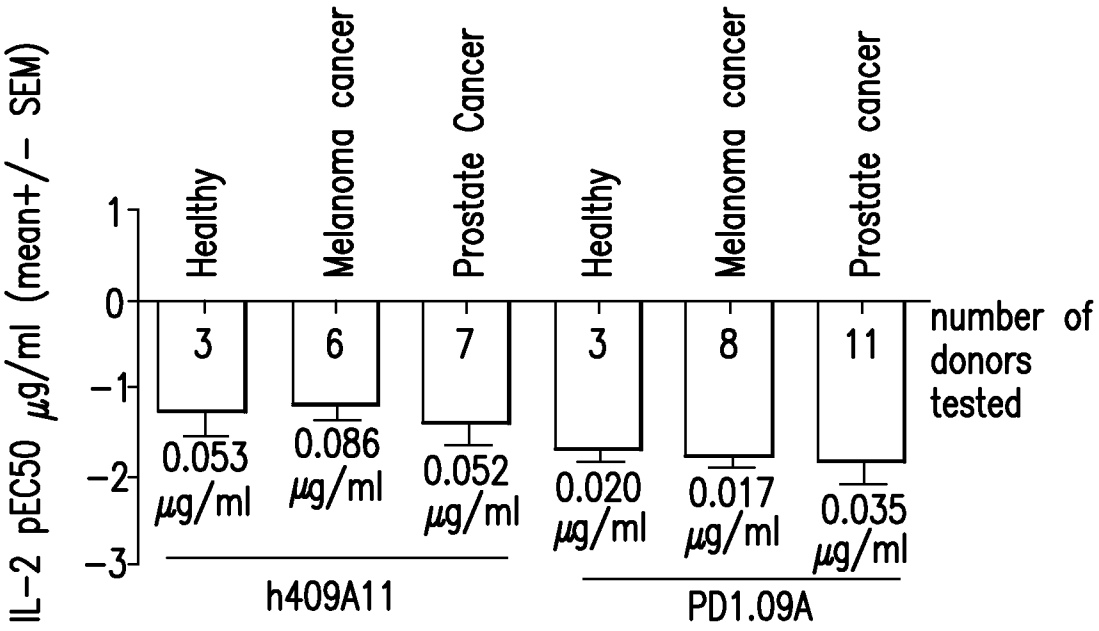

IL-2 pEC50 µg/ml (mean+/- SEM)
1
0
-1
-2
-3
Healthy
Melanoma cancer
Prostate Cancer
Healthy
Melanoma cancer
Prostate cancer
3
6
7
3
8
11
number of donors tested
0.053 µg/ml
0.086 µg/ml
0.052 µg/ml
0.020 µg/ml
0.017 µg/ml
0.035 µg/ml
h409A11
PD1.09A

