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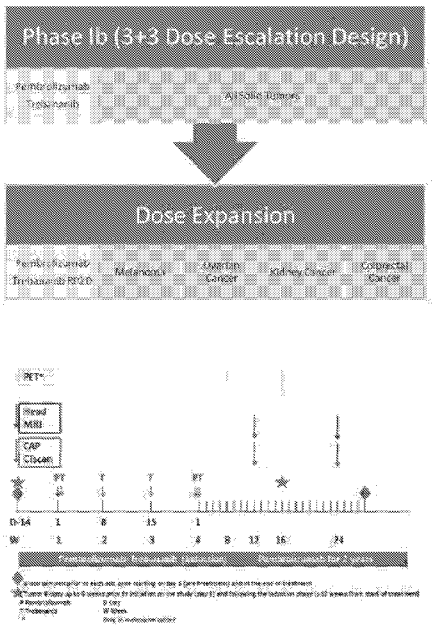
(54) Title: METHODS OF USING PEMBROLIZUMAB AND TREBANANIB

(57) Abstract: The present invention relates to methods of treating cancer with pembrolizumab and trebananib.

FIG. 38

STUDY SCHEMA



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METHODS OF USING PEMBROLIZUMAB AND TREBANANIB

RELATED APPLICATIONS

This application claims the benefit of priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No: 62/539,176, filed July 31, 2017, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Human immune responses against cancer can be suppressed through various mechanisms during disease progression such that cancers evade immune recognition and anti-tumor functions. Immune checkpoint blockade can result in clinical benefit; however, some cancers exhibit limited efficacy to checkpoint blockade alone. As such, prior to the invention described herein, there was a pressing need to design treatment modality combinations that could improve efficacy of immune checkpoint blockade.

SUMMARY OF THE INVENTION

The invention is based, at least in part, upon the discovery that the combination of pembrolizumab and trebananib is unexpectedly more effective at treating cancer than either drug alone.

While cancer treatment sometimes entails combination therapy using two cytotoxic drugs; in general, such combination therapy targets two different pathways on the same cancer cell. It is believed that such combinations have the advantage of overcoming biological redundancy, thereby allowing the cancer cell to avoid developing drug resistance through mutation or upregulation of expression of a single signaling pathway.

By contrast, as described herein, the combination of a PD-1 inhibitor and an Ang-2 inhibitor is based upon a completely different rationale. Although not wishing to be bound by a particular theory, the combination therapy disclosed herein takes advantage of an increased $CD8^+$ T cell cytotoxicity, which has a direct and potent antitumor effect. It has been reported that many tumors have increased expression of PD-L1, which makes tumor cells less susceptible to $CD8^+$ T cell-mediated lysis. Accordingly, a PD-1 inhibitor suppresses PD-1 signaling, and, in turn, enhances $CD8^+$ T-mediated killing.

However, anti-PD-1 treatment also has some limitations. In particular, as described herein, the presence of tumor vessels could negatively impact the therapeutic effect of the anti-PD-1 treatment. As shown in the Examples below, it was identified that Ang-2, a vascular growth factor, contributes to PD-1 resistance by increasing angiogenesis and suppressing $CD8^+$ T cell activity in the tumor microenvironment. Therefore, as described in detail below, the administration of an Ang-2 inhibitor in combination with the PD-1 inhibitor may help overcome this limitation and augment the effect of the PD-1 inhibitor.

Accordingly, provided are methods of treating neoplasia in a subject in need thereof. First, a subject with neoplasia or at risk of developing neoplasia is identified. An effective amount of an angiopoietin-2 (Ang-2) inhibitor and an effective amount of a programmed cell death protein 1 (PD-1) inhibitor are administered to the subject, thereby treating neoplasia in the subject.

For example, the Ang-2 inhibitor comprises a small molecule inhibitor, an antibody, or a peptibody, i.e., peptide-Fc domain fusion proteins. An exemplary Ang-2 peptibody comprises trebananib (AMG386), an Fc fusion protein directed against Ang-1 and Ang-2. In some cases, the effective amount of trebananib is 3 mg/kg, 10 mg/kg, 15 mg/kg, or 30 mg/kg. In other cases, the effective amount of trebananib is between 1 mg/kg and 500 mg/kg, e.g., 10 mg/kg, 20 mg/kg, 30 mg/kg, 40 mg/kg, 50 mg/kg, 60 mg/kg, 70 mg/kg, 80 mg/kg, 90 mg/kg, 100 mg/kg, 150 mg/kg, 200 mg/kg, 250 mg/kg, 300 mg/kg, 350 mg/kg, 400 mg/kg, 450 mg/kg, or 500 mg/kg.

In one aspect, the Programmed Cell Death protein 1 (PD-1) inhibitor comprises a small molecule inhibitor, an antibody, or a peptibody. Exemplary anti-PD-1 antibodies include nivolumab, pembrolizumab, atezolizumab, avelumab, durvalumab, pidilizumab, and cemiplimab. For example, the anti-PD-1 antibody comprises pembrolizumab. Other suitable PD-1 inhibitors include AMP-224, AMP-514, and PDR001.

An exemplary dose of pembrolizumab is 200 mg every three weeks. Alternatively, the effective amount of the pembrolizumab is 2 mg/kg. In another aspect, the effective amount of pembrolizumab is between 1 mg/kg and 500 mg/kg, e.g., 10 mg/kg, 20 mg/kg, 30 mg/kg, 40 mg/kg, 50 mg/kg, 60 mg/kg, 70 mg/kg, 80 mg/kg, 90 mg/kg, 100 mg/kg, 150 mg/kg, 200 mg/kg, 250 mg/kg, 300 mg/kg, 350 mg/kg, 400 mg/kg, 450 mg/kg, or 500 mg/kg.

In one aspect, the pembrolizumab is administered every three weeks for twelve weeks and the trebananib is administered once per week for twelve weeks. Thereafter, the pembrolizumab is administered every three weeks for two additional years. For example, the pembrolizumab and trebananib are administered simultaneously or sequentially. Preferably, the trebananib is administered immediately after the pembrolizumab.

Suitable modes of administration for the pembrolizumab and trebananib include systemic, intravenous, subcutaneous, intramuscular, and oral administration.

In some cases, the neoplasia comprises a solid tumor. For example, the neoplasia comprises melanoma, ovarian cancer, kidney cancer (renal cell carcinoma), or colorectal cancer.

Preferably, the neoplasia, e.g., a tumor, is inhibited by at least 1%, e.g., at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or 100%.

In some aspects, clinical benefit in the subject is evaluated by response evaluation criteria in solid tumors (RECIST) or immune-related response criteria (irRC).

Optionally, the methods further comprise obtaining a sample from the subject before and after administration of the Ang-2 inhibitor and the PD-1 inhibitor. Treatment efficacy is evaluated by analyzing a blood sample or a tumor biopsy from the subject. In one aspect, the subject is human.

Method of determining whether inhibition of Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA4) and/or inhibition of PD-1 in a subject with melanoma will result in clinical benefit in the subject are carried out by obtaining a test sample from a subject having or at risk of developing melanoma; determining the expression level of Ang-2 in the test sample; comparing the expression level of Ang-2 in the test sample with the expression level of Ang-2 in a reference sample; and determining whether CTLA4 and PD-1 blockade will inhibit melanoma in the subject if the expression level of the Ang-2 in the test sample is differentially expressed as compared to the level of the Ang-2 in the reference sample. For example, the test sample is obtained from the melanoma tissue or from the tumor microenvironment.

Clinical benefit in a subject comprises complete or partial response as defined by response evaluation criteria in solid tumors (RECIST), stable disease as defined by RECIST, or long-term survival in spite of disease progression or response as defined by irRC criteria.

For example, the test sample is obtained from the melanoma; and it is determined that inhibition of CTLA4 and/or PD-1 in a subject with melanoma will not result in clinical benefit in the subject if the expression level of Ang-2 in the test sample is higher than the level of Ang-2 in the reference sample. In some aspects, the reference sample is obtained from healthy normal tissue, melanoma that received a clinical benefit from CTLA4 and/or PD-1 inhibition, or melanoma that did not receive a clinical benefit from CTLA4 and PD-1 inhibition.

Also provided are kits for treatment of cancer comprising a therapeutically effective amount of pembrolizumab and a therapeutically effective amount of trebananib and instructions for use.

Definitions

Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. "About" can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term "about."

The term "antibody" (Ab) as used herein includes monoclonal antibodies, polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments, so long as they exhibit the desired biological activity. The term "immunoglobulin" (Ig) is used interchangeably with "antibody" herein.

By "control" or "reference" is meant a standard of comparison. In one aspect, as used herein, "changed as compared to a control" sample or subject is understood as having a level that is statistically different than a sample from a normal, untreated, or control sample. Control samples include, for example, cells in culture, one or more laboratory test animals, or one or more human subjects. Methods to select and test control samples are within the ability of those in the art. An analyte can be a naturally occurring substance that is characteristically expressed or produced by the cell or organism (e.g., an antibody, a protein) or a substance produced by a reporter construct (e.g, β-galactosidase or luciferase). Depending on the method used for detection, the amount and measurement of the change can vary. Determination of statistical significance is within the ability of those skilled in the art, e.g., the number of standard deviations from the mean that constitute a positive result.

By the terms "effective amount" and "therapeutically effective amount" of a formulation or formulation component is meant a sufficient amount of the formulation or component, alone or in a combination, to provide the desired effect. For example, by "an effective amount" is meant an amount of a compound, alone or in a combination, required to ameliorate the symptoms of a disease, e.g., neoplasia, relative to an untreated patient. The effective amount of active compound(s) used to practice the present invention for therapeutic treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian decides the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

Nucleic acid molecules useful in the methods of the invention include any nucleic acid molecule that encodes a polypeptide of the invention or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity, e.g., at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule.

By "neoplasia" is meant a disease or disorder characterized by excess proliferation or reduced apoptosis. Illustrative neoplasms for which the invention can be used include, but are not limited to pancreatic cancer, leukemias (e.g., acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemia, acute myeloblastic leukemia, acute promyelocytic leukemia, acute myelomonocytic leukemia, acute monocytic leukemia, acute erythroleukemia, chronic leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia), polycythemia vera, lymphoma (Hodgkin's disease, non-Hodgkin's disease), Waldenstrom's macroglobulinemia, heavy chain disease, and solid tumors such as sarcomas and carcinomas (e.g., fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, nile duct carcinoma, choriocarcinoma, seminoma, embryonal

carcinoma, Wilms' tumor, cervical cancer, uterine cancer, testicular cancer, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, glioblastoma multiforme, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodenroglioma, schwannoma, meningioma, melanoma, neuroblastoma, and retinoblastoma).

By "peptibody" is meant a specific binding agent that is a molecule comprising an antibody Fc domain attached to at least one peptide. The production of peptibodies is generally described in PCT publication WO 00/24782 (incorporated herein by reference). Exemplary peptides may be generated by any of the methods set forth therein, such as carried in a peptide library (e.g., a phage display library), generated by chemical synthesis, derived by digestion of proteins, or generated using recombinant DNA techniques.

The phrase "pharmaceutically acceptable carrier" is art recognized and includes a pharmaceutically acceptable material, composition or vehicle, suitable for administering the PD-1 inhibitor and/or Ang-2 inhibitor of the present invention to mammals. The carriers include liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agent from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations.

The term "sample" as used herein refers to a biological sample obtained for the purpose of evaluation *in vitro*. Exemplary tissue samples for the methods described herein include tissue samples from neoplasias. With regard to the methods disclosed herein, the sample or patient

sample preferably may comprise any body fluid or tissue. In some embodiments, the bodily fluid includes, but is not limited to, blood, plasma, serum, lymph, breast milk, saliva, mucous, semen, vaginal secretions, cellular extracts, inflammatory fluids, cerebrospinal fluid, feces, vitreous humor, or urine obtained from the subject. In some aspects, the sample is a composite panel of at least two of a blood sample, a plasma sample, a serum sample, and a urine sample. In exemplary aspects, the sample comprises blood or a fraction thereof (e.g., plasma, serum, fraction obtained via leukopheresis). Preferred samples are whole blood, serum, plasma, or urine. A sample can also be a partially purified fraction of a tissue or bodily fluid.

A reference sample can be a "normal" sample, from a donor not having the disease or condition fluid, or from a normal tissue in a subject having the disease or condition. A reference sample can also be from an untreated donor or cell culture not treated with an active agent (e.g., no treatment or administration of vehicle only). A reference sample can also be taken at a "zero time point" prior to contacting the cell or subject with the PD 1 inhibitor and/or Ang-2 inhibitor to be tested or at the start of a prospective study.

By "specifically binds" is meant a compound or antibody that recognizes and binds a polypeptide of the invention, but which does not substantially recognize and bind other molecules in a sample, for example, a biological sample, which naturally includes a polypeptide of the invention.

The term "subject" as used herein includes all members of the animal kingdom prone to suffering from the indicated disorder. In some aspects, the subject is a mammal, and in some aspects, the subject is a human. The methods are also applicable to companion animals such as dogs and cats as well as livestock such as cows, horses, sheep, goats, pigs, and other domesticated and wild animals.

The terms "treating" and "treatment" as used herein refer to the administration of PD-1 inhibitor and/or Ang-2 inhibitor to a clinically symptomatic individual afflicted with an adverse condition, disorder, or disease, so as to effect a reduction in severity and/or frequency of symptoms, eliminate the symptoms and/or their underlying cause, and/or facilitate improvement or remediation of damage. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated. Treatment may also refer to prophylactic treatment (i.e., preventative treatment) and/or management of treatment. For example, prophylactic treatment or preventative

treatment refers to the administration of the PD-1 inhibitor and/or Ang-2 inhibitor to a clinically asymptomatic individual who is at risk of developing, susceptible, or predisposed to a particular adverse condition, disorder, or disease, and thus relates to the prevention of the occurrence of symptoms and/or their underlying cause.

Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

In some cases, a composition of the invention is administered orally or systemically. Other modes of administration include rectal, topical, intraocular, buccal, intravaginal, intracisternal, intracerebroventricular, intratracheal, nasal, transdermal, within/on implants, or parenteral routes. The term "parenteral'' includes subcutaneous, intrathecal, intravenous, intramuscular, intraperitoneal, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis. They could, however, be preferred in emergency situations. Compositions comprising a composition of the invention can be added to a physiological fluid, such as blood. Oral administration can be preferred for prophylactic treatment because of the convenience to the patient as well as the dosing schedule. Parenteral modalities (subcutaneous or intravenous) may be preferable for more acute illness, or for therapy in patients that are unable to tolerate enteral administration due to gastrointestinal intolerance, ileus, or other concomitants of critical illness. Inhaled therapy may be most appropriate for pulmonary vascular diseases (e.g., pulmonary hypertension).

Pharmaceutical compositions may be assembled into kits or pharmaceutical systems for use in arresting cell cycle in rapidly dividing cells, e.g., cancer cells. Kits or pharmaceutical systems according to this aspect of the invention comprise a carrier means, such as a box, carton, tube, having in close confinement therein one or more container means, such as vials, tubes, ampoules, bottles, syringes, or bags. The kits or pharmaceutical systems of the invention may also comprise associated instructions for using the kit.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All published foreign patents and patent applications cited herein are incorporated herein by reference. Genbank and NCBI submissions indicated by accession number cited herein are incorporated herein by reference. All other published references, documents, manuscripts and scientific literature cited herein are incorporated herein by reference. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a series of line plots depicting baseline vascular endothelial growth factor (VEGF) value correlated with patient overall survival. Kaplan-Meier curves demonstrating the difference in OS for patients with VEGFlow and VEGFhi are statistically significant: 3 mg/kg dose of ipilimumab (median OS 14.33 vs 7.44 months, p=0.0367); 10 mg/kg dose of ipilimumab (median OS 10.85 vs 6.16 months, p=0.0477); all patients (median OS 12.87 vs 6.56 months p=0.006).

FIG. 2 is a series of images of melanoma. Left: Melanoma tumor deposit post-ipilimumab demonstrating extensive hemorrhagic tumor necrosis with rim of viable tumor heavily infiltrated with granulocytes and lymphocytes. Right: Melanoma tumor deposit post-ipilimumab with severe tumor vasculopathy accompanied by perivascular and intramural lymphoid infiltrates associated with luminal thrombosis. (Magnification: ×125).

FIG. 3 is a series of images depicting clinical activity of ipilimumab plus bevacizumab by positron emission tomography–computed tomography (PET-CT) (CT images on left, PET images on right). Pretreatment demonstrates an fluorodeoxyglucose (FDG) avid liver metastasis. With treatment the metastasis is no longer metabolically avid, but anatomically still present. With continued treatment and follow up, this lesion regressed approximately four months later without evidence of additional disease.

FIG. 4 is a histogram of activity in treated patients by cohort according to RECIST criteria. Arrows indicate alive at time of analysis. Crosses indicate death. Black bars indicate discontinuation of treatment other than due to progressive disease. Five patients came off trial due to toxicity requiring systemic steroids. One patient withdrew consent after week 12 without dose-limiting toxicity. PD= progressive disease. SD = stable disease. PR = partial response. CR = complete response.

FIG. 5A-FIG. 5B is a series of graphs showing response kinetics in treated patients. Baseline tumor measurements are standardized to zero. FIG. 5A shows an entire treatment population (cohorts 1-4). FIG. 5B shows cohort 2 patients (MTD). Horizontal line PD = progressive disease representing 20% increase. Horizontal line PR represents 30% decrease from baseline.

FIG. 6 is a graph depicting Kaplan-Meier estimates of overall survival.

FIG. 7 a series of images depicting changes in tumor deposits resulting from treatment with bevacizumab plus ipilimumab. Phenotypic characterization of immune cell infiltrates in biopsies from responders before and after initiation of therapy. Significant infiltration by $CD3^+CD8^+$ T cells and $CD163^+$ dendritic macrophages with minimal change in forkhead box P3 ($Foxp3^+$) component were observed.

FIG. 8 is a series of images of lymphoid aggregates and morphologic changes in endothelial cells.

FIG. 9 is a series of images of blood vessels before and after initiation of therapy. Endothelial cells lining small vessels within melanomas of ipilimumab plus bevacizumab treated patients were plump as assessed by hematoxylin and eosin stain (H&E) and CD31 staining, in contrast to pre-treatment samples and on treatment samples from patients receiving ipilimumab alone (column to extreme right; v = vessel). Endothelial cells in tumor deposits of patients receiving ipilimumab plus bevacizumab were also associated with increased expression of E-selectin, and adhesion and diapedesis of $CD8^+$ T cells. Enlarged central panels highlight the focally occlusive appearance of this endothelial activation (top H&E, bottom CD31, [inset, E-selectin]). Base membrane of vessels approximated by dotted line.

FIG. 10A- FIG. 10C are a series of graphs of T cell responses of melanoma patients after treatment with ipilimumab alone or ipilimumab plus bevacizumab. FIG. 10A is a series of graphs depicting an example of changes as a function of treatment in $CD4^+CCR7^+CD45RO^+$ and

$CD4^+CCR7^-CD45RO^+$ T cell populations to ipilimumab plus bevacizumab treatment, compared to changes with ipilimumab treatment alone. FIG. 10B is a series of graphs depicting an example of changes as a function of treatment to $CD8^+CCR7^+CD45RO^+$ and $CD8^+CCR7^-CD45RO^+$ T cell populations to ipilimumab plus bevacizumab treatment, compared to the responses to ipilimumab treatment alone. FIG. 10C is a chart depicting numbers of melanoma patients that have at least 50% enhancement in $CD4^+/CD8^+$ $CCR7^+CD45RO^+$ and $CD4^+/CD8^+$ $CCR7^-CD45RO^+$ T cell populations following treatment with ipilimumab (3mg/kg), or ipilimumab (3mg/kg) plus bevacizumab, or ipilimumab (10mg/kg) plus bevacizumab. * indicates $P<0.05$ between ipilimumab and ipilimumab plus bevacizumab. ** indicates $P<0.01$.

FIG. 11 is a chart showing that humoral responses to angiopoietins are associated with clinical benefits. Specifically, the chart shows humoral immune response to Ang-2 in treated melanoma patients with favorable clinical outcomes. Eight out of eleven patients showed long-term survival (≥4.5 yrs).

FIG. 12 is a series of graphs and images showing that patient humoral responses to Ang-2 are functional in TIE-2 binding assays. Specifically, anti-angiopoietin Abs in sera block Tie-2-mediated signaling in endothelial cells.

FIG. 13 is a series of images of patient humoral responses to Ang-2 are functional in tube forming assays. Specifically, anti-angiopoietin Abs in sera block tube formation by endothelial cells.

FIG. 14 is a histogram of ratios of post/pre-treatment Ang-2 level and clinical outcomes to ipilimumab. Comparison is from pre-treatment to week 12 of treatment in melanoma patients.

FIG. 15 is a line plot of an example of changes in Ang-2 humoral immunity as a function of treatment with ipilimumab plus bevacizumab in a patient with metastatic melanoma.

FIG. 16 is a series of histograms of changes from baseline Ang-2 antibody titers to clinical outcomes in ipilimumab treated melanoma patients.

FIG. 17A-FIG. 17D is a series of graphs depicting identification and enumeration of circulating endothelial cells (CEC) and circulating progenitor cells (CPC) in whole blood samples of melanoma patients by multicolor flow cytometric analyses. Mononuclear cellular events were gated on the forward-side scatter plot (red in FIG. 17A). CEC in the mononuclear cellular population were identified as $CD31brightCD45^-CD34^+CD133^-$(green in FIG. 17B-FIG17. D) and CPC as $CD133^+CD34brightCD31^+CD45dim$ (black in FIG. 17B-FIG. 17D). CEC

and CPE were 0.6% and 0.15% of blood mononuclear cells, respectively, within the typical ranges of CEC (from 0.1% to 6.0%) and CPE (from 0.01 to 0.20%) in blood mononuclear cells from a normal donor.

FIG. 18 is a histogram showing an example of serum cytokine profiling of ipilimumab plus bevacizumab treated patients using Luminex technology. Thirty-nine cytokines were analyzed. The levels of many cytokines altered as function of the treatment.

FIG. 19 is a series of line plots showing that treatment with ipilimumab plus bevacizumab decreased circulating CEC in the blood of melanoma patients.

FIG. 20 is a series of western blot images showing that treatment with ipiliumab plus bevacizumab resulted in humoral immune recognition of targets on tumor associated endothelial cells (TEC) isolated from fresh post-treatment biopsies as well as targets on melanoma cells. Specifically, the photographs show changes in serum recognition of TEC and melanoma cell line as function of treatment.

FIG. 21 is a series of graphs depicting expression of CD14 and Tie-2 on monocytes. Monocytes were isolated by adherent cell selections. Expressions of CD14 and Tie-2 on the monocytes were analyzed by cytometry.

FIG. 22A-FIG. 22B are a series of histograms showing the effects of Ang1 and AMG386 on T cell proliferation. Pan T cells were stained with carboxyfluorescein succinimidyl ester (CSFE) and further cocultured with $CD14^{+}Tie\text{-}2^{+}$ monocytes in the presence or absence of anti-$CD3^{+}CD28$ beads and/or ML4-3 and L1-7, as indicated. ML4-3 and L1-7 are two different anti-Ang-1/2 peptibodies (AMG386). FIG. 22A shows $CD14^{+}Tie\text{-}2^{+}$ monocytes. FIG. 22B shows $CD14^{+}Tie\text{-}2^{+}$ monocytes transduced with Tie-2 expressing Lenti virus. Data represent percentage of proliferated T cells. The percentage of anti-$CD3^{+}CD28$ bead group are presented as 100%.

FIG. 23 is a series of graphs showing expression of PD-L1 on $CD14^{+}Tie\text{-}2^{+}$ monocytes. Expression of PD-L1 on $CD14^{+}Tie\text{-}2^{+}$ monocytes and $CD14^{+}Tie\text{-}2^{+}$ monocytes infected with Tie-2 expressing Lenti virus were analyzed by cytometry.

FIG. 24 is a series of graphs showing expression of inducible T-cell co-stimulator ligand (ICOSL) on $CD14^{+}Tie\text{-}2^{+}$ monocytes. Expression of ICOSL on $CD14^{+}Tie\text{-}2^{+}$ monocytes and $CD14^{+}Tie\text{-}2^{+}$ monocytes infected with Tie-2 expressing Lenti virus were analyzed by cytometry.

FIG. 25 is an image of control Hodgkin lymphoma stained for Programmed death-ligand 1 (PDL1) (left) and mediastinal large B-cell lymphoma stained for PDL2 (right).

FIG. 26 is an image of control tonsil tissue stained for PD1.

FIG. 27 is a histogram showing semi-quantitative assessment of malignant and non-malignant cell expression of PDL1 in Hodgkin lymphoma, from Chen BJ et al.

FIG. 28 is a histogram depicting aperio-base quantitative assessment of ALK protein expression in lung cancers, from Mino-Kenudson et al.

FIG. 29 is a series of images showing the immunohistochemical staining of CD279, CD274 and CD273 in advanced melanoma.

FIG. 30 is a series of graphs of expression of Tie-2, PD-L1, PD-L2, Fas Ligand (FASL), CD11b, human leukocyte antigen – antigen D related (HLA-DR), and human leukocyte antigen (HLA) A*02 on human $CD14^+$ monocytes. Expression of Tie-2, PD-L1, PD-L2, FASL, CD11b, HLA-DR, and HLA A*02 on $CD14^+$ monocytes were analyzed by flow cytometry.

FIG. 31A- FIG. 31C are a series of histograms and graphs depicting the effects of toxic shock syndrome toxin (TSST), phytohemagglutinin (PHA), toll-like receptors (TLR) activators, and cytokines on human $CD14^+$ monocytes; effects of $CD14^+$ monocytes on T cell activation. FIG. 31A is a series of histograms showing the effects of TSST, PHA, and TLR activators on expressions of PD-L, PD-L2, FASL, interleukin-10 (IL-10), transforming growth factor beta (TGFβ), and Arginase-1. Peripheral blood mononuclear cells (PBMC) were treated with 5 μg/ml TSST (Toxic shock syndrome toxin), 5 μg/ml PHA (Phytohemagglutinin), 20 μg/ml Zymozan, 10 μg/ml pI:C, 10 μg/ml LPS (Lipopolysaccharide), 1 μg/ml FLA-ST (Flagellin), 100 ng/ml FSL-1, 5 μg/ml GDQ (Gardimoquid), 5 μg/ml single stranded RNA (ssRNA) 40/LyoVecTM , and 5 μM ODN2006, respectively, for 2 days. Expressions of PD-L, PD-L2, FASL on $CD14^+$ monocytes were analyzed by flow cytometry. Expressions of IL-10, TGFβ, and Arginase-1 in $CD14^+$ monocytes were analyzed by intracellular staining and flow cytometry. Data are represented as MFI, and their dot plots are shown in FIG. 34 and FIG. 35. FIG. 31B is a series of histograms showing the effects of cytokines on expressions of PD-L, PD-L2, and FASL. PBMC were treated with 200 U/ml interferon gamma (IFNγ), 200 ng/ml interferon alpha (IFNα), 10 ng/ml tumor necrosis factor alpha (TNFα), respectively, for 2 days. Expressions of PD-L, PD-L2, and FASL on $CD14^+$ monocytes were examined by flow cytometry. Data are represented as MFI, and their dot plots are shown in FIG. 36. FIG. 31C is a series of graphs depicting the effects of $CD14^+$ monocytes on T cell activation. Both $CD4^+$ and $CD8^+$ T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and further treated with anti-CD3 and CD28

beads in presence of either $CD14^-$ or $CD14^+$ monocytes for 3 days. Proliferation of T cells was analyzed by flow cytometry. $CD4^+$ $CD8^+$ $CD19^+$ dep: $CD4^+$ $CD8^+$ $CD19^+$ cells of PBMC were depleted. $CD4^+$ $CD8^+$ $CD19^+$ $CD14^+$ dep: $CD4^+$ $CD8^+$ $CD19^+$ $CD14^+$ cells of PBMC were depleted. $CD4^+$ Treg: $CD4^+$ $CD25^+$ T cells.

FIG. 32A- FIG. 32C is a series of histograms, graphs, and images depicting the effects of anti-Ang peptibody on human $CD14^+$ monocytes and T cell activation. FIG. 32A is a series of histograms depicting the effects of anti-Ang peptibody on $CD14^+$ monocytes. Adherent monocytes and PBMC were treated with Ang-1 and-2 in the presence or absence of anti-Ang peptibody. $CD14^+$ monocytes were analyzed by flow cytometry. Data are represented as % of $CD14^+$ $PD\text{-}L1^+$ cells (Left panel) or % of $CD14^+$ (Right panel), and their dot plots are shown in FIG. 37A and FIG. 37B. Left panel: adherent monocytes; right panel: PBMC. M4-3 and L1-7 are two different anti-Ang-1/2 peptibodies (AMG386). FIG. 32B is a series of graphs showing the effects of anti-Ang peptibody on T cell activation. Both $CD4^+$ and $CD8^+$ T cells were stained with CFSE and activated with anti-CD3 and CD28 beads, and cocultured with adherent monocytes in the presence or absence of Ang and anti-Ang peptibodies as indicated for 3 days. Proliferation of both $CD4^+$ and $CD8^+$ T cells were analyzed by flow cytometry. FIG. 32C is a series of images illustrating the effects of anti-Ang peptibody in generation of antigen specific T cells. PBMC were stimulated with 10 μg/ml Mart-1 peptide for 7 days in presence or absence of 100 μg/ml peptibodies, and the cells were restimulated with 10 μg/ml Mart-1 in presence of autologous PBMC during enzyme-linked immunospot (ELISPOT) assay.

FIG. 33A- FIG. 33B are a series of graphs depicting human $CD14^+$ $Tie\text{-}2^+$ $PD\text{-}L1^+$ monocytes in PBMC and tumor infiltration cells. $CD14^+$ $Tie\text{-}2^+$ $PD\text{-}L1^+$ monocytes were examined by flow cytometry. FIG. 33A is box plot showing $CD14^+$ $Tie\text{-}2^+$ $PD\text{-}L1^+$ monocytes in PBMC of healthy donors and stage IV melanoma patients. P=0.038. FIG. 33B is are dot plot examples $CD14^+$ $Tie\text{-}2^+$ $PD\text{-}L1^+$ monocytes from PBMC (left panel) and tumor infiltration cells (right panel).

FIG. 34 is a series of graphs of the effects of TSST, PHA, and TLR activators on expressions of PD-L, PD-L2, and FASL in human $CD14^+$ monocytes. PBMC were treated with 5 μg/ml TSST, 5 μg/ml PHA, 20 μg/ml Zymozan, 10 μg/ml pI:C, 10 μg/ml LPS, 1 μg/ml FLA-ST, 100 ng/ml FSL-1, 5 μg/ml GDQ, 5 μg/ml ssRNA40/LyoVecTM , and 5 μM ODN2006,

respectively, for 2 days. Expressions of PD-L, PD-L2, and FASL on $CD14^+$ monocytes were analyzed by flow cytometry. Data are represented as dot plots.

FIG. 35 is a series of graphs showing the effects of TSST, PHA, and TLR activators on expressions of IL-10, TGFβ, and arginase-1 in human $CD14^+$ monocytes. PBMC were treated with 5 μg/ml TSST, 5 μg/ml PHA, 20 μg/ml Zymozan, 10 μg/ml pI:C, 10 μg/ml LPS, 1 μg/ml FLA-ST, 100 ng/ml FSL-1, 5 μg/ml GDQ, 5 μg/ml ssRNA40/LyoVecTM , and 5 μM ODN2006, respectively, for 2 days. Expressions of IL-10, TGFβ, and arginase-1 in $CD14^+$ monocytes were analyzed by intracellular staining and flow cytometry. Data are represented as dot plots.

FIG. 36 is a graph depicting the effects of cytokines on expressions of PD-L, PD-L2, and FASL. PBMC were treated with 200 U/ml IFNγ, 200 ng/ml IFNα, 10 ng/ml TNFα, respectively, for 2 days. Expressions of PD-L, PD-L2, and FASL on $CD14^+$ monocytes were examined by flow cytometry. Data are represented as dot plots.

FIG. 37A- FIG. 37B are a series of graphs depicting the effects of anti-Ang on human $CD14^+$ monocytes. Adherent monocytes or PBMC were treated with Ang-1/2 in the presence or absence of anti-Ang peptibody. $CD14^+$ monocytes were analyzed by flow cytometry. FIG. 37A is a series of graphs showing adherent monocytes. FIG. 37B is a series of graphs showing PBMC. M4-3 and L1-7 are two different anti-Ang-1/2 peptibodies (AMG386). Data are represented as dot plots.

FIG. 38 is a series of images and charts depicting a study schema examples for Phase 1b dual drug clinical trial.

FIG. 39A-FIG. 39F is a series of graphs depicting high pretreatment angiopoietin 2 (ANGPT2; ANG-2) concentrations and increases in serum ANGPT2 were associated with poor clinical outcomes to immune checkpoint therapy in metastatic melanoma. FIG. 39A and FIG. 39B show Kaplan–Meier survival curves of pooled data from patients receiving ipilimumab or ipilimumab plus bevacizumab, based on ANGPT2 pretreatment concentrations (FIG. 39A, n ¼ 91) and fold changes (FIG. 39B, n ¼ 84). FIG. 39C shows ANGPT2 fold changes and clinical responses in pooled patients receiving ipilimumab or ipilimumab plus bevacizumab (n ¼ 84). Each bar represents a patient and its color indicates clinical response of the patient. FIG. 39D shows Kaplan–Meier survival curves of PD-1 blockade–treated patients by pretreatment ANGPT2 levels (n ¼ 43). FIG. 39E shows proportions of PD-1 blockade–treated patients with

PR, SD, and PD by ANGPT2 fold changes (n ¼ 43). FIG> 39F shows ANGPT2 fold changes and clinical responses to PD-1 blockade (n ¼ 43).

FIG. 40A-FIG. 40E is a series of graphs depicting high pretreatment serum ANGPT2 concentrations followed by treatment-induced increases were associated with the worst OS and progressive disease. Data sets from patients receiving ipilimumab, ipilimumab plus bevacizumab or PD-1 blockade were combined and analyzed. FIG 40A shows Kaplan–Meier survival curves based on pretreatment ANGPT2 levels (n ¼ 134). FIG. 40B shows Kaplan–Meier survival curves by ANGPT2 fold changes (n ¼ 127). FIG. 40C shows proportions of patients with complete remission/partial remission (CR/PR), stable disease (SD) and progressive disease (PD) according to ANGPT2 fold changes (n ¼ 127). FIG. 40D shows Kaplan–Meier survival curves based on pretreatment ANGPT2 concentrations and fold changes (n ¼ 127). FIG. 40E shows proportions of patients with CR/PR, SD, and PD by the combination of pretreatment ANGPT2 levels and fold changes (n ¼ 127).

FIG. 41A-FIG. 41D is a series of graphs and images depicting PD-1 blockade and ipilimumab increased, whereas ipilimumab plus bevacizumab (Ipi-Bev) decreased serum ANGPT2 in significant proportions of patients. FIG. 41A shows proportions of patients displayed increase (fold change 1.25), decrease (fold change 0.75) or no change (0.75 < fold change < 1.25) in ANGPT2 in response to immune checkpoint therapy. FIG. 41B shows ipilimumab plus bevacizumab-treated patients (n ¼ 43) displayed smaller fold changes than patients receiving ipilimumab (n ¼ 41) or PD-1 blockade (n ¼ 43). The diamonds, horizontal lines, and upper and lower boundaries of the boxes represent the sample average, median, 75th and 25th percentiles, respectively. FIG. 41C shows bevacizumab (Bev) downregulated ANGPT2 expression in TEC. FIG. 41D shows VEGF promoted ANGPT2 expression and bevacizumab blocked VEGF-induced ANGPT2 expression in TEC. Representative images of two experiments are shown.

FIG. 42A-FIG. 42E is a series of graphs and images depicting ipilimumab and ipilimumab plus bevacizumab influenced tumor ANGPT2 expression and macrophage infiltration. Paired and sequential pretreatment and posttreatment tumor biopsies were stained with anti-ANGPT2, anti-CD68, and anti-CD163, respectively. FIG. 42A shows ANGPT2 upregulation was accompanied by increased infiltration of CD68þ and CD163þ macrophages in posttreatment tumor of an ipilimumab-treated patient. FIG. 42B and FIG. 42C show ANGPT2

downregulation and upregulation in posttreatment tumor vasculature of ipilimumab plus bevacizumab-treated patients was respectively accompanied by decreased and increased infiltration of CD68þ and CD163þ macrophages. FIG. 42D and FIG. 42E, show semiquantitative analysis of macrophage infiltration in tumors with increased (D, n ¼ 4) and decreased (E, n ¼ 3) vascular ANGPT2 expression.