FIG.6

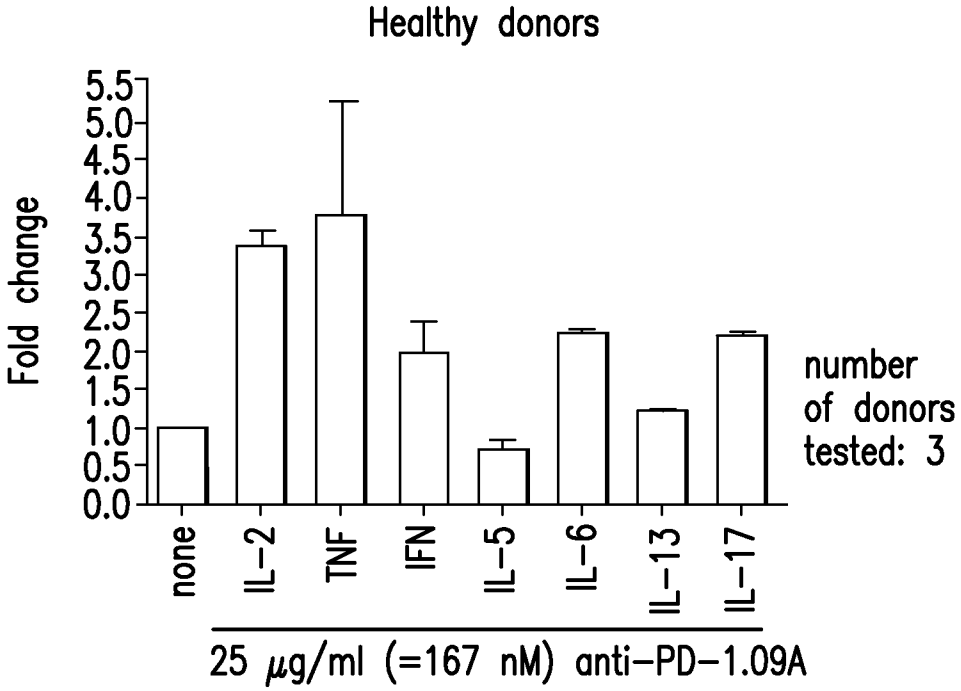

Healthy donors
Fold change
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
none
IL-2
TNF
IFN
IL-5
IL-6
IL-13
IL-17
number of donors tested: 3
25 μg/ml (=167 nM) anti-PD-1.09A

FIG.7A

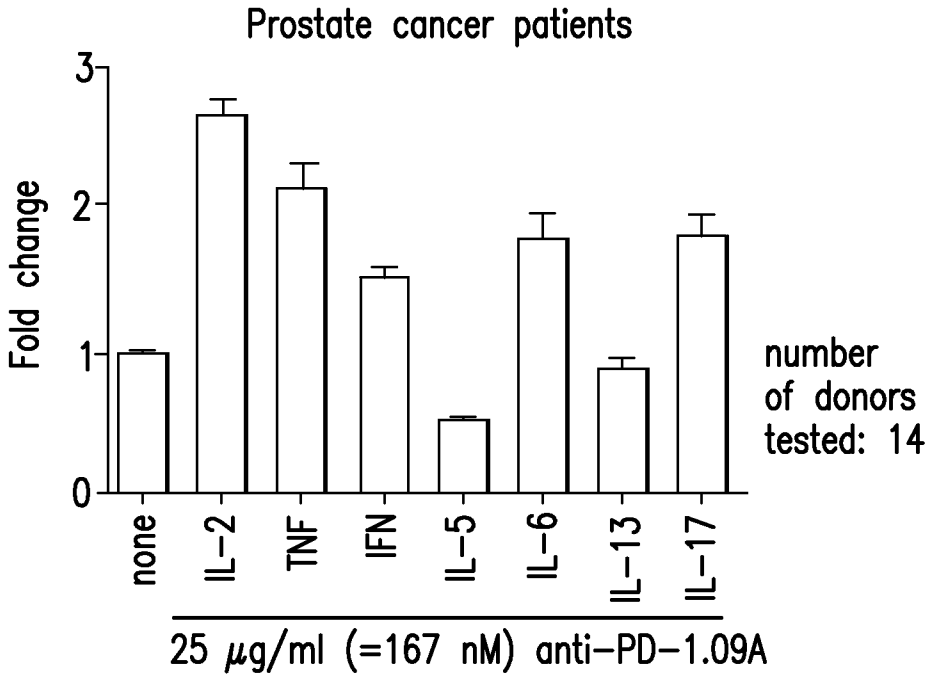

Prostate cancer patients
Fold change
3
2
1
0
none
IL-2
TNF
IFN
IL-5
IL-6
IL-13
IL-17
number of donors tested: 14
25 μg/ml (=167 nM) anti-PD-1.09A

FIG.7B

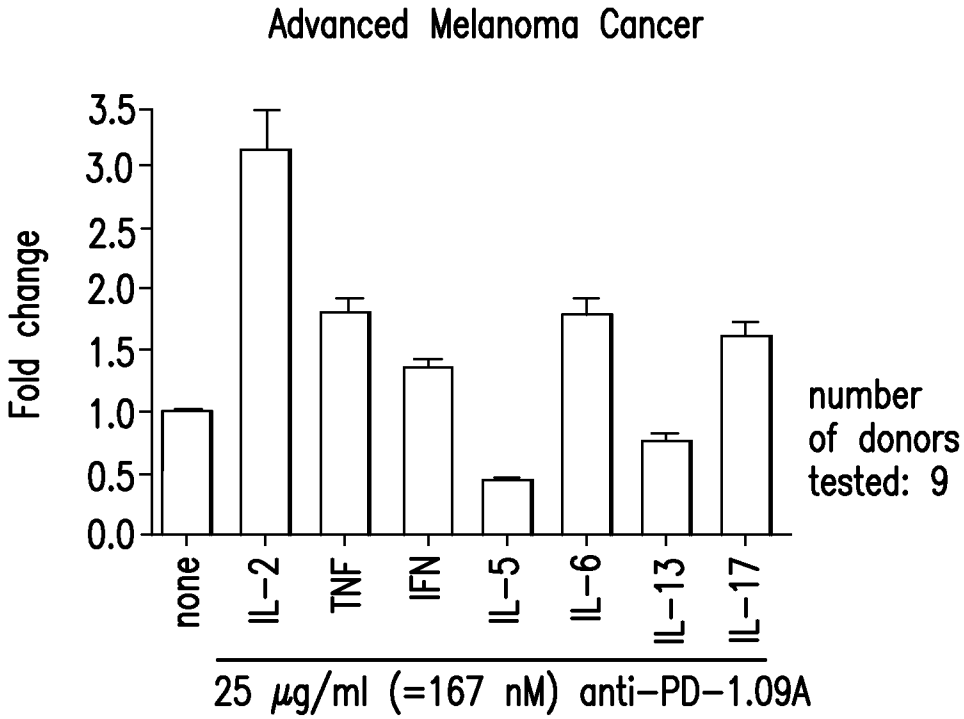

Advanced Melanoma Cancer
Fold change
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
none
IL-2
TNF
IFN
IL-5
IL-6
IL-13
IL-17
number of donors tested: 9
25 μg/ml (=167 nM) anti-PD-1.09A

FIG.7C

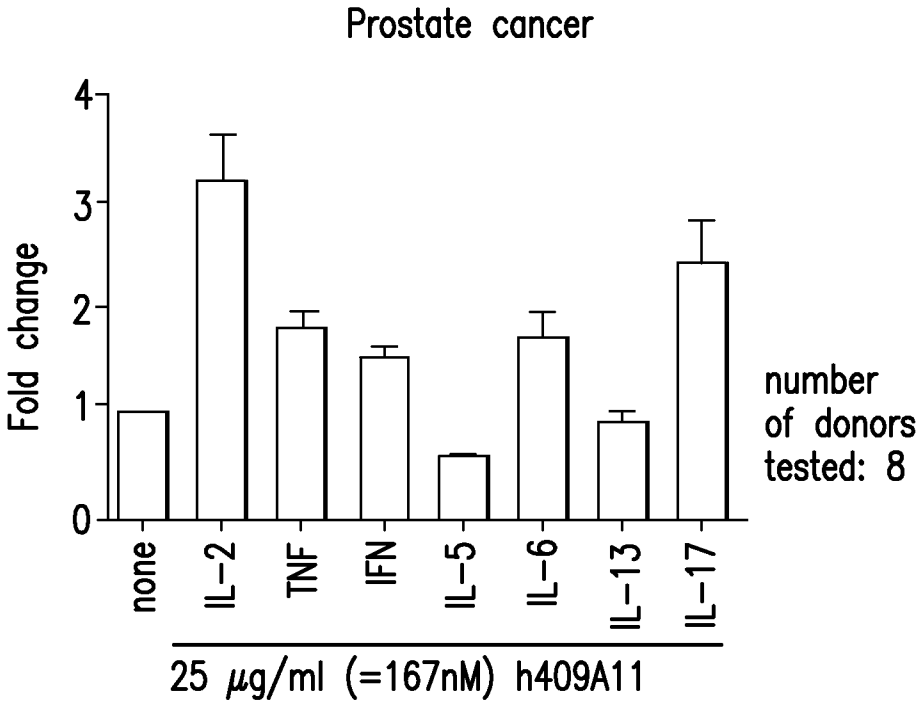

Prostate cancer
Fold change
4
3
2
1
0
none
IL-2
TNF
IFN
IL-5
IL-6
IL-13
IL-17
number of donors tested: 8
25 μg/ml (=167nM) h409A11