FIG. 43A-FIG. 43C is a series of graphs depicting ANGPT2 induces PD-L1 expression on M2-polarized monocyte derived macrophages (MDMs). FIG. 43A–FIG. 43C show that MDMs were differentiated from monocytes with colony stimulating factor 1 (CSF1) and then treated with ANGPT2 (300 ng/mL) for 3 days in the presence of CSF1 (FIG. 43A) or for 2 days in the presence of IL10 (FIG. 43B) or IL4 (FIG. 43C). MDMs were sequentially stained with PE-conjugated PD-L1 antibody and fluorescein isothiocyanate (FITC)-conjugated CD68 antibody. Macrophages were gated on forward scatter/side scatter and analyzed for CD68 and PD-L1 expression (FIG. 43A) or gated on CD68þ cells and analyzed for PD-L1 expression (FIG. 43B and FIG. 43C). Representative results of at least 4 independent experiments are shown.

FIG. 44A- FIG. 44F is a series of graphs and images showing immune checkpoint therapy elicited antibody responses to ANGPT2. FIG. 44A is an immunoblot assay showing that ANGPT2 antibodies were detected in pretreatment and posttreatment plasma samples of ipilimumab plus bevacizumab-treated patients. FIG. 44B is a graph depicting ELISA data showing that ANGPT2 antibodies were detected in pretreatment and posttreatment plasma samples of ipilimumab plus bevacizumab-treated patients. Clinical responses are also indicated. FIG. 44C is a graph depicting proportions of patients receiving ipilimumab plus bevacizumab (n ¼ 43), ipilimumab (n ¼ 36), and PD-1 blockade (n ¼ 38) displayed an increase by 40% or more in ANGPT2 antibody concentrations. FIG. 44D is a graph of longitudinal analysis of serum ANGPT2 and/or ANGPT2 antibodies in patients receiving ipilimumab plus bevacizumab. FIG. 44E is a graph of longitudinal analysis of serum ANGPT2 and/or ANGPT2 antibodies in patients receiving ipilimumab. FIG. 44F is a graph of longitudinal analysis of serum ANGPT2 and/or ANGPT2 antibodies in patients receiving PD-1 blockade. Dosing of ipilimumab, bevacizumab, or nivolumab was indicated on the x-axis. Day 0 is pretreatment.

FIG. 45A-FIG. 45D are a series of graphs depicting Kaplan-Meier survival curves of patients receiving ipilimumab or ipilimumab plus bevacizumab. FIG. 45A shows ipilimumab-treated patients according to pre-treatment ANGPT2 levels (n = 47). FIG. 45B shows

ipilimumab plus bevacizumab-treated patients based on pre-treatment Ang-2 levels (n = 43). FIG. 45C shows ipilimumab-treated patients according to ANGPT2 fold changes (n = 41). FIG. 45D shows ipilimumab plus bevacizumab-treated patients based on ANGPT2 fold changes (n = 43).

FIG. 46A-FIG. 46B is a series of graphs showing clinical responses and survival of PD-1 blockade-treated patients based on serum ANGPT2 fold changes. FIG. 46A shows fold changes of SD (n = 11) and PD (n = 16) patients were significantly larger than those of PR (n = 16) patients. The diamonds and horizontal lines within the boxes, and the upper and lower boundaries of the boxes represent the sample average, median, 75th and 25th percentiles, respectively. PR vs. PD, P = 0.007; PR vs. SD, P = 0.002; SD vs. PD, P = 0.87. FIG. 46B shows overall survival of PD-1 blockade-treated patients by ANGPT2 fold change (n = 43).

FIG. 47 is a series of westernblot images depicting hypoxia upregulated ANGPT2 expression in melanoma cells. Melanoma cells were treated with VEGF and/or bevacizumab (Bev) in Dulbecco's Modified Eagle Medium (DMEM) containing 1% Fetal Bovine Serum (FBS) for 48 hours under normoxic (21% O2) and hypoxic (1% O2) conditions. ANGPT2 expression was determined by immunoblot analysis.

FIG. 48A-FIG. 48C are a series of graphs depicting ANGPT2 enhanced PD-L1 expression on CSF1-activated MDM from healthy donors. FIG. 48A shows surface marker expression of CSF-1, IL4, or IL10-activated MDM. Monocytes were isolated from PBMC by adhesion and differentiated into macrophages with CSF1 treatment for 6 days. MDM were then treated with CSF1, IL4 or IL10 for 2 more days and stained with anti-CD80 (APC-conjugated) and anti-CD163 (PE-conjugated) antibodies. MDM were gated on Side SCatter/Forward SCatter (SSC/FSC) and analyzed for CD80 and CD163 expression. FIG. 48B shows CSF1-activated MDM were treated with ANGPT2 (300 ng/ml) for 3 days and stained with anti-human PD-L1 (PE-conjugated) antibody and then with anti-CD68 (FITC-conjugated) antibody after permeabilization/Fixation. MDM were gated on FSC/SSC and analyzed for CD68 and PD-L1 expression. FIG. 48C shows CSF1 activated MDM were treated with ANGPT2 for 24 hours and stained with PD-L1 (PE-conjugated). MDM were gated on FSC/SSC and analyzed for PD-L1 expression.

FIG. 49A-FIG. 49C are a series of graphs depicting antibody responses to ANGPT2 and clinical responses of melanoma patients receiving immune checkpoint therapy. Patients were

plotted based on their ANGPT2 antibody fold changes. Each bar represents a patient and its color indicates clinical response of the patient. FIG. 49A shows ipilimumab plus bevacizumab-treated patients (n = 43). FIG. 49B shows ipilimumab-treated patients (n = 36). FIG. 49C shows PD-1 blockade-treated patients (n = 38).

FIG. 50A-FIG. 50B are a series of graphs showing enriched endogenous anti-ANGPT2 antibodies inhibited ANGPT2-mediated extracellular signal-regulated kinases 1 and 2 (Erk1/2) phosphorylation. Endogenous anti-ANGPT2 antibodies were enriched from post-treatment plasma samples of patient P26 using recombinant human ANGPT2 coupled to magnetic beads. FIG. 50A shows enriched anti-ANGPT2 antibodies recognized ANGPT2. The enriched antibodies were incubated with equal amount of recombinant human ANGPT1, ANGPT2 and VEGF spotted onto a membrane and detected with HRP-conjugated anti-human IgG antibody. FIG. 50B shows enriched anti- ANGPT2 antibodies inhibited ANGPT2-mediated Erk1/2 phosphorylation in human umbilical vein endothelial cells (HUVECs). Serum-starved HUVEC were treated with ANGPT2 (400 ng/ml) that had been pre-incubated with normal human IgG or enriched ANGPT2 antibodies (1200 ng/ml) for 15 min. Erk1/2 phosphorylation was determined by immunoblot analysis of whole cell lysates.

DETAILED DESCRIPTION OF THE INVENTION

The invention is based, at least in part, upon the discovery that the combination of pembrolizumab and trebananib is unexpectedly more effective at treating cancer than either drug alone.

Treatment protocol overview

Described herein is a treatment for subjects with cancer, e.g., solid tumors. Also described herein is the evaluation of the safety, clinical, and immunological effect of the combination of pembrolizumab (MK-3475) and trebananib (AMG386). As described in detail below, the treatment includes an induction phase of pembrolizumab and trebananib for 4 cycles (12 wks) followed by pembrolizumab alone for 2 years. The study accrues up to 60 subjects. This trial is conducted in 2 parts. Part I uses a standard $3^{+}3$ dose escalation design in all solid tumors. The goal of Part I is to identify the recommended part 2 (expansion cohort) doses (RP2D) for the combination of pembrolizumab plus trebananib (AMG386). Dose escalation begins in dose cohort $^{+}1$. If two or more patients in dose cohort $^{+}1$ experience a dose limiting

toxicity (DLT), the next cohort of patients are enrolled into dose cohort -1. Should dose cohort -1 prove too toxic, enrollment to the study stops. If the toxicity profile of dose cohort $^{+}1$ is acceptable, the next cohort are enrolled into dose cohort $^{+}2$. Should dose cohort $^{+}2$ have acceptable toxicity, that is the RP2D; otherwise, dose cohort $^{+}1$ is the RP2D.

Part II proceeds with four dose expansion cohorts: melanoma, renal cell carcinoma, ovarian cancer, and colorectal cancer. For each disease type, 12 patients are enrolled and treated at the RP2D of pembrolizumab and trebananib (AMG386). Safety assessments include all patients receiving one or more doses of the study drug combinations. Secondary and correlative endpoints are based on the cohorts of patients enrolled in Part II of the trial. Secondary and correlative endpoints are summarized according to disease indication and, in an exploratory fashion, with all patients combined. Pre-and post-treatment biopsies are obtained in at least 20 patients enrolled in the dose expansion cohorts with different disease types.

Described herein is a determination of the safety, tolerability, and RP2D for trebananib when given with pembrolizumab in patients with metastatic solid tumor (Part I). Also described herein is a determination of the safety and tolerability of the RP2D of trebananib, determined in Part 1, when given with pembrolizumab in patients with unresectable stage III or stage IV melanoma, metastatic renal cell, ovarian, or colorectal cancer (Part 2 (expansion cohort)). Also described herein is the identification of preliminary estimates of progression free survival (PFS) at 6 months; the rate of 1-year overall (OS); the response rate (RR); and of time to progression. Also described herein is an assessment of positron emission tomography (PET) response versus RECIST versus irRC criteria.

As described in detail below, the effect of the combination of the PD-1 inhibitor and/or Ang-2 inhibitor on vasculopathy, immune infiltration, and tumor necrosis is identified by staining pathologic specimens for VEGF/VEGFR expression, phosphoTie-2 and other targets. Described herein is the investigation of immune responses in the periphery to VEGFR, Tie-2, and other angiogenic molecules and tumor-specific antigens as a function of treatment.

Immune checkpoint blockade

Immune checkpoint therapies targeting CTLA-4 and PD-1 have proven effective in cancer treatment. However, prior to the invention described herein, the identification of biomarkers for predicting clinical outcomes and mechanisms to overcome resistance remained as critical needs. Angiogenesis is increasingly appreciated as an immune modulator. Angiopoietin-2

(Ang-2; ANGPT2) is an immune target in patients and is involved in resistance to anti-VEGF treatment with the monoclonal antibody bevacizumab. As described herein, the predictive and prognostic value of circulating ANGPT2 in metastatic melanoma patients receiving immune checkpoint therapy was investigated. As described in detail below, high pretreatment serum ANGPT2 was associated with reduced overall survival in CTLA-4 and PD-1 blockade–treated patients. These treatments also increased serum ANGPT2 in many patients early after treatment initiation, whereas ipilimumab plus bevacizumab treatment decreased serum concentrations. ANGPT2 increases were associated with reduced response and/or overall survival. Ipilimumab increased, and ipilimumab plus bevacizumab decreased, tumor vascular ANGPT2 expression in a subset of patients, which was associated with increased and decreased tumor infiltration by $CD68^+$ and $CD163^+$ macrophages, respectively. In vitro, bevacizumab blocked VEGF-induced ANGPT2 expression in tumor-associated endothelial cells, whereas ANGPT2 increased PD-L1 expression on M2-polarized macrophages. As described herein, treatments elicited long-lasting and functional antibody responses to ANGPT2 in a subset of patients receiving clinical benefit. The results presented herein suggest that serum ANGPT2 is a predictive and prognostic biomarker for immune checkpoint therapy and contributes to treatment resistance via increasing proangiogenic and immunosuppressive activities in the tumor microenvironment. Accordingly, as described herein, targeting ANGPT2 provides a rational combinatorial approach to improve the efficacy of immune therapy.

Pharmaceutical and Therapeutic Background of Pembrolizumab

The importance of intact immune surveillance in controlling outgrowth of neoplastic transformation has been known for decades. Accumulating evidence shows a correlation between tumor-infiltrating lymphocytes (TILs) in cancer tissue and favorable prognosis in various malignancies. In particular, the presence of $CD8^+$ T-cells and the ratio of $CD8^+$ effector T-cells / $FoxP3^+$ regulatory T-cells seems to correlate with improved prognosis and long-term survival in many solid tumors.

The PD-1 receptor-ligand interaction is a major pathway hijacked by tumors to suppress immune control. The normal function of PD-1, expressed on the cell surface of activated T-cells under healthy conditions, is to down-modulate unwanted or excessive immune responses, including autoimmune reactions. PD-1 (encoded by the gene Pdcd1) is an Ig superfamily member related to CD28 and CTLA-4 which has been shown to negatively regulate antigen

receptor signaling upon engagement of its ligands (PD-L1 and/or PD-L2). The structure of murine PD-1 has been resolved. PD-1 and family members are type I transmembrane glycoproteins containing an Ig Variable-type (V-type) domain responsible for ligand binding and a cytoplasmic tail which is responsible for the binding of signaling molecules. The cytoplasmic tail of PD-1 contains 2 tyrosine-based signaling motifs, an immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). Following T-cell stimulation, PD-1 recruits the tyrosine phosphatases Src homology region 2 domain-containing phosphatase-1/2 (SHP-1 and SHP-2) to the ITSM motif within its cytoplasmic tail, leading to the dephosphorylation of effector molecules such as CD3ζ, PKCθ and ZAP70 which are involved in the CD3 T-cell signaling cascade. The mechanism by which PD-1 down modulates T-cell responses is similar to, but distinct from that of CTLA-4 as both molecules regulate an overlapping set of signaling proteins. PD-1 was shown to be expressed on activated lymphocytes including peripheral $CD4^+$ and $CD8^+$ T-cells, B-cells, T regs and Natural Killer cells.

Binding of either PD-1 ligand to PD-1 inhibits T-cell activation triggered through the T-cell receptor. PD-L1 is expressed at low levels on various non-hematopoietic tissues, most notably on vascular endothelium, whereas PD-L2 protein is only detectably expressed on antigen-presenting cells found in lymphoid tissue or chronic inflammatory environments. PD-L2 is thought to control immune T-cell activation in lymphoid organs, whereas PD-L1 serves to dampen unwarranted T-cell function in peripheral tissues. Although healthy organs express little (if any) PD-L1, a variety of cancers were demonstrated to express abundant levels of this T-cell inhibitor. PD-1 has been suggested to regulate tumor-specific T-cell expansion in subjects with melanoma (MEL). This suggests that the PD-1/PD-L1 pathway plays a critical role in tumor immune evasion and should be considered as an attractive target for therapeutic intervention.

Immune checkpoint blockade (CTLA-4) and anti-angiogenesis

Human immune responses against cancer can be suppressed through various mechanisms during disease progression such that cancers evade immune recognition and anti-tumor effector functions. The expression of immune regulatory molecules such as cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and programmed death 1 (PD-1) inhibits the proliferation and function of conventional T cells. Immune checkpoint blockade with ipilimumab (CTLA-4 blockade) has revealed improved survival in patients with metastatic melanoma. Blockade of

PD-1 and PD-L1 interactions has also revealed durable clinical benefits in patients with a variety of cancers including melanoma, non-small cell lung cancer, and renal cell carcinoma. Prior to the invention described herein, efforts were needed to better understand treatment modality combinations that could improve efficacy of immune checkpoint blockade. This would include clinical benefits in cancers that exhibit limited efficacy to checkpoint blockade alone. There is increasing evidence of the role that angiogenic factors play in affecting immune regulation as well as immune effector cell trafficking into tumors. Soluble VEGF (sVEGF) predicts clinical benefit to ipilimumab therapy.

Anti-CTLA-4 blockade

Ipilimumab is a fully human monoclonal antibody that blocks the costimulatory checkpoint molecule CTLA-4. The anti-tumor mechanism of action involves amplification of T cells by blocking endogenous CTLA-4 with resultant T cell proliferation and tumor cell killing. Activity has been observed when administered as a single agent or in combination with other immunotherapies such as vaccines or interleukin-2 (IL-2) as well as when combined with chemotherapy, and in multiple indications including melanoma.

CTLA-4 blockade with ipilimumab leads to improved overall survival in patients with advanced melanoma as documented in two phase III studies, emphasizing the antitumor activity of immune checkpoint blockade. The published response rate is approximately 18%, with a substantial number of responses being durable and/or complete. Recent long-term analyses of follow up for patients treated with ipilimumab reveal a durable 22% survival rate with an inflection and flattening of the survival curves at approximately 3 years, demonstrating long term benefits for patients. As described herein, anti-angiogenesis is one modality pursued to combine with checkpoint blockade to improve efficacy.

Pembrolizumab

Pembrolizumab is a potent and highly selective humanized monoclonal antibody (mAb) of the IgG4/kappa isotype designed to directly block the interaction between PD-1 and its ligands, PD-L1 and PD-L2. Keytruda® (pembrolizumab) has been approved in the United Stated for the treatment of unresectable or metastatic melanoma, metastatic non-small cell lung cancer (NSCLC) whose tumors express programmed death ligand 1 (PD-L1), and recurrent metastatic squamous cell carcinoma of the head and neck.

More specifically, pembrolizumab is a humanized anti-PD-1 mAb of the IgG4/kappa isotype with a stabilizing S228P sequence alteration in the fragment crystallizable (Fc) region. Pembrolizumab binds to human PD-1 and blocks the interaction between PD-1 and its ligands. The theoretical molecular weight of the polypeptide is 146,288 Da and its theoretical pI is 7.5. Additional information on pembrolizumab nomenclature is detailed in the following table:

Table 1

Code Name	MK-3475 (Anti–PD-1)
Other Code Name	MK3, 02P106, ORG 307448-0, SCH 900475 (Anti–PD-1)
Chemical Name	Humanized X PD-1-mAb (H409A11) IgG4
CAS Number	1374853-91-4
CAS Name	Anti-(human protein PDCD1 (programmed cell death 1)) immunoglobulin G4 (human-Mus musculus monoclonal heavy chain) disulfide with human-Mus musculus monoclonal light chain, dimer
Trade Name	KEYTRUDA®

The sequence of pembrolizumab is set forth below.

> Heavy chain sequence (SEQ ID NO: 2)

QVQLVQSGVE VKKPGASVKV SCKASGYTFT NYYMYWVRQA PGQGLEWMGG INPSNGGTNF
NEKFKNRVTL TTDSSTTTAY MELKSLQFDD TAVYYCARRD YRFDMGFDYW GQGTTVTVSS
ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS
GLYSLSSVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES KYGPPCPPCP APEFLGGPSV
FLFPPKPKDT LMISRTPEVT CVVVDVSQED PEVQFNWYVD GVEVHNAKTK PREEQFNSTY
RVVSVLTVLH QDWLNGKEYK CKVSNKGLPS SIEKTISKAK GQPREPQVYT LPPSQEEMTK
NQVSLTCLVK GFYPSDIAVE WESNGQPENN YKTTPPVLDS DGSFFLYSRL TVDKSRWQEG
NVFSCSVMHE ALHNHYTQKS LSLSLGK

> Light chain sequence (SEQ ID NO: 24)

EIVLTQSPAT LSLSPGERAT LSCRASKGVS TSGYSYLHWY QQKPGQAPRL LIYLASYLES
GVPARFSGSG SGTDFTLTIS SLEPEDFAVY YCQHSRDLPL TFGGGTKVEI KRTVAAPSVF
IFPPSDEQLK SGTASVVCLL NNFYPREAKV QWKVDNALQS GNSQESVTEQ DSKDSTYSLS
STLTLSKADY EKHKVYACEV THQGLSSPVT KSFNRGEC

(Dimer; disulfide bridges: H22-H96, H134-L218, H147-H203, H226-H'226, H229-H'229, H261-H321, H367-H425, H'22-H'96, H'134-L'218, H'147-H'203, H'261-H'321, H'367-H'425, L23-L92, L138-L198, L'23-L'92, L'138-L'198.)

Two drug product (DP) dosage forms are available for pembrolizumab: a white to off-white lyophilized powder, 50 mg/vial, and a liquid, 100 mg/vial, both in Type I glass vials intended for single use only.

Pembrolizumab Powder for Solution for Infusion, 50 mg/vial is a lyophilized powder that is reconstituted with sterile water for injection prior to use. It is manufactured using either the

fully formulated DS or the partially formulated DS. The fully formulated DS uses L-histidine as a buffering agent, polysorbate 80 as surfactant, and sucrose as stabilizer/tonicity modifier. Pembrolizumab DP using the partially formultated DS is formulated with L-histidine as a buffering agent, polysorbate 80 as a surfactant, and sucrose as a stabilizer/tonicity modifier, and may contain hydrochloric acid and/or sodium hydroxide for pH adjustment (if necessary). Pembrolizumab Solution for Infusion 100 mg/vial is a liquid DP (manufactured using the fully formulated DS with L-histidine as a buffering agent, polysorbate 80 as a surfactant, and sucrose as a stabilizer/tonicity modifier).

Trebananib

Trebananib is an Fc fusion protein directed against Ang1 and Ang2, expressed recombinantly in *Escherichia coli* (*E. coli*). The molecule is a non-glycosylated homodimer engineered by fusing an immunoglobulin G1 (IgG1) Fc domain to 4 copies of an anti-Ang1 / anti-Ang2 peptide. Each monomeric unit contains 10 cysteine residues that are involved in 4 intrachain disulfide bonds and 2 interchain disulfide bonds. Trebananib contains 287 amino acids. The molecular weight is approximately 63.5 kilodalton (kDa). The sequence of trebananib is set forth below (SEQ ID NO: 1):

MDKTHTCPPC PAPELLGGPS VFLFPPKPKD TLMISRTPEV TCVVVDVSHE DPEVKFNWYV
DGVEVHNAKT KPREEQYNST YRVVSVLTVL HQDWLNGKEY KCKVSNKALP APIEKTISKA
KGQPREPQVY TLPPSRDELT KNQVSLTCLV KGFYPSDIAV EWESNGQPEN NYKTTPPVLD
SDGSFFLYSK LTVDKSRWQQ GNVFSCSVMH EALHNHYTQK SLSLSPGKGG GGGAQQEECE
WDPWTCEHMG SGSATGGSGS TASSGSGSAT HQEECEWDPW TCEHMLE

Trebananib is provided as a sterile, preservative-free, lyophilized powder for reconstitution with sterile water for injection (sWFI) and dilution in normal saline (0.9% sodium chloride) for IV administration. Each sterile vial contains specified amount of deliverable drug product, that when reconstituted with a specified volume of sWFI contains an isotonic formulation of 30 mg/mL trebananib formulated with 10 mM histidine, 4% (weight/volume [w/v]) mannitol, 2% (w/v) sucrose, 10 mM arginine hydrochloride, and 0.01% (w/v) polysorbate 20 to a pH of 7.1. Each vial is for single use only. Lyophilized vials are manufactured in 4 presentations based on the deliverable drug product. The vial presentations, vial sizes, deliverable amount, and reconstitution volume are provided in the table below.

Table 5

Vial Presentation	Vial size	Deliverable Amount	Reconstitution Volume (mL sWFI)
54 mg	5 cc	54 mg	2.0
150 mg	20 cc	150 mg	5.0
240 mg	20 cc	240 mg	8.0
600 mg	50 cc	600 mg	20.0

cc = cubic centimeters; mg = milligram; sWFI = sterile water for injection

Anti-ANG-2 antibodies

Although much evidence points to the usefulness of inhibiting Ang-2 levels in treatment of unwanted angiogenesis (or any subset of conditions involving unwanted generation of blood vessels, like arteriogenesis), simultaneous inhibition of Ang-1 may also be beneficial in such therapies. Accordingly, the Ang-2 inhibitor of the present disclosure may inhibit both Ang-1 and Ang-2 signaling.

In one aspect, the Ang-2 inhibitor is an antibody that specifically binds to Ang-1 and/or Ang-2, and thereby inhibiting Ang-1 and/or Ang-2 binding to Tie1 and/or Tie2 receptors. The antibody may be a chimeric antibody, a humanized antibody, or a fully human antibody. The antibody may bind Ang-1 and/or Ang-2 with a Kd value of less than about 100 pM, 30 pM, 20 pM, 10 pM, 5 pM or 1 pM. In certain embodiments, the antibody is of IgG type, e.g., IgG1, IgG2, IgG3, and IgG4.

Exemplary anti-Ang2 antibodies can be found, e.g., in WO 2009105269, which is incorporated herein by reference. The following exemplary antibodies are disclosed in WO2009105269.

Table 6

Antibody*	Antibody Heavy Chain	Antibody Light Chain
H6L7	H6 HC	L7 LC
H5L7	H5 HC	L7 LC
H4L13	H4 HC	L13 LC
H11L7	H11 HC	L7 LC
H10L7	H10 HC	L7 LC
H4L7	H4 HC	L7 LC
H5L6	H5 HC	L6 LC
H2L7	H2 HC	L7 LC
H5L8	H5 HC	L8 LC
H6L8	H6 HC	L8 LC
H3L7	H3 HC	L7 LC
H5L4	H5 HC	L4 LC
H4L12	H4 HC	L12 LC

H6L6	H6 HC	L6 LC
H4L2	H4 HC	L2 LC
H4L6	H4 HC	L6 LC
H4L4	H4 HC	L4 LC
H5L11	H5 HC	L11 LC
H5L1	H5 HC	L1 LC
H4L11	H4 HC	L11 LC
H5L12	H5 HC	L12 LC
H5L9	H5 HC	L9 LC

Sequences of the variable regions of these antibodies are provided below. The antibody can further comprise any constant region known in the art. The light chain constant region can be, for example, a kappa or lambda light chain constant region, e.g., a human kappa or lambda light chain constant region. The heavy chain constant region can be, for example, an alpha, delta, epsilon, gamma, or mu heavy chain constant region, e.g., a human alpha, delta, epsilon, gamma, or mu heavy chain constant region. In one embodiment, the light or heavy chain constant region is a fragment, derivative, variant, or mutein of a naturally occurring constant region.

Techniques are known for deriving an antibody of a different subclass or isotype from an antibody of interest, i.e., subclass switching. Thus, IgG antibodies may be derived from an IgM antibody, for example, and vice versa. Such techniques allow the preparation of new antibodies that possess the antigen-binding properties of a given antibody (the parent antibody), but also exhibit biological properties associated with an antibody isotype or subclass different from that of the parent antibody. Recombinant DNA techniques may be employed. Cloned DNA encoding particular antibody polypeptides may be employed in such procedures, e.g., DNA encoding the constant domain of an antibody of the desired isotype. See also, Lantto et al., Methods Mol. Biol. 178:303-16 (2002).

In certain embodiments, the antibody comprises the IgG1 heavy chain constant domain or a fragment of the IgG1 heavy chain constant domain. In certain embodiments, the antibody comprises the constant light chain kappa or lambda domains or a fragment thereof. In certain embodiments, the antibody comprises an IgG2 heavy chain constant domain, or a fragment thereof.

Table 7

Heavy Chain Variable Regions	
Antibody HC	Sequence
536 HC	EVQLVQSGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGK

(SEQ ID NO: 3)	GLEWVSYISSSGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRA
	EDTAVYYCARDLLDYDILTGYGYWGQGTLVTVSS
H2	EVQLVQSGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGK
(SEQ ID NO: 4)	GLEWVSYISSSGSTIEYADSVKGRFTISRDNAKNSLYLQMNSLRA
	EDTAVYYCARDLLDYDILTGYGYWGQGTLVTVSS
H3	EVQLVQSGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGK
(SEQ ID NO: 5)	GLEWVSYISSSGSTIQYADSVKGRFTISRDNAKNSLYLQMNSLRA
	EDTAVYYCARDLLDYDILTGYGYWGQGTLVTVSS
H4	EVQLVQSGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGK
(SEQ ID NO: 6)	GLEWVSYISSSGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRA
	EDTAVYYCARDLLDYDLLTGYGYWGQGTLVTVSS
H6	EVQLVQSGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGK
(SEQ ID NO: 7)	GLEWVSYISSSGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRA
	EDTAVYYCARDLLDYDIYTGYGYWGQGTLVTVSS
H10	EVQLVQSGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGK
(SEQ ID NO: 8)	GLEWVSYISSSGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRA
	EDTAVYYCARDLLDYDILTGYGLWGQGTLVTVSS
H11	EVQLVQSGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGK
(SEQ ID NO: 9)	GLEWVSYISSSGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRA
	EDTAVYYCARDLLDYDILTGYGMWGQGTLVTVSS
H5P	EVQLVQSGGGVVQPGRSLRLSCAASGFTFSSYGMHWVRQAPGK
(SEQ ID NO: 10)	GLEWVSYISSSGSTIYYADSVKGRFTISRDNAKNSLYLQMNSLRA
	EDTAVYYCARDLLDYDIWTGYGYWGQGTLVTVSS

Table 8

Light Chain Variable Regions	
Antibody LC	Sequence
536 kappa	DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNYLDWYLQKP
(Ref)	GQSPQLLIYLGSNRASGVPDRFSGSGSGTDFTLKISRVEAEDVGV
(SEQ ID NO: 11)	YYCMQGTHWPPTFGQGTKLEIK
L1	DIVMTQSPLSLPVTPGEPASISCRSIQSLLQSNGYNYLDWYLQKP
(SEQ ID NO: 12)	GQSPQLLIYLGSNRASGVPDRFSGSGSGTDFTLKISRVEAEDVGV
	YYCMQGTHWPPTFGQGTKLEIK
L2	DIVMTQSPLSLPVTPGEPASISCRSSQSLLLSNGYNYLDWYLQKP
(SEQ ID NO: 13)	GQSPQLLIYLGSNRASGVPDRFSGSGSGTDFTLKISRVEAEDVGV
	YYCMQGTHWPPTFGQGTKLEIK
L4	DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSHGYNYLDWYLQKP
(SEQ ID NO: 14)	GQSPQLLIYLGSNRASGVPDRFSGSGSGTDFTLKISRVEAEDVGV
	YYCMQGTHWPPTFGQGTKLEIK
L6	DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSVGYNYLDWYLQKP
(SEQ ID NO: 15)	GQSPQLLIYLGSNRASGVPDRFSGSGSGTDFTLKISRVEAEDVGV

	YYCMQGTHWPPTFGQGTKLEIK
L7	DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNFLDWYLQKP
(SEQ ID NO: 16)	GQSPQLLIYLGSNRASGVPDRFSGSGSGTDFTLKISRVEAEDVGV
	YYCMQGTHWPPTFGQGTKLELK
L8	DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNMLDWYLQKP
(SEQ ID NO: 17)	GQSPQLLIYLGSNRASGVPDRFSGSGSGTDFTLKISRVEAEDVGV
	YYCMQGTHWPPTFGQGTKLEIK
L9	DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNYLDWYLQKP
(SEQ ID NO: 18)	GQSPQLLIYAGSNRASGVPDRFSGSGSGTDFTLKISRVEAEDVGV
	YYCMQGTHWPPTFGQGTKLEIK
L11	DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNYLDWYLQKP
(SEQ ID NO: 19)	GQSPQLLIYLGSDRASGVPDRFSGSGSGTDFTLKISRVEAEDVGV
	YYCMQGTHWPPTFGQGTKLEIK
L12	DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNYLDWYLQKP
(SEQ ID NO: 20)	GQSPQLLIYLGSNRASGVPDRFSGSGSGTDFTLKISRVEAEDVGV
	YYCMQATHWPPTFGQGTKLEIK
L13	DIVMTQSPLSLPVTPGEPASISCRSSQSLLHSNGYNYLDWYLQKP
(SEQ ID NO: 21)	GQSPQLLIYLGSNRASGVPDRFSGSGSGTDFTLKISRVEAEDVGV
	YYCMQVTHWPPTFGQGTKLEIK

Kappa Light Constant domain (SEQ ID NO: 22):

RTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQES
VTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

IgG2 Heavy Constant domain (SEQ ID NO: 23):

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA
VLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCP
APPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVH
NAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKT
KGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Methods of treatment

One aspect of the invention relates to a method of treating cancer in a patient (e.g., a human patient), the method comprising administering to the patient a prophylactically effective regimen or a therapeutically effective regimen, the regimen comprising administering to the patient a PD-1 inhibitor and/or Ang-2 inhibitor of the invention or a composition of the invention, e.g., a combination of a PD-1 inhibitor and an Ang-2 inhibitor, wherein the patient has been diagnosed with cancer. The amount of a PD-1 inhibitor and/or Ang-2 inhibitor of the

invention used in the prophylactic and/or therapeutic regimens which will be effective in the treatment of cancer can be based on the currently prescribed dosage of the PD-1 inhibitor and/or Ang-2 inhibitor as well as assessed by methods disclosed herein.

In one example, the cancer is a hematologic cancer. For instance, the cancer is leukemia, lymphoma or myeloma. In another example, the cancer is a solid tumor. In some cases, the patient has undergone a primary therapy to reduce the bulk of a solid tumor prior to therapy with the compositions and methods described herein. For example, the primary therapy to reduce the tumor bulk size is a therapy other than a PD-1 inhibitor and/or Ang-2 inhibitor of the invention. For example, the solid tumor is fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon cancer, colorectal cancer, kidney cancer, pancreatic cancer, bone cancer, breast cancer, ovarian cancer, prostate cancer, esophageal cancer, stomach cancer, oral cancer, nasal cancer, throat cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, uterine cancer, testicular cancer, small cell lung carcinoma, bladder carcinoma, lung cancer, epithelial carcinoma, glioma, glioblastoma multiforme, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, skin cancer, melanoma, neuroblastoma, retinoblastoma, embryonal brain tumor, primitive neuroectodermal tumor (PNET), or choroid plexus tumor.

Melanoma

Melanoma is a cancer that usually starts in a certain type of skin cell, i.e., melanocytes. Other names for “melanoma” include malignant melanoma and cutaneous melanoma. Most melanoma cells still make melanin, so melanoma tumors are usually brown or black. However, some melanomas do not make melanin and can appear pink, tan, or even white. Melanomas can develop anywhere on the skin, but they are more likely to start on the trunk (chest and back) in men and on the legs in women. The neck and face are other common sites. Having darkly pigmented skin lowers the risk of melanoma at these more common sites, but anyone can get

melanoma on the palms of the hands, soles of the feet, and under the nails. Melanomas can also form in other parts of the body such as the eyes, mouth, genitals, and anal area, but these are much less common than melanoma of the skin. Melanoma is much less common than basal cell and squamous cell skin cancers. However, melanoma is more dangerous because it is much more likely to spread to other parts of the body if not caught early.

Melanoma may spread to other sites in the body by metastasis. Metastatic melanoma may cause nonspecific paraneoplastic symptoms, including loss of appetite, nausea, vomiting and fatigue. Metastasis of early melanoma is possible, but relatively rare: less than a fifth of melanomas diagnosed early become metastatic. Brain metastases are particularly common in patients with metastatic melanoma. Melanoma may also spread to the liver, bones, abdomen or distant lymph nodes.

Melanoma diagnosis

Visual inspection is the most common diagnostic technique. Moles that are irregular in color or shape are typically treated as candidates. To detect melanomas (and increase survival rates), it is recommended to regularly examine moles for changes (shape, size, color, itching or bleeding) and to consult a qualified physician when a candidate appears.

Early signs of melanoma are changes to the shape or color of existing moles or, in the case of nodular melanoma, the appearance of a new lump anywhere on the skin. At later stages, the mole may itch, ulcerate or bleed. Early signs of melanoma are summarized by the mnemonic "ABCDE":

- **A**symmetry
- **B**orders (irregular with edges and corners)
- **C**olor (variegated)
- **D**iameter (greater than 6 mm (0.24 in), about the size of a pencil eraser)
- **E**volving over time

These classifications do not, however, apply to the most dangerous form of melanoma, nodular melanoma, which has its own classifications:

- **E**levated above the skin surface
- **F**irm to the touch
- **G**rowing

Following a visual examination and a dermatoscopic exam, or *in vivo* diagnostic tools such as a confocal microscope, the doctor may biopsy the suspicious mole. A skin biopsy performed under local anesthesia is often required to assist in making or confirming the diagnosis and in defining severity. Elliptical excisional biopsies may remove the tumor, followed by histological analysis and Breslow scoring. Punch biopsies are contraindicated in suspected melanomas, for fear of seeding tumor cells and hastening the spread of malignant cells.

Lactate dehydrogenase (LDH) tests are often used to screen for metastases, although many patients with metastases (even end-stage) have a normal LDH; extraordinarily high LDH often indicates metastatic spread of the disease to the liver.

It is common for patients diagnosed with melanoma to have chest X-rays and an LDH test, and in some cases CT, MRI, PET and/or PET/CT scans. Although controversial, sentinel lymph node biopsies and examination of the lymph nodes are also performed in patients to assess spread to the lymph nodes.

A diagnosis of melanoma is supported by the presence of the S-100 protein marker. Additionally, HMB-45 is a monoclonal antibody that reacts against an antigen present in melanocytic tumors such as melanomas. It is used in anatomic pathology as a marker for such tumors. The antibody was generated to an extract of melanoma. It reacts positively against melanocytic tumors, but not other tumors, thus demonstrating specificity and sensitivity.

The following are melanoma stages with 5 year survival rates. Stage 0: melanoma in situ (99,9% survival); Stage I/II: invasive melanoma (89-95% survival); Stage II: high risk melanoma (45-79% survival); Stage III: regional metastasis (24-70% survival); Stage IV: distant metastasis (7-19% survival).