FIG.7D

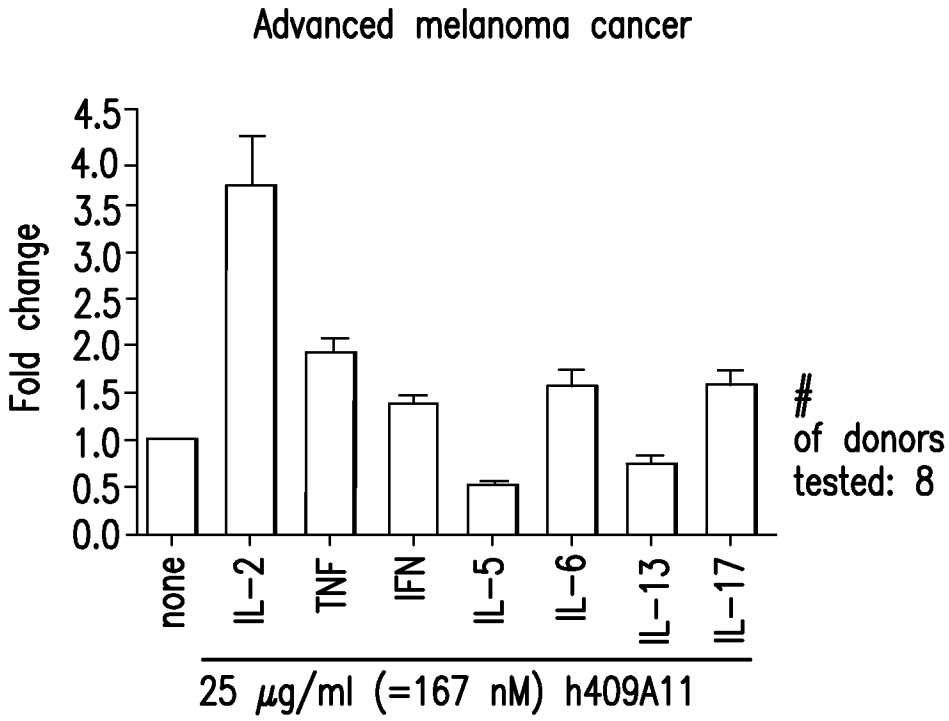

Advanced melanoma cancer
Fold change
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
none
IL-2
TNF
IFN
IL-5
IL-6
IL-13
IL-17
# of donors tested: 8
25 μg/ml (=167 nM) h409A11

FIG.7E

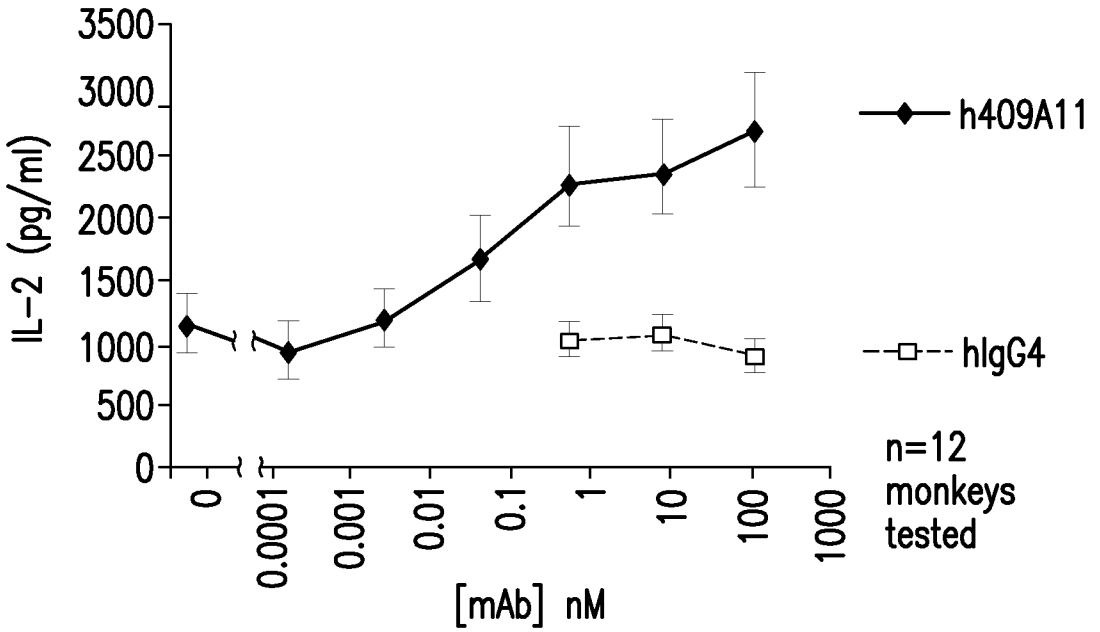

3500
3000
2500
2000
1500
1000
500
0
IL-2 (pg/ml)
0
0.0001
0.001
0.01
0.1
1
10
100
1000
[mAb] nM
h409A11
hIgG4
n=12
monkeys
tested