Recent evidence suggests that the prognosis of melanoma patients with regional metastases is influenced by tumor stroma immunobiology (Akbani et al., 2015 Cell (161), 1681-1696, incorporated herein by reference).

Renal Cell Carcinoma

Renal cell carcinoma (RCC) is a kidney cancer that originates in the lining of the proximal convoluted tubule, a part of the very small tubes in the kidney that transport primary urine. Based on the symptoms presented, a range of biochemical tests (using blood and/or urine samples) may be considered as part of the screening process to provide sufficient quantitative

analysis of any differences in electrolytes, renal and liver function, and blood clotting times. Upon physical examination, palpation of the abdomen may reveal the presence of a mass or an organ enlargement. Exemplary diagnostic tools for detecting renal cell carcinoma are ultrasound, computed tomography (CT) scanning, and magnetic resonance imaging (MRI) of the kidneys.

The staging of renal cell carcinoma is as follows:

Stage I: Tumor of a diameter of 7 cm (approx. 2 3⁄4 inches) or smaller, and limited to the kidney. No lymph node involvement or metastases to distant organs.

Stage II: Tumor larger than 7.0 cm, but still limited to the kidney. No lymph node involvement or metastases to distant organs.

Stage III: Tumor of any size with involvement of a nearby lymph node, but no metastases to distant organs. Tumor of this stage may be with or without spread to fatty tissue around the kidney, with or without spread into the large veins leading from the kidney to the heart; or

Tumor with spread to fatty tissue around the kidney and/or spread into the large veins leading from the kidney to the heart, but without spread to any lymph nodes or other organs.

Stage IV: Tumor that has spread directly through the fatty tissue and the fascia ligament-like tissue that surrounds the kidney;

Involvement of more than one lymph node near the kidney;

Involvement of any lymph node not near the kidney; or

Distant metastases, such as in the lungs, bone, or brain.

Ovarian Cancer

Ovarian cancer is a cancer that forms in or on an ovary. Symptoms may include bloating, pelvic pain, abdominal swelling, and loss of appetite. Common areas to which the cancer may spread include the lining of the abdomen, lymph nodes, lungs, and liver.

Diagnosis of ovarian cancer starts with a physical examination (including a pelvic examination), a blood test (for CA-125 and sometimes other markers), and transvaginal ultrasound. Diagnosis must be confirmed with surgery to inspect the abdominal cavity, take biopsies (tissue samples for microscopic analysis), and look for cancer cells in the abdominal fluid. Ovarian cancers are staged using the International Federation of Gynecology and Obstetrics (FIGO) staging system.

Colorectal Cancer

Colorectal cancer (CRC), also known as bowel cancer and colon cancer, is the development of cancer from the colon or rectum (parts of the large intestine). Signs and symptoms may include blood in the stool, a change in bowel movements, weight loss and tiredness. Colorectal cancer diagnosis is performed by sampling of areas of the colon suspicious for possible tumor development, typically during colonoscopy or sigmoidoscopy, depending on the location of the lesion. It is confirmed by microscopical examination of a tissue sample. Staging of colorectal cancer is usually made according to the TNM staging system from the WHO organization, the UICC and the AJCC.

World Health Organization Criteria

The WHO Criteria for evaluating the effectiveness of anti-cancer agents on tumor shrinkage, developed in the 1970s by the International Union Against Cancer and the World Health Organization, represented the first generally agreed specific criteria for the codification of tumor response evaluation. These criteria were first published in 1981 (Miller et al., 1981 Clin Cancer Res., 47(1): 207–14, incorporated herein by reference). WHO Criteria proposed >50% tumour shrinkage for a Partial Response and >25% tumour increase for Progressive Disease.

Response Evaluation Criteria in Solid Tumors (RECIST)

RECIST is a set of published rules that define when tumors in cancer patients improve ("respond"), stay the same ("stabilize"), or worsen ("progress") during treatment (Eisenhauer et al., 2009 European Journal of Cncer, 45: 228-247, incorporated herein by reference). Only patients with measureably disease at baseline should be included in protocols where objective tumor response is the primary endpoint.

The response criteria for evaluation of target lesions are as follows:

- Complete Response (CR): Disappearance of all target lesions.
- Partial Response (PR): At least a 30% decrease in the sum of the longest diameter (LD) of target lesions, taking as reference the baseline sum LD.
- Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum LD since the treatment started.
- Progressive Disease (PD): At least a 20% increase in the sum of the LD of target lesions, taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions.

The response criteria for evaluation of non-target lesions are as follows:

- Complete Response (CR): Disappearance of all non-target lesions and normalization of tumor marker level.
- Incomplete Response/ Stable Disease (SD): Persistence of one or more non-target lesion(s) or/and maintenance of tumor marker level above the normal limits.
- Progressive Disease (PD): Appearance of one or more new lesions and/or unequivocal progression of existing non-target lesions.

The response criteria for evaluation of best overall response are as follows. The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for PD the smallest measurements recorded since the treatment started). In general, the patient's best response assignment will depend on the achievement of both measurement and confirmation criteria.

- Patients with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be classified as having "symptomatic deterioration". Every effort should be made to document the objective progression even after discontinuation of treatment.
- In some circumstances, it may be difficult to distinguish residual disease from normal tissue. When the evaluation of complete response depends on this determination, it is recommended that the residual lesion be investigated (fine needle aspirate/biopsy) to confirm the complete response status.

Immune-Related Response Criteria

The immune-related response criteria (irRC) is a set of published rules that define when tumors in cancer patients improve ("respond"), stay the same ("stabilize"), or worsen ("progress") during treatment, where the compound being evaluated is an immuno-oncology drug. The Immune-Related Response Criteria, first published in 2009 (Wolchok J.D., et al., Clin. Cancer Res. 15:7412-20 (2009), incorporated herein by reference), arose out of observations that immuno-oncology drugs would fail in clinical trials that measured responses using the WHO or RECIST Criteria, because these criteria could not account for the time gap in many patients between initial treatment and the apparent action of the immune system to reduce the tumor burden. The key driver in the development of the irRC was the observation that, in studies of various cancer therapies derived from the immune system such as cytokines and

monoclonal antibodies, the looked-for Complete and Partial Responses as well as Stable Disease only occurred after an increase in tumor burden that the conventional RECIST Criteria would have dubbed ''Progressive Disease'. RECIST failed to take account of the delay between dosing and an observed anti-tumour T cell response, so that otherwise 'successful' drugs - that is, drugs which ultimately prolonged life - failed in clinical trials.

The irRC are based on the WHO Criteria; however, the measurement of tumor burden and the assessment of immune-related response have been modified as set forth below.

Measurement of tumor burden

In the irRC, tumor burden is measured by combining 'index' lesions with new lesions. Ordinarily, tumor burden would be measured with a limited number of 'index' lesions (that is, the largest identifiable lesions) at baseline, with new lesions identified at subsequent timepoints counting as 'Progressive Disease'. In the irRC, by contrast, new lesions are a change in tumor burden. The irRC retained the bidirectional measurement of lesions that had originally been laid down in the WHO Criteria.

Assessment of immune-related response

In the irRC, an immune-related Complete Response (irCR) is the disappearance of all lesions, measured or unmeasured, and no new lesions; an immune-related Partial Response (irPR) is a 50% drop in tumor burden from baseline as defined by the irRC; and immune-related Progressive Disease (irPD) is a 25% increase in tumor burden from the lowest level recorded. Everything else is considered immune-related Stable Disease (irSD). Even if tumor burden is rising, the immune system is likely to "kick in" some months after first dosing and lead to an eventual decline in tumor burden for many patients. The 25% threshold accounts for this apparent delay.

Therapy

Another aspect of the invention relates to a method of treating cancer, wherein the patient received another therapy. In some embodiments, the prior therapy is, for example, chemotherapy, radioimmunotherapy, toxin therapy, prodrug-activating enzyme therapy, antibody therapy, surgical therapy, immunotherapy, radiation therapy, targeted therapy or any combination thereof. In some embodiments, the prior therapy has failed in the patient. In some cases, the therapeutically effective regimen comprising administration of a composition of the invention is administered to the patient immediately after patient has undergone the prior

therapy. For instance, in certain cases, the outcome of the prior therapy may be unknown before the patient is administered a PD-1 inhibitor and/or Ang-2 inhibitor of the invention.

In some cases, the therapeutic regimen described herein results in a reduction in the cancer cell population in the patient. In one example, the patient undergoing the therapeutic regimen is monitored to determine whether the regimen has resulted in a reduction in the cancer cell population in the patient. Typically, the monitoring of the cancer cell population is conducted by detecting the number or amount of cancer cells in a specimen extracted from the patient. Methods of detecting the number or amount of cancer cells in a specimen are known in the art. This monitoring step is typically performed at least 1, 2, 4, 6, 8, 10, 12, 14, 15, 16, 18, 20, or 30 days after the patient begins receiving the regimen.

In one aspect, the specimen may be a blood specimen, wherein the number or amount of cancer cells per unit of volume (e.g., 1 mL) or other measured unit (e.g., per unit field in the case of a histological analysis) is quantitated. The cancer cell population, in certain embodiments, can be determined as a percentage of the total blood cells. In other cases, the specimen extracted from the patient is a tissue specimen (e.g., a biopsy extracted from suspected cancerous tissue), where the number or amount of cancer cells can be measured, for example, on the basis of the number or amount of cancer cells per unit weight of the tissue. The number or amount of cancer cells in the extracted specimen can be compared with the numbers or amounts of cancer cells measured in reference samples to assess the efficacy of the regimen and amelioration of the cancer under therapy. For example, the reference sample is a specimen extracted from the patient undergoing therapy, wherein the specimen from the patient is extracted at an earlier time point (e.g., prior to receiving the regimen, as a baseline reference sample, or at an earlier time point while receiving the therapy). In another example, the reference sample is extracted from a healthy, noncancer-afflicted patient.

In other cases, the cancer cell population in the extracted specimen can be compared with a predetermined reference range. In a specific embodiment, the predetermined reference range is based on the number or amount of cancer cells obtained from a population(s) of patients suffering from the same type of cancer as the patient undergoing the therapy.

Pharmaceutical Therapeutics

For therapeutic uses, the PD-1 inhibitor and/or Ang-2 inhibitor described herein may be administered systemically, for example, formulated in a pharmaceutically-acceptable buffer such

as physiological saline. Preferable routes of administration include, for example, subcutaneous, intravenous, interperitoneal, intramuscular, or intradermal injections that provide continuous, sustained levels of the drug in the patient. Treatment of human patients or other animals will be carried out using a therapeutically effective amount of a therapeutic identified herein in a physiologically-acceptable carrier. Suitable carriers and their formulation are described, for example, in Remington's Pharmaceutical Sciences by E. W. Martin. The amount of the PD-1 inhibitor and/or Ang-2 inhibitor to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the clinical symptoms of the neoplasia. Generally, amounts will be in the range of those used for other agents used in the treatment of other diseases associated with neoplasia, although in certain instances lower amounts will be needed because of the increased specificity of the PD-1 inhibitor and/or Ang-2 inhibitor. For example, a PD-1 inhibitor and/or Ang-2 inhibitor is administered at a dosage that is cytotoxic to a neoplastic cell.

Formulation of Pharmaceutical Compositions

Human dosage amounts can initially be determined by extrapolating from the amount of the PD-1 inhibitor and/or Ang-2 inhibitor used in animal models, as a skilled artisan recognizes it is routine in the art to modify the dosage for humans compared to animal models. In certain embodiments it is envisioned that the dosage may vary from between about 1 μg compound/Kg body weight to about 5000 mg compound/Kg body weight; or from about 5 mg/Kg body weight to about 4000 mg/Kg body weight or from about 10 mg/Kg body weight to about 3000 mg/Kg body weight; or from about 50 mg/Kg body weight to about 2000 mg/Kg body weight; or from about 100 mg/Kg body weight to about 1000 mg/Kg body weight; or from about 150 mg/Kg body weight to about 500 mg/Kg body weight. In other cases, this dose may be about 1, 5, 10, 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1050, 1100, 1150, 1200, 1250, 1300, 1350, 1400, 1450, 1500, 1600, 1700, 1800, 1900, 2000, 2500, 3000, 3500, 4000, 4500, or 5000 mg/Kg body weight. In other aspects, it is envisaged that doses may be in the range of about 5 mg compound/Kg body to about 20 mg compound/Kg body. In other embodiments, the doses may be about 8, 10, 12, 14, 16 or 18 mg/Kg body weight. Of course, this dosage amount may be adjusted upward or downward, as is routinely done in such treatment protocols, depending on the results of the initial clinical trials and the needs of a particular patient.

In some cases, the PD-1 inhibitor and/or Ang-2 inhibitor of the invention is administered at a dose that is lower than the human equivalent dosage (HED) of the no observed adverse effect level (NOAEL) over a period of three months, four months, six months, nine months, 1 year, 2 years, 3 years, 4 years or more. The NOAEL, as determined in animal studies, is useful in determining the maximum recommended starting dose for human clinical trials. For instance, the NOAELs can be extrapolated to determine human equivalent dosages. Typically, such extrapolations between species are conducted based on the doses that are normalized to body surface area (i.e., mg/m^2). In specific embodiments, the NOAELs are determined in mice, hamsters, rats, ferrets, guinea pigs, rabbits, dogs, primates, primates (monkeys, marmosets, squirrel monkeys, baboons), micropigs or minipigs. For a discussion on the use of NOAELs and their extrapolation to determine human equivalent doses, see Guidance for Industry Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers, U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), Pharmacology and Toxicology, July 2005, incorporated herein by reference.

The amount of a PD-1 inhibitor and/or Ang-2 inhibitor of the invention used in the prophylactic and/or therapeutic regimens which will be effective in the treatment of cancer can be based on the currently prescribed dosage of the PD-1 inhibitor and/or Ang-2 inhibitor as well as assessed by methods disclosed herein and known in the art. The frequency and dosage will vary also according to factors specific for each patient depending on the specific PD-1 inhibitor and/or Ang-2 inhibitor administered, the severity of the cancerous condition, the route of administration, as well as age, body, weight, response, and the past medical history of the patient. For example, the dosage of a PD-1 inhibitor and/or Ang-2 inhibitor of the invention which will be effective in the treatment of cancer can be determined by administering the PD-1 inhibitor and/or Ang-2 inhibitor to an animal model such as, e.g., the animal models disclosed herein or known to those skilled in the art. In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges.

In some aspects, the prophylactic and/or therapeutic regimens comprise titrating the dosages administered to the patient so as to achieve a specified measure of therapeutic efficacy. Such measures include a reduction in the cancer cell population in the patient.

In certain cases, the dosage of the PD-1 inhibitor and/or Ang-2 inhibitor of the invention in the prophylactic and/or therapeutic regimen is adjusted so as to achieve a reduction in the number or amount of cancer cells found in a test specimen extracted from a patient after undergoing the prophylactic and/or therapeutic regimen, as compared with a reference sample. Here, the reference sample is a specimen extracted from the patient undergoing therapy, wherein the specimen is extracted from the patient at an earlier time point. In one aspect, the reference sample is a specimen extracted from the same patient, prior to receiving the prophylactic and/or therapeutic regimen. For example, the number or amount of cancer cells in the test specimen is at least 2%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 99% lower than in the reference sample.

In some cases, the dosage of the PD-1 inhibitor and/or Ang-2 inhibitor of the invention in the prophylactic and/or therapeutic regimen is adjusted so as to achieve a number or amount of cancer cells that falls within a predetermined reference range. In these embodiments, the number or amount of cancer cells in a test specimen is compared with a predetermined reference range.

In other embodiments, the dosage of the PD-1 inhibitor and/or Ang-2 inhibitor of the invention in prophylactic and/or therapeutic regimen is adjusted so as to achieve a reduction in the number or amount of cancer cells found in a test specimen extracted from a patient after undergoing the prophylactic and/or therapeutic regimen, as compared with a reference sample, wherein the reference sample is a specimen is extracted from a healthy, noncancer-afflicted patient. For example, the number or amount of cancer cells in the test specimen is at least within 60%, 50%, 40%, 30%, 20%, 15%, 10%, 5%, or 2% of the number or amount of cancer cells in the reference sample.

In treating certain human patients having solid tumors, extracting multiple tissue specimens from a suspected tumor site may prove impracticable. In these cases, the dosage of the PD-1 inhibitor and/or Ang-2 inhibitor of the invention in the prophylactic and/or therapeutic regimen for a human patient is extrapolated from doses in animal models that are effective to reduce the cancer population in those animal models. In the animal models, the prophylactic and/or therapeutic regimens are adjusted so as to achieve a reduction in the number or amount of cancer cells found in a test specimen extracted from an animal after undergoing the prophylactic and/or therapeutic regimen, as compared with a reference sample. The reference sample can be a specimen extracted from the same animal, prior to receiving the prophylactic and/or therapeutic

regimen. In specific embodiments, the number or amount of cancer cells in the test specimen is at least 2%, 5%, 10%, 15%, 20%, 30%, 40%, 50% or 60% lower than in the reference sample. The doses effective in reducing the number or amount of cancer cells in the animals can be normalized to body surface area (e.g., mg/m^2) to provide an equivalent human dose.

The prophylactic and/or therapeutic regimens disclosed herein comprise administration of a PD-1 inhibitor and/or Ang-2 inhibitor of the invention or pharmaceutical compositions thereof to the patient in a single dose or in multiple doses (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, or more doses).

In one aspect, the prophylactic and/or therapeutic regimens comprise administration of the PD-1 inhibitor and/or Ang-2 inhibitor of the invention or pharmaceutical compositions thereof in multiple doses. When administered in multiple doses, the PD-1 inhibitor and/or Ang-2 inhibitor or pharmaceutical compositions are administered with a frequency and in an amount sufficient to treat the condition. For example, the frequency of administration ranges from once a day up to about once every eight weeks. In another example, the frequency of administration ranges from about once a week up to about once every six weeks. In another example, the frequency of administration ranges from about once every three weeks up to about once every four weeks.

Generally, the dosage of a PD-1 inhibitor and/or Ang-2 inhibitor of the invention administered to a subject to treat cancer is in the range of 0.01 to 500 mg/kg, e.g., in the range of 0.1 mg/kg to 100 mg/kg, of the subject's body weight. For example, the dosage administered to a subject is in the range of 0.1 mg/kg to 50 mg/kg, or 1 mg/kg to 50 mg/kg, of the subject's body weight, more preferably in the range of 0.1 mg/kg to 25 mg/kg, or 1 mg/kg to 25 mg/kg, of the patient's body weight. In another example, the dosage of a PD-1 inhibitor and/or Ang-2 inhibitor of the invention administered to a subject to treat cancer in a patient is 500 mg/kg or less, preferably 250 mg/kg or less, 100 mg/kg or less, 95 mg/kg or less, 90 mg/kg or less, 85 mg/kg or less, 80 mg/kg or less, 75 mg/kg or less, 70 mg/kg or less, 65 mg/kg or less, 60 mg/kg or less, 55 mg/kg or less, 50 mg/kg or less, 45 mg/kg or less, 40 mg/kg or less, 35 mg/kg or less, 30 mg/kg or less, 25 mg/kg or less, 20 mg/kg or less, 15 mg/kg or less, 10 mg/kg or less, 5 mg/kg or less, 2.5 mg/kg or less, 2 mg/kg or less, 1.5 mg/kg or less, or 1 mg/kg or less of a patient's body weight.

In another example, the dosage of a PD-1 inhibitor and/or Ang-2 inhibitor of the invention administered to a subject to treat cancer in a patient is a unit dose of 0.1 to 50 mg, 0.1 mg to 20 mg, 0.1 mg to 15 mg, 0.1 mg to 12 mg, 0.1 mg to 10 mg, 0.1 mg to 8 mg, 0.1 mg to 7 mg, 0.1 mg to 5 mg, 0.1 to 2.5 mg, 0.25 mg to 20 mg, 0.25 to 15 mg, 0.25 to 12 mg, 0.25 to 10 mg, 0.25 to 8 mg, 0.25 mg to 7 mg, 0.25 mg to 5 mg, 0.5 mg to 2.5 mg, 1 mg to 20 mg, 1 mg to 15 mg, 1 mg to 12 mg, 1 mg to 10 mg, 1 mg to 8 mg, 1 mg to 7 mg, 1 mg to 5 mg, or 1 mg to 2.5 mg.

In another example, the dosage of a PD-1 inhibitor and/or Ang-2 inhibitor of the invention administered to a subject to treat cancer in a patient is in the range of 0.01 to 10 g/m^2, and more typically, in the range of 0.1 g/m^2 to 7.5 g/m^2, of the subject's body weight. For example, the dosage administered to a subject is in the range of 0.5 g/m^2 to 5 g/m^2, or 1 g/m^2 to 5 g/m^2 of the subject's body's surface area.

In another example, the prophylactic and/or therapeutic regimen comprises administering to a patient one or more doses of an effective amount of a PD-1 inhibitor and/or Ang-2 inhibitor of the invention, wherein the dose of an effective amount achieves a plasma level of at least 0.1 μg/mL, at least 0.5 μg/mL, at least 1 μg/mL, at least 2 μg/mL, at least 5 μg/mL, at least 6 μg/mL, at least 10 μg/mL, at least 15 μg/mL, at least 20 μg/mL, at least 25 μg/mL, at least 50 μg/mL, at least 100 μg/mL, at least 125 μg/mL, at least 150 μg/mL, at least 175 μg/mL, at least 200 μg/mL, at least 225 μg/mL, at least 250 μg/mL, at least 275 μg/mL, at least 300 μg/mL, at least 325 μg/mL, at least 350 μg/mL, at least 375 μg/mL, or at least 400 μg/mL of the PD-1 inhibitor and/or Ang-2 inhibitor of the invention.

In another example, the prophylactic and/or therapeutic regimen comprises administering to a patient a plurality of doses of an effective amount of a PD-1 inhibitor and/or Ang-2 inhibitor of the invention, wherein the plurality of doses maintains a plasma level of at least 0.1 μg/mL, at least 0.5 μg/mL, at least 1 μg/mL, at least 2 μg/mL, at least 5 μg/mL, at least 6 μg/mL, at least 10 μg/mL, at least 15 μg/mL, at least 20 μg/mL, at least 25 μg/mL, at least 50 μg/mL, at least 100 μg/mL, at least 125 μg/mL, at least 150 μg/mL, at least 175 μg/mL, at least 200 μg/mL, at least 225 μg/mL, at least 250 μg/mL, at least 275 μg/mL, at least 300 μg/mL, at least 325 μg/mL, at least 350 μg/mL, at least 375 μg/mL, or at least 400 μg/mL of the PD-1 inhibitor and/or Ang-2 inhibitor of the invention for at least 1 day, 1 month, 2 months, 3 months, 4

months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 15 months, 18 months, 24 months or 36 months.

In other embodiments, the prophylactic and/or therapeutic regimen comprises administering to a patient a plurality of doses of an effective amount of a PD-1 inhibitor and/or Ang-2 inhibitor of the invention, wherein the plurality of doses maintains a plasma level of at least 0.1 μg/mL, at least 0.5 μg/mL, at least 1 μg/mL, at least 2 μg/mL, at least 5 μg/mL, at least 6 μg/mL, at least 10 μg/mL, at least 15 μg/mL, at least 20 μg/mL, at least 25 μg/mL, at least 50 μg/mL, at least 100 μg/mL, at least 125 μg/mL, at least 150 μg/mL, at least 175 μg/mL, at least 200 μg/mL, at least 225 μg/mL, at least 250 μg/mL, at least 275 μg/mL, at least 300 μg/mL, at least 325 μg/mL, at least 350 μg/mL, at least 375 μg/mL, or at least 400 μg/mL of the PD-1 inhibitor and/or Ang-2 inhibitor of the invention for at least 1 day, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 15 months, 18 months, 24 months or 36 months.

Combination Therapy

In one example, the PD-1 inhibitor and/or Ang-2 inhibitor are administered in combination therapy, i.e., combined with other agents, e.g., therapeutic agents, that are useful for treating pathological conditions or disorders, such as various forms of cancer. The term "in combination" in this context means that the PD-1 inhibitor and/or Ang-2 inhibitor are given substantially contemporaneously, either simultaneously or sequentially. If given sequentially, at the onset of administration of the second compound, the first of the two compounds is in some cases still detectable at effective concentrations at the site of treatment.

The administration of a compound or a combination of compounds for the treatment of a neoplasia may be by any suitable means that results in a concentration of the therapeutic that, combined with other components, is effective in ameliorating, reducing, or stabilizing a neoplasia. The PD-1 inhibitor and/or Ang-2 inhibitor may be contained in any appropriate amount in any suitable carrier substance, and is generally present in an amount of 1-95% by weight of the total weight of the composition. The PD-1 inhibitor and/or Ang-2 inhibitor may be provided in a dosage form that is suitable for parenteral (e.g., subcutaneously, intravenously, intramuscularly, or intraperitoneally) administration route. The PD-1 inhibitor and/or Ang-2 inhibitor may be formulated according to conventional pharmaceutical practice (see, e.g., Remington: The Science and Practice of Pharmacy (20th ed.), ed. A. R. Gennaro, Lippincott

Williams & Wilkins, 2000 and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York).

Accordingly, in some examples, the prophylactic and/or therapeutic regimen comprises administration of a PD-1 inhibitor and/or Ang-2 inhibitor of the invention in combination with one or more additional anticancer therapeutics. In one example, the dosages of the one or more additional anticancer therapeutics used in the combination therapy is lower than those which have been or are currently being used to treat cancer. The recommended dosages of the one or more additional anticancer therapeutics currently used for the treatment of cancer can be obtained from any reference in the art including, but not limited to, Hardman et al., eds., Goodman & Gilman's The Pharmacological Basis Of Basis Of Therapeutics, 10th ed., McGraw-Hill, New York, 2001; Physician's Desk Reference (60.sup.th ed., 2006), which is incorporated herein by reference in its entirety.

The PD-1 inhibitor and/or Ang-2 inhibitor of the invention and the one or more additional anticancer therapeutics can be administered separately, simultaneously, or sequentially. In various aspects, the PD-1 inhibitor and/or Ang-2 inhibitor of the invention and the additional anticancer therapeutic are administered less than 5 minutes apart, less than 30 minutes apart, less than 1 hour apart, at about 1 hour apart, at about 1 to about 2 hours apart, at about 2 hours to about 3 hours apart, at about 3 hours to about 4 hours apart, at about 4 hours to about 5 hours apart, at about 5 hours to about 6 hours apart, at about 6 hours to about 7 hours apart, at about 7 hours to about 8 hours apart, at about 8 hours to about 9 hours apart, at about 9 hours to about 10 hours apart, at about 10 hours to about 11 hours apart, at about 11 hours to about 12 hours apart, at about 12 hours to 18 hours apart, 18 hours to 24 hours apart, 24 hours to 36 hours apart, 36 hours to 48 hours apart, 48 hours to 52 hours apart, 52 hours to 60 hours apart, 60 hours to 72 hours apart, 72 hours to 84 hours apart, 84 hours to 96 hours apart, 96 hours apart, 120 hours part, or 168 hours apart. In another example, two or more anticancer therapeutics are administered within the same patient visit.

In certain aspects, the PD-1 inhibitor and/or Ang-2 inhibitor of the invention and the additional anticancer therapeutic are cyclically administered. Cycling therapy involves the administration of one anticancer therapeutic for a period of time, followed by the administration of a second anticancer therapeutic for a period of time and repeating this sequential administration, i.e., the cycle, in order to reduce the development of resistance to one or both of

the PD-1 inhibitor and/or Ang-2 inhibitor, to avoid or reduce the side effects of one or both of the PD-1 inhibitor and/or Ang-2 inhibitor, and/or to improve the efficacy of the therapies. In one example, cycling therapy involves the administration of a first anticancer therapeutic for a period of time, followed by the administration of a second anticancer therapeutic for a period of time, optionally, followed by the administration of a third anticancer therapeutic for a period of time and so forth, and repeating this sequential administration, i.e., the cycle in order to reduce the development of resistance to the PD-1 inhibitor and/or Ang-2 inhibitor, to avoid or reduce the side effects of one of the PD-1 inhibitor and/or Ang-2 inhibitor, and/or to improve the efficacy of the PD-1 inhibitor and/or Ang-2 inhibitor.

In another example, the PD-1 inhibitor and/or Ang-2 inhibitor are administered concurrently to a subject in separate compositions. The combination the PD-1 inhibitor and/or Ang-2 inhibitor of the invention may be administered to a subject by the same or different routes of administration.

When a PD-1 inhibitor and/or Ang-2 inhibitor of the invention and the additional anticancer therapeutic are administered to a subject concurrently, the term "concurrently" is not limited to the administration of the PD-1 inhibitor and/or Ang-2 inhibitor at exactly the same time, but rather, it is meant that they are administered to a subject in a sequence and within a time interval such that they can act together (e.g., synergistically to provide an increased benefit than if they were administered otherwise). For example, the PD-1 inhibitor and/or Ang-2 inhibitor may be administered at the same time or sequentially in any order at different points in time; however, if not administered at the same time, they should be administered sufficiently close in time so as to provide the desired therapeutic effect, preferably in a synergistic fashion. The combination of the PD-1 inhibitor and/or Ang-2 inhibitor of the invention can be administered separately, in any appropriate form and by any suitable route. When the components of the combination the PD-1 inhibitor and/or Ang-2 inhibitor are not administered in the same pharmaceutical composition, it is understood that they can be administered in any order to a subject in need thereof. For example, a PD-1 inhibitor and/or Ang-2 inhibitor of the invention can be administered prior to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (e.g., 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6

hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of the additional anticancer therapeutic, to a subject in need thereof. In various aspects, the PD-1 inhibitor and/or Ang-2 inhibitor are administered 1 minute apart, 10 minutes apart, 30 minutes apart, less than 1 hour apart, 1 hour apart, 1 hour to 2 hours apart, 2 hours to 3 hours apart, 3 hours to 4 hours apart, 4 hours to 5 hours apart, 5 hours to 6 hours apart, 6 hours to 7 hours apart, 7 hours to 8 hours apart, 8 hours to 9 hours apart, 9 hours to 10 hours apart, 10 hours to 11 hours apart, 11 hours to 12 hours apart, no more than 24 hours apart or no more than 48 hours apart. In one example, the PD-1 inhibitor and/or Ang-2 inhibitor are administered within the same office visit. In another example, the combination the PD-1 inhibitor and/or Ang-2 inhibitor of the invention are administered at 1 minute to 24 hours apart.

Release of pharmaceutical compositions

Pharmaceutical compositions according to the invention may be formulated to release the PD-1 inhibitor and/or Ang-2 inhibitor substantially immediately upon administration or at any predetermined time or time period after administration. The latter types of compositions are generally known as controlled release formulations, which include (i) formulations that create a substantially constant concentration of the drug within the body over an extended period of time; (ii) formulations that after a predetermined lag time create a substantially constant concentration of the drug within the body over an extended period of time; (iii) formulations that sustain action during a predetermined time period by maintaining a relatively, constant, effective level in the body with concomitant minimization of undesirable side effects associated with fluctuations in the plasma level of the active substance (sawtooth kinetic pattern); (iv) formulations that localize action by, e.g., spatial placement of a controlled release composition adjacent to or in contact with the thymus; (v) formulations that allow for convenient dosing, such that doses are administered, for example, once every one or two weeks; and (vi) formulations that target a neoplasia by using carriers or chemical derivatives to deliver the PD-1 inhibitor and/or Ang-2 inhibitor to a particular cell type (e.g., neoplastic cell). For some applications, controlled release formulations obviate the need for frequent dosing during the day to sustain the plasma level at a therapeutic level.

Any of a number of strategies can be pursued in order to obtain controlled release in which the rate of release outweighs the rate of metabolism of the PD-1 inhibitor and/or Ang-2

inhibitor. In one example, controlled release is obtained by appropriate selection of various formulation parameters and ingredients, including, e.g., various types of controlled release compositions and coatings. Thus, the therapeutic is formulated with appropriate excipients into a pharmaceutical composition that, upon administration, releases the therapeutic in a controlled manner. Examples include single or multiple unit tablet or capsule compositions, oil solutions, suspensions, emulsions, microcapsules, microspheres, molecular complexes, nanoparticles, patches, and liposomes.

Parenteral Compositions

The pharmaceutical composition may be administered parenterally by injection, infusion or implantation (subcutaneous, intravenous, intramuscular, intraperitoneal, or the like) in dosage forms, formulations, or via suitable delivery devices or implants containing conventional, non-toxic pharmaceutically acceptable carriers and adjuvants. The formulation and preparation of such compositions are well known to those skilled in the art of pharmaceutical formulation. Formulations can be found in Remington: The Science and Practice of Pharmacy, supra.

Compositions for parenteral use may be provided in unit dosage forms (e.g., in single-dose ampoules), or in vials containing several doses and in which a suitable preservative may be added (see below). The composition may be in the form of a solution, a suspension, an emulsion, an infusion device, or a delivery device for implantation, or it may be presented as a dry powder to be reconstituted with water or another suitable vehicle before use. Apart from the PD-1 inhibitor and/or Ang-2 inhibitor that reduces or ameliorates a neoplasia, the composition may include suitable parenterally acceptable carriers and/or excipients. The PD-1 inhibitor and/or Ang-2 inhibitor may be incorporated into microspheres, microcapsules, nanoparticles, liposomes, or the like for controlled release. Furthermore, the composition may include suspending, solubilizing, stabilizing, pH-adjusting agents, tonicity adjusting agents, and/or dispersing, agents.

As indicated above, the pharmaceutical compositions according to the invention may be in the form suitable for sterile injection. To prepare such a composition, the suitable active antineoplastic therapeutic(s) are dissolved or suspended in a parenterally acceptable liquid vehicle. Among acceptable vehicles and solvents that may be employed are water, water adjusted to a suitable pH by addition of an appropriate amount of hydrochloric acid, sodium hydroxide or a suitable buffer, 1,3-butanediol, Ringer's solution, and isotonic sodium chloride

solution and dextrose solution. The aqueous formulation may also contain one or more preservatives (e.g., methyl, ethyl or n-propyl p-hydroxybenzoate). In cases where one of the compounds is only sparingly or slightly soluble in water, a dissolution enhancing or solubilizing agent can be added, or the solvent may include 10-60% w/w of propylene glycol.

Controlled Release Parenteral Compositions

Controlled release parenteral compositions may be in form of aqueous suspensions, microspheres, microcapsules, magnetic microspheres, oil solutions, oil suspensions, or emulsions. Alternatively, the active drug may be incorporated in biocompatible carriers, liposomes, nanoparticles, implants, or infusion devices.

Materials for use in the preparation of microspheres and/or microcapsules are, e.g., biodegradable/bioerodible polymers such as polygalactin, poly-(isobutyl cyanoacrylate), poly(2-hydroxyethyl-L-glutam- nine) and, poly(lactic acid). Biocompatible carriers that may be used when formulating a controlled release parenteral formulation are carbohydrates (e.g., dextrans), proteins (e.g., albumin), lipoproteins, or antibodies. Materials for use in implants can be non-biodegradable (e.g., polydimethyl siloxane) or biodegradable (e.g., poly(caprolactone), poly(lactic acid), poly(glycolic acid) or poly(ortho esters) or combinations thereof).

Kits or Pharmaceutical Systems

The present compositions may be assembled into kits or pharmaceutical systems for use in ameliorating a neoplasia. Kits or pharmaceutical systems according to this aspect of the invention comprise a carrier means, such as a box, carton, tube or the like, having in close confinement therein one or more container means, such as vials, tubes, ampoules, or bottles. The kits or pharmaceutical systems of the invention may also comprise associated instructions for using the PD-1 inhibitor and/or Ang-2 inhibitor of the invention.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as, “Molecular Cloning: A Laboratory Manual”, second edition (Sambrook, 1989); “Oligonucleotide Synthesis” (Gait, 1984); “Animal Cell Culture” (Freshney, 1987); “Methods in Enzymology” “Handbook of Experimental Immunology” (Weir, 1996); “Gene Transfer Vectors for Mammalian Cells” (Miller and Calos, 1987); “Current Protocols in Molecular Biology” (Ausubel, 1987); “PCR: The Polymerase

Chain Reaction", (Mullis, 1994); "Current Protocols in Immunology" (Coligan, 1991). These techniques are applicable to the production of the polynucleotides and polypeptides of the invention, and, as such, may be considered in making and practicing the invention. Particularly useful techniques for particular embodiments will be discussed in the sections that follow.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the assay, screening, and therapeutic methods of the invention, and are not intended to limit the scope of what the inventors regard as their invention.