FIG.8A

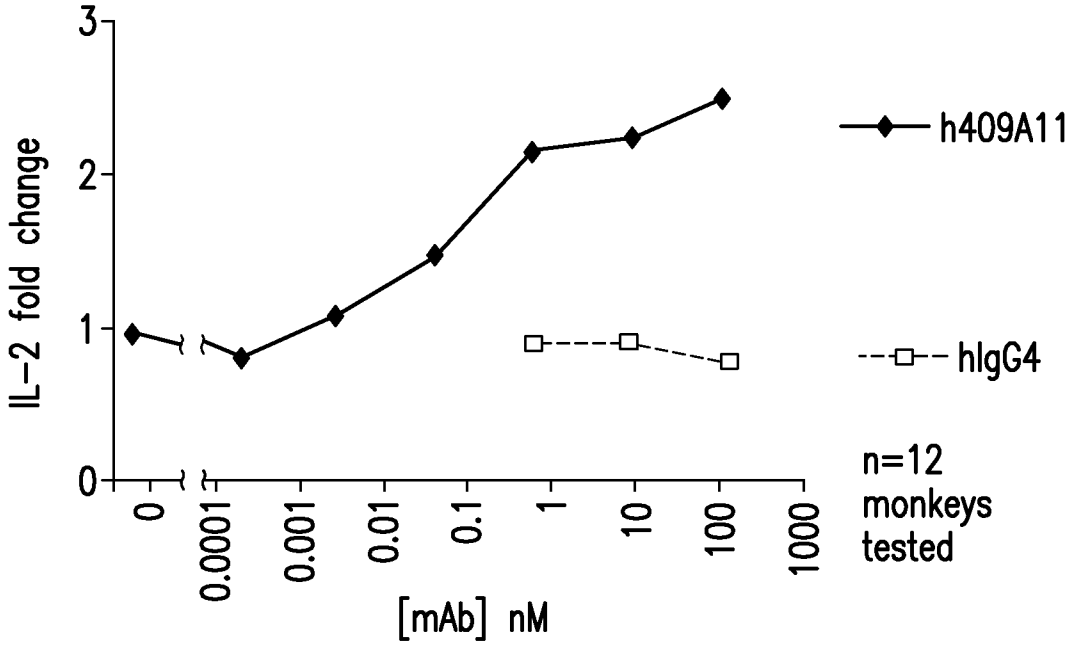

3
2
1
0
IL-2 fold change
0
0.0001
0.001
0.01
0.1
1
10
100
1000
[mAb] nM
h409A11
hIgG4
n=12
monkeys
tested

FIG.8B

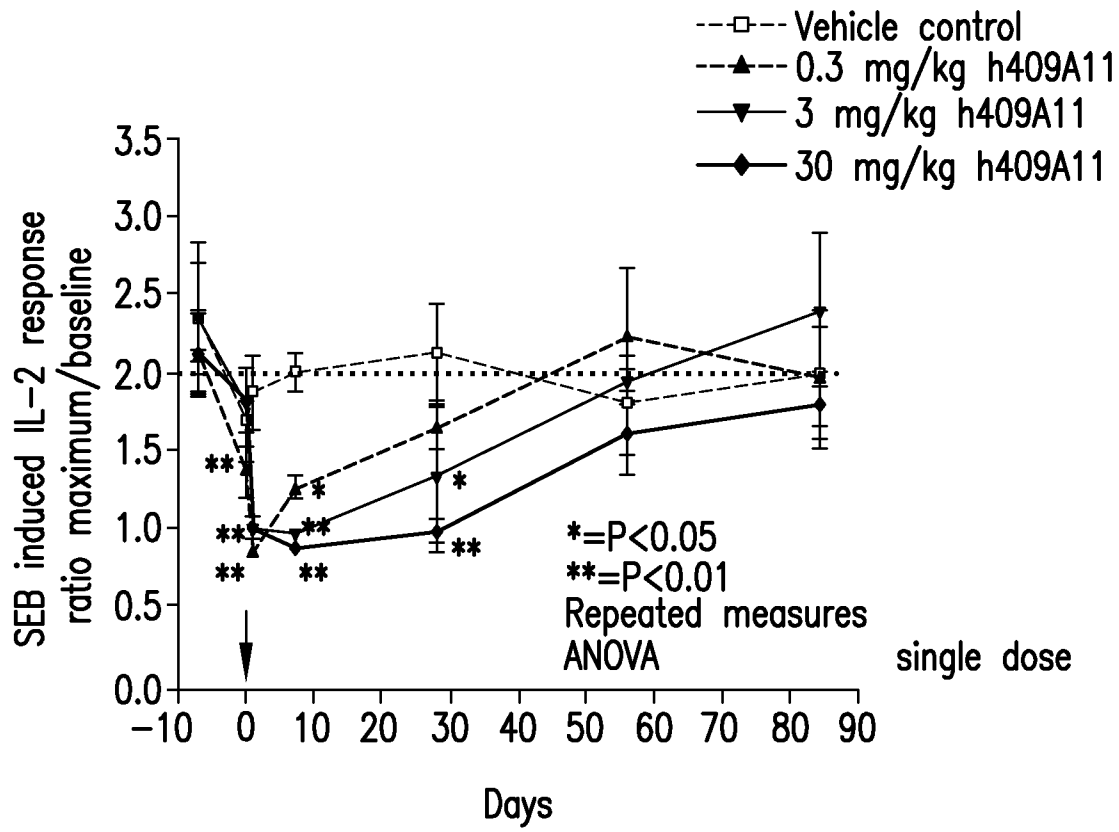

Vehicle control
0.3 mg/kg h409A11
3 mg/kg h409A11
30 mg/kg h409A11
SEB induced IL-2 response
ratio maximum/baseline
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
−10 0 10 20 30 40 50 60 70 80 90
Days
*=P<0.05
**=P<0.01
Repeated measures
ANOVA
single dose