EXAMPLES

Example 1: Phase Ib study to test the safety and potential synergy of pembrolizumab (anti-PD-1) and AMG386 (angiopoietin-2 (Ang-2)) in patients with advanced solid tumor

Study Disease(s):

1. Solid Tumors
2. Melanoma
3. Ovarian Cancer
4. Kidney cancer
5. Colorectal Adenocarcinoma

Agent(s): Pembrolizumab (KEYTRUDA®):
Trebananib (AMG386)

Table 9

STUDY SUMMARY

Abbreviated Title	Pembrolizumab and Trebananib in advanced solid tumors
Trial Phase	Ib
Clinical Indication	Phase Ib: all solid tumors Expansion cohorts: melanoma, ovarian, renal, and colorectal cancer
Trial Type	Interventional
Type of control	No control
Route of administration	Intravenous
Trial Blinding	Unblinded Open Label
Treatment Groups	Phase Ib: one cohort for all solid tumors Expansion cohorts: melanoma, ovarian, renal, and colorectal cancer
Number of trial subjects	60 patients
Estimated enrollment period	1.5 years
Estimated duration of trial	3-4 years
Duration of Participation	2 years
Estimated average length of treatment per patient	1-2 years

Described herein is the safety and augmented anti-tumor activity of dual blockade of PD-1 checkpoint and angiopoietin-2 (Ang-2) angiogenic signaling with the combination of pembrolizumab and AMG386 (Trebananib).

The following is noted for this Example:

a. Developed biopsy and serum biomarker findings from prior clinical studies of patients with advanced malignancies treated with therapeutic administration of granulocyte-macrophage colony-stimulating factor (GMCSF) expressing autologous vaccines followed by anti-CTLA-4 treatment. The studies showed intra and peri-tumoral immune-mediated vasculopathy in long term responders, and also detected elevated titers of autoantibodies to angiogenic factors, including members of the VEGF family as well as the angiopoietins. The auto-antibodies were found to have functionally neutralizing activity. Accordingly, described herein is whether combination angiogenesis and checkpoint blockade augments anti-tumor activity.

b. Led clinical study that demonstrated potential augmented combination activity of anti-CTLA-4 (ipilimumab) and anti-VEGF-A (bevacizumab) in patients with advanced

melanoma. The combination was well tolerated with sufficient activity to warrant formal comparison of the combination vs. ipilimumab alone in a phase 2 study about to complete accrual under Eastern Cooperative Oncology Group (ECOG) oversight. Notable tissue and serum findings in the study included evidence of enhanced intra-tumor DC activation, formation of intra-tumor tertiary lymphoid aggregates including endothelial morphology consistent with high-endothelial venules of lymph nodes.

c. Described herein are initial preclinical studies revealing the importance of Ang2 as a target and combinatorial approach with checkpoint blockade. In addition, described herein is preclinical work with AMG386 revealing mechanisms of action regarding immune regulation.

d. These studies have set the stage to explore the combination of PD-1/PD-L1 blockade and angiogenesis inhibition, with several potential mechanisms that may account for augmented and durable anti-tumor activity.

e. The combination of pembrolizumab and AMG386 is explored. AMG386 is a peptibody designed specifically to block angiopoietin-2 mAb. Specific Ang-2 inhibition may provide additional immune-supportive clinical benefit when added to PD-1.

f. The study includes correlative tissue biopsy and circulating biomarker studies (as well as other circulating biomarker analyses).

g. In the dose escalation phase, the agents are administered every 3 weeks to patients with advanced solid malignancies with measurable disease, and safety expansion cohorts to demonstrate RP2D are planned for targeted tumor types of melanoma, renal cell carcinoma (RCC), colorectal, and ovarian cancer.

Rationale and Background:

Human immune responses against cancer can be suppressed through various mechanisms during disease progression such that cancers evade immune recognition and anti-tumor effector functions. The expression of immune regulatory molecules such as cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and programmed death 1 (PD-1), etc. inhibits the proliferation and function of conventional T cells. Immune checkpoint blockade with ipilimumab (CTLA-4 blockade) has revealed improved survival in patients with metastatic melanoma (Robert, C., et al., N. Engl. J. Med. 364(26): 2517-2526 (2011); Hodi FS, et al., N. Engl. J. Med 363: 711-723 (2010)). Blockade of PD-1 and PD-L1 interactions has also revealed durable clinical benefits in

patients with a variety of cancers including melanoma, non-small cell lung cancer, and renal cell carcinoma (Topalian S.L., et al., N. Engl. J. Med. 366(26): 2443-2454 (2012), Hamid O, et al., N. Engl. J. Med. 369(2): 134-144 (2013)). Prior to the invention described herein, efforts were needed to better understand treatment modality combinations that could improve efficacy of immune checkpoint blockade. This would include clinical benefits in cancers that exhibit limited efficacy to checkpoint blockade alone. There is increasing evidence of the role that angiogenic factors play in affecting immune regulation as well as immune effector cell trafficking into tumors. Soluble VEGF (sVEGF) predicts clinical benefit to ipilimumab therapy (Yuan J., et al., Cancer Immunology Research 2:127-32 (2014)).

Accordingly, described herein is an evaluation of the safety and tolerability and a determination of the recommended phase II dosing for the combination of pembrolizumab plus AMG386 in patients with colorectal cancer. Also described herein is a determination of progression free survival (PFS) at 6 months; rate of 1-year OS; Response Rate; and Time to Progression.

Correlative sciences include biopsy of pre-existing sites of disease, and whenever possible following treatment to assess histologically for VEGF/VEGFR expression, phosphoTie-2. Described herein is the investigation of immune responses to other angiogenic molecules as a function naïve and memory CD4, CD8 and other lymphocyte populations. Also described herein are cellular and humoral immune responses to established antigens as a function of treatment well as melanoma antigen targets, Mucin-1 (Muc-1), carcinoembryonic antigen (CEA), cancer antigen (CA)-125, and cancer-testis antigen (NY-Eso-1) as examples. Described herein are PET response versus RECIST versus irRC criteria.

Safety Measures

Safety is evaluated for all treated patients using the National Cancer Institute (NCI) Common Terminology Criteria review of adverse event (AE) reports and the results of vital sign measurements, physical examinations and clinical laboratory tests. The incidence of AEs period for safety data is from the date of first on-study dose to 70 days after the last dose is received. Additionally, from the time of consent.

A patient is classified as having a dose-limiting toxicity (DLT) for any of the following: an unexpected toxicity of grade 3 or higher; a toxicity of grade 3 or higher that does not resolve with or without expected intervention within 7 days; eye pain of grade 2 or higher; hypertension

difficult to control requiring two increases or addition of another medication to control; urine protein:creatinine > 3.5 or >2g protein on 24 hour urine collection; two delays of treatment (not due to scheduling non-compliance) each lasting more than 10 days within 4 cycles of drug.

The DLT period is the first 4 weeks of study therapy.

Inclusion criteria are the following: measureable unresectable or metastatic disease; ECOG Performance Status (PS) 0,1; normal white blood cell (WBC), platelets, renal function; LFT < 2x ULN; two or fewer prior therapies; ovarian cancer patients are platinum resistant; renal cell patients may have had one prior VEGF tyrosine kinase inhibitor (TKI); in the dose escalation, any solid tumor with measureable disease in dose expansion, melanoma, renal cell carcinoma, ovarian cancer, or colorectal cancer patients.

Exclusion criteria are the following: brain metastases – treated central nervous system (CNS) disease that is stable for >2 months may be considered eligible; history of autoimmunity, GI (colon) metastases, skin ulcerated lesions, anti-coagulant therapy, poorly controlled hypertension; pregnant or nursing women; prior therapy with PD-1/PD-L1 antibodies or AMG386.

Study Design

This is a prospective trial which accrues subjects with solid tumors to evaluate the safety, clinical, and immunological effect of the combination of pembrolizumab (MK-3475) and trebananib (AMG386). The treatment includes an induction phase of pembrolizumab and trebananib for 4 cycles (12 wks) followed by pembrolizumab alone for 2 years. The study accrues up to 60 subjects. This trial is conducted in 2 parts. Part I uses a standard $3^{+}3$ dose escalation design in all solid tumors. The goal of Part I is to identify the recommended part 2 (expansion cohort) doses (RP2D) for the combination of pembrolizumab plus trebananib (AMG386). Dose escalation begins in dose cohort $^{+}1$. If two or more patients in dose cohort $^{+}1$ experience a DLT, the next cohort of patients are enrolled into dose cohort -1. Should dose cohort -1 prove too toxic, enrollment to the study stops. If the toxicity profile of dose cohort $^{+}1$ is acceptable, the next cohort are enrolled into dose cohort $^{+}2$. Should dose cohort $^{+}2$ have acceptable toxicity that is the RP2D, otherwise, dose cohort $^{+}1$ is the RP2D.
Part II proceeds with four dose expansion cohorts: melanoma, renal cell carcinoma, ovarian cancer, and colorectal cancer. For each disease type, 12 patients are enrolled and treated at the RP2D of pembrolizumab and trebananib (AMG386). Safety assessments include all patients

receiving one or more doses of the study drug combinations. Secondary and correlative endpoints are based on the cohorts of patients enrolled in Part II of the trial. Secondary and correlative endpoints are summarized according to disease indication and, in an exploratory fashion, with all patients combined. Pre-and post-treatment biopsies are obtained in at least 20 patients enrolled in the dose expansion cohorts with different disease types.

Primary Objectives

- Part 1: To determine the safety, tolerability, and RP2D for trebananib when given with pembrolizumab in patients with metastatic solid tumor.
- Part 2 (expansion cohort): To determine the safety and tolerability of the RP2D of trebananib, determined in Part 1, when given with pembrolizumab in patients with unresectable stage III or stage IV melanoma, metastatic renal cell, ovarian, or colorectal cancer.

Secondary Objectives

- To obtain preliminary estimates of progression free survival (PFS) at 6 months.
- To obtain preliminary estimates of the rate of 1-year overall (OS).
- To obtain preliminary estimates of the response rate (RR).
- To obtain preliminary estimates of time to progression.
- To perform a pilot assessment of PET response versus RECIST versus irRC criteria.

Exploratory Objectives

- To determine the effect of the combination on vasculopathy, immune infiltration, and tumor necrosis by staining pathologic specimens for VEGF/VEGFR expression, phosphoTie-2 and other targets.
- To investigate immune responses in the periphery to VEGFR, Tie-2, and other angiogenic molecules and tumor-specific antigens as a function of treatment.

BACKGROUND

Pharmaceutical and Therapeutic Background

The PD-1 receptor-ligand interaction is a major pathway hijacked by tumors to suppress immune control. The normal function of PD-1, expressed on the cell surface of activated T-cells under healthy conditions, is to down-modulate unwanted or excessive immune responses, including autoimmune reactions. PD-1 (encoded by the gene Pdcd1) is an Ig superfamily member related to CD28 and CTLA-4 which has been shown to negatively regulate antigen

receptor signaling upon engagement of its ligands (PD-L1 and/or PD-L2) (Sharpe, A.H., et al., Nat Rev Immunol. 2(2): p. 116-26 (2002)).

The mechanism by which PD-1 down modulates T-cell responses is similar to, but distinct from that of CTLA-4 as both molecules regulate an overlapping set of signaling proteins. PD-1 was shown to be expressed on activated lymphocytes including peripheral $CD4^+$ and $CD8^+$ T-cells, B-cells, T regs and Natural Killer cells.

Expression has also been shown during thymic development on $CD4^-CD8^-$ (double negative) T-cells as well as subsets of macrophages and dendritic cells. The ligands for PD-1 (PD-L1 and PD-L2) are constitutively expressed or can be induced in a variety of cell types, including non-hematopoietic tissues as well as in various tumors. Both ligands are type I transmembrane receptors containing both IgV- and IgC-like domains in the extracellular region and contain short cytoplasmic regions with no known signaling motifs. Binding of either PD-1 ligand to PD-1 inhibits T-cell activation triggered through the T-cell receptor (Sharpe, A.H., et al., Nat Rev Immunol. 2(2): p. 116-26 (2002)). PD-L1 is expressed at low levels on various non-hematopoietic tissues, most notably on vascular endothelium, whereas PD-L2 protein is only detectably expressed on antigen-presenting cells found in lymphoid tissue or chronic inflammatory environments. PD-L2 is thought to control immune T-cell activation in lymphoid organs, whereas PD-L1 serves to dampen unwarranted T-cell function in peripheral tissues. Although healthy organs express little (if any) PD-L1, a variety of cancers were demonstrated to express abundant levels of this T-cell inhibitor. PD-1 has been suggested to regulate tumor-specific T-cell expansion in subjects with melanoma (MEL). This suggests that the PD-1/PD-L1 pathway plays a critical role in tumor immune evasion and should be considered as an attractive target for therapeutic intervention.

Pembrolizumab is a potent and highly selective humanized monoclonal antibody (mAb) of the IgG4/kappa isotype designed to directly block the interaction between PD-1 and its ligands, PD-L1 and PD-L2. Keytruda® (pembrolizumab) has been approved in the United Stated for the treatment of unresectable or metastatic melanoma, metastatic non-small cell lung cancer (NSCLC) whose tumors express programmed death ligand 1 (PD-L1), and recurrent metastatic squamous cell carcinoma of the head and neck.

Previous experience with immune checkpoint blockade (CTLA-4) and anti-angiogenesis

The expression of immune regulatory molecules such as cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and programmed death 1 (PD-1), etc. inhibits the proliferation and function of conventional T cells. Immune checkpoint blockade with ipilimumab (CTLA-4 blockade) has revealed improved survival in patients with metastatic melanoma (Robert, C., et al., N. Engl. J. Med. 364(26): 2517-2526 (2011); Hodi FS, et al., N. Engl. J. Med 363: 711-723 (2010)). Blockade of PD-1 and PD-L1 interactions has also revealed durable clinical benefits in patients with a variety of cancers including melanoma, non-small cell lung cancer, and renal cell carcinoma (Topalian S.L., et al., N. Engl. J. Med. 366(26): 2443-2454 (2012), Hamid O, et al., N. Engl. J. Med. 369(2): 134-144 (2013)). Prior to the invention described herein, efforts were needed to better understand treatment modality combinations that could improve efficacy of immune checkpoint blockade. This would include clinical benefits in cancers that exhibit limited efficacy to checkpoint blockade alone. There is increasing evidence of the role that angiogenic factors play in affecting immune regulation as well as immune effector cell trafficking into tumors. Soluble VEGF (sVEGF) predicts clinical benefit to ipilimumab therapy (Yuan J., et al., Cancer Immunology Research 2:127-32 (2014)).

Anti-CTLA-4 blockade

Ipilimumab is a fully human monoclonal antibody that blocks the costimulatory checkpoint molecule CTLA-4. The anti-tumor mechanism of action involves amplification of T cells by blocking endogenous CTLA-4 with resultant T cell proliferation and tumor cell killing. Activity has been observed when administered as a single agent or in combination with other immunotherapies such as vaccines or interleukin-2 (IL-2) as well as when combined with chemotherapy, and in multiple indications including melanoma.

CTLA-4 blockade with ipilimumab leads to improved overall survival in patients with advanced melanoma as documented in two phase III studies, emphasizing the antitumor activity of immune checkpoint blockade. The published response rate is approximately 18%, with a substantial number of responses being durable and/or complete. Recent long-term analyses of follow up for patients treated with ipilimumab reveal a durable 22% survival rate with an inflection and flattening of the survival curves at approximately 3 years (ECCO 2013), demonstrating long term benefits for patients.

As described herein, angiogenesis is one modality pursued to combine with checkpoint blockade to improve efficacy.

Combination of anti-CTLA-4 and anti-VEGF therapy in patients with advanced melanoma

Experience with ipilimumab includes its administration to patients following a therapeutic vaccine. In patients in whom pre-existing sites of disease were biopsied following treatment, the consistent presence of an immune mediated vasculopathy around the vasculature feeding the tumor deposit associated with extensive tumor necrosis was observed (FIG. 1 and FIG. 2; Hodi FS, et al., Proc. Natl. Acad. Sci. USA 100(8):4712-4717 (2003)).

In addition to this evidence, VEGF is known to be a potent inhibitor of dendritic cell maturation. VEGF inhibition has also been demonstrated to facilitate T cell trafficking across endothelia. As a result of these observations, a phase 1 trial of the combination of bevacizumab and ipilimumab in patients with unresectable stage III or IV melanoma was initiated and completed. The results of the trial provide the first experience of combining anti-angiogenesis with immune checkpoint blockade.

The primary endpoints of the trial were the safety and preliminary activity of the combination of the two treatments for patients with advanced melanoma. Patients received ipilimumab every 3 weeks for four doses then every 12 weeks, and bevacizumab every 3 weeks. Patients could continue treatment with good performance status (PS), $\leq$ 40% increase in sum of the longest diameter, and $\leq$ 2 new target lesions. Cohort 1 comprised 10 mg/kg ipilimumab plus 7.5 mg/kg bevacizumab. Following the induction dosing every three weeks for four cycles, bevacizumab was continued every 3 weeks as tolerated, and ipilimumab was administered every 12 weeks as tolerated. With $\geq$ 3/5 patients not experiencing DLT, Cohort 2 enrolled at 10 mg/kg ipilimumab plus 15 mg/kg bevacizumab. Twelve additional patients were treated at MTD. With the FDA approval of ipilimumab at 3 mg/kg additional Cohorts (12 patients each) were added and received ipilimumab 3 mg/kg with 7.5 or 15 mg/kg of bevacizumab (Cohorts 3 and 4, respectively). A total of forty-six patients were treated. The combination showed promising evidence of activity, including a 32% overall response rate (ORR) (6 PR, 1 CR) and an additional 32% rate of durable (>6 months) stable disease. Inflammatory events included giant cell arteritis (1), hepatitis (2), and uveitis (2). Median follow-up at the time of latest analysis was 17.3 months (FIG. 3; 95% CI: 11.1 to 30.2 months).

Radiographic examples of pseudo-progression and delayed best response were also observed. Thirty-one patients reported a best response of CR, PR, or SD, resulting in a disease-control percentage of 67.4% (FIG. 4; 95% exact CI: 52% to 81%).

The highest percentage with disease control was 76.5%, which was reported in Cohort 2 (95% exact CI: 50% to 93%). A number of patients had durable responses, with several achieving best response after months of therapy. A patient in Cohort 2 (MTD) experienced approximately 7 months of stable disease before a partial response and subsequently had a complete response beginning approximately 17 months following the initiation of therapy. Eight patients in Cohorts 1 and 2 remain alive for months after discontinuation of therapy. There is significantly shorter follow-up time for Cohorts 3 and 4. The median time to progression (based on mWHO) was 9.0 months, 95% CI (5.5 to 14.5 months). Median overall survival was 25.1 months (FIG. 5A- FIG. 5B; 95% CI: 12.7 to ∞).

For this trial (FIG. 6), the Kaplan-Meier estimate of 1-year OS was 79% (95% CI 62% to 89%) and the Kaplan-Meier estimate of 6-month progression-free survival (PFS) was 63% (95% CI 47% to 75%). This compares favorably with the expected 1-year survival rate of 25% in 2nd line, and 35% in 1st line patients. Both 6 month PFS and 1-year OS values are in excess of the 95% confidence limit upper boundary to be expected for trials of similar size as described in the Korn meta-analysis of phase II trials for metastatic melanoma (Korn EL, J. Clin. Oncol. 26(4): 527-534 (2008)). This is also superior to the overall survival for the phase II and III trials previously reported. These data reveal that anti-angiogenic therapy with bevacizumab VEGF-A blockade and immune checkpoint blockade with ipilimumab can be safely administered and resulted in a significant proportion of patients receiving clinical benefit.

Correlatives Revealing Mechanisms of Action for Combination Therapy of Bevacizumab and Ipilimumab

A number of notable observations were made in correlative laboratory and pathological investigations in this trial. Marked infiltration with $CD3^+$, $CD4^+$, and $CD8^+$ T-cells as well as $CD163^+$ cells (monocyte/macrophage lineage) were observed after treatment with ipilimumab plus bevacizumab. In contrast, patients treated only with ipilimumab demonstrated a lesser degree of immune cell infiltration while on therapy (FIG. 7).

The immune infiltrate appeared to form tertiary lymphoid aggregates (FIG. 8), which was associated with dendritic cell infiltration and evidence of local endothelial activation similar to that seen in high endothelial venules in lymph nodes (FIG. 9).

In addition, flow cytometry analysis on peripheral PBMC indicated a marked increase in the number of patients exhibiting a $\geq$ 50% increase in levels of circulating $CD4^+$ and $CD8^+$ memory cells (CD45Ro), a phenomenon that has not been previously observed in studies of ipilimumab alone (FIG. 10A- FIG. 10C).

Angiopoietin-2 and its relevance to immune checkpoint blockade

Angiopoietin-1 (Ang-1) is constitutively expressed in many adult tissues and is required for normal vascular homeostasis, whereas Ang-2 is predominantly expressed in tissues undergoing vascular remodeling and in hypoxic tumor microenvironments (Nasarre, P., et al., Cancer Res 69(4): 1324-1333 (2009)). Ang-2 is a critical regulator of blood vessel maturation (Augustin HG, et al., Nat. Rev. Mol. Cell Biol. 10(3):165-177 (2009)). The molecule, which is in normal tissue almost exclusively produced by endothelial cells (EC), functions as a vessel-destabilizing molecule that facilitates the activities of other endothelial-acting cytokines by controlling the Ang-2/Tie-2 signaling pathway (Wong, A. L., et al., Circ. Res 81(4): 567-574 (1997); Augustin HG, et al., Nat. Rev. Mol. Cell Biol. 10(3):165-177 (2009)). Several studies have demonstrated that elevated levels of Ang-2 and higher Ang-2/Ang1 ratios compared to levels in normal tissues are associated with a worse prognosis in a number of different tumor types. As described herein, the expression patterns of Ang-2 in normal tissues versus tumor suggest that Ang-2 is a target for cancer therapy. Circulating Ang-2 was identified as a biomarker for progression and metastasis in melanoma (Helfrich I, et al., Clin Cancer Res. 15(4):1384-92 (2009)) Furthermore, Ang-2 was found to be expressed by tumor-associated endothelial cells and melanoma cells; siRNA silencing of Ang-2 lead to strongly reduced invasive and migratory capacity of melanoma cells.

Angiopoietin-2 in ipilimumab studies

In previous experience in early ipilimumab studies, serologic screening of cDNA expression libraries identified angiopoietin-2 (Ang-2) as a target of high titer antibodies in treated patients. Furthermore, a number of patients who experienced favorable clinical outcomes from these studies have developed high titer antibodies to Angiopoietin-2 (Ang-2) as a function of treatment (FIG. 11).

These antibodies in patients have proven to be functional in TIE-2 binding assays as well as tube formation assays (TIE-2 receptor signaling in endothelial cells), suggesting that synergy of immune checkpoint blockade may go beyond VEGF and include the family of angiogeneic factors including angiopoietin 2 (FIG. 12 and FIG. 13).

To investigate the potential influence of Ang-2 relative to immune checkpoint activity, pre-treatment and post-treatment Ang-2 levels were assessed by Luminex relative to clinical outcomes of patients receiving ipilimumab therapy. There was a correlation with increased post-treatment levels of Ang-2 and clinical responses in a cohort of ipilimumab treated patients, suggesting that Ang-2 may play a role in disease progression in these patients (FIG. 14).

Angiopoietin-2 in ipilimumab combined with bevacizumab studies

The development of antibodies to Ang-2 as a function of ipilimumab or ipilimumab plus bevacizumab in melanoma patients was assessed. In a cohort of 48 patients, 16 patients developed high-titer antibodies to Ang-2 as a function of treatment by ELISA and confirmed by immunoblotting (FIG. 15).

Percent changes from baseline Ang-2 antibody titers to clinical outcomes in ipilimumab treated melanoma patients demonstrating a trend in patients experiencing a response and magnitude of antibody titer changes were correlated (FIG. 16).

Cytokines, circulating endothelial cells, and tumor associated endothelial cells were assessed. Assays for biologically active molecules involved in tumor related angiogenesis and the means to assess the immune responses to these molecules have been developed in the laboratory. A means to assay for VEGF, basic fibroblast growth factor (bFGF), as well as circulating endothelial cells (CEC) and circulating progenitor cells (CPC) was established (FIG. 17A- FIG. 17D; Duda, D.G., et al., Nat. Protoc. 2(4): 805-810 (2007)).

A Luminex platform for circulating cytokine analyses was established. Analyses of samples from ipilimumab treated and ipilimumab plus bevacizumab treated patients are in process (FIG. 18, FIG. 19, and FIG. 20).

Trebananib (AMG386)

Trebananib (AMG386) is an angiopoietin-1/antiopoietin-2 neutralizing peptibody. Preclinical studies with AMG386 showed significant inhibitions of several tumor types (Neal, J., et al., Curr. Opin. Mol. Ther. 12(4): 487-495 (2010); Coxon, A., et al., J. Mol. Cancer Ther. 9(10): 2641-2651 (2010)). Recently, the TRINOVA-1 phase 3 trials investigated the combination

of AMG386 and paclitaxel and showed significant reduction in disease progression, but no improvement in overall survival in ovarian cancer patients (Monk, BJ, et al., Lancet Oncol. 15:799-808 (2014); Monk, BJ, et al., Gyn. Onc. 143:27¬34 (2016)). In metastatic colorectal cancer AMG386 in combination with leucovorin calcium (folinic acid), fluorouracil, irinotecan hydrochloride (FOLFIRI) regimen did not improve PFS (Peeters, A., et al., BJC 108: 503-511 (2013)), however, the ongoing VENGEANCE study in combination with bevacizumab suggests clinical activity in this setting (Mooi, J, et al., J. Clin. Oncol. 33(no.15_suppl):3533 (2015)). The RCC studies suggested improved responses and PFS of AMG386 in combination with sunitinib but not with sorafenib (Atkins, MB et al., JCO 33:3431-3438 (2015); Rini, B., et al., Cancer 118: 6152-616 (2012)).

These indicate important roles of angiopoietin-Tie-2 axis in tumor progression. However, prior to the invention described herein, mechanisms of AMG386 on tumor immunity were not clearly elucidated, in particular, its roles in T cell and $CD14^+Tie\text{-}2^+$ monocyte immunity. The results presented herein suggest that interactions of Ang2 with $CD14^+Tie\text{-}2^+$ monocytes exert inhibitory effects on T cell activation, and AMG386 shows partially restoration of suppression of T cell activation. In addition, initial studies showed that Tie-2 pathway increases PD-L1 expression and decrease ICOSL in $CD14^+Tie\text{-}2^+$ monocytes. These strongly suggest complicated suppression mechanism of T cell activation by Tie-2 signaling.

Generation of $CD14^+Tie\text{-}2^+$ monocytes

$CD14^+Tie\text{-}2^+$ monocytes play critical roles in T cell via Ang2/Tie-2 axis (Coffelt, S.B., et al., J Immunol. 186(7): 4183-4190 (2011)). As shown in FIG. 21, approximately 20% $CD14^+Tie\text{-}2^+$ monocytes were generated for studies of T cell suppression and AMG386. Tie-2 expressing Lenti virus was also generated for investigations of signals of Tie-2 and Ang2. Monocytes were further transduced with the virus. Approximately 36% $CD14^+Tie\text{-}2^+$ monocytes were generated after the infection.

Effects of Ang2 and AMG386 on T cell proliferation via enriched and Tie-2-infected $CD14^+Tie\text{-}2^+$ monocytes

Based on $CD14^+Tie\text{-}2^+$ monocytes and Tie-2-infected $CD14^+Tie\text{-}2^+$ monocytes, effects of Ang2, ML4-3, and L1-7 on T cell proliferation were further examined. ML4-3 and L1-7 are two different anti-Ang-1/2 peptibodies (AMG386). As shown in FIG. 22A- FIG. 22B, Ang2 exerts inhibitory effects on T cell proliferation, and the inhibitions were Tie-2 level dependent of

$CD14^+$ monocytes. ML4-3 and L1-7 partially restored Ang2 induced suppression. These suggest neutralizing effects of AMG386 on Ang2 in T cell suppression.

Tie-2 signaling of $CD14^+Tie\text{-}2^+$ monocytes involved in T cell suppression

To further investigate the inhibitory mechanism of $CD14^+Tie\text{-}2^+$ monocytes on T cell activation, PD-L1 and ICOSL are examined. As shown in FIG. 23, expression of PD-L1 was remarkably increased in Tie-2 infected $CD14^+Tie\text{-}2^+$ monocytes, in comparison with parental cells. Interestingly, expression of ICOSL was decreased over 50% in Tie-2 infected $CD14^+Tie\text{-}2^+$ monocytes, in comparison with parental cells (FIG. 24).

It is shown that Ang2 stimulates Tie-2-expressing monocytes to suppress T cell activation (Coffelt, S.B., et al., J Immunol. 186(7): 4183-4190 (2011)). Current studies are consistent with this notion. AMG386 partially reversed Ang2 induced suppression. These data support the notion that AMG386 functions as neutralizing peptibodies.

Furthermore, the inhibitory effects are dependent on Tie-2 levels of $CD14^+$ monocytes. It suggests critical roles of Tie-2 signaling in effector memory (TEM) induced T cell suppression. Analyses with gene signature, phenotype, and superior proangiogenic abilities indicate that TEM and major histocompatibility complex class II^{low} ($MHC\text{-}II^{low}$) tumor-associated macrophages (TAM) are analogous (Pucci, F., et al., Blood 114(4): 901-914 (2009); Movahedi, K., et al., Cancer Res 70(14): 5728-5739 (2010)). Functionally, both $MHC\text{-}II^{high}$ and $MHC\text{-}II^{low}$ cells are capable of suppressing T cell activation. $MHC\text{-}II^{high}$ cell-mediated suppression is inducible nitric oxide synthase (iNOS) dependent, whereas, TEM derived IL10, which is induced by Ang2, suppresses T cell activation in both in vitro and mouse tumor in vivo models (Coffelt, S.B., et al., J Immunol. 186(7): 4183-4190 (2011)). Whether AMG386 reverse the suppression via IL10 pathway is described herein.

PD-L1 is a membrane bound protein, primarily expressed on dendritic cells and monocytes (Keir, M. E., et al., Annu. Rev. Immunol 26:677-704 (2008)). The receptor for the ligand is PD1, which is expressed on activated T cells and B cells, DC, and monocytes (Keir, M. E., et al., Annu. Rev. Immunol 26:677-704 (2008)). During the engagement of T cells with antigen/MHC complex, interaction of PDL1 with PD1 exerts inhibitory effects on T cell activation, leading to immune suppression (Sharpe, A.H., et al., Nat Rev Immunol. 2(2): p. 116-26 (2002); Keir, M. E., et al., Annu. Rev. Immunol 26:677-704 (2008)). The data show that Tie-

2 signaling is associated with PD-L1 expression on $CD14^+Tie\text{-}2^+$ monocytes. It is of interest whether Ang2 and AMG386 affects PD-L1 expression on $CD14^+Tie\text{-}2^+$ monocytes.

Inducible T-cell co-stimulator (ICOS) is a member of CD28 immunoglubulin superfamily, expressed upon T cell activation (Sharpe, A.H., et al., Nat Rev Immunol. 2(2): p. 116-26 (2002)). Its ligand is ICOSL, which expressed on B cells, dendritic cells, monocytes/macrophages, and T cells (Sharpe, A.H., et al., Nat Rev Immunol. 2(2): p. 116-26 (2002); Coyle, A. J., et al., Nat Immunol. 2(3): 203-209 (2001); Chambers, C. A., et al., 22(4): 217-223 (2001)). ICOS pathway is involved in functions of T helper cells, formation of germinal centers, and collaboration of T/B cells (Sperling, Bluestone et al. 2001; Mak, T. W. et al., Immunol 4(8): 765-772 (2003)). Disruption of ICOS/ICOSL pathway by genetic depletion showed important roles in anti-tumor therapy by CTLA4 blockade (Fu, T., et. al., Cancer Res 71(16): 5445-5454 (2011)). The data showed that Tie-2 signaling is associated with down-regulation of ICOSL expression on $CD14^+Tie\text{-}2^+$ monocytes. It is of interest whether Ang2 and AMG386 affects ICOSL expression on $CD14^+Tie\text{-}2^+$ monocytes.

Soluble (s)PD-L1 was recently identified and characterized. It is also secreted from mature DC, melanoma and renal tumor cells (Frigola, X., et al., Immunol. Lett. 142(1-2): 78-82. (2012); Frigola, X., et al., Clin Cancer. Res. 17(7):1915-1923 (2011)). It showed suppression of T cell activation (Frigola, X., et al., Clin Cancer. Res. 17(7):1915-1923 (2011)). Elevated sPD-L1 is associated with tumor progression in patients with renal cell carcinoma (Frigola, X., et al., Clin Cancer. Res. 17(7):1915-1923 (2011)). The data indicated that higher levels of sPD-L1 are associated with progressive diseases in advanced melanoma patients, and expression of PD-L1 is associated with secretion of sPD-L1. Whether sPD-L1 secretion occurs and how Ang2 and AMG386 affects sPD-L1 secretion in $CD14^+Tie\text{-}2^+$ monocytes are worthy of further investigation.

In summary, Tie-2 pathway plays roles in the regulation of expression of PD-L1, ICOSL, and IL10, which are involved in the modulations of T cell and tumor immunity. Impacts of Ang2 and its neutralizing AMG386 on Tie-2 pathway are described herein.

Rationale for the Trial and Selected Subject Population

The combination of CTLA-4 and VEGF blockade appears to be well tolerated in patients with advanced melanoma. The clinical efficacy data and correlative studies of the immune response suggest that the combination has enhanced antitumor immunostimulatory effects

beyond those observed with CTLA-4 blockade alone. One potential mechanism is that VEGF blockade promotes normalization of tumor blood vessels and permits enhanced egress of tumor specific lymphocytes and other immune effectors. Another, non-exclusive mechanism is that VEGF may inhibit some aspects of an effective adaptive immune response itself, including endothelial cell activation and dendritic cell maturation, and that blockade of VEGF further augments the anti-tumor immune response. The emergence of antibodies to Ang-2 after vaccination or CTLA-4 blockade observed in the studies suggests that synergy of immune checkpoint blockade may go beyond VEGF and include the family of angiogenic factors including Ang-2. There also remains the potential role for Ang2 blockade at influencing immune suppressor cells such as myeloid and M2 macrophages. These data in conjunction with the increasing evidence for Ang-2 as an important target for cancer therapy provide the rationale for the experiments of combined blockade described herein.

Ang-2 inhibition has been tested in a number of cancers as a single agent and has demonstrated activity in combination with paclitaxel in ovarian cancer (Gerald D, et al., Cancer Res 73(6): 1649-1657 (2013)), and ongoing studies have generated clinical responses in colorectal and ovarian cancer.

Given the currently available data and clinical experiences, there are several cancer types that are reasonable to target based on this biology and clinical activity when considering combination therapies of immune checkpoint blockade and anti-angiogenesis. Pembrolizumab has demonstrated significant clinical activity in melanoma patients who have previously been treated with ipilimumab or are ipilimumab-naïve (Hamid O, et al., N. Engl. J. Med. 369(2): 134-144 (2013)). Given the clinical activity and iplimumab-bevacizumab combination data, melanoma is one disease to target. Immune checkpoint blockade with ipilimumab and PD-1 agents have been seen in renal cell carcinoma and ant-VEGF agents are a mainstay of treatment for this disease. Therefore, including renal cell carcinoma in such combinations should also be considered. Anti-VEGF therapies including bevacizumab and ziv-aflibercept have been approved in colorectal cancer and improved understanding of checkpoint blockade as well as combinations is warranted in this disease with unmet need. Finally, ipilimumab has demonstrated activity in ovarian cancer. Ovarian cancer can also be responsive to bevacizumab and early clinical activity with Ang-2 inhibition. Prior to the invention described herein, there was an unmet need in platinum-resistant ovarian cancer.

The observation of the vascular effects of ipilimumab on tumor deposits further supports the critical importance of angiogenesis to tumor growth and to influencing the anti-tumor immune response. The clinical efficacy of targeting VEGF and its effect on pathologic angiogenesis has been extensively studied with the use of bevacizumab. Given the profound effects on tumor vasculature witnessed in melanoma patients being treated with ipilimumab, along with initial promising data of the combination of bevacizumab and ipilimumab summarized above, there is promise in the synergies with combinations of immune checkpoint blockade and anti-angiogenesis, as described herein. A phase I study testing the combination of pembrolizumab and the angiopoietin-2 peptibody, AMG386, in patients with metastatic melanoma, ovarian cancer, renal cell carcinoma, or colorectal adenocarcinoma is described in detail below.

The clinical experience of combining ipilimumab plus bevacizumab has demonstrated that it can be administered safely. Accumulating clinical experience with AMG386 has demonstrated both activity and a favorable safety profile.

As described herein, targeting angiogenic factors normalizes blood flow supporting T cell ingress. There is also the ability to modify immune responses via effects on dendritic cells. VEGF and PD-1 blockade has also shown successful combination in pre-clinical animal models. Ang-2 and VEGF blockade together has revealed synergy in pre-clinical animal models and have successfully been combined in clinical trials. Ang-2 plays an important role in the proangiogenic and immune inhibitory effects of TIE-2 positive monocytes.

The addition of Ang-2 inhibition to PD-1 blockade with pembrolizumab may further complement the reversal of angiogenic immune suppression and improve immune cell trafficking. Whether the combination of Ang-2 inhibition with PD-1 blockade is tolerable and safe is evaluated. Described herein is a determination of whether the combination augments anti-tumor activity through evidence of clinical responses and biomarker responses.

In order to gain greater safety as well as preliminary efficacy data and correlatives to determine mechanisms of potential synergy in these patient populations, expanded cohortsare treated at the recommended phase II dose for pembrolizumab plus AMG386.

Overall response rate is approximately 26-38% with evidence for durable benefit. The anti-PD-1 antibody nivolumab has also demonstrated significant clinical activity with a response rate of approximately 31%, median overall survival of 16.8 months, and median response

duration of two years. Furthermore with this clinical activity, improved outcomes and understanding of combination approaches are needed. In addition, single agent activity of ziv-aflibercept as anti-angiogenesis in metastatic cutaneous or uveal melanoma included a 7.5% response rate and a median overall survival of 16.3 months (Tarhini Frankel, and Margolin, 2011). With the ipilimumab and bevacizumab combination clinical experience (Hodi, Lawrence et al. 2014), the single agent activity of zivaflibercept in melanoma, as well as known clinical activity for pembrolizumab in melanoma, melanoma is one disease to include investigations with this combination. Clinical activity with immune checkpoint blockade with ipilimumab and PD-1 agents has also been seen in renal cell carcinoma, and anti-VEGF agents are a mainstay of treatment for this disease (Choueiri 2013, Escudier, Albiges et al. 2013, McDermott and Atkins 2013, Mooney, Paluri et al. 2014). Currently available are a number of VEGF TKI agents that have demonstrated clinical activity but with the development of resistance. Therefore, treatment of renal cell carcinoma patients with such combinations should also be considered as improvement of patient outcomes is still warranted. Anti-angiogenesis with bevacizumab and Ziv-aflibercept has become a mainstay treatment for colorectal cancer in combination with chemotherapy (Damin and Lazzaron 2014) (Patel and Sun 2014) (Dietvorst and Eskens 2013). There remains a need for improving patient outcomes from these combinatorial approaches. Improved understanding of checkpoint blockade as well as combinations is warranted in this disease with unmet need. Finally, ipilimumab has demonstrated activity in ovarian cancer (Hodi FS, et al., Proc. Natl. Acad. Sci. USA 100(8):4712-4717 (2003)). Bevacizumab is an active agent used in combination therapy for ovarian cancer. As part of the mainstay in this disease, there remains an unmet need in platinum-resistant ovarian cancer (Jayson, Kohn et al. 2014, Syrios, Banerjee et al. 2014).

Accumulating clinical experience with AMG386 has demonstrated both activity and a favorable safety profile. VEGF and PD-1 blockade has also shown successful combination in pre-clinical animal models. Ang-2 and VEGF blockade together has revealed synergy in pre-clinical animal models and have successfully been combined in clinical trials. Ang-2 plays an important role in the proangiogenic and immune inhibitory effects of TIE-2 positive monocytes. The addition of Ang-2 inhibition to PD-1 blockade with pembrolizumab may further complement the reversal of angiogenic immune suppression and improve immune cell trafficking. Whether the combination of Ang-2 inhibition with PD-1 blockade is tolerable and

safe is tested. If the combination augments anti-tumor activity through evidence of clinical responses and biomarker responses is determined.

In order to gain greater safety as well as preliminary efficacy data and correlatives to determine mechanisms of potential synergy in these patient populations, expanded cohortsare treated at the recommended part 2 (expansion cohort) dose for pembrolizumab plus AMG386.

Rationale Pembrolizumab Dose Selection

The dose of pembrolizumab planned to be studied in this study is 200 mg every 3 weeks (Q3W). The dose recently approved in the United States for treatment of melanoma subjects is 2 mg/kg Q3W. Information on the rationale for selecting 200 mg Q3W is summarized below.

Available pharmacokinetics (PK) results in subjects with melanoma, non-small cell lung cancer (NSCLC), and other solid tumor types support a lack of meaningful difference in PK exposures obtained at a given dose among tumor types. An open-label Phase I trial (Protocol 001) has been conducted to evaluate the safety and clinical activity of single agent pembrolizumab. The dose escalation portion of this trial evaluated three dose levels, 1 mg/kg, 3 mg/kg, and 10 mg/kg, administered every 2 weeks (Q2W) in subjects with advanced solid tumors. All three dose levels were well tolerated and no dose limiting toxicities were observed. This first in human study of pembrolizumab showed evidence of target engagement and objective evidence of tumor size reduction at all dose levels (1 mg/kg, 3 mg/kg and 10 mg/kg Q2W). No maximum tolerated dose (MTD) has been identified.

In KEYNOTE-001, two randomized cohort evaluations of melanoma subjects receiving pembrolizumab at a dose of 2 mg/kg versus 10 mg/kg Q3W have been completed. The clinical efficacy and safety data demonstrate a lack of clinically important differences in efficacy response or safety profile at these doses. For example, in Cohort B2, advanced melanoma subjects who had received prior ipilimumab therapy were randomized to receive pembrolizumab at 2 mg/kg versus 10 mg/kg Q3W. The overall response rate (ORR) was 26% (21/81) in the 2mg/kg group and 26% (25/79) in the 10 mg/kg group (full analysis set (FAS)). The proportion of subjects with drug-related adverse events (AEs), grade 3-5 drug-related AEs, serious drug related AEs, death or discontinuation due to an AE was comparable between groups or lower in the 10 mg/kg group.

Population PK data analysis of pembrolizumab administered Q2W confirmed the expectation that intrinsic factors do not affect exposure to pembrolizumab to a clinically

meaningful extent and also revealed slow systemic clearance, limited volume of distribution, and a long half-life (refer to IB). Pharmacodynamic data (IL-2 release assay) suggested that peripheral target engagement is durable (>21 days). This early PK and pharmacodynamic data provides scientific rationale for testing a Q3W dosing schedule. Taken together, these data also support the use of lower doses (with similar exposure to 2 mg/kg Q3W) in all solid tumor indications. 2 mg/kg Q3W is being evaluated in NSCLC in PN001, Cohort F30 and PN010, and 200 mg Q3W is being evaluated in head and neck cancer in PN012, which are expected to provide additional data supporting the dose selection.

Selection of 200 mg as the appropriate dose for a switch to fixed dosing is based on simulation results indicating that 200 mg provides exposures that are reasonably consistent with those obtained with the 2 mg/kg dose and importantly maintains individual patient exposures within the exposure range established in melanoma as associated with maximal clinical response. A population PK model, which characterized the influence of body weight and other patient covariates on exposure, has been developed using available data from 476 subjects from PN001. The distribution of exposures from the 200 mg fixed dose are predicted to considerably overlap those obtained with the 2 mg/kg dose, with some tendency for individual values to range slightly higher with the 200 mg fixed dose. The slight increase in PK variability predicted for the fixed dose relative to weight-based dosing is not expected to be clinically important given that the range of individual exposures is well contained within the range of exposures shown inthe melanoma studies of 2 and 10 mg/kg to provide similar efficacy and safety. The population PK evaluation revealed that there was no significant impact of tumor burden on exposure. In addition, exposure was similar between the NSCLC and melanoma indications. Therefore, there are no anticipated changes in exposure between different tumor types and indication settings. A fixed dose regimen simplifies the dosing regimen to be more convenient for physicians and reduce potential for dosing errors. Additionally, a fixed dosing scheme reduces complexity in the logistical chain at treatment facilities and reduce wastage.

Rationale Trebananib Dose Selection

The safety, pharmacokinetics, and antitumor activity of Trebananib (AMG 386) were evaluated in Phase I study in patients with advanced solid tumors. Thirty-two patients received weekly intravenous AMG 386 doses of 0.3, 1, 3, 10, or 30 mg/kg in sequential cohorts. One DLT was reported in the 30 mg/kg cohort (respiratory arrest), which likely was caused by tumor

burden that was possibly related to AMG 386. The most common toxicities were fatigue and peripheral edema. Proteinuria (n = 11) was observed without clinical sequelae. Only four patients (12%) experienced treatment-related toxicities greater than grade 1. A maximum-tolerated dose was not reached. PK was dose-linear and the mean terminal-phase elimination half-life values ranged from 3.1 to 6.3 days. Serum AMG 386 levels appeared to reach steady-state after four weekly doses, and there was minimal accumulation. No anti-AMG 386 neutralizing antibodies were detected (Herbst RS, et al., J. Clin. Oncol. 27(21):3557-65 (2009)). The safety of AMG 386 was also evaluated in a Phase I study in the Japanese population using dose escalation of 3, 10, or 30 mg/kg. Trebananib was well tolerated at all dose levels and no DLT was observed (Doi T., et al., Cancer Chemother. Pharmacol. 71(1):227-35 (2013)). Trebananib has been evaluated as monotherapy and in combination with chemotherapy or other biologic agents across tumor types, including mixed solid tumors, ovarian, breast, renal, gastric, hepatic, and colorectal cancers. As of the study-specific data cutoff dates, 3611 subjects have been enrolled into 20 studies in the trebananib clinical program, of whom 3561 subjects have received ≥ 1 dose of trebananib or trebananib placebo, at doses ranging from 0.3 mg/kg to 30 mg/kg intravenously (IV) once weekly (QW). No maximum tolerated dose for trebananib monotherapy has been identified at doses up to 30 mg/kg IV QW. Most of early phase 1b/2 studies (in combination with other agents) were conducted with 3 and 10 mg/kg every week (QW) doses. Later, based on exposure response modeling the 15 mg/kg QW dose was elected in all 3 of the large Phase 3 trials and this dose was explored further in a few Phase 1b/2. Few small studies using 30 mg/kg QW in combination with chemotherapy or other agents are currently ongoing and there is no controlled studies comparing 15 to 30 mg/kg.

Efficacy Endpoints

The primary endpoint of this study is to determine the safety, tolerability and recommended dosing for the combination of pembrolizumab plus AMG386. A chart depicting a study schema for Phase 1b clinical drug trial combining pembrolizumab plus AMG386 is shown in FIG. 38. The secondary objective are to obtain in preliminary fashion the efficacy of the combination including PFS at 6 months, the rate of 1-year overall (OS), the response rate (RR) and time to progression in diseases where anti-angiogenesis had shown to be effective. FDG-PET imaging are obtained at baseline, at eight weeks, and at sixteen weeks following the beginning of treatment in the melanoma cohort in the expansion phase. This is utilized to assess

for metabolic changes as a function of this combination therapy at tumor sites in attempt to get an early sense of tumor activity/response versus immune inflammation. Chest, abdomen, and pelvic CT scan and head MRI or CT is obtained every twelve weeks for determination of therapeutic efficacy. Standard solid tumor response criteria (RECIST) obtained. To further explore the clinical observations in some patients receiving ipilimumab of delayed responses and disease burden increasing before stable disease or response attained, proposed immune response criteria is incorporated to assess for clinical activity. These immune response criteria (irRC) (Hodi F, et al., J. Clin. Oncol. 26 (no. 15_suppl):3008 (2008)) (Wolchok J.D., et al., Clin. Cancer Res. 15:7412-20 (2009)) is captured and compared to standard response criteria for solid tumors.

Correlative Studies Background

Antigen-specific T cell responses are controlled by co-stimulatory and co-inhibitory molecules positively and negatively. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1, CD279) are among the key co-inhibitory molecules, broadly categorized as "checkpoint molecules" (Pardoll D.M., Nat Rev Cancer 12: 252-64 (2012)). CD279 is up-regulated on activated T lymphocytes and mediate immunosuppression when binding to its ligands B7-H1 (CD274) and B7-DC (CD273). Blockade of CD279 or CD274 induced durable objective response in patients with advanced melanoma, renal cell carcinoma and non-small cell lung cancers in clinical trials (Topalian S.L., et al., N. Engl. J. Med. 366(26): 2443-2454 (2012); Brahmer JR, et. al, N. Engl. J. Med. 366(26):2455-65 (2012); Hamid O, et al., N. Engl. J. Med. 369(2): 134-144 (2013)). Moreover, immunohistochemical staining performed on pretreatment tumor tissues from patients with anti-CD279 treatment showed that none of 17 patients with CD274 negative tumors had an objective response whereas 36% (9/25) patients with CD274 positive tumors had an objective response (P=0.006). This striking difference suggests that CD274 expression on tumor specimen might be a useful biomarker to predict response to anti- CD279 treatment.

Anti-vascular endothelial growth factor pathway therapies preferentially target immature tumor blood vessels and leave behind normalized and resistant blood vessels. Angiopoietin-2 (ANGPT2)/TIE pathway is largely confined to vasculature. It has two receptors TIE1 and TIE2 as well as three ligands, angiopoietin-1, angiopoietin-2 (ANGPT2) and angiopoietin-4. ANGPT2 plays an important role in vascular remodeling and angiogenesis. It acts as context-specific

antagonist of angiopoietin-1/TIE2 signaling, destabilizes the quiescent blood vessels as a prerequisite to sprouting angiogenesis in the presence of proangiogenic stimulation or vascular regression in the absence of such stimuli. Therapeutics targeting the ANGPT2/TIE pathways including selective anti-ANGPT2 antibodies is in development (Gerald D, et al., Cancer Res 73(6): 1649-1657 (2013); Karlan BY, et al., J. Clin. Oncol. 30(4):362-71 (2012); Hashizume H, et al., Cancer Res. 70:6 2213-2223 (2010)). Serum ANGPT2 is found to be a biomarker for tumor progression and survival in various malignancies (Helfrich I, et al., Clin Cancer Res. 15(4):1384-92 (2009)).

As described herein, not only CD274, but also CD279 and CD273 protein expression in tumor tissues, is associated with favorable clinical response, and serve as biomarkers for patient selection for CD279 blockade in clinical treatment. As described herein, ANGPT2 protein expression in tumor tissue is a biomarker to identify a group of patients who might have objective responses on anti-ANGPT2 or/with anti-vascular endothelial growth factor therapies.

Treatment Regimen

This is a prospective trial which accrues subjects with solid tumors to evaluate the safety, clinical, and immunological effect of the combination of pembrolizumab (MK-3475) and trebananib (AMG386). The treatment includes an induction phase of pembrolizumab and trebananib for 4 cycles (12 wks) followed by pembrolizumab alone for up to 2 years. This trial are conducted in 2 parts:

Part I uses a standard $3^{+}3$ dose escalation design in all solid tumors (refer to Table 2. Dose escalation begins in dose cohort $^{+}1$ (refer to Table 3). If two or more patients in dose cohort $^{+}1$ experience a DLT, the next cohort of patients are enrolled into dose cohort -1. Should dose cohort -1 prove too toxic (i.e. two or more patients experience a DLT), enrollment to the study stops. If the toxicity profile of dose cohort $^{+}1$ is acceptable per the escalation decision rules in Table 2, the next cohort are enrolled into dose cohort $^{+}2$. Should dose cohort $^{+}2$ have acceptable toxicity per the escalation decision rules in Table 2, that are the RP2D; otherwise, dose cohort $^{+}1$ are the RP2D.

Table 2. Dose Escalation Schema

Number of Patients with DLT at a Given Dose Level	Escalation Decision Rule
0 out of 3	Enter 3 patients at the next dose level.
≥2	Dose escalation will be stopped. This dose level will be declared the maximally administered dose (highest dose administered). Three (3) additional patients will be entered at the next lowest dose level if only 3 patients were treated previously at that dose.
1 out of 3	Enter 3 more patients at this dose level. • If 0 of these 3 patients experience DLT, proceed to the next dose level. • If 1 or more of this group suffer DLT, then dose escalation is stopped, and this dose is declared the maximally administered dose. Three (3) additional patients will be entered at the next lowest dose level if only 3 patients were treated previously at that dose.
≤1 out of 6 at highest dose level below the maximally administered dose	This is generally the recommended phase 2 dose. At least 6 patients must be entered at the recommended phase 2 dose.

Table 3. Part I Dose Escalation Schedule

Dose Cohort	Pembrolizumab every 3 weeks IV	Trebananib weekly IV	N
- 1	200mg	3 mg/kg	3+3
+1	200mg	15 mg/kg	3+3
+2	200mg	30 mg/kg	3+3

Dose Escalation Schema:

Dose cohort	PEMBROLIZUMAB every three weeks	AMG386 weekly	N
-1	2 mg/kg	3 mg/kg	3+3
+1	2 mg/kg	10mg/kg	3+3
+2	2 mg/kg	30 mg/kg	3+3

Part II proceeds with four dose expansion cohorts: melanoma, renal cell carcinoma, ovarian cancer, and colorectal cancer. For each disease type, 12 patients are enrolled and treated at the RP2D of pembrolizumab and trebananib (AMG386). Safety assessments include all patients receiving one or more doses of the study drug combinations. Secondary and correlative endpoints are summarized according to disease indication and, in an exploratory fashion with all patients combined.

Treatment

PEMBROLIZUMAB is tested at 2 mg/kg. The AMG386 combination has been tested in patients every week. Patients may continue treatment with up to four new lesions in the absence of a decline in performance status. Evaluation are by standard response criteria.

FDG-PET imaging is obtained at baseline, at eight weeks, and at sixteen weeks following the beginning of treatment. This is utilized to assess for metabolic changes as a function of this combination therapy at tumor sites in attempt to get an early sense of tumor activity/response versus immune inflammation. Chest, abdomen, and pelvic Computed tomography (CT) scan and head Magnetic resonance imaging (MRI) or CT is obtained every twelve weeks for determination of therapeutic efficacy. Standard solid tumor response criteria (RECIST) is obtained. To further explore the clinical observations in some patients receiving ipilimumab of delayed responses and disease burden increasing before stable disease or response attained, proposed immune response criteria is incorporated to assess for clinical activity. These immune

response criteria (irRC) (Hodi F, et al., J. Clin. Oncol. 26(no.15_suppl):3008 (2008)) (Wolchok J.D., et al., Clin. Cancer Res. 15:7412-20 (2009)) are captured and compared to standard response criteria for solid tumors.

The investigator shall take responsibility for and shall take all steps to maintain appropriate records and ensure appropriate supply, storage, handling, distribution and usage of trial treatments in accordance with the protocol and any applicable laws and regulations.

Trial Treatments

The treatment to be used in this trial is outlined below in Table 4. Each treatment cycle are 3 weeks (21 days) long.

The treatment includes an induction phase of pembrolizumab and trebananib for 4 cycles (12 wks) followed by pembrolizumab alone for 2 years.

Table 4. Regimen Description for Parts I and II

Regimen Description					
Agent	**Pre-medications**	**Dose**	**Route**	**Schedule *****	**Cycle Length**
Pembrolizumab	Not routinely necessary unless prior infusion reaction	200mg at a final concentration of 1 mg/mL to 10 mg/mL in NS or D5	IV over approximately 30 minutes (range: 25-40 minutes)	Day 1	21 days (3 weeks)
Trebananib	Not routinely necessary unless prior infusion reaction	** mg/kg at a final concentration of 30mg/mL in	****IV over approximately 30 minutes	Day 1,8,15	
Doses as appropriate for assigned dose level. * The treatment will include an induction phase of pembrolizumab and trebananib for 4 cycles (12 wks) followed by pembrolizumab alone for 2 years. **** First dose of trebananib should be administered over 60 minutes.					

**Doses as appropriate for assigned dose level.

*** The treatment includes an induction phase of pembrolizumab and trebananib for 4 cycles (12 wks) followed by pembrolizumab alone for 2 years.

**** First dose of trebananib should be administered over 60 minutes.

Agent Administration

Treatment is administered on an outpatient basis. Dose for weight-based drugs should be flagged for recalculation at the start of each cycle should the weight of a subject change by more than 5% from the previous cycle's Day 1 weight. No investigational or commercial agents or therapies other than those described below may be administered with the intent to treat the patient's malignancy.

Timing of Dose Administration: Pembrolizumab

Pembrolizumab should be administered on Day 1 of each cycle after all procedures/assessments have been completed as detailed on the Trial Flow Chart (Section 9). Pembrolizumab may be administered with trebananib up to 2 days before or after the scheduled Day 1 of each cycle due to administrative reasons. Pembrolizumab is administered before trebananib.

Pembrolizumab (200 mg) is administered as a 30 minute IV infusion every 3 weeks. Sites should make every effort to target infusion timing to be as close to 30 minutes as possible. However, given the variability of infusion pumps from site to site, a window of -5 minutes and $^{+}$10 minutes is permitted (i.e., infusion time is 30 minutes: -5 min/$^{+}$10 min).

The Pharmacy Manual contains specific instructions for the preparation of the pembrolizumab infusion fluid and administration of infusion solution.

Timing of Dose Administration: Trebananib

Trebananib should be administered on Day 1, 8, and 15 of each 21-day cycle after all procedures/assessments have been completed. Trebananib may be administered with pembrolizumab up to 2 days before or after the scheduled Day of each cycle due to administrative reasons (±2 days).

The first dose of Trebananib is administered by IV infusion over a 60-minute period. If the initial dose administration is well tolerated, future administrations may be given over approximately 30 minutes (treatment cycle intervals may be increased due to toxicity a described in section 5). A window of -5 to $^{+}$10 minutes is permitted (i.e. infusion time is 30 minutes: -5 min/$^{+}$10 min). Trebananib is administered within 2 hours after Pembrolizumab.

The Investigational Product Instruction Manual (IPIM) contains specific instructions for the preparation of the Trebananib infusion fluid and administration of infusion solution.

Pembrolizumab Dose Modification

There are no dose reductions for an individual patient for pembrolizumab. Only dose holds are permitted per protocol.

Trebananib Dose Modification and Toxicity Management

Doses may be modified based on toxicity. Such modification can be carried out based on the knowledge of a skilled artisan.

Definition of Maximum Tolerated Dose

The MTD is based on the assessment of DLTs as defined in section 5.5 and does not exceed the 30mg/kg trebanabib weekly dose. The MTD is defined as the dose at which fewer than one-third of participants experience a DLT to pembrolizumab and trebananib.

PHARMACEUTICAL INFORMATION

Pembrolizumab Description

Pembrolizumab is a humanized anti-PD-1 mAb of the IgG4/kappa isotype with a stabilizing S228P sequence alteration in the fragment crystallizable (Fc) region. Pembrolizumab binds to human PD-1 and blocks the interaction between PD-1 and its ligands. The theoretical molecular weight of the polypeptide is 146,288 Da and its theoretical pI is 7.5. Additional information on pembrolizumab nomenclature is detailed in the following table:

Table 10

Code Name	MK-3475 (Anti–PD-1)
Other Code Name	MK3, 02P106, ORG 307448-0, SCH 900475 (Anti–PD-1)
Chemical Name	Humanized X PD-1-mAb (H409A11) IgG4
CAS Number	1374853-91-4
CAS Name	Anti-(human protein PDCD1 (programmed cell death 1)) immunoglobulin G4 (human-Mus musculus monoclonal heavy chain) disulfide with human-Mus musculus monoclonal light chain, dimer
Trade Name	KEYTRUDA®

Trebananib Description

Trebananib is an Fc fusion protein directed against Ang1 and Ang2, expressed recombinantly in *Escherichia coli* (*E coli*). The molecule is a non-glycosylated homodimer engineered by fusing an immunoglobulin G1 (IgG1) Fc domain to 4 copies of an anti-Ang1 / anti-Ang2 peptide. Each monomeric unit contains 10 cysteine residues that are involved in 4 intrachain disulfide bonds and 2 interchain disulfide bonds. Trebananib contains 287 amino

acids. The molecular weight is approximately 63.5 kDa. The molecular weight is approximately 63.5 kDa.

Form

Pembrolizumab Form

Two drug product (DP) dosage forms are availaible for pembrolizumab: a white to off-white lyophilized powder, 50 mg/vial, and a liquid, 100 mg/vial, both in Type I glass vials intended for single use only. The drug products are manufactured using facilities and practices under Good Manufacturing Practice (GMP) requirements.

Pembrolizumab Powder for Solution for Infusion, 50 mg/vial is a lyophilized powder that is reconstituted with sterile water for injection prior to use. It is manufactured using either the fully formulated DS or the partially formulated DS. The fully formulated DS uses L-histidine as a buffering agent, polysorbate 80 as surfactant, and sucrose as stabilizer/tonicity modifier. Pembrolizumab DP using the partially formultated DS is formulated with L-histidine as a buffering agent, polysorbate 80 as a surfactant, and sucrose as a stabilizer/tonicity modifier, and may contain hydrochloric acid and/or sodium hydroxide for pH adjustment (if necessary).

Pembrolizumab Solution for Infusion 100 mg/vial is a liquid DP (manufactured using the fully formulated DS with L-histidine as a buffering agent, polysorbate 80 as a surfactant, and sucrose as a stabilizer/tonicity modifier).

Pembrolizumab is provided as summarized in the following table:

Table 11

Product Name & Potency	Dosage Form
Pembrolizumab 50 mg	Lyophilized Powder for Injection
Pembrolizumab 100 mg/ 4mL	Solution for Injection

Trebananib Form

Trebananib is provided as a sterile, preservative-free, lyophilized powder for reconstitution with sterile water for injection (sWFI) and dilution in normal saline (0.9% sodium chloride) for IV administration. Each sterile vial contains specified amount of deliverable drug product, that when reconstituted with a specified volume of sWFI contains an isotonic formulation of 30 mg/mL trebananib formulated with 10 mM histidine, 4% (weight/volume [w/v]) mannitol, 2% (w/v) sucrose, 10 mM arginine hydrochloride, and 0.01% (w/v) polysorbate

20 to a pH of 7.1. Each vial is for single use only. Lyophilized vials are manufactured in 4 presentations based on the deliverable drug product. The vial presentations, vial sizes, deliverable amount, and reconstitution volume are provided in the table below.

Table 12

Vial Presentation	Vial size	Deliverable Amount	Reconstitution Volume (mL sWFI)
54 mg	5 cc	54 mg	2.0
150 mg	20 cc	150 mg	5.0
240 mg	20 cc	240 mg	8.0
600 mg	50 cc	600 mg	20.0

cc = cubic centimeters; mg = milligram; sWFI = sterile water for injection

Administration

Pembrolizumab Administration

Pembrolizumab 200 mg is administered as a 30 minute IV infusion every 3 weeks. Sites should make every effort to target infusion timing to be as close to 30 minutes as possible. However, given the variability of infusion pumps from site to site, a window of -5 minutes and $^{+}$10 minutes is permitted (i.e., infusion time is 30 minutes: -5 min/$^{+}$10 min).

Trebananib

Trebananib should be administered on Day 1, 8, and 15 of each 21-day cycle after all procedures/assessments have been completed. Trebananib may be administered up to 2 days before or after the scheduled Day of each cycle due to administrative reasons (±2 days).

The first dose of Trebananib is administered by IV infusion over a 60-minute period. If the initial dose administration is well tolerated, future administrations may be given over approximately 30 minutes (treatment cycle intervals may be increased due to toxicity a described in section 5). Trebananib is administered immediately after Pembrolizumab.

MEASUREMENT OF EFFECT

Antitumor Effect – Solid Tumors

Although the clinical benefit of these drugs is described herein, the intent of offering this treatment is to provide a therapeutic benefit, and thus the patient is carefully monitored for tumor response and symptom relief in addition to safety and tolerability. Patients with measurable disease are assessed by standard criteria. For the purposes of this study, patients are re-evaluated every 12 weeks. In addition to a baseline scan, confirmatory scans are also obtained 4-6 weeks following initial documentation of an objective response.

Response and progression are evaluated in this study using the new international criteria proposed by the Response Evaluation Criteria in Solid Tumors (RECIST) guideline (version 1.1) [Eur J Ca 45:228-247, 2009]. Changes in the largest diameter (unidimensional measurement) of the tumor lesions and the shortest diameter in the case of malignant lymph nodes are used in the RECIST criteria.

Definitions

Evaluable for Target Disease response. Only those participants who have measurable disease present at baseline, have received at least one cycle of therapy, and have had their disease re-evaluated are considered evaluable for target disease response. These participants have their response classified according to the definitions stated below. (Note: Participants who exhibit objective disease progression prior to the end of cycle 1 are also considered evaluable.)

Evaluable Non-Target Disease Response. Participants who have lesions present at baseline that are evaluable but do not meet the definitions of measurable disease, have received at least one cycle of therapy, and have had their disease re-evaluated are considered evaluable for non-target disease. The response assessment is based on the presence, absence, or unequivocal progression of the lesions.

Disease Parameters

Measurable disease

Measurable lesions are defined as those that can be accurately measured in at least one dimension (longest diameter to be recorded) as $\geq$ 20 mm by chest x-ray or $\geq$10 mm with CT scan, MRI, or calipers by clinical exam. All tumor measurements must be recorded in millimeters (or decimal fractions of centimeters).Note: Tumor lesions that are situated in a previously irradiated area might or might not be considered measurable.

Malignant lymph nodes

To be considered pathologically enlarged and measurable, a lymph node must be $\geq$ 15 mm in short axis when assessed by CT scan (CT scan slice thickness recommended to be no greater than 5 mm). At baseline and in follow-up, only the short axis is measured and followed.

Non-measurable disease

All other lesions (or sites of disease), including small lesions (longest diameter < 10 mm or pathological lymph nodes with $\geq$ 10 to < 15 mm short axis), are considered non-measurable disease. Bone lesions, leptomeningeal disease, ascites, pleural/pericardial effusions, lymphangitis

cutis/pulmonitis, inflammatory breast disease, abdominal masses (not followed by CT or MRI), and cystic lesions are all considered non-measurable. Note: Cystic lesions that meet the criteria for radiographically defined simple cysts should not be considered as malignant lesions (neither measurable nor non-measurable) since they are, by definition, simple cysts.

'Cystic lesions' thought to represent cystic metastases can be considered as measurable lesions, if they meet the definition of measurability described above. However, if non-cystic lesions are present in the same participant, these are preferred for selection as target lesions.

Target lesions

All measurable lesions up to a maximum of 2 lesions per organ and 5 lesions in total, representative of all involved organs, are identified as target lesions and recorded and measured at baseline. Target lesions should be selected on the basis of their size (lesions with the longest diameter), be representative of all involved organs, but in addition should be those that lend themselves to reproducible repeated measurements. It may be the case that, on occasion, the largest lesion does not lend itself to reproducible measurement in which circumstance the next largest lesion which can be measured reproducibly should be selected. A sum of the diameters (longest for non-nodal lesions, short axis for nodal lesions) for all target lesions is calculated and reported as the baseline sum diameters. If lymph nodes are to be included in the sum, then only the short axis is added into the sum. The baseline sum diameters are used as reference to further characterize any objective tumor regression in the measurable dimension of the disease.

Non-target lesions

All other lesions (or sites of disease) including any measurable lesions over and above the 5 target lesions are identified as non-target lesions and should also be recorded at baseline. Measurements of these lesions are not required, but the presence, absence, or in rare cases unequivocal progression of each should be noted throughout follow up.

Methods for Evaluation of Disease

All measurements should be taken and recorded in metric notation using a ruler, calipers, or a digital measurement tool. All baseline evaluations should be performed as closely as possible to the beginning of treatment and never more than 4 weeks before the beginning of the treatment.

The same method of assessment and the same technique should be used to characterize each identified and reported lesion at baseline and during follow-up. Imaging-based evaluation is

preferred to evaluation by clinical examination unless the lesion(s) being followed cannot be imaged but are assessable by clinical exam.

Clinical lesions

Clinical lesions are only considered measurable when they are superficial (e.g., skin nodules and palpable lymph nodes) and ≥ 10 mm in diameter as assessed using calipers (e.g., skin nodules). In the case of skin lesions, documentation by color photography, including a ruler to estimate the size of the lesion, is recommended.

Chest x-ray

Lesions on chest x-ray are acceptable as measurable lesions when they are clearly defined and surrounded by aerated lung; however, CT is preferable.

Conventional CT and MRI

This guideline has defined measurability of lesions on CT scan based on the assumption that CT thickness is 5mm or less. If CT scans have slice thickness greater than 5 mm, the minimum size of a measurable lesion should be twice the slice thickness. MRI is also acceptable in certain situations (e.g. for body scans).

Use of MRI remains a complex issue

MRI has excellent contrast, spatial, and temporal resolution; however, there are many image acquisition variables involved in MRI, which greatly impact image quality, lesion conspicuity, and measurement. Furthermore, the availability of MRI is variable globally. As with CT, if an MRI is performed, the technical specifications of the scanning sequences used should be optimized for the evaluation of the type and site of disease. Furthermore, as with CT, the modality used at follow-up should be the same as was used at baseline and the lesions should be measured/assessed on the same pulse sequence. It is beyond the scope of the RECIST guidelines to prescribe specific MRI pulse sequence parameters for all scanners, body parts, and diseases. Ideally, the same type of scanner should be used and the image acquisition protocol should be followed as closely as possible to prior scans. Body scans should be performed with breath-hold scanning techniques, if possible.

Fluorodeoxyglucose (FDG)-positron emission tomography (PET)

While FDG-PET response assessments need additional study, it is sometimes reasonable to incorporate the use of FDG-PET scanning to complement CT scanning in assessment of

progression (particularly possible 'new' disease). New lesions on the basis of FDG-PET imaging can be identified according to the following algorithm:

a) Negative FDG-PET at baseline, with a positive FDG-PET at follow-up is a sign of PD based on a new lesion.

b) No FDG-PET at baseline and a positive FDG-PET at follow-up: If the positive FDG-PET at follow-up corresponds to a new site of disease confirmed by CT, this is PD. If the positive FDG-PET at follow-up is not confirmed as a new site of disease on CT, additional follow-up CT scans are needed to determine if there is truly progression occurring at that site (if so, the date of PD is the date of the initial abnormal FDG-PET scan). If the positive FDG-PET at follow-up corresponds to a pre-existing site of disease on CT that is not progressing on the basis of the anatomic images, this is not PD.

c) FDG-PET may be used to upgrade a response to a CR in a manner similar to a biopsy in cases where a residual radiographic abnormality is thought to represent fibrosis or scarring. The use of FDG-PET in this circumstance should be prospectively described in the protocol and supported by disease-specific medical literature for the indication. However, it must be acknowledged that both approaches may lead to false positive CR due to limitations of FDG-PET and biopsy resolution/sensitivity. FDG-PET imaging is obtained at baseline, at eight weeks, and at sixteen weeks following the beginning of treatment in the melanoma cohort. This is utilized to assess for metabolic changes as a function of this combination therapy at tumor sites in attempt to get an early sense of tumor activity/response versus immune inflammation and is compared in exploratory fashion to RECIST and irRECIST criteria.

- Note: A ‘positive’ FDG-PET scan lesion means one which is FDG avid with an uptake greater than twice that of the surrounding tissue on the attenuation corrected image. PET-CT. At present, the low dose or attenuation correction CT portion of a combined PET-CT is not always of optimal diagnostic CT quality for use with RECIST measurements. However, if the site can document that the CT performed as part of a PET-CT is of identical diagnostic quality to a diagnostic CT (with IV and oral contrast), then the CT portion of the PET-CT can be used for RECIST measurements and can be used interchangeably with conventional CT in accurately measuring cancer lesions over time. Note, however, that the PET

portion of the CT introduces additional data which may bias an investigator if it is not routinely or serially performed.

Ultrasound

Ultrasound is not useful in assessment of lesion size and should not be used as a method of measurement. Ultrasound examinations cannot be reproduced in their entirety for independent review at a later data and, because they are operator dependent, it cannot be guaranteed that the same technique and measurements are taken from one assessment to the next. If new lesions are identified by ultrasound in the course of the study, confirmation by CT or MRI is advised. If there is concern about radiation exposure from CT, MRI may be used instead of CT in selected instances.

Endoscopy, Laparoscopy

The utilization of these techniques for objective tumor evaluation is not advised. However, such techniques may be useful to confirm complete pathological response when biopsies are obtained or to determine relapse in trials where recurrence following complete response (CR) or surgical resection is an endpoint.

Tumor markers

Tumor markers alone cannot be used to assess response. If markers are initially above the upper normal limit, they must normalize for a participant to be considered in complete clinical response. Specific guidelines for both CA-125 response (in recurrent ovarian cancer) and PSA response (in recurrent prostate cancer) have been published [JNCI 96:487-488, 2004; J Clin Oncol 17, 3461-3467, 1999; J Clin Oncol 26:1148-1159, 2008]. In addition, the Gynecologic Cancer Intergroup has developed CA-125 progression criteria which are to be integrated with objective tumor assessment for use in first-line trials in ovarian cancer [JNCI 92:1534-1535, 2000].

Cytology, Histology

These techniques can be used to differentiate between partial responses (PR) and complete responses (CR) in rare cases (e.g., residual lesions in tumor types, such as germ cell tumors, where known residual benign tumors can remain).