FIG.9A

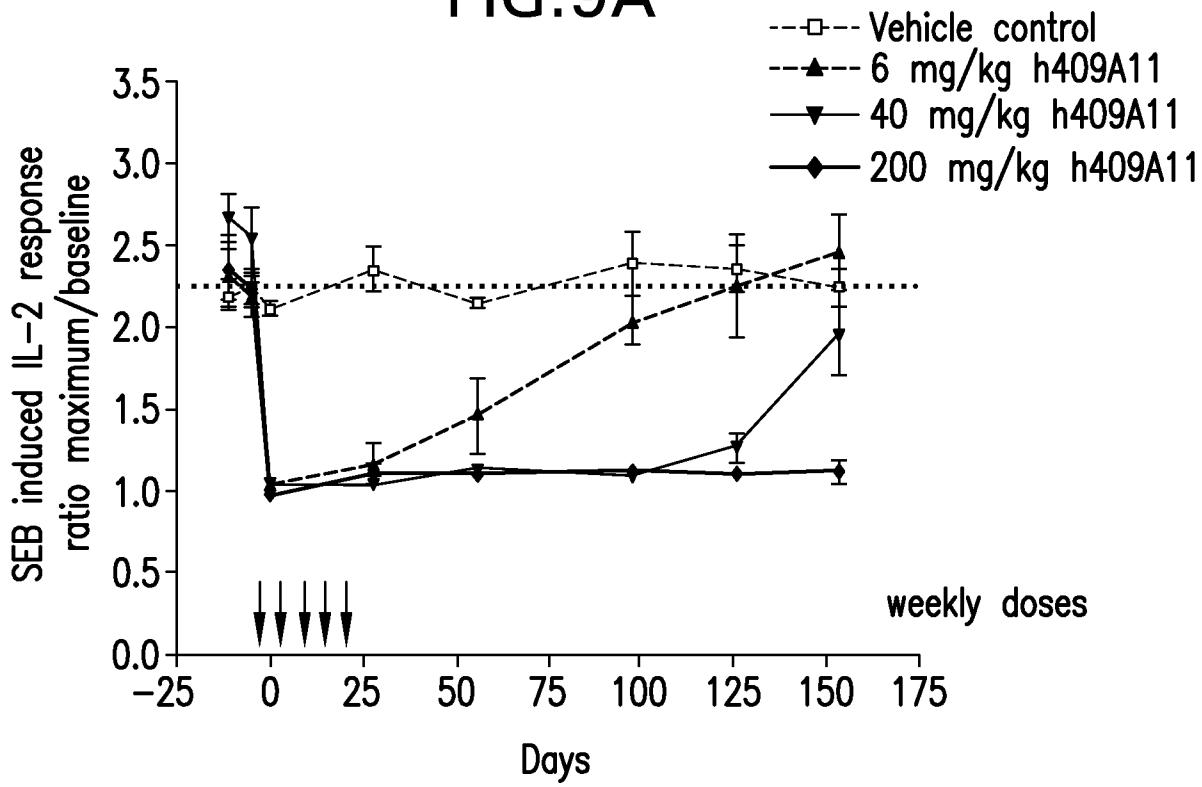

Vehicle control
6 mg/kg h409A11
40 mg/kg h409A11
200 mg/kg h409A11
SEB induced IL-2 response
ratio maximum/baseline
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
−25 0 25 50 75 100 125 150 175
Days
weekly doses

FIG.9B

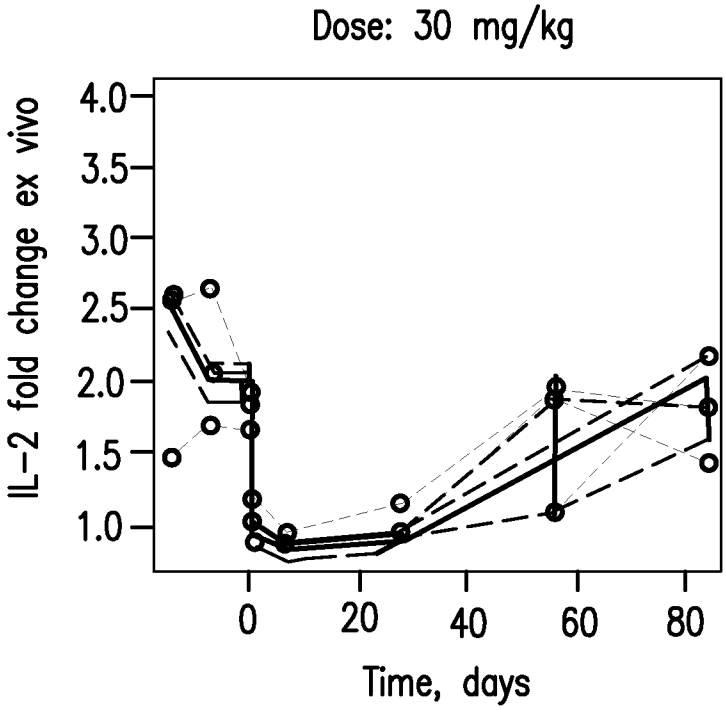


FIG.10A

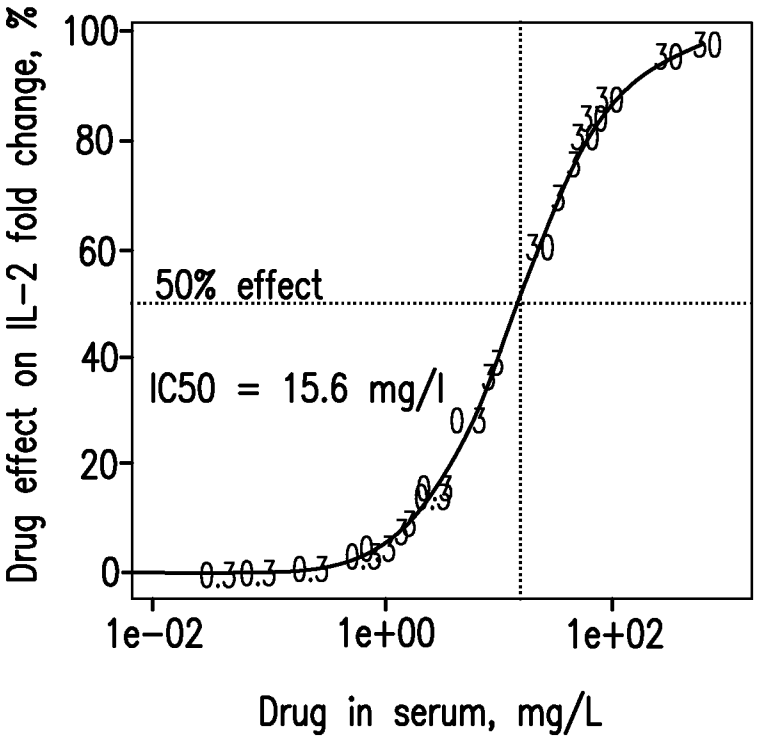


FIG.10B