The cytological confirmation of the neoplastic origin of any effusion that appears or worsens during treatment when the measurable tumor has met criteria for response or stable

disease is mandatory to differentiate between response or stable disease (an effusion may be a side effect of the treatment) and progressive disease.

Response Criteria

Evaluation of Target Lesions

Complete Response (CR): Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to <10 mm.

Partial Response (PR): At least a 30% decrease in the sum of the diameters of target lesions, taking as reference the baseline sum diameters.

Progressive Disease (PD): At least a 20% increase in the sum of the diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm. (Note: the appearance of one or more new lesions is also considered progressions).

Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study.

Evaluation of Non-Target Lesions

Complete Response (CR): Disappearance of all non-target lesions and normalization of tumor marker level. All lymph nodes must be non-pathological in size (<10 mm short axis).

Note: If tumor markers are initially above the upper normal limit, they must normalize for a patient to be considered in complete clinical response.

Non-CR/Non-PD: Persistence of one or more non-target lesion(s) and/or maintenance of tumor marker level above the normal limits.

Progressive Disease (PD): Appearance of one or more new lesions and/or unequivocal progression of existing non-target lesions. Unequivocal progression should not normally trump target lesion status. It must be representative of overall disease status change, not a single lesion increase.

Although a clear progression of "non-target" lesions only is exceptional, the opinion of the treating physician should prevail in such circumstances, and the progression status should be confirmed at a later time by the review panel (or Principal Investigator).

Evaluation of Best Overall Response

The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for progressive disease the smallest measurements recorded since the treatment started). The patient's best response assignment depends on the achievement of both measurement and confirmation criteria.

Table 13

For Participants with Measurable Disease (*i.e.*, Target Disease)

Target Lesions	Non-Target Lesions	New Lesions	Overall Response	Best Overall Response when Confirmation is Required*
CR	CR	No	CR	>4 wks Confirmation**
CR	Non-CR/Non-PD	No	PR	>4 wks Confirmation**
CR	Not evaluated	No	PR	
PR	Non-CR/Non-PD/not evaluated	No	PR	
SD	Non-CR/Non-PD/not evaluated	No	SD	Documented at least once >4 wks from baseline**
PD	Any	Yes or No	PD	no prior SD, PR or CR
Any	PD***	Yes or No	PD	
Any	Any	Yes	PD	

□ Se RECIST 1.1 manuscript for further details on what is evidence of a new lesion. non-randomized trials with response as primary endpoint.

** Only for circumstances, unequivocal progression in non-target lesions may

*** Incomplete be as disease progression.

assessment

with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be as reported "*symptomatic deterioration.*" Every effort should be made to document the objective progression even after discontinuation of treatment.

Table 14

For Participants with Non-Measurable Disease (*i.e.*, Non-Target Disease)

Non-Target Lesions	New Lesions	Overall Response
CR	No	CR
Non-CR/non-PD	No	Non-CR/non-PD*
Not all evaluated	No	not evaluated
Unequivocal PD	Yes or No	PD
Any	Yes	PD
□ 'Non-CR/non-PD' is preferred over 'stable disease' for non-target disease since SD is increasingly used as an endpoint for assessment of efficacy in some trials so to assign this category when no lesions can be measured is not advised		

Duration of Response

Duration of overall response: The duration of overall response is measured from the time measurement criteria are met for CR or PR (whichever is first recorded) until the first date that recurrent or progressive disease is objectively documented (taking as reference for progressive disease the smallest measurements recorded since the treatment started, or death due to any cause. Participants without events reported are censored at the last disease evaluation).

Duration of overall complete response: The duration of overall CR is measured from the time measurement criteria are first met for CR until the first date that progressive disease is objectively documented, or death due to any cause. Participants without events reported are censored at the last disease evaluation.

Duration of stable disease: Stable disease is measured from the start of the treatment until the criteria for progression are met, taking as reference the smallest measurements recorded since the treatment started, including the baseline measurements.

Treatment Beyond Progression

Immunotherapeutic agents such as Pembrolizumab may produce antitumor effects by potentiating endogenous cancer-specific immune responses. The response patterns seen with such an approach may extend beyond the typical time course of responses seen with cytotoxic agents, and can manifest as a clinical response after an initial increase in tumor burden or even the appearance of new lesions.

If radiologic imaging shows progressive disease (PD), tumor assessment may be repeated by the site approximately 4 weeks later in order to confirm continued PD with the option of continuing treatment per below while awaiting radiologic confirmation of progression. If repeat

imaging shows a reduction or stabilization in the tumor burden compared to the initial scan demonstrating PD, treatment may be continued as per treatment calendar. If repeat imaging confirms continued PD, patients are discontinued from study therapy. If reimaging is no worse than the prior scan 4 weeks prior, patients may continue therapy and be reimaged in another 8 weeks. If evidence for continued, increasing progression with subsequent imaging, the patient is discontinued. In determining whether or not the tumor burden has increased or decreased, investigators should consider all target lesions as well as non-target lesions. The decision to continue study treatment after the first evidence of disease progression determined by radiologic imaging is at the Investigator's discretion based on the clinical status of the patient as described in the table below.

Patients may receive study treatment while waiting for confirmation of continued PD if they are clinically stable as defined by the following criteria:

- Absence of signs and symptoms (including worsening of laboratory values) indicating disease progression
- No decline in ECOG performance status
- Absence of rapid progression of disease
- Absence of progressive tumor at critical anatomical sites (e.g., cord compression) requiring urgent alternative medical intervention

Treatment beyond progression is shown in the table below.

Table 15

	Clinically Stable		Clinically Unstable	
	Imaging	**Treatment**	**Imaging**	**Treatment**
1st radiologic evidence of PD	Repeat imaging at approximately 4 weeks to confirm PD	May continue study treatment at the Investigator's discretion while awaiting confirmatory scan	Repeat imaging at approximately 4 weeks to confirm PD if possible	Discontinue treatment
Repeat scan confirms PD	No additional imaging required	Discontinue treatment	No additional imaging required	N/A
Repeat scan shows SD, PR, or CR	Continue regularly scheduled imaging assessments every 6 weeks	Continue study treatment at the Investigator's discretion	Continue regularly scheduled imaging assessments every 6 weeks	May restart study treatment if condition has improved and/or clinically stable per Investigator's discretion
Tumor imaging will be assessed every 12 weeks (84 ±7 days).				

Progression-Free Survival

Overall Survival: Overall Survival (OS) is defined as the time from randomization (or registration) to death due to any cause, or censored at date last known alive.

Progression-Free Survival: Progression-Free Survival (PFS) is defined as the time from randomization (or registration) to the earlier of progression or death due to any cause. Participants alive without disease progression are censored at date of last disease evaluation.

Time to Progression: Time to Progression (TTP) is defined as the time from randomization (or registration) to progression, or censored at date of last disease evaluation for those without progression reported.

Antitumor Effect

Anti-Tumor Effect Using irRECIST

Definition of Tumor Response Using Immune-Related Response Criteria (irRC):

The sum of the longest diameter of lesions (SPD) at tumor assessment using the immune-related response criteria (irRC) for progressive disease incorporates the contribution of new measurable lesions. Each net Percentage Change in Tumor Burden per assessment using irRC criteria accounts for the size and growth kinetics of both old and new lesions as they appear.

Definition of Target Lesions Response Using irRC:

irComplete Response (irCR): Complete disappearance of all target lesions. This category encompasses exactly the same subjects as "CR" by the mWHO criteria.

irPartial Response (irPR): Decrease, relative to baseline, of 50% or greater in the sum of the products of the two largest perpendicular diameters of all target and all new measurable lesions (i.e., Percentage Change in Tumor Burden). Note: the appearance of new measurable lesions is factored into the overall tumor burden, but does not automatically qualify as progressive disease until the SPD increases by > 25% when compared to SPD at nadir.

irStable Disease (irSD): Does not meet criteria for irCR or irPR, in the absence of progressive disease.

irProgressive Disease (irPD): At least 25% increase Percentage Change in Tumor Burden (i.e., taking SPD of all target lesions and any new lesions) when compared to SPD at nadir.

Definition of Non-Target Lesions Response Using irRC

irComplete Response (irCR): Complete disappearance of all non-target lesions. This category encompasses exactly the same subjects as "CR" by the mWHO criteria.

irPartial Response (irPR) or irStable Disease (irSD): non-target lesion(s) are not considered in the definition of PR; these terms do not apply.

irProgressive Disease (irPD): Increases in number or size of non-target lesion(s) does not constitute progressive disease unless/until the Percentage Change in Tumor Burden increases by 25% (i.e., the SPD at nadir of the target lesions increases by the required amount).

Impact of New Lesions on irRC:

New lesions in and by themselves do not qualify as progressive disease. However their contribution to total tumor burden is included in the SPD which in turn feeds into the irRC criteria for tumor response. Therefore, new non-measurable lesions do not discontinue any subject from the study.

Definition of Overall Response Using irRC

Overall response using irRC is based on these criteria:

Immune-Related Complete Response (irCR): Complete disappearance of all tumor lesions (target and non-target together with no new measurable/unmeasurable lesions) for at least 4 weeks from the date of documentation of complete response.

Immune-Related Partial Response (irPR): The sum of the products of the two largest perpendicular diameters of all target lesions is measured and captured as the SPD baseline. At each subsequent tumor assessment, the SPD of the two largest perpendicular diameters of all target lesions and of new measurable lesions are added together to provide the Immune Response Sum of Product Diameters (irSPD). A decrease, relative to baseline of the irSPD compared to the previous SPD baseline, of 50% or greater is considered an immune Partial Response (irPR). Immune-Related Stable Disease (irSD): irSD is defined as the failure to meet criteria for immune complete response or immune partial response, in the absence of progressive disease. Immune-Related Progressive Disease (irPD): It is recommended in difficult cases to confirm PD by serial imaging. Any of the following constitutes progressive disease:

At least 25% increase in the SPD of all target lesions over baseline SPD calculated for the target lesions.

At least a 25% increase in the SPD of all target lesions and new measurable lesions (irSPD) over the baseline SPD calculated for the target lesions. (Hodi F, et al., J. Clin. Oncol. 26(no.15_suppl):3008 (2008); Wolchok J.D., et al., Clin. Cancer Res. 15:7412-20 (2009)). Immune-related response criteria definitions are set forth in the table below.

Table 16

Target Lesion Definition	Non-Target Lesion Definition	New Measurable Lesions	New Unmeasurable Lesions	Percent change in tumor burden (including measurable new lesions when present)	Overall irRC Response
Complete Response	Complete Response	No	No	−100%	irCR
Partial Response	Any	Any	Any	> −50%	irPR
				<−50% to <+25%	irSD
				>+25%	irPD
Stable Disease	Any	Any	Any	<−50% to <+25%	irSD
				>+25%	irPD
Progressive Disease	Any	Any	Any	>+25%	irPD

Immune-Related Best Overall Response Using irRC (irBOR):

irBOR is the best confirmed irRC overall response over the study as a whole, recorded between the date of first dose until the last tumor assessment before subsequent therapy (except for local palliative radiotherapy for painful bone lesions) for the individual subject in the study. For the assessment of irBOR, all available assessments per subject are considered.

irCR or irPR determinations included in the irBOR assessment must be confirmed by a second (confirmatory) evaluation meeting the criteria for response and performed no less than 4 weeks after the criteria for response are first met.

PET EORTC Guidelines for PET Scan Reporting (Young, H et al. Euro J of Cancer. 1999; 35(13):1773-1782; Shankar LK., et al., J. Nucl. Med. 47(6):1059-66 (2006))

Target Lesions

- 5 most FDG-avid lesions according to SUVmax
- Representative of all involved organs
- Should correspond to target/non-target lesions on CT/MRI assessment

Evaluation of Target Lesions

- Complete Response (CR): Complete resolution of FDG uptake within the tumor so that it is indistinguishable from surrounding normal tissue.
- Partial Response (PR): At least a 25% decrease in SUVmax of target lesions, taking as reference the baseline sum LD.
- Progressive Disease (PD): At least a 25% increase in the SUVmax, taking as reference the nadir SUVmax, and/or appearance of new FDG uptake in suspected metastatic lesions.
- Stable Disease (SD): Neither sufficient decrease in SUVmax to qualify for PR nor sufficient increase to qualify for PD.
- Unknown (UN): Assessment of target lesions cannot be made due to insufficient or unevaluable data. In this case, a concise explanation must be given.

  Note: If tumor response data is missing, an overall assessment cannot be done. However, if there is missing or unevaluable data for non-target lesions, but data is available for all target lesions, the overall response for that time point are assigned based on the SUVmax of all target lesions. Additionally, the assessment of CR cannot be made if there is

missing or unevaluable data for non-target lesions. In this case, the overall assessment would be PR.

TUMOR TISSUE COLLECTION AND CORRELATIVE STUDIES BLOOD SAMPLING

Correlative sciences include fresh biopsies of pre-existing sites of disease and following treatment to assess histologically for vasculopathy, immune infiltration, and tumor necrosis; stain pathologic specimens for VEGF/VEGFR expression, phosphoTie2; monitor circulating levels of and development of anti-trebananib antibodies as a function of treatment. Baseline and post-treatment values of a number of inflammatory and angiogenic cytokines are monitored. Pilot studies include the investigation of immune responses to other angiogenic molecules as a function of treatment. Flow cytometry of PBMC is monitored for changes in levels of naïve and memory CD4, CD8 and other lymphocyte populations. Cellular and humoral immune responses to established antigens as a function of treatment are performed. These include melanosomal differentiation antigens as well as melanoma antigen targets, Muc-1, CEA, CA-125, and NY-Eso-1 as examples.

Fresh tumor biopsies:

Biopsies of fresh tumor are obtained whenever possible prior to treatment initiation on day 1 and post-treatment (approximately 12 weeks). Dedicated funds are currently available at the institution for obtaining post-treatment biopsies in patients receiving immune based therapies.

Formalin fixed-paraffin embedded (FFPE) tumor slices are prepared and H&E stained for assessment of TIL in pre- and post-treatment tumor samples. To identify different immune cell populations (effector/memory/ CD8 cells, T regulatory cells, dendritic cells, tumor associated macrophages, NK cells, TEM) immunohistochemical staining is performed on FFPE tumor slices using the following antibodies:CD3, CD4, CD8, CD25, FoxP3, Indoleamine 2,3 deoxygenase-1 (IDO), CD11c, CD83, CD86, CD56, CD14, CD16, and Tie-2.

Immunohistochemical staining on paraffin embedded tissues was developed for PD-L1, PD-L2, TIM-3 and LAG-3 through the Center for Immuno-oncology Pathology Core (Scott Rodig, M.D., Ph.D. Core Director, collaboration letter included). PD-L1 immunohistochemistry (IHC) has recently been established in a Clinical Laboratory Improvement Amendments (CLIA) approved laboratory and the remaining assays for CLIA laboratory conduct are being finalized.

Methods, protocols, and data (FIG. 25) establishing the sensitivity and specificity of immunohistochemical staining (IHC) assays using the monoclonal antibodies recognizing PD-L1 (CD274, B7-H1, antibody clone 7G11, generated in the laboratory of Gordon Freeman, DFCI) and PD-L2 (CD273, B7-DC, clone 9E5, generated in the laboratory of Gordon Freeman, DFCI) are published in two recent manuscripts, each of which is incorporated herein by reference:

1. Chen BJ, et al., Clin. Cancer Res. 19(13):3462-73 (2013)
2. Shi M et al., Am J Surg Pathol. 2014, Jul 14, PMID: 25025450.

As part of the validation of the assays in a CLIA-certified laboratory, identical cases were stained multiple times and under a variety of staining conditions and the results reviewed by two certified pathologists. A positive control sample (classical Hodgkin lymphoma for PD-L1 expression; primary mediastinal large B-cell lymphoma for PD-L2 expression) and negative control sample (benign lymph node) is stained with each experimental tissue biopsy sample. The controls are reviewed by a certified pathologist at the time of review of the experimental sample.

An IHC assay (FIG. 26) for PD-1 (CD279, clone NAT105, Cell Marque Inc.) expression has been in standard surgical pathology diagnostic practice for several years and used to confirm the diagnosis of angioimmunoblastic T-cell lymphoma (AITL).

3. Yu H et al., Am J Clin Pathol. 2009, Jan;131(1):33-41, incorporated herein by reference. PMID:19095563.

PD-1 IHC is performed routinely in the CLIA-certified laboratory and interpreted by a certified pathologist with an appropriate control (reactive lymph node, intra-follicular T-cells are positive for PD-1) as described above.

Manuscript #1 above describes a semi-quantitative scoring method, which is in accordance with typical biomarker scoring in anatomic and surgical pathology. Briefly, staining is scored according to intensity (0= no staining, 1= weak staining, 2= moderate staining, 3= strong staining), staining pattern (M= predominantly cell membrane; C= predominantly cell cytoplasm), and the percentage of cells showing positive staining (0-100%). The semi-quantitative scoring is performed for: 1) the neoplastic tumor cells and 2) the non-neoplastic infiltrating immune cells. In the research setting, all cases are reviewed by two pathologists and any discordant results resolved by consensus review. Significantly discordant scoring results have been rare during case evaluations (FIG. 27; Chen BJ, et al., Clin. Cancer Res. 19(13):3462-73 (2013)).

Digital, quantitative scoring of stained tissue is performed using the Aperio slide scanning and analysis platform (FIG. 28). Quantitative assessment of positive staining uses the commercially provided algorithm for cell identification and positive pixels counting within a pre-defined DAB (brown, chromogenic) channel. This method of analysis shows good correlation with pathologists' scoring:

4. Mino-Kenudson M et al., Clin Cancer Res. 2010 Mar 1;16(5):1561-71, incorporated herein by reference. PMID:20179225.

This method was used to score PD-L1 expression in tumor cells:

5. Green MR et al., Blood. 2010 Oct 28;116(17):3268-77. PMID: 20628145, incorporated herein by reference.

The correlation between quantitative IHC as determined by Aperio analysis, and semi-quantitative scoring, as determined by visual interpretation, is determined as part of this study.

The scoring for markers (such as the PD-Ligands) that stain macrophages, dendritic cells, and other cells of heterogeneous morphology is semi-quantitative and performed by a pathologist using a modified H-score to capture 1) the percentage of neoplastic cells positive for biomarker expression, intensity of expression, and membrane or cytoplasmic expression, and 2) the percentage of non-neoplastic cells (macrophages, dendritic cells, endothelial cells) positive for biomarker expression, intensity of expression, and membrane or cytoplasmic expression.

Scoring for PD-1 and other markers that stain lymphoid cells (CD3, CD4, CD8, CD25, FOXP3, IDO, CD16, CD56, Lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin domain 3 (TIM-3)) is primarily performed by automated analysis using the Aperio system.

Aperio scoring for PD-1^+ (and other lymphoid markers) lymphocytes is accomplished using a standard Aperio algorithm, developed for quantifying nuclear stains, but is applicable to quantifying membrane staining of cells with a very high N:C ratio- such as lymphocytes (Nuclear algorithm). The output is number of positive-staining cells per unit area (microns2).

The data derived from the analyses above are used as individual data points compared to other clinical (response to treatment) and pathological (histomorphological) data in the study. A goal is to determine whether individual data points (i.e. number of PD-1^+ T-cells/ unit area) are of prognostic value, or if combined data using two or more data (an "immuno-score") provides

prognostic data. These investigations are exploratory and are performed in conjunction with the biostatisticians associated with this study.

For image analysis:

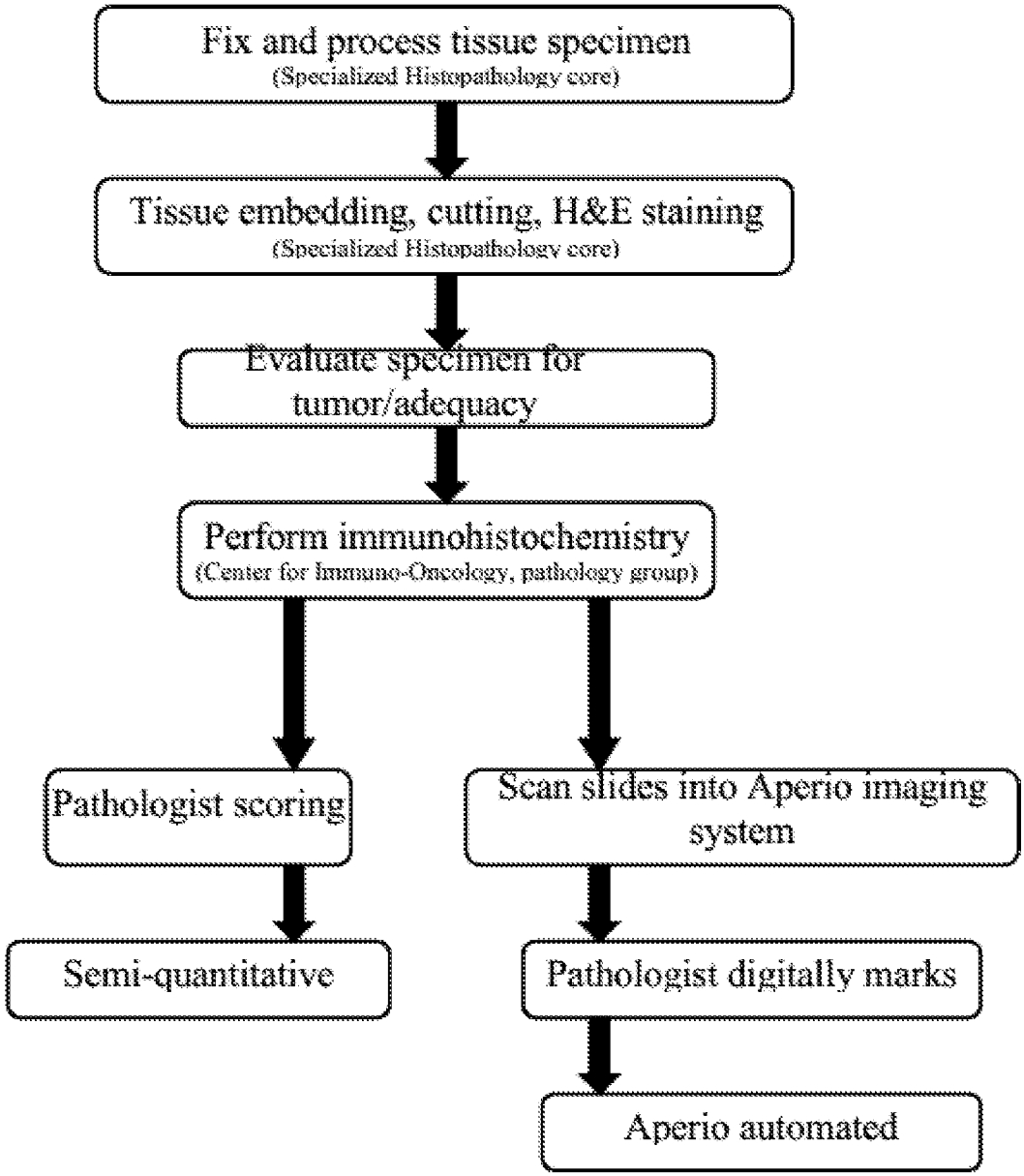
1. IHC stained slides are digitally scanned using the Aperio ScanScope XT (Leica Microsystems, Buffalo Grove, IL). The instrumentation is housed in the Tissue Microarray and Imaging Core (TMI) facility of the Dana-Farber/ Harvard Cancer Center (DF-/HCC). This facility is located adjacent to the office of Dr. Scott Rodig in the Department of Pathology. All digital images are stored on servers owned by the TMI core facility and accessed via the internet using a password-protected log-in.

2. Digital images are viewed using ImageScope Software (version 10.0.35.1800; Leica) on standard PCs. Slides are digitally annotated by the pathologist (Dr. S. Rodig) to identify the region of interest and analysis.

3. Quantitative analysis is performed using analytical software associated with ImageScope, specifically Aperio Color Deconvolution V.9 (for PD-Ligands) and nuclear algorithm (for PD-1^+ lymphocytes) and the results given as the percentage of positive pixels per unit area (for PD-Ligands) or number of positive cells per unit area (for PD-1^+ lymphocytes). Intensity of staining is also captured automatically using the above algorithms and assigned a score (0, 1, 2, or 3) based upon the average optical density of the region or cells. All results are exported into an excel spreadsheet.

4. Individual scoring data are compared to clinical parameters to determine if there is an association with outcome. Scores using a combination of biomarker data are considered.

Below, is a schematic of the workflow for the tissue-based biomarker analysis:



The list of antibodies to be used for correlative studies is included in the table below, which includes the prioritization of the markers (1=highest, 2=intermediate, 3=lowest), as well as the antibody clones, source of antibody, and status of validation.

Table 17

IHC Biomarkers	Priority	Clone/ Cat #	Source	Host species	Dilution	Optimized?
PD-L1	1	9A11	G. Freeman	Mouse	1/125	Yes
PD-L2	1	9E.6	G. Freeman	Mouse	1/10000	Yes
PD-1	1	EH33	G. Freeman	Mouse	1/600	Yes
CD3	1	IS503	Dako	Rabbit	1/250	Yes
CD4	1	4B12	Vector Labs	Mouse	1/200	Yes
CD8	1	144B	Dako	Mouse	1/100	Yes
FOXP3	1	206D	BioLegend	Mouse	1:50	Yes
TIM3	1	AF2365	R&D Systems	Goat	1:50	Yes
LAG3	1	17B4	LifeSpan BioSc	Mouse	1/200	Yes
Tie2	2	AF313	R&D Systems	Goat	1/500	Yes
ANGPT2	2	sc-74403	Santa Cruz Bio	Mouse	1/200	Yes
IDO1	2	ab55305	Abcam	Mouse	1/100	No
CD38	3	SPC32	Abcam	Mouse	1/300	Yes
CD56	3	123C3	Dako	Mouse	1/100	Yes
CD14	3	ab49755	Abcam	Mouse	1/100	Yes
CD16	3	ab183354	Abcam	Rabbit	1/100	No
CD11c	3	EP1347Y	Abcam	Rabbit	1/500	Yes

The cut-off of 5% for PD-L1 tumor positivity is in accordance the criteria used in a prior study examining the use of this biomarker to predict clinical response in patients treated with a PD-1 antibody (Topalian S.L., et al., N. Engl. J. Med. 366(26): 2443-2454 (2012)).

The semi-quantitative scoring for this study is in accordance with those published previously (Topalian S.L., et al., N. Engl. J. Med. 366(26): 2443-2454 (2012); Chen BJ, et al., Clin. Cancer Res. 19(13):3462-73 (2013)) and, as described above, includes scores for both the neoplastic and non-neoplastic cells within the tumor microenvironment. Data derived from pathologist visual scoring (semi-quantitative, but with increased specificity for delineating neoplastic and non-neoplastic cells) and pathologist-assisted, automated scoring (quantitative, but without accurately delineating neoplastic and non-neoplastic cells) for each marker of interest is assessed for its clinical value. As necessary, the data from combinations of makers are considered (i.e. combined scores from PD-L1 and PD-L2 expression). All data are analyzed in conjunction with the biostatistics group.

Peripheral blood:

Serial blood/serum samples are collected prior to each odd cycle (Cycle 1, Cycle 3, Cycle 5, etc.) prior to pembrolizumab administration starting on day 1 (pre-treatment) and at the end of the treatment. A panel of cytokines and chemokines is tested in serum using Luminex cytokine assay. Changes in cytokine production in immune cell subsets as a function of treatment are

determined by ELISA and intracellular cytokine staining. Absolute lymphocyte count (ALC) is monitored.

Peripheral blood mononuclear cells (PBMCs) are collected from whole blood to assess immune cell populations. Surface staining with a panel of antibodies (CD3, CD4, CD8, CD19, CD25, FoxP3, CD11c, CD83, CD86, CD56) and intracytoplasmatic cytokine staining, followed by flow cytometry is performed in order to identify different T cell populations, their activation status, myeloid-derived suppressor cells (CD11b, CD14, CD19, CD33, HLA-DR) and the production of different cytokines as well as other immune cell population as described in the table below.

Table 18

		FITC	PE	ECD	PE-Cy5	PE-Cy7
1	Treg (1): intracellular	FoxP3	CTLA-4	CD3	CD25	CD4
2	T, B, monocyte, NK, NKT	CD19	CD56	CD14	CD45	CD3
3	T cell subset	CD8	TCR γδ	CD4	TCR αβ	CD3
4	CD4 T cell naive memory	CCR7	CD45	CD45RO	CD28	CD4
5	CD8 T cell naive memory	CCR7	CD45	CD45RO	CD28	CD8
6	Myeloid DC	Lineage (CD3,CD14, CD16,CD19,CD56)	CD86	HLA-DR	CD11c	CD45
7	Plasmacytoid DC	Lineage (CD3,CD14, CD16,CD19,CD56)	CD86	HLA-DR	CD45	CD123
8	PD1-CD28	CD28	PD-1	CD3	CD8	CD4
9	41BB-OX40	CD8	4-1BB	CD3	OX-40	CD4
10	CD127 Treg	CD3	CD127	CD25	CD27	CD4
11	NK,NKT	CD16	NKG2D	CD3	CD56	CD8
12	BDCA DC	BDCA-2	BDCA-1	CD14,CD19	BDCA-3	CD45

Given the results from the study of ipilimumab plus bevacizumab, changes are analyzed as a function of treatments for $CCR7^{+/-}/CD45RO^{+}$ cell populations for both the $CD4^{+}$ and $CD8^{+}$ compartments.

Serologic changes to antibody responses are assessed for tumor antigens NY-ESO-1, melanoma-associated antigen recognized by T cells (MART-1), MUC-1, and melanoma-associated antigen 3 (MAGE-3) when appropriate.

Table 19: Biomarkers

Biomarker name	Assay	Tissue/Body Fluid Tested
CD274 (PD-L1)	Immunohistochemistry	Tissue
CD273 (PD-1)	Immunohistochemistry	Tissue
CD279 (PD-1)	Immunohistochemistry	Tissue
ANGPT2	Immunohistochemistry	Tissue
Circulating endothelial cells (CEC) and progenitor cells (CPC)	Surface staining and FACS	Blood
Circulating cytokines/Chemokines (as indicated in figure 17)	Luminex assay, ELISA	Blood
Circulating angiogenic factors (VEGF, Ang-2, PLGF, PDGF, HGF, FGF, BMP-9, endothelin, endoglin, leptin)	Luminex assay	Blood
Humoral response to angiogenic factors	ELISA and Western blot	Blood
Humoral response to immunosuppressive molecules	ELISA and Western blot	Blood
Expression of angiogenic factors and immunosupresive molecules	IHC	Tumor tissues
Circulating Tie-2 expressing Monocytes (TEM)	Surface staining and FACS	Blood
Tumor infiltrating TEM	IHC and/or Surface staining and FACS	Tumor tissues

Antigen-specific T cell responses are controlled by co-stimulatory and co-inhibitory molecules positively and negatively. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1, CD279) are among the key co-inhibitory molecules, broadly categorized as “checkpoint molecules” (Pardoll D.M., Nat Rev Cancer 12: 252-64 (2012)). CD279 is up-regulated on activated T lymphocytes and mediate immunosuppression when binding to its ligands B7-H1 (CD274) and B7-DC (CD273). Blockade of CD279 or CD274 induced durable objective response in patients with advanced melanoma, renal cell carcinoma and non-small cell lung cancers in clinical trials (Topalian S.L., et al., N. Engl. J. Med. 366(26): 2443-2454 (2012); Brahmer JR, et. al, N. Engl. J. Med. 366(26):2455-65 (2012); Hamid O, et al., N. Engl. J. Med. 369(2): 134-144 (2013)). Moreover, immunohistochemical staining performed on pretreatment tumor tissues from patients with anti-CD279 treatment showed that none of 17 patients with CD274 negative tumors had an objective response whereas 36% (9/25) patients with CD274 positive tumors had an objective response (P=0.006). This striking difference

suggests that CD274 expression on tumor specimen might be a useful biomarker to predict response to anti-CD279 treatment.

Anti-vascular endothelial growth factor pathway therapies preferentially target immature tumor blood vessels and leave behind normalized and resistant blood vessels. Angiopoietin-2 (ANGPT2)/TIE pathway is largely confined to vasculature. It has two receptors TIE1 and TIE2 as well as three ligands, angiopoietin-1, angiopoietin-2 (ANGPT2) and angiopoietin-4.

Peripheral Blood Mononuclear Cell isolation and staining procedure for Flow Cytometry of Peripheral Blood Mononuclear Cells (PBMCs) are isolated from 25 ml of blood by Ficoll centrifugation. Aliquots of 5-10 million cells are immediately frozen with 500ul of Fetal Bovine Serum (FBS) containing 15% Dimethyl Sulfoxide (DMSO). The cryovials containing cells are placed in special freezing containers at -80C overnight. After which, cryovials are transferred to liquid nitrogen. After a minimum of 48 hours in liquid nitrogen storage, cells are thawed in a 37C water bath. A single vial is resuspended in approximately 10ml of warm Roswell Park Memorial Institute medium (RPMI) with 10% FBS. Cells are centrifuged at 1800 RPM for 5 minutes, and supernatant is aspirated. The cell pellet is resuspended in 2ml of ice cold Phosphate Buffered Saline (PBS) containing 2.5% FBS (staining solution) and human anti-CD16/CD32 blocking antibodies and incubated on ice. After 15 minutes, cells are aliquoted into a V-bottom 96 well plate (approx. 500,000 cells/100ul/well) and incubated with specific antibodies at manufacturer's recommended concentrations for 45 minutes in the dark. In the case of intracellular markers (e.g. FoxP3) plates are incubated with antibodies targeting membrane markers, fixed with 1% Formaldehyde and treated with a cellular permeabilization reagent (e.g., saponin) prior to the addition of the intracellular protein targeting antibodies. Cells are spun down at 1800 RPM at 4C for 5 minutes, and washed twice with cold staining solution. After washing, plates are incubated on ice in the dark with 3uM of DAPI for 10 minutes. Cells are washed once more with staining solution and then finally resuspended in 150ul of staining solution.

Antibody panels for Flow Cytometry

The following immune cell populations are detected in PBMCs using specific marker antibodies and flow cytometry gating strategies: Regulatory T cells ($CD4^+/CD25^+/FoxP3^+$), Effector T cells ($CD4^+/CD69^+$), Naïve T cells ($CD4^+/CD69^-$), Memory T cells ($CCR7^+/CD45RO^+$), CD8 Cytotoxic cells ($CD8^+/CD3^+$), Plasmocytoid Dendritic cells

($CD123^+/CD303^+$), Myeloid Dendritic cells ($CD11c^+/CD141^+$), Natural Killer cells ($CD3^-/CD56^+$), Natural Killer T cells ($a/bTCR^+/NKG2D^+$), Classic Monocytes ($CD14^+$), and Monocytic Myeloid-Derived Suppressor cells ($CD14^+/HLADR^-$).

Detection of soluble biomarkers

Plasma from heparin treated blood is collected, aliquoted and stored at -80C. Via Cytokine/Chemokine Multiplex assays, concentration levels are assessed for up to 50 biomarkers including (but not limited to) the following inflammatory mediators: IL-6, IFNg, TNFa, IL-10, interferon gamma-induced protein 10 (IP-10), IL-1b, Chemokine (C-X-C motif) ligand 16 (CXCL16), VEGF, and angiopoietin 1 (Ang1; Ang-1). The assays are performed following manufacturer Standard Operating Procedures for each biomarker group panel.

Integrated Correlative Studies

Immunohistochemical Staining for CD274, CD273, CD279, and ANGP2

Not only CD274, but also CD279 and CD273, protein expression in tumor tissues might be associated with favorable clinical response, and might be served as biomarkers for patient selection for CD279 blockade in clinical treatment. ANGPT2 protein expression in tumor tissue might be a biomarker to identify a group of patients who might have objective responses with anti-vascular endothelial growth factor therapies.

Assay, patient and specimen information

Immunohistochemical (IHC) staining of CD274, CD273, CD279 and ANGPT2 are used as integrated markers in the clinical trial, which are used in the future phase II trials to identify a group of patients who have a good response to the treatment as a stratification variable. Tumor specimens are be collected from metastatic deposits of melanoma, ovarian cancer, colorectal cancer and renal cell carcinoma. Pre-treatment archived specimens are retrieved if no fresher tumor are obtained prior to treatment initiation on day 1. Post-treatment tissues are collected and fixed by 10% neutral buffered formalin overnight, dehydrated and paraffin embedded. Four-micrometer-thick sections are cut. The paraffin blocks and unstained slides are stored at room temperature. All IHC staining is performed in the Center for Immuno-Oncology Pathology Core at Dana-Farber/Harvard Cancer Center Specialized Histopathology Core, which is a central research laboratory for this multiple-center clinical trial. Unstained slides from other two centers are shipped to Dr Xiaoyun Liao, Thorn building 603B, Brigham and Women Hospital, 75 Francis Street, Boston, MA, 02215.

Design of immunohistochemical assay

The IHC assay for CD274, CD273 and ANGPT2 is semi-quantitative while CD279 stained slides are scanned by an automated scanning microscope and quantitatively analyzed by Aperio image analysis system (Leica Biosystems) after they are evaluated and positive cells are manually counted by a pathologist.

Standard EnVision two-step (indirect) staining method is utilized. Four-micrometer-thick sections are cut, deparaffinized, rehydrated and subjected to heat mediated antigen retrieval in citrate buffer (pH 6) (Invitrogen) by steaming for 30 minutes. After cooling, tissue sections areincubated with peroxidase block (DAKO, Carpinteria, CA) for five minutes, then serum free protein block (DAKO) for 20 minutes. Slides are incubated at room temperature for one hour with a primary antibody. Antibodies are diluted in Da Vinci Green Diluent (Biocare Medical, Concord, CA). EnvisionTM anti-mouse HRP-labeled polymer (DAKO) are applied to the sections for 30 minutes, followed by visualization by using the chromogen 3, 3-diaminobenzidine (DAKO). All the sections are counterstained with haematoxylin, dehydrated, mounted and coverslipped. Positive and negative controls are included in each staining. Known positive stained Hodgkin lymphoma (CD274), tonsil (CD279 and ANGPT2) and melanoma (CD273) slides are used as external control (separate slides). Stained slides are stored at room temperature.

In the study, immunoreactivity for CD274 was detected in the cytoplasm and cell membrane while CD273 and CD279 expression was observed in the cytoplasm. ANGPT2 expression is present in the cytoplasm (Buehler D., et al., Mod. Pathol. 26(8):1032–1040 (2013)). Scoring for CD274, CD273 and ANGPT2 is semi-quantitative/ordered categorical. The percentage of the tumor cells staining positive for CD274, CD273 or ANGPT2 and the intensity of the tumor cells are recorded as 0 (no staining), 1 (weak staining), 2 (moderate staining) and 3 (intense staining). Absolute CD279 positive cells are counted under microscope lens x 20 power field. Representative 5 areas are chosen to count. The average number from 5 areas are recorded and compared with data from image analysis (FIG. 29).

Assay performance

The results are obtained on retrospective data sets. Protocols of these three antibodies have been optimized, standardized to minimize staining variance. Positive control and negative controls are used and stained separately with each batch of slides. The IHC staining of three

markers (CD274, CD273 and CD279) has been performed in two different labs by three different technicians on whole tissue sections of Hodgkin lymphomas, melanomas, lung cancers and renal cell carcinomas. Three readers were involved, confirming the good reproducibility of the assay.

Tumor is considered positive if > 5% (CD274) (Topalian S.L., et al., N. Engl. J. Med. 366(26): 2443-2454 (2012)) or 10% (CD273 and ANGPT2) of the tumor cell population demonstrates unequivocally staining, respectively. CD279 positivity was defined as > 3% positive cells/HPF (Bachireddy P., et al., Blood 123(9):1412-21 (2014)).

All IHC stained slides are evaluated and scored by a pathologist (Dr Xiaoyun Liao). A subset of slides is reviewed by a second pathologist (Dr Scott Rodig) to ensure concordance of interpretation.

For CD279 staining, slides are scanned by an automated scanning microscope and analyzed by Aperio image analysis system (Leica Biosystems). Tumor areas are marked by a pathologist to exclude non-neoplastic areas, such as stroma, normal epithelial and necrotic regions. The software is used to count the number of positive cells in each tissue. The percentage of CD279 positive cells is calculated. Data are compared with that of manual counting by a pathologist to exclude tissue artifacts that cannot be recognized by computer image software.

Exploratory/Ancillary Correlative Studies

Monitoring Peripheral Blood for Changes in Immune Function and Angiogeneic Factors

Correlative sciences are expanded from the previous determined biology and analyses developed through experience thus far with ipilimumab and ipilimumab-bevacizumab treated patients. Subpopulations of PBMCs are isolated, including but not limited to dendritic cells, T cells, and B cells. Phenotype changes in these cell populations by flow cytometry are determined as a function of treatment. These include regulatory and effector immune panels, naïve and memory CD4, CD8 and NK lymphocyte populations. Given its importance in immune regulation and association, Tie-2 expressing monocytes (TEM) are evaluated. Changes in antigen specific responses to known melanoma antigen epitopes (MART-1, NY-ESO-1) are determined utilizing HLA-A20201 peptide system for APC (including DC maturation and presentation) and targets.

Endpoint Definitions

Rate of DLT: The proportion of patients with DLT in each dose escalation cohort.

Objective response rate (ORR): Objective response is determined by the best overall response designation (per RECIST 1.1) recorded between the date of first dose of trial therapy

and the date of objectively documented disease progression or cessation of trial therapy, whichever occurs first. The objective response rate is the proportion of patients achieving complete or partial response as their best response to therapy.

Progression-free survival (PFS): Time from start of trial treatment until objective disease progression (per RECIST 1.1) or death, whichever occurs first. Deaths that occur after 12 weeks following the date last known progression-free are not counted as PFS events. For patients without progression or death within 12 weeks following the date last known progression-free, follow-up is censored at the date of last disease assessment.

Overall survival (OS): Time from start of trial treatment to death from any cause. For patients who are lost to follow-up or who have no documentation of death at the time of final analysis, follow-up is censored at the date of last assessment of vital status.

Time-to-progression (TTP): Time interval between the dates of the start of trial treatment and first documentation of progressive disease. In the absence of documented progressive disease, follow-up is censored at date of last disease assessment. Deaths without prior progression is censored events.

Efficacy Analysis

Secondary endpoints are used to gain preliminary estimates of efficacy. The analyses of secondary endpoints are based on patients enrolled in the expansion cohorts and are summarized within disease type. ORR is estimated for each disease cohort and summarized with 90% confidence intervals estimated using exact binomial methods. For samples of size 12, the confidence intervals are no wider than 0.5. Time-to-event endpoints (i.e., PFS, OS, TTP) are summarized using the product-limit method of Kaplan-Meier; confidence intervals are based on log(-log(survival) methodology. Six-month PFS, 12-month OS, and median TTP are presented, by disease, with 90% confidence intervals. With 12 patients in each disease, statistical testing is of low power. Therefore, the analyses within disease are primarily descriptive and do not rely on p-values.

Proposed analyses of correlative endpoints

For the analysis of cytokines, chemokines, and immune cell populations from serum or blood, data are combined from all four disease groups according to treatment, resulting in longitudinal measurements for approximately 48 patients. Serum marker levels are summarized descriptively and graphically. The time course of expression levels are summarized graphically

by patient, noting disease group and times of disease progression. Since patients may have rapid disease progression and terminate treatment early, the use of linear mixed models includes partial data in the analysis allowing characterization of outcome. Transformations are applied to the outcome measures to stabilize variability and normalize the distributions, when appropriate.

In an exploratory analysis, changes in biomarker levels in the subsets of patients who complete 6 months of treatment are summarized, recognizing that this may be a select group of participants with less severe disease. Fold-changes are calculated comparing 6-month and pre-treatment levels (6-month/pre) and summarized descriptively. Approximately 40% of patients complete 24 weeks of treatment, resulting in 19-20 patients. Biomarkers from tissue are assessed using IHC. Analyses relating baseline expression levels with response are based on approximately 48 patient samples from newly obtained excisional biopsies or archival tissue. Paired biopsies are needed from 20 patients in the dose expansion cohort, ideally 5 per disease indication. An endpoint of interest in the tissue analysis would be the proportion of patients with at least a 50% decrease in CD137 M2 macrophages. A null proportion of 0.20 with at least a 50% decrease is anticipated. The combination of AMG386 with pembrolizumab would show important biomarker response if the proportion with 50% decrease in CD137 M2 macrophages is at least 0.45. With 20 paired biopsies, an exact binomial test with nominal, two-sided, 0.1-significance level has at least 80% power to detect the difference between proportions of 0.20 and 0.47.

Table 20

PERFORMANCE STATUS CRITERIA

ECOG Performance Status

Grade	Description
0	Normal activity. Fully active, able to carry on all pre-disease performance without restriction.
1	Symptoms, but ambulatory. Restricted in physically strenuous activity, but ambulatory and able to carry out work of a light or sedentary nature (e.g., light housework, office work).
2	In bed <50% of the time. Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	In bed >50% of the time. Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	100% bedridden. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Dead.
* As published in Am. J. Clin. Oncol.: *Oken, M.M., Creech, R.H., Tormey, D.C., Horton, J., Davis, T.E., McFadden, E.T., Carbone, P.P.: Toxicity And Response Criteria Of The Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649-655, 1982.* The Eastern Cooperative Oncology Group, Robert Comis M.D., Group Chair.	

Example 2: Studies on $CD14^+$ monocytes and AMG386 peptibody

Introduction

CD14 was preferentially expressed on monocytes/macrophages, and it serves as a pattern recognition receptor for a variety of ligands from apoptotic cells to bacterial products and plays important roles in innate immunity (Jersmann, H.P., Immunology and cell biology 83: 462-467 (2005)).

Previously, human myeloid derived suppress cells (MDSC) were typically defined as $CD11b^+$ CD14- cells (Schmid, M.C., et al., Journal of Oncology 201026: DOI 10.1155/2010/201026 (2016); Gabrilovich, D.I., et al., Nature Reviews Immunology 9:162-174 (2009)). Recently, $CD11b^+$ $CD14^+$ $HLA\text{-}DR^{-/low}$ cells are also regarded as mononuclear MDSC (M-MDSC) (Marvel, D., et al., The Journal of clinical investigation 125: 3356-3364 (2015); Condamine, T., et al. Annual review of medicine 66: 97-110 (2015)). Immune suppressive mechanism of MDSC is related to local presence of arginase-1, matrix metalloproteinase-9, indoleamine 2,3-dioxygenase, cyclooxygnase 2, inducible nitric oxide, IL-10, and TGF-β, and

the suppression is associated with local microenvironment in tumor (Condamine, T., et al. Annual review of medicine 66: 97-110 (2015)).

In addition, $CD14^+$ $CD16^+$ subpopulation of monocytes was found to express Tie-2, and this subset is called Tie-2 expressing monocytes (TEM) (De Palma, M. et al., Nature medicine 9: 789-795 (2003); De Palma M., et al., Cancer cell 8: 211-226 (2005); Coffelt, S.B., et al., Journal of Immunology 186: 4183-4190 (2011)). Tie-2 is a receptor for angiopointin (Ang)-1 and -2. Tie-2/Ang2 signaling augments the ability of TEM in angiogenesis and facilitates TEM toward an M2-like macrophage phenotype (Coffelt, S.B., et al., Cancer research 70: 5270- 5280 (2010); Pucci, F., et al., Blood 114: 901-914 (2009); De Palma, M. et al., Trends in Immunology 28: 519-524 (2007)). Furthermore, Ang2 induces the immunno-suppressive properties of TEM via suppression of T cell activation, promotion of Treg expansion, and upregulations of IL-10 and CCR17 (Coffelt, S.B., et al., Journal of Immunology 186: 4183-4190 (2011); Coffelt, S.B., et al., Cancer research 70: 5270- 5280 (2010)). TEM also existed in colon adenocarcinoma of mouse model and colonrectal adenocarcinoma patients (Venneri, M.A, et al., Blood 109(12): 5276-5285 (2007), Goede, V. et al., Cancer investigation 30: 225-230 (2012)). A subset of $CD14^+$ $IL4R\alpha^+$ monocytes were able to inhibited T cell proliferation and found in melanoma and colon cancer patients (Mandruzzato, S., Journal of immunology 182: 6562-6568 (2009)). Another subset of $CD14^+$ monocytes was found to express PD-L1 (Heeren, A.M., et al., Cancer Immunology Research 3: 48-58 (2015)). The subset was increased in lymph nodes (LN) of patients with cervical cancer and significantly correlated with frequencies of Treg. In vitro studies showed that the subset was able to produce IL-10, IL-6, and TNFα. Early occurrences of lymphatic tumor spread are associated with the patients with $CD14^+$ $PD\text{-}L1^+$ cells in LN (LN^+), compared to LN^-. These suggest that current markers for MDSC are limited.

AMG386 is an Ang-1/-2 neutralizing peptibody. Preclinical studies with AMG386 showed significant inhibitions of several tumor types (Neal, J. et al., Current Opinion in Molecular Therapeutics 12:487-495 (2010); Coxon, A., et al., Molecular Cancer Therapeutics 9:2641-2651 (2010)). Recent AMG386 TRINOVA-1 phase 3 trials show significant reduction in disease progression and death in ovarian cancer patients. These indicate important roles of angiopoietin/Tie-2 axis in tumor development.

However, (1) roles of $CD14^+$ monocytes in tumor immunity have not been clearly elucidated; (2) roles of angiopoietin/Tie-2 axis in $CD14^+$ monocytes are not fully studied; (3)

action mechanism of AMG386 in T cell and $CD14^+$ monocyte immunity has not been explored. Current studies show that $CD14^+$ monocytes are able to express a variety of immune suppressive factors such as PD-L1, PD-L2, FASL, IL-10, TGF-β, and arginase-1. $CD14^+$ monocytes exerted stronger inhibitory effects on T cell activation in comparison with $CD14^-$ monocytes. Ang-2 inhibited T cell activation by increasing survival of $CD14^+$ monocytes. AMG386 abolished Ang-2 increased $CD14^+$ monocytes and partially restored of T cell activation. In addition, $CD14^+$ $Tie\text{-}2^+$ $PD\text{-}L1^+$ monocytes were found in PBMC and tumor infiltration cells of stage IV melanoma patients.

Materials and methods

The following materials and methods were utilized in generating the results presented

Characterization of human $CD14^+$ monocytes

$CD14^+$ $Tie\text{-}2^+$ monocytes play critical roles in inhibition T cell activation via Ang-2/Tie-2 axis (Coffelt, S.B., et al., Journal of Immunology 186: 4183-4190 (2011)). Expression of Tie-2, PD-L1, PD-L2, and FASL on $CD14^+$ cells was examined.

As shown in FIG. 30, $CD14^+$ monocytes express Tie-2, PD-L1, and PD-L2 but not FASL, and they are CD11b, HLA-DR and A*02 positive. Toxic shock syndrome toxin (TSST), phytohaemagglutinin (PHA), and Toll-like receptor (TLR) activators are shown to induce monocyte activation and differentiation (Kiener, P.A., Journal of Immunology 159, 1594-1598 (1997); Krutzik, S.R., et al., Nature Medicine 11: 653-660 (2005)).

Therefore, it is of interest to examine effects of TSST, PHA, and TLR activators on expression of PD-L1, PD-L2, and FASL in $CD14^+$ monocytes. Both TSST and PHA increased expressions of PD-L1, PD-L2, and FASL (FIG. 31A and FIG. 34). Activators of TLR2 to 9 differentially increased expression of PD-L1, PD-L2, and FASL except FLA for PD-L1, FLA and ODN2006 for PD-L2, and poly I:C and FLA for FASL (FIG. 31A and FIG. 34). Expression of IL-10, TGFβ, and arginase-1 were also examined. TSST, PHA and zymozan are able to enhance expression of IL-10, TGFβ, and arginase-1 (FIG. 31A and FIG. 35). Notably, PD-L1 was most highly expressed in response to TSST, PHA, and TLR activators.

Effects of cytokines on expression PD-L1, PD-L2, and FASL were investigated. IFNγ, IFNα, and TNFα were able to increase PD-L1 expression, IFNα enhanced PD-L2 expression, and all the cytokines had no effects on FASL expression (FIG. 31B and FIG. 36). Again, PD-L1 expression was most sensitive to cytokine stimulation. Taken together, $CD14^+$ monocytes have

abilities to express PD-L1, PD-L2, FASL, IL-10, TGFβ, and arginase-1 in response to TSST, PHA, TLR activators, and cytokines. In comparison, $CD14^-$ monocytes had less or no abilities.

Effects of $CD14^+$ monocytes on T cell activation

To further investigate effects of $CD14^+$ monocytes on T cell activation, $CD14^+$ and $CD14^-$ monocytes were enriched by depleting $CD4^+$ $CD8^+$ $CD19^+$ or $CD4^+$ $CD8^+$ $CD19^+$ $CD14^+$ cells, respectively. $CD14^+$ monocytes and $CD4^+$ $CD25^+$ Treg were generated by CD14 positive selection and Treg isolation kits (Miltenyi biotec, San Diego, CA). Enriched $CD14^+$ and $CD14^-$ monocytes, $CD14^+$ monocytes, and Treg were further co-cultured with $CD4^+$ and $CD8^+$ T cells in presence of anti-CD3 and CD28 beads for 3 days. $CD14^+$ and CD14- monocytes, and Treg inhibited T cell proliferation. Furthermore, $CD14^+$ monocytes exerted more inhibitory effects on T cell proliferation, in comparison with CD14- monocytes and $CD4^+$ Treg (FIG. 31C).

Since $CD14^+$ monocytes express Tie-2, effects of Ang-1/2 and anti-Ang-1/2 neutralizing peptibodies on $CD14^+$ monocytes and T cell proliferation were examined. As shown in FIG. 32A and FIG. 37A and FIG. 37B, Ang-1/2 increased $CD14^+$ monocytes, and AMG386 partially reduced the increases induced by Ang-1/2. CFSE assay failed to prove that the increases in $CD14^+$ monocytes were due to proliferation (data not shown), suggesting that Ang-1/2 sustained survival of $CD14^+$ monocytes. Furthermore, Ang-1/2 exerts inhibitory effects on T cell proliferation, and AMG386 partially restored Ang-2 induced suppression (FIG. 32B). These suggest neutralizing effects of AMG386 in Ang-1/2 suppressed T cell proliferation. In addition, AMG386 increased antigen specific Mart-1 $CD8^+$ T cells (FIG. 32C).

Existence of $CD14^+$ $Tie\text{-}2^+$ $PD\text{-}L1^+$ in melanoma

As $CD14^+$ monocytes expressed Tie-2 and PD-L1, and PD-L1 was most highly expressed in response to a variety of factors shown in FIG. 30 and FIG. 31A- FIG. 31C, frequencies of $CD14^+$ $Tie\text{-}2^+$ $PD\text{-}L1^+$ monocytes were examined in PBMC from both healthy donors and melanoma patients, and in tumor infiltration cells. As shown in FIG. 33A and 33B-left panel, $CD14^+$ monocytes expressed higher levels of Tie-2 and PD-L1 in stage IV melanoma patients than in healthy donor. Furthermore, high levels of PD-L1 and Tie-2 on $CD14^+$ monocytes were seen in tumor infiltration cells (FIG. 33B-right panel).

Subsets of human $CD14^+$ monocytes with either $Tie\text{-}2^+$ or $IL4R\alpha^+$, or PD^-L1^+ showed association with immune suppression, possibly via IL-10 production (8 offelt, S.B., et al., Journal of Immunology 186: 4183-4190 (2011); Mandruzzato, S., Journal of Iimmunology 182: 6562-

6568 (2009); Heeren, A.M., et al., Cancer Immunology Research 3: 48-58 (2015)). However, features of $CD14^+$ monocytes are not fully elucidated.

There are a variety of factors involved in immune suppression, such as PD-L1, PD-L2, FASL, IL-10, arginase-1, and TGF-β (3 Gabrilovich, D.I., et al., Nature Reviews Immunology 9:162-174 (2009); Keir, M.J., et al., Annual review of Immunology 26: 677-704 (2008); Sharpe, A.H., et al., Nature Reviews Immunology 2: 116-126 (2002), Latchman, Y., at al., Nature Immunology 2: 261-268 (2001); Nagata, S., Advances in Immunology 57:129-144 (1994); Ramsdell, F. et al., European Journal of Immunology 24: 928-933 (1994); Takahashi, T., et al., Cell 76: 969-976 (1994); Zea, A.H., et al., Cancer Research 65: 3044-3048 (2005)). The results presented herein show that $CD14^+$ monocytes are able to express PD-L1, PD-L2, FASL, arginase-1, and TGF-β besides IL-10, suggesting complicated suppression mechanism by $CD14^+$ monocytes. Furthermore, increases in $CD14^+$ monocytes lead to more inhibition in T cell activation in comparison with $CD14^-$ cells. These strongly suggest that $CD14^+$ monocytes function as MDSC and play critical roles in immune regulation. Phenotype of $CD14^+$ monocytes showed Tie-2^+, $CD11b^+$, HLA DRhigh , PD-$L1^+$, and PD-$L2^+$. Obviously, current known surface markers are not sufficient to define MDSC.

PHA and TSST are shown to activate monocytes (Kiener, P.A., Journal of Immunology 159, 1594-1598 (1997)). TLR is co-receptor of CD14 in triggering down-stream signaling in inflammatory responses, and it play important roles in innate immunity (Triantafilou, M., et al., Trends in Immunology 23:301-304 (2002); Raby, A.C., et al., Science Translational Medicine 5: 185ra164; Akira, S., et al., Nature Reviews Immunology 4: 499-511 (2004)). Inflammation can be critical factor in cancer progression, and cytokines can also exert anti-tumor responses (Hanahan, D et al., Cell 144: 646-674 (2011); Dranoff, D., Nature Reviews Cancer 4:11- 22 (2004); Coussens, L.M., et al., Nature 420: 860-867 (2002)). Current data clearly showed involvement of varieties of factors such as TSST, PHA, TLR activators, and cytokines in expression of PD-L1/2, FASL, arginase-1, IL-10, and TGF-β in $CD14^+$ monocytes. These suggest important roles of antigen, TLR, and cytokines in regulation of $CD14^+$ monocyte activation. Notably, PD-L1 was most highly expressed in response to these factors. Therefore, $CD14^+$ PD-$L1^+$ could be a potential prognostic and pharmacodynamic biomarker for estimation of immune regulation statues. In addition, impacts of tumor antigen on $CD14^+$ monocytes are worthy of investigation.

Ang-2 activated subset of $CD14^+$ $CD16^+$ $Tie\text{-}2^+$ monocytes (TEM) and inhibit T cell activation through TEM derived IL-10 (Coffelt, S.B., et al., Journal of Immunology 186: 4183-4190 (2011)). The data also show that almost all $CD14^+$ monocytes express Tie-2, and Ang-1/2 increased population of $CD14^+$ monocytes and suppressed T cell activation. AMG386, Ang neutralizing peptibodies abrogated Ang-induced $CD14^+$ monocytes and partially reverse the suppression induced by Ang-1/2. These suggest that Ang/Tie-2 signaling inhibits T cell activation by increasing survival of $CD14^+$ monocytes. Notably, in spite that the suppression was significantly reversed by neutralizing anti-IL-10 antibody, contact of TEM-T cells was required for Ang-2 induced inhibition (Coffelt, S.B., et al., Journal of Immunology 186: 4183-4190 (2011)). As $CD14^+$ monocytes expressed PD-L1 and PD-L2 in the studies, roles of PD-L1 and PD-L2 in the inhibition need to be studied.

$CD14^+$ $PD\text{-}L1^+$ monocytes in lymph node were associated with early occurrences of lymphatic tumor spread in cervical cancer patients (Heeren, A.M., et al., Cancer Immunology Research 3: 48-58 (2015)). The data showed existence of $CD14^+$ $Tie\text{-}2^+$ $PD\text{-}L1^+$ monocytes in PBMC and tumor infiltration cells of melanoma patients, suggesting potential role in immune suppression in melanoma. Therefore, roles of $CD14^+$ $Tie\text{-}2^+$ $PD\text{-}L1^+$ monocytes plus Ang-1/2 in melanoma, such as in vivo mouse model and melanoma patients need to be further investigated.

Given the fact that PD-L1/PD-L2, FASL, arginase-1, IL-10, and TGF-β are expressed in $CD14^+$ monocytes, significance of $CD14^+$ monocytes in immune regulation and tumor immunity is certainly worthy of further study. Impacts of Ang-1/2 and its neutralizing AMG386 peptibody in melanoma need to be clarified accordingly.

Example 3: Angiopoietin-2 as a Biomarker and Target for Immune Checkpoint Therapy

Abstract

Immune checkpoint therapies targeting CTLA-4 and PD-1 have proven effective in cancer treatment. However, prior to the invention described herein, the identification of biomarkers for predicting clinical outcomes and mechanisms to overcome resistance remained as critical needs. Angiogenesis is increasingly appreciated as an immune modulator with potential for combinatorial use with checkpoint blockade. Angiopoietin-2 (ANGPT2) is an immune target in patients and is involved in resistance to anti-VEGF treatment with the monoclonal antibody bevacizumab. The predictive and prognostic value of circulating ANGPT2 in metastatic melanoma patients receiving immune checkpoint therapy was investigated. High pretreatment

serum ANGPT2 was associated with reduced overall survival in CTLA-4 and PD-1 blockade–treated patients. These treatments also increased serum ANGPT2 in many patients early after treatment initiation, whereas ipilimumab plus bevacizumab treatment decreased serum concentrations. ANGPT2 increases were associated with reduced response and/or overall survival. Ipilimumab increased, and ipilimumab plus bevacizumab decreased, tumor vascular ANGPT2 expression in a subset of patients, which was associated with increased and decreased tumor infiltration by CD68þ and CD163þ macrophages, respectively. In vitro, bevacizumab blocked VEGF-induced ANGPT2 expression in tumor-associated endothelial cells, whereas ANGPT2 increased PD-L1 expression on M2-polarized macrophages. Treatments elicited long-lasting and functional antibody responses to ANGPT2 in a subset of patients receiving clinical benefit. These findings suggest that serum ANGPT2 may be considered as a predictive and prognostic biomarker for immune checkpoint therapy and may contribute to treatment resistance via increasing proangiogenic and immunosuppressive activities in the tumor microenvironment. Targeting ANGPT2 provides a rational combinatorial approach to improve the efficacy of immune therapy. Cancer Immunol Res; 5(1); 17–28. Ó2016 AACR.

Recent developments in immune checkpoint therapy have changed the way patients with cancer are treated. Ipilimumab treatment, which targets CTLA-4, improves overall survival (OS) in patients with metastatic melanoma (Hodi, F.S., et al., N. Engl. J. Med. 363:711–23 (2010); Robert, C. et al., N. Engl. J. Med. 364: 2517–26 (2011)). A phase I trial combining bevacizumab, a humanized monoclonal antibody targeting VEGF, with ipilimumab demonstrated favorable clinical activity compared with ipilimumab alone (Hodi, F.S., et al., Cancer Immunol. Res. 2: 632–42 (2014)). Anti–PD-1 therapy with nivolumab or pembrolizumab, monoclonal antibodies that block interactions of PD-1 with PD-L1 and PD-L2, improve survival or have significant activity in a variety of cancer types, including metastatic melanoma, non–small cell lung cancer, renal cell cancer, bladder cancer, and Hodgkin disease (Topalian, S.L., et al., N. Engl. J. Med. 366: 2443–54 (2012); Hamid, O., et al., N. Engl. J Med 369:134–44 (2013); Topalian, S.L. et al., J. Clin. Oncol. 32:1020–30 (2014); Postow, M.A., et al., J. Clin. Oncol. 33: 1974–82 (2015); Powles, T., et. al., Nature 515: 558–62 (2014); Ansell, SM, et. al., N. Engl. J. Med. 372: 311–19 (2015)). The combination of CTLA-4 and PD-1 blockade yields significantly longer progressionfree survival and higher response rates than monotherapy in melanoma patients (Wolchok, J.D., N. Engl. J. Med. 369:122–33 (2013); Postow, M.A., et. al., N. Engl. J. Med.

372: 2006–17 (2015); Larkin, J., et al., N. Engl. J. Med. 373:23–34 (2014)). Yet identification of biomarkers for predicting clinical outcomes to treatments and to search for mechanisms to overcome resistance are an unmet need. Increasing evidence suggests that angiogenic factors play important roles in immune regulation and have immunoinhibitory activities (Rivera, L.B., et al., Trends Immunol. 36: 240–49 (2015)). VEGF inhibits dendritic cell maturation and antigen presentation and tumor infiltration by lymphocytes, while promoting regulatory T cell (Treg) and myeloid-derived suppressor cell (MDSC) expansion in the tumor microenvironment (Ohm, J.E., et al., Immunol. Res. 23:263–72 (2001); Oyama, T., et al., J. Immunol. 160: 1224–32 (1998); Vanneman, M., Nat. Rev. Cancer. 12:237–51 (2012); Shrimali, R.K., et al., Cancer Res. 70: 6171–80 (2010); Huang, H., et al., FASEB J 29:227–38 (2015)). Higher pretreatment serum VEGF is associated with decreased survival in ipilimumab-treated metastatic melanoma patients (Yuan, J, et al., Cancer. Immunol. Res. 2:127–32. (2014)). Angiopoietin-2 (ANGPT2), a ligand of the receptor tyrosine kinase Tie-2, functions as a vessel-destabilizing molecule and is a critical regulator of blood vessel maturation (Fiedler, U., et al., Trends Immunol. 27:552–8 (2006); Huang, H. et al., Nat. Rev. Cancer 10:575–85 (2010)). ANGPT2 is primarily produced by endothelial cells and facilitates angiogenesis. ANGPT2 is low in normal tissues but often highly upregulated in the tumor vasculature (Tait, C.R. et al., J. Pathol. 204:1–10 (2004); Thurston, G., et al, Cold Spring Harb Perspect Med 2:a006550 (2012)).

Elevated circulating ANGPT2 has been associated with poor prognosis and more invasive tumors in a variety of cancers, including melanoma (Huang, H. et al., Nat. Rev. Cancer 10:575–85 (2010); Tait, C.R. et al., J. Pathol. 204:1–10 (2004); Thurston, G., et al, Cold Spring Harb Perspect Med 2: a006550 (2012); Helfrich, I., et al., Clin. Cancer Res. 15: 1384–92 (2009); Jary, M., et al., Cancer Epidemiol. Biomarkers Prev. 24: 603–12 (2015); Dreikhausen, L., at al., BMC Cancer 15: 121 (2015); Goede, V. et al., Br. J. Cancer 103: 1407–14 (2010)). ANGPT2 can also play a role in inflammation (Scholz, A. et al., Blood 118: 5050–9 (2011); Scholz, A., et, al., Ann. N.Y. Acad. Sci. 1347:45–51 (2015)). Patients receiving immune therapy can make antibodies to ANGPT2 as the result of treatment (Schoenfeld, J. et al, Cancer Res. 70: 10150–60 (2010)). ANGPT2 can confer compensatory resistance to antiangiogenesis therapy targeting VEGF (Scholz, A., et, al., Ann. NY Acad Sci 1347:45–51 (2015); Huang. H. et al., Clin. Cancer Res. 17:1001–11 (2011); Rigamonti, N. et al, Cell Rep. 8:696–706 (2014); Daly, C. et al., Cancer Res.73:108–18 (2013)), and high pretreatment serum ANGPT2 is associated with reduced

response rate and survival in metastatic colorectal cancer patients receiving antiangiogenesis therapy with bevacizumab (Goede, V. et al., Br. J. Cancer 103: 1407–14 (2010)). The possible prognostic/predictive role of ANGPT2 and its potential as a target for immune therapy requires further investigation.

The results presented herein investigate the predictive and prognostic value of serum ANGPT2 concentrations for immune checkpoint therapy as well as investigating any synergistic effects of ANGPT2 on immune regulation. A high baseline circulating ANGPT2 concentrations was found, and early increases in ANGPT2 during treatment, were associated with shortened OS and/or reduced response rates. Immune checkpoint therapy elicited functional humoral immune responses to ANGPT2. Pathologic analyses revealed that immune checkpoint therapy increased or decreased the infiltration of tumor macrophages in association with elevated or reduced tumor vascular ANGPT2 expression. Additionally, ANGPT2 promoted PD-L1 expression on M2-polarized macrophages. As described herein, these findings suggest serum ANGPT2 as a potential biomarker for predicting clinical outcomes to immune checkpoint therapy as well as a role for ANGPT2 in resistance to these therapies and possible target for synergistic combination treatments.

Materials and Methods

Tissue and blood collection

Patients with metastatic melanoma were treated and biospecimens were collected per Dana-Farber/Harvard Cancer Center Institutional Review Board (IRB)-approved protocols. Informed consent was obtained from all the patients involved in this study after the nature and possible consequences of the studies were explained. Patients with advanced melanoma enrolled in the phase I Ipi-Bev trial have been described previously (Hodi, F.S., et al., Cancer Immunol. Res. 2: 632–42 (2014)). Demographics, disease status, and prior treatments of patients with metastatic melanoma receiving ipilimumab or PD-1 blockade treatment are summarized in Supplementary Table S1. For serum collection, blood samples collected in Vacutainer tubes with serum separator were centrifuged at 1,000 g for 15 minutes at room temperature, and the supernatant (serum) was collected and stored at 20° C. For plasma collection, blood samples collected in Vacutainer tubes containing heparin were diluted with equal volume of RPMI1640 and subjected to Ficoll density gradient separation of PBMCs. The supernatant (plasma) above the PBMC layer was collected and stored at 20° C.

Measurement of circulating ANGPT2

ANGPT2 in plasma/serum samples was measured using Magnetic Luminex Screening Assay kits (R&D Systems) per manufacturer's instructions.

Culture and treatment of endothelial cells and melanoma cells

Tumor-associated endothelial cells (TEC) were isolated using Dynabeads CD31 Endothelial Cell as guided by the manufacturer (Life Technologies) and confirmed by surface expression of CD31 and VEGFR2 and tube formation (Wu, X., et. al., Cancer Immunol. Res 4:858–68 (2016)). HUVECs werepurchased from Lonza. TECs and HUVECs were cultured in EGM-2 (Lonza). Melanoma cell lines K008, K033, and M23 were established approximately 25 years ago from harvested fresh tissues on Dana-Farber/Harvard Cancer Center Institutional Review Board (IRB) approved protocols as described previously (Wu, X. et al., PLoS One 8: e56134 (2013)). Melanoma A375 cells were obtained from American Type Culture Collection (ATCC) approximately 10 years ago. They were not authenticated but had confirmed expression of MITF and melanocytic markers. Melanoma cells were cultured in DMEM containing 10% FBS, penicillin (50 mg/mL), and streptomycin (100 mg/mL). In some experiments, EC and melanoma cells were cultured in a hypoxic chamber with 1% O2. To examine the effect of VEGF and bevacizumab on ANGPT2 expression, EC and melanoma cells were incubated with VEGF (100 ng/mL; Cell Guidance Systems) and/or bevacizumab (25 mg/mL; Genetech) in serum and angiogenesis factor reduced EBM/EGM-2 (3:1, v/v) medium and DMEM containing 1% FBS, respectively. To examine the effect of enriched endogenous ANGPT2 antibodies on ANGPT2-mediated Erk1/2 phosphorylation, HUVECs were serum starved for 6 hours and treated with ANGPT2 (400 ng/mL; R&D Systems) preincubated with human normal IgG (Life Technologies) or enriched ANGPT2 antibodies (1.2 mg/mL) for 15 minutes at 37° C and 5% CO2.

Generation and polarization of monocyte-derived macrophages

Frozen PBMCs isolated from healthy donors were thawed briefly at 37°C in a water bath, washed in R-PS [RPMI1640 containing 50 penicillin (mg/mL) and streptomycin (100 mg/mL)], and incubated in R-PS containing 5% FBS (R-PS5) on cell culture dishes for 1.5 hours. Floating cells were removed by washing with R-PS at least 5 times. The attached monocytes were cultured in R-PS10 medium (R-PS supplemented with 10% FBS) containing CSF1 (15–100 ng/mL; Biolegend) for 3 days to differentiate into macrophages. After being washed with R-PS,

the attached monocyte-derived macrophages (MDM) were incubated with fresh R-PS10 containing CSF1 for 3 more days. MDMs were activated with CSF1 (100 ng/mL), IL4 (10 or 20 ng/mL; R&D Systems), or IL10 (10 or 20 ng/mL; R&D Systems) for 2 days. In some experiments, ANGPT2 (300 ng/mL; R&D Systems and EMD Millipore) was added to MDMs after 3 days of differentiation with CSF1 or when they were activated with IL4 or IL10 to examine its effect on PD-L1 expression. Phenotypes of polarized MDMs were analyzed by FACS after staining with APC conjugated CD80 (Clone 2D-10; Biolegend) and PE-conjugated CD163 antibodies (Clone GHI/61; Biolegend). Detection of PD-L1 expression on macrophages MDMs were detached from culture dishes using Accutase (Life Technologies), incubated with FcR blocker (Miltenyi Biotec) for 30 minutes at 4° C, and stained with PE-conjugated PD-L1 antibody (Clone 29E.2A3; Biolegend) in PBS containing 1% BSA for 30 minutes at 4 C. In some experiments, macrophages were stained with FTIC-conjugated CD68 antibody (Clone FA-11; Biolegend) after PD-L1 staining and fixation/ permeabilization. Macrophages were analyzed using FACS and the FlowJo software.

Detection of ANGPT2 antibodies in patient plasma samples ANGPT2 antibodies in plasma samples were determined by immunoblot analysis and ELISA using recombinant humanANGPT2 (R&D Systems). Immunoblot analysis of ANGPT2 antibodies with plasma samples was performed as previously described with minor modifications (Hodi, F.S., et al., Cancer Immunol. Res. 2: 632–42 (2014)). Briefly, ANGPT2 was run in SDS gels and transferred onto PVDF membranes. After blocking with 5% BSA in PBS, the membranes were incubated overnight with paired pretreatment and posttreatment plasma samples diluted by 1 103 folds. Antibodies bound to ANGPT2 were detected with HRP-conjugated goat anti-human IgG antibody (Life Technologies) and visualized with ECL. For ELISA measurement of ANGPT2 antibodies, recombinant human ANGPT2 was coated in TBS onto 96-well plates overnight. The plates were rinsed and blocked with a protein-free blocking solution (Thermo Scientific) for 1.5 hours at room temperature. Plasma samples were diluted by 500- to 2,000-fold in the blocking solution containing 0.1% Tween-20 and incubated with coated ANGPT2 for 1 hour at 4° C. Wells coated with His tag were used as background controls (named as "His Tag" background). To make sure signals were from plasma antibodies, additional wells coated with ANGPT2 and incubated with the Tween-20 containing blocking solution without plasma were also included (named as "No Plasma" background). The plates were washed extensively with PBST (PBS plus

0.05% Tween-20) and incubated with diluted rabbit F(ab')2 HPR anti-human IgG (SouthernBiotech) for 1 hour at room temperature. The plates were washed thoroughly with PBST and incubated with diluted biotinyl-tyramide (PerkinElmer) for 15 minutes at room temperature. After another thorough washing with PBST, the plates were incubated with streptavidin-HRP diluted in PBST plus 1% BSA for 30 minutes at room temperature. The plates were washed thoroughly with PBST and developed with TMB. OD at 450 and 570 nm was recorded using a microplate reader. Antibody titer was calculated by subtracting OD 570 from OD 450 and subtracting "His Tag" background and "No Plasma" background from ANGPT2 reading.

Purification of ANGPT2 antibodies from plasma

Recombinant human ANGPT2 (6 mg) was coupled to activated NHS magnet beads (40 mL; Thermo Scientific). Plasma samples (600 mL) were diluted with equal volume of PBS and incubated with the ANGPT2-coupled beads with rotation at 4° C overnight. The beads were pulled down with a magnet and washed with PBS 5 times. The antibodies bound to ANGPT2 were eluted with 0.1 mol/L glycine (pH 2.5) from the beads and neutralized with 1/10 volume of 1 mol/L Tris-Cl (pH 9.0). The antibodies were concentrated using an Amicon Ultra filter and stored in PBS supplemented with 0.02% BSA at 4° C. IgG content was determined by ELISA against normal human IgG (Life Technologies).

Immunohistochemical (IHC) staining

For IHC staining of ANGPT2 and CD163, 5-mm-thick paraffinembedded sections were pre-baked at 60° C for 1 hour, deparaffinized, and rehydrated. Antigen retrieval was induced by heating sections in citrate buffer (pH 6.0, Invitrogen) for 30 minutes using a steamer. After cooling for 30 minutes, sections were treated with peroxidase block (DAKO) for 5 minutes, followed by serum-free protein block (DAKO) for 20 minutes. Slides were then incubated overnight at 4° C with primary antibodies against ANGPT2 (1:25, sc-74403; Santa Cruz Biotechnology) or CD163 (1:200, 10D6; NeoMarkers) diluted in Da Vinci Green Diluent (Biocare Medical). For secondary reagents, Envision anti-mouse HRP-labeled polymer (DAKO) was applied for 30 minutes to sections for CD163 staining. ANGPT2 sections were incubatedwith Novocastra Post Primary (Leica Biosystems) for 30 minutes, followed by Novolink Polymer (Leica Biosystems) for 30 minutes. Sections were then developed with diaminobenzidine (DAKO), counterstained with hematoxylin, dehydrated, and mounted.

CD68 (PG-M1; DAKO) staining was performed using an automated staining system (Bond III; Leica Biosystems) following the manufacturer's protocols for the Bond Polymer Refine detection system (Leica Biosystems). Heat-induced antigen retrieval was performed using ER1 solution (pH 6.0; Leica Biosystems) for 30 minutes. Anti-CD68 antibody was diluted 1:200 in Da Vinci Green Diluent and incubated for 30 minutes. Slides were removed from the autostainer to be dehydrated and mounted. ANGPT2 expression was observed in cytoplasm of tumor cells and endothelia of small blood vessels. The expression was considered positive if 10% of cells had cytoplasmic staining. The intensity and the percentage of positive stained cells were assessed and recorded separately. Scoring was performed twice with a 1-week interval. For CD163 and CD68 staining, all slides were scanned using the Aperio Scan Scope (Aperio Technologies). After saving of each digital image, one to five representative areas of tumor (excluding areas of necrosis, artifact and other poor quality regions) were selected for analysis. Aperio ImageScope software (Aperio) was used, including a positive pixel count algorithm. Average percentage of area for positive staining was recorded as a final result for each case. All the slides were evaluated and scored by a pathologist (X. Liao) blinded to clinical data.

Immunoblot analyses

Cells were lysed in 1 lysis buffer (Cell Signaling Technology) supplemented with proteinase inhibitor cocktail (Roche), and centrifuged for 10 minutes at 14,000 rpm. Supernatants were collected, run on SDS gels, and transferred onto membranes. The membranes were blocked and probed with ANGPT2 antibody (Clone F-1; Santa Cruz Biotechnology), Erk1/2 antibody, or pErk1/2 antibody (Cell Signaling Technology). Representative results from one of the two experiments are shown.

Statistical analysis

The algorithm of Contal-O'Quigley (Contal, C., et al., Comput. Stat Data Anal. 30: 253–70 (1999)) was used to estimate the optimal division points of pretreatment ANGPT2 and fold changes in ANGPT2. This algorithm divides the sample into high and low based on all possible values of pretreatment ANGPT2 (or ANGPT2 fold change) and assesses OS based on the resulting two categories. The division point with the largest log-rank statistic was considered to be the "best" division point for the respective ANGPT2 measurement. OS was defined as the time from trial enrollment to death from any cause. The survival distribution was summarized using the method of Kaplan–Meier; confidence intervals (CI) were estimated using log (log

(survival)) methodology. To address the potential for guarantee–time bias, three-month conditional landmark analyses were used to explore the relationship between fold change in ANGPT2 and survival. Patients who were alive and had pretreatment and subsequent ANGPT2 measurements within 3 months were followed forward in time. Cox proportion hazards models were used to describe the relationship between ANGPT2 categories and response or survival. Cox models were stratified by trial (ipilimumab, ipilimumab plus bevacizumab, PD-1 blockade) to allow for differences between trials in the baseline hazard of death. Hazard ratios are shown with 95% CIs. Statistical significance of Cox model results is based on the Wald test. The association between pretreatment serum ANGPT2 levels or ANGPT2 fold changes and clinical responses, and the association between immune therapy and serum ANGPT2 changes, were evaluated using Fisher exact tests. The correlation between immune therapy and serum ANGPT2 fold changes was evaluated using the Kruskal–Wallis test. Holm–Bonferroni correction was used to preserve overall 0.05 type-1 error for multiple comparisons. $P < 0.05$ was considered statistically significant for all comparisons.

Culture and treatment of endothelial cells and melanoma cells

Tumor samples were obtained from patients on Dana-Farber/Harvard Cancer Center Institutional Review Board approved protocols. Tumor associated endothelial cells (TEC) were isolated using Dynabeads CD31 Endothelial Cell as guided by the manufacturer (Life Technologies) and confirmed by surface expression of CD31 and VEGFR2 and tube formation (Wu, 2016). HUVEC were purchased from Lonza (Allendale, NJ). TEC and HUVEC were cultured in EGM-2 (Lonza). Melanoma cell lines K008, K033 and M23 were established approximately 25 years ago from harvested fresh tissues on Dana-Farber/Harvard Cancer Center Institutional Review Board approved protocols as described previously (Wu, X. et al., PLoS One 8: e56134 (2013)). Melanoma A375 cells were obtained from ATCC (Manassas, VA) approximately 10 years ago. They were not authenticated, but have confirmed expression of MITF and melanocytic markers. Melanoma cells were cultured in DMEM containing 10% FBS, 50 μg/mL penicillin and 100 μg/mL streptomycin. In some experiments, EC and melanoma cells were cultured in a hypoxic chamber with 1% O2. To examine the effect of VEGF and bevacizumab on ANGPT2 expression, EC and melanoma cells were incubated with VEGF (100 ng/ml) and/or bevacizumab (25 μg/mL) in serum and angiogenesis factor reduced EBM/EGM-2 (3:1, v/v) medium and DMEM containing 1% FBS, respectively. To examine the effect of

enriched endogenous ANGPT2 antibodies on ANGPT2-mediated Erk1/2 phosphorylation, HUVEC were serum starved for 6 h and treated with ANGPT2 (400 ng/mL) preincubated with human normal IgG (Life Technologies) or enriched ANGPT2 antibodies (1.2 μg/mL) for 15 min at 37°C and 5% CO2.

Generation and polarization of monocyte derived macrophages (MDM). Frozen PBMC isolated from healthy donors were thawed briefly at 37°C in a water bath, washed in RPMI1640 containing 50 μg/mL penicillin and 100 μg/mL streptomycin (R-PS), and incubated in R-PS containing 5% FBS (R-PS5) on cell culture dishes for 1.5 hours. Floating cells were removed by washing with R-PS at least 5 times. The attached monocytes were cultured in R-PS10 medium (R-PS supplemented with 10% FBS) containing CSF1 (Biolegend, 15-100 ng/ml) for 3 days to differentiate into macrophages. After being washed with R-PS, the attached monocyte derived macrophages (MDM) were incubated with fresh R-PS10 containing CSF1 for 3 more days. MDM were activated with CSF1 (100 ng/mL), IL4 (10 or 20 ng/mL, R&D Systems), or IL10 (10 or 20 ng/ml, R&D Systems) for 2 days. In some experiments, ANGPT2 (300 ng/mL; R&D Systems, Minneapolis, MN; EMD Millipore, Temecula, CA) was added to MDM after 3 days of differentiation with CSF1 or when they were activated with IL4 or IL10 to examine its effect on PD-L1 expression. Phenotypes of polarized MDM were analyzed by FACS after staining with APC-conjugated anti CD80 (Clone 2D-10, Biolegend) and PE-conjugated anti-CD163 antibodies (Clone GHI/61, Biolegend).

Purification of ANGPT2 antibodies from plasma. Recombinant human ANGPT2 (6 μg) was coupled to activated NHS magnet beads (40 μL) (Thermo Scientific). Plasma samples (600 μL) were diluted with equal volume of PBS and incubated with the ANGPT2 -coupled beads with rotation at 4 ^{0}C overnight. The beads were pulled down with a magnet and washed with PBS 5 times. The antibodies bound to ANGPT2 were eluted with 0.1 M glycine (pH 2.5) from the beads and neutralized with 1/10 volume of 1 M Tris-Cl (pH 9.0). The antibodies were concentrated using an Amicon Ultra filter and stored in PBS supplemented with 0.02% BSA at 4^{0} C. IgG content was determined by ELISA against normal human IgG (Life Technologies).

Immunoblot analyses

Cells were lysed in 1x lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with proteinase inhibitor cocktail (Roche, Indianapolis, IN), and centrifuged for 10 min at 14,000 rpm. Supernatants were collected, run on SDS gels, and transferred onto

membranes. The membranes were blocked and probed with anti-ANGPT2 antibody (Clone F-1, Santa Cruz Biotechnology), Erk1/2 antibody, or pErk1/2 antibody (Cell Signaling Technology). Representative results from one of the two experiments are shown.

Results

Patients

A total of 48, 43, and 43 patients with advanced melanoma on immune checkpoint therapy with ipilimumab, ipilimumab plus bevacizumab, or PD-1 blockade, respectively, were analyzed for serum ANGPT2 concentrations before and during treatment. Patients enrolled in the phase I ipilimumab plus bevacizumab trial have been described previously (Hodi, F.S., et al., Cancer Immunol. Res. 2: 632–42 (2014)). Demographics, disease status, and prior treatment of the patients on ipilimumab or PD-1 blockade treatment are summarized in Supplementary Table S1. Approximately 16.7%, 19.6%, and 37.2% of patients on ipilimumab, ipilimumab plus bevacizumab, or PD-1 blockade treatment, respectively, achieved complete or partial responses. In addition, 33.3%, 47.8%, and 25.6% of them had stable disease. The median follow-up time in the current dataset for all data combined was 33 months (95% CI, 22–40).

Poor survival in ANGPT2-high patients receiving ipilimumab alone or with bevacizumab

To determine if pretreatment serum ANGPT2 levels were associated with clinical outcomes, the patients were divided into two groups, based on their pretreatment serum concentrations of ANGPT2. The division point was determined using the Contal– O'Quigley algorithm (Contal, C., et al., Comput. Stat Data Anal. 30: 253–70 (1999)) and found to be 3,175 pg/mL for all three groups of patients combined. High (>3175 pg/mL) or low (3175 pg/mL) pretreatment ANGPT2 concentrations were not associated with pretreatment lactose dehydrogenase (LDH) concentrations, gender, or stage of pooled patients receiving ipilimumab or ipilimumab plus bevacizumab (Supplementary Table S2). The median OS of patients with high or low pretreatment serum ANGPT2 was 12.2 (95% CI, 5.7–∞) versus 28.2 (95% CI, 13.5–∞) months (P ¼ 0.165), respectively, for patients treated with ipilimumab alone (FIG. 45A). High pretreatment serum ANGPT2 was associated with reduced OS also in patients treated with ipilimumab plus bevacizumab [median survival (high vs. low): 10.9 (95% CI, 3.1–19.8) vs. 19.3 (95% CI, 16.1–∞) months, P ¼ 0.0125; FIG. 45B]. This pattern held when data from patients treated with either ipilimumab or ipilimumab plus bevacizumab were pooled [10.9 (95% CI, 6–20) vs. 19.7 (95% CI, 16–55) months, P ¼ 0.004; FIG. 39A]. In the ipilimumab plus

bevacizumab treated patients, none of the 10 with high serum ANGPT2 achieved complete or partial remissions, whereas 8 out of the 33 (24.2%) with low ANGPT2 did. For ipilimumab alone, patients with low or high pretreatment ANGPT2 levels had similar response rates (17.6% vs. 16.1%).

Reduced OS associated with ipilimumab-induced early increases of serum ANGPT2

To examine whether dynamic changes in serum ANGPT2 were associated with treatment outcomes, posttreatment samples collected within 3 months after treatment initiation were analyzed. The division point for fold change of serum ANGPT2 within this time frame was 1.25 in all patients combined, as determined using the Contal–O'Quigley algorithm. The median OS of ipilimumab-treated patients based on this cutoff (1.25 vs. < 1.25) was 12.4 (95% CI, 5–55) versus 28.1 (95% CI, 14–∞) months (P ¼ 0.019; FIG. 45C). Ipilimumab plus bevacizumab-treated patients with fold changes 1.25 also had shortened OS (10.9 months, 95% CI, 5–∞) compared with those with fold changes < 1.25 (18.0 months, 95% CI, 14–), although this did not reach statistical significance due to small number of patients (n ¼ 4) with fold changes 1.25 (P ¼ 0.59; FIG. 45D). ANGPT2 increases were significantly associated with reduced OS when data from patients receiving ipilimumab or ipilimumab plus bevacizumab were pooled [median survival: 12.2 (95% CI, 5–55) vs. 19.3 (95% CI, 16–35) months, P ¼ 0.02; FIG. 39B]. All patients treated with ipilimumab or ipilimumab plus bevacizumab with ANGPT2 increases of at least 25% had either stable disease or progressive disease, except for one ipilimumabtreated patient with a 26.5% ANGPT2 increase who achieved a partial response (FIG. 39C).

Reduced OS in ANGPT2-high patients treated with PD-1 blockade

Among the PD-1 blockade-treated patients, 34 had low and 9 had high pretreatment serum ANGPT2. High or low pretreatment ANGPT2 was not associated with patient characteristics except for LDH concentrations (Supplementary Table S2). High pretreatment serum ANGPT2 was significantly associated with reduced OS (P ¼ 0.004; FIG. 39D). The median OS of patients with high pretreatment ANGPT2 was 7.3 (95% CI, 3.4–25.9) months, whereas that of patients with low pretreatment ANGPT2 was not reached because more than half of the patients were still alive. Patients with high or low pretreatment ANGPT2 had comparable response rates (33.3% and 38.2%, respectively).

Reduced response to PD-1 blockade if early increases of serum ANGPT2 were induced

Forty-three PD-1 blockade-treated patients with posttreatment samples collected within a 3-month time frame were analyzed for association of ANGPT2 fold changes and clinical outcomes. Patients with progressive (PD) and stable disease (SD) had significantly larger ANGPT2 fold changes than patients with partial responses (PR; PR vs. PD, P ¼ 0.007; PR vs. SD, P ¼ 0.002; SD vs. PD, P ¼ 0.87; FIG. 46A). Fold changes were significantly associated with clinical responses (P ¼ 0.002), and small fold changes were significantly associated with a higher response rate (58% vs. 6%; FIG. 39E). Similar to ipilimumab-treated patients, all patients with ANGPT2 fold change 1.25 had SD or PD, except one patient with an ANGPT2 fold change of 1.25 who achieved PR (FIG. 39F). ANGPT2 increases also appeared to be associated with reduced OS [median survival 16.3 (95% CI, 6.8–∞) vs. 36.7 (95% CI, 13.7–∞) months; FIG. 46B], although it did not reach statistical significance (P ¼ 0.22).

High initial serum ANGPT2, then therapy-induced increase, predicts poor OS and PD

Whether the combination of pretreatment serum ANGPT2 concentrations and the fold change after immune checkpoint therapy would enhance the predictive power of serum ANGPT2 was investigated. To do this, datasets of all three groups of patients were combined. High pretreatment ANGPT2 was associated with reduced OS in the pooled data [median survival: 10.9 (95% CI, 6.8–17.6) vs. 28.2 (95% CI, 18.6–∞) months, P < 0.0001; FIG. 40A]. The hazard ratio estimated from the Cox model stratified by trial is 2.48 (95% CI, 1.5–4.1; P ¼ 0.0003). In addition, large ANGPT2 fold changes were associated with shortened OS [median survival: 12.4 (95% CI, 7.9– 54.8) vs. 22.9 (95% CI, 17.6–40.6) months, P ¼ 0.002; FIG. 40B]. ANGPT2 fold changes were also significantly associated with clinical response (P ¼ 0.001; FIG. 40C), and response was significantly higher among patients with fold change < 1.25 (<1.25 vs. 1.25, 29.8% vs. 6.1%). Furthermore, the combination of pretreatment ANGPT2 serum concentrations and fold changes was associated with OS (P ¼ 0.001; Fig. 40D). Patients with high pretreatment ANGPT2 and large fold changes had the worst survival, whereas those with low pretreatment ANGPT2 and small fold changes had the best survival [median survival 7.9 (95% CI, 3.8–∞) vs. 34.6 (95% CI, 18.7–∞) months]. Patients with high pretreatment ANGPT2 and small fold changes or low pretreatment and large fold changes had intermediate survival [13.6 (95% CI, 7.3–22.9) and 16.3 (95% CI, 10–54.8) months, respectively]. The combination of pretreatment

ANGPT2 and fold changes was also significantly associated with clinical responses (P ¼ 0.006; Fig. 40E). One of the 11 patients (9.1%) with high pretreatment ANGPT2 and large fold changes achieved PR/CR, in comparison with 23 of the 72 patients (31.9%) with low pretreatment ANGPT2 and small fold changes (P ¼ 0.002). In contrast, 9 of the 11 patients (81.8%) with high pretreatment ANGPT2 and large fold changes had PD compared with 20 of the 72 patients (27.8%) with low pretreatment ANGPT2 and small fold changes. Patients with low pretreatment ANGPT2 and large ANGPT2 fold changes also had a low response rate (4.6%) than patients with low pretreatment ANGPT2 and small fold changes (P ¼ 0.01). Patients with low pretreatment ANGPT2 and large ANGPT2 fold changes or high pretreatment ANGPT2 and small fold changes had intermediate progression rates (54.5% and 36.4%, respectively). These observations suggest that the combination of high pretreatment serum ANGPT2 and large fold change following the initiation of treatment is a stronger predictor for PD and poor OS than either alone.

Immune checkpoint therapy influenced serum ANGPT2 concentrations

The effects of ipilimumab, ipilimumab plus bevacizumab, and PD-1 blockade on circulating ANGPT2 were compared. The effect of ipilimumab plus bevacizumab on serum ANGPT2 was significantly different from that of ipilimumab and PD-1 blockade (P ¼ 0.0001; Fig. 41A). Although 7.1%, 30.9%, and 39.5% of patients receiving ipilimumab plus bevacizumab, ipilimumab, and PD-1 blockade, respectively, displayed an increase in serum ANGPT2 by 25% or more, 38.1%, 16.7%, and 4.6% of patients, respectively, displayed a decrease by at least 25% within 3 months after treatment initiation (Fig. 41A). Furthermore, ipilimumab plus bevacizumabtreated patients displayed smaller ANGPT2 fold changes than ipilimumab and PD-1 blockade-treated patients (P ¼ 0.0001; Fig. 41B; Supplementary Table S3).

Bevacizumab blocked VEGF-induced tumor vascular ANGPT2 expression

To further address the effect of bevacizumab on ANGPT2 expression, ANGPT2 expression in cultured TECs and tumor cells was examined (detailed protocols are described in Materials and Methods), as well as in paired pretreatment and posttreatment tumor biopsies from patients treated with ipilimumab or ipilimumab plus bevacizumab. Bevacizumab decreased ANGPT2 expression in TEC after 96 hours (Fig. 41C). VEGF enhanced ANGPT2 expression in TEC under normoxic and hypoxic conditions, while bevacizumab blocked VEGF-induced

ANGPT2 expression (Fig. 41D). In melanoma cells, hypoxia increased ANGPT2 expression, whereas VEGF appeared to have no or minimal inhibitory effects (Fig. 47). Among 5 ipilimumab- treated patients whose tumors were analyzed, ANGPT2 was barely detected in the pretreatment tumors but highly expressed in both tumor cells and endothelia of posttreatment tumors in two of them (Fig. 42A; Ipi-P1 and Ipi-P2; Supplementary Table S4). Another ipilimumab-treated patient also displayed increased ANGPT2 expression in endothelial cells but not in melanoma cells in posttreatment biopsies (Ipi-P3; Supplementary Table S4). In comparison, ANGPT2 expression was significantly decreased in tumor vessels of the posttreatment biopsies of 2 patients among the 7 ipilimumab plus bevacizumab-treated patients analyzed (Fig. 42B; P1 and P28; Supplementary Table S4). These in vitro and in vivo findings support the inhibitory effect of bevacizumab on VEGF-induced ANGPT2 expression in tumor-associated endothelia. Nonetheless, ANGPT2 expression in response to ipilimumab and ipilimumab plus bevacizumab is heterogeneous, with modest decreases (Ipi-P4), increases (P20 and P27), or no change (P4, P9, and P31) in its expression having also been observed (Fig. 42C; Supplementary Table S4). This may reflect heterogeneity in the tumor microenvironment and the complex regulation of ANGPT2 expression in tumors by multiple factors (Thurston, G., et al, Cold Spring Harb Perspect Med 2:a006550 (2012)).

Tumor vascular ANGPT2 was associated with macrophage infiltration

Given the known expression of Tie-2 (ANGPT2 receptor) on monocytes/macrophages (Garcia, S. et al., PLoS One 9: e82088 (2014); Forget, M.A., et al., PLoS One 9: e98623 (2014)), it was wondered if the addition of bevacizumab to ipilimumab treatment resulting in decreased ANGPT2 expression had an impact on tumor macrophage infiltration. Examination of the tumors from ipilimumab-treated patients with robust ANGPT2 induction revealed an increase in $CD68^+$ and $CD163^+$ macrophages as a function of treatment (Fig. 42A; Supplementary Table S4). Similarly, an increased infiltration of $CD68^+$ and $CD163^+$ macrophages was observed in the posttreatment tumor biopsies of the ipilimumab plus bevacizumab patients with increased vascular ANGPT2 expression (Fig. 42C; Supplementary Table S4). In contrast, substantially fewer CD68þ and CD163þ macrophages were detected in posttreatment biopsies where ANGPT2 wasmsignificantly downregulated in both tumor cells and TECsm (Fig. 42B). Additional paired biopsy analyses revealed that changes in tumor CD68þ and/or CD163þ macrophage infiltration overall correlated with changes in tumor endothelial ANGPT2

expression: increased $CD68^+$ and/or $CD163^+$ macrophages were observed in three of the four cases with elevated vascular ANGPT2 expression in the posttreatment biopsies (Supplementary Table S4; Fig. 42D), while decreased $CD68^+$ and $CD163^+$ macrophages were detected in three of the three cases with reduced vascular ANGPT2 expression in the posttreatment biopsies (Supplementary Table S4; Fig. 42E). Nonetheless, increased and decreased macrophage infiltration was also observed in cases where vascular ANGPT2 was not alteredby the treatment (Supplementary Table S4), suggesting that other chemoattractants (such as CXCL12 and chemokine (C-C) ligand 2 (CCL2)) may also be involved in tumor macrophage recruitment (Huang. H. et al., Clin. Cancer Res. 17:1001–11 (2011); Srivastava, K. et al., Cancer Cell 26:880–95 (2014)), as well as inherent sampling bias and heterogeneity associated with human sample collection.

ANGPT2 upregulates PD-L1 expression on M2-polarized macrophages

The association of serum ANGPT2 concentration and clinical outcomes to immune checkpoint therapy suggested that ANGPT2 may play additional roles in immune regulation. Examined the effect of ANGPT2 on PD-L1 expression on MDMs that were activated with CSF1, IL4, or IL10 (Zajac, E. et al., Blood 122: 4054–67 (2013); Martinez, F.O, et al. Prime Rep. 6:13 (2014); Vogel, D.Y., et al., Immunobiology 219:695–703 (2014)) was examined. CSF1, IL4, and IL10-activated MDMs were derived from normal donors (described in Materials and Methods) and expressed M2 marker CD163, no or low M1 marker CD80 (FIG. 48A), and have been shown to have prometastatic, proangiogenic, and immunosuppressive activities (Zajac, E. et al., Blood 122: 4054–67 (2013); Martinez, F.O, et al. Prime Rep. 6:13 (2014); Mantovani, A. et al., Curr Opin Immunol 22:231–7 (2010)). ANGPT2 increased PD-L1 expression on CSF1, IL10, and IL4-activated macrophages (FIG. 43A– FIG. 43C). This effect was somewhat heterogeneous in magnitude among donors (FIG. 48B and FIG. 48C).

Immune checkpoint therapy elicited antibody responses to ANGPT2

Ipilimumab plus bevacizumab can elicit humoral immune responses to target antigens in patients with advanced melanoma (Hodi, F.S., et al., Cancer Immunol. Res. 2: 632–42 (2014); Wu, X., et. al., Cancer Immunol. Res 4:858–68 (2016)). Therefore, antibody responses to ANGPT2 were investigated in patients receiving ipilimumab, ipilimumab plus bevacizumab, and PD-1 blockade using immunoblot analyses and ELISA. ANGPT2 antibody concentrations in the pretreatment and posttreatment plasma samples of representative ipilimumab plus bevacizumab-

treated patients were measured (FIG. 44A and FIG. 44B). Approximately 8%, 19%, and 21% of the patients, including responders and nonresponders (FIG. 49A–FIG. 49C), displayed an increase in the ANGPT2 antibody level by 40% or more in response to PD-1 blockade, ipilimumab, and ipilimumab plus bevacizumab, respectively (FIG. 44C). Robust ANGPT2 antibody increases were detected in two ipilimumab plus bevacizumab-treated patients (P16 and P26) who survived for more than 3 years with stable disease (FIG. 44A, FIG. 44B and FIG. 44D). Of note, the increase in ANGPT2 antibody appeared to parallel a rise in circulating ANGPT2 in patient P26 (FIG. 44D). A significant ANGPT2 antibody increase wasalso observed in a long-term responder of ipilimumab (FIG. 44E) and PD-1 blockade (FIG. 44F). Longitudinal analyses revealed that ANGPT2 antibody levels increased following initial treatment and lasted for months to years (FIG. 44D– FIG. 44F). To determine the functionality of the endogenous ANGPT2 antibodies, ANGPT2 antibodies were purified from the posttreatment plasma of patient P26 using ANGPT2 coupled beads (detailed protocols are provided in Materials and Methods). The enriched antibodies recognized ANGPT2 and inhibited ANGPT2-mediated Erk1/2 phosphorylation in HUVEC (FIG. 50A and FIG. 50B), demonstrating their capability of neutralizing the biological activity of ANGPT2.

Identification of predictive and prognostic biomarkers as well as mechanisms of resistance to immune therapy help not only in selecting patients who may benefit from treatment, but also in finding combinatorial approaches that offer hope for improved patient outcomes. Both high pretreatment concentrations and increases in serum ANGPT2 early during treatment were associated with reduced survival and/or response in patients receiving immune checkpoint blockade. Although previous studies have identified serum ANGPT2 as a prognostic biomarker for a number of types of cancers, including melanoma and colon cancer being treated with anti-VEGF containing therapy (Thurston, G., et al, Cold Spring Harb Perspect Med 2:a006550 (2012); Helfrich, I., et al., Clin. Cancer Res. 15:1384–92 (2009); Jary, M., et al., Cancer Epidemiol. Biomarkers Prev. 24:603–12 (2015); Dreikhausen, L., at al., BMC Cancer 15:121 (2015); Goede, V. et al., Br. J. Cancer 103: 1407–14 (2010)), the results presented herein suggest that pretreatment serum ANGPT2 concentration, ANGPT2 fold change, and their combination can be used as a prognostic and/or predictive biomarker for immune checkpoint therapy.

Predictive and prognostic biomarker candidates for checkpoint blockade have been difficult to reliably validate. Recent candidates have included tumor and immune cell PD-L1

expression for anti–PD-1/PD-L1 therapy in many tumor types, as well as the significance of a preexisting inflamed tumor microenvironment to predict clinical benefit (Mahoney, K.M. et al., Clin. Ther. 37: 764–82 (2015)). Tumor heterogeneity and the focal and dynamic nature of PD-L1 expression makes such biomarker evaluation challenging (Mahoney, K.M. et al., Clin. Ther. 37: 764–82 (2015)). Serologic markers may provide a global assessment of immune activation and provide an immediate snapshot in the dynamic process. Serum ANGPT2 can be easily measured and monitored. It could be an additional parameter to consider for prognostic and predictive evaluation of immune checkpoint blockade in conjunction with other factors or on its own. Additional prospective studies to confirm these initial observations are warranted as well as further understanding of the complex biology influencing patient outcomes to treatment. ANGPT2 is well known to have proangiogenic and protumoral activity, as well as function in resistance to anti-VEGF therapy (Fiedler, U., et al., Trends Immunol. 27:552–8 (2006); Huang, H. et al., Nat. Rev. Cancer 10:575–85 (2010); Tait, C.R. et al., J. Pathol. 204:1–10 (2004); Rigamonti, N. et al, Cell Rep. 8:696–706 (2014); Coffelt, S.B. et al., Cancer Res 70: 5270–80 (2010)). The association of serum ANGPT2 level with poor clinical outcomes to immune checkpoint therapy suggests that ANGPT2 may also contribute to resistance to immune checkpoint therapy. This may be attributed to its role in the recruitment of monocytes/macrophages into the tumor microenvironment and induction of PD-L1 expression in $M2^{-}$ polarized macrophages. An association of tumor vascular ANGPT2 expression and macrophage infiltration in patient tumors was observed, suggesting that tumor vascular ANGPT2 may play a significant role in tumor macrophage recruitment. This is consistent with previous findings from animal studies that tumor-derived ANGPT2 and endothelial cell–specific overexpression of ANGPT2 promote tumor recruitment of macrophages (Scholz, A. et al., Blood 118: 5050–9 (2011); Scholz, A., et, al., Ann. NY Acad Sci 1347:45–51 (2015); Coffelt, S.B. et al., Cancer Res 70: 5270–80 (2010); Scholz, A. et al., EMBO Mol Med 8: 39–57 (2018)). In addition, ANGPT2 promoted PD-L1 expression on M2-polarized macrophages. Tumor-associated macrophages (TAM) promote tumor initiation, invasion, metastasis, angiogenesis, and immune suppression (Noy, R. et al., Immunity 41: 49–61 (2014)). High TAM infiltration correlates with a poor prognosis in most human tumor types (De Palma, M., et al., Cancer Cell 23: 277–86 (2013); Riabov, V. et al., Front Physiol. 5:75 (2014); Ostuni, R., et al., Trends Immunol. 36: 229–39 (2015)). Specifically, PD-L1þ monocytes/macrophages

effectively suppress tumorspecific T-cell immunity, and tumor infiltration of PD-L1þ monocytes/macrophages is associated with disease progression and reduced survival in patients (Kuang, D.M., et al., J. Exp. Med. 206: 1327–37 (2009)). Because PD-1 blockadenand ipilimumab target distinct immune checkpoints and act on different stages of T-cell activation, upregulation of PD-L1 may confer resistance to ipilimumab-based therapy and limit effectiveness of PD-1 or PD-L1 directed treatment. These studies together may suggest a critical role for ANGPT2 in TAM recruitment and in shaping the proangiogenic and immunosuppressive environment of tumors.

The potential role of ANGPT2 in resistance to anti–CTLA-4 or anti–PD-1 therapy is also supported by the ipilimumab and PD-1 blockade–induced increase in serum ANGPT2 in substantial proportions of the nonresponders. Increased ANGPT2 expression in tumors was also observed in ipilimumab-treated patients. Ipilimumab plus bevacizumab decreased ANGPT2 expression in sera and in tumors, most pronounced in the tumor vasculature. Together with the in vitro data, these findings reveal an important role for VEGF in upregulation of tumor vascular ANGPT2 expression, and prevention of such expression by bevacizumab, leading to decreased endothelial ANGPT2 expression. This mechanism may prevent infiltration of M2 macrophages into tumors. Such a phenomenon is in agreement with animal studies showing that dual inhibition of VEGF and ANGPT2 led to reprogramming of macrophages in glioblastoma (Kloepper, J., Proc. Natl. Acad. Sci USA 113: 4476–81 (2016); Peterson, T.E, et al., Proc Natl Acad Sci USA 113: 4470–5 (2016)). Anti-VEGF may also reduce ANGPT2 expression in tumor cells by normalizing tumor vessels and making the tumor microenvironment less hypoxic. Anti-VEGF may reduce tumor vascular ANGPT2 expression at least with initial treatment, thereby further contributing to the antitumor effect of immune therapy. In addition, the ANGPT2-resistant mechanism for anti-VEGF therapy may be a long-term consequence and not significant during initiation of therapy.

Extending the previous findings (Hodi, F.S., et al., Cancer Immunol. Res. 2: 632–42 (2014); Wu, X., et. al., Cancer Immunol. Res 4:858–68 (2016)), immune checkpoint therapy elicited humoral immune responses to ANGPT2 were shown. These responses were long lasting and robust in several long-term survivors experiencing clinical benefit. ANGPT2 antibodies induced by immune therapy are functional in neutralization of biological activity of ANGPT2 (Schoenfeld, J. et al, Cancer Res. 70: 10150–60 (2010)). Together with the antitumor effect of

ANGPT2 antibodies observed in animal studies and clinical trials (Kienast, Y. et. al., Clin Cancer Res 19: 6730–40 (2013); Herbst, R.S., et al., J. Clin. Oncol. 27: 3557–65 (2009); Thomas, M., et al., PLoS One 8: e54923 (2013); Mazzieri, R., et al., Cancer Cell 19: 512–26 (2011)), antibody responses to ANGPT2 may potentially contribute to the antitumor activity of immune checkpoint therapy, as described herein.

In summary, serum ANGPT2 may be used as a prognostic and/or predictive biomarker for immune checkpoint therapy. ANGPT2 may constitute a resistance mechanism for immune checkpoint therapy by enhancing tumor recruitment of monocytes/macrophages and upregulating PD-L1 expression in TAM. Additionally, reduction in tumor vascular ANGPT2 expression by anti-VEGF and antibody responses to ANGPT2 elicited by immune checkpoint blockade may enhance efficacy of immune therapy. Therefore, ANGPT2 should be considered a pertinent target for therapeutic intervention particularly in combination with immune checkpoint blockade. These findings have immediate clinical implications for improving the efficacy of current and developing cancer treatments.

Supplementary Tables

Table S1. Patient characteristics

Treatment	CTLA-4 blockade		PD-1 blockade	
	Ipilimumab (N=48)		Nivolumab (N=13) / Pembrolizumab (N=30)	
	N	%	N	%
Gender				
Female	18	37.5	24	56
Male	30	62.5	19	44
Stage				
M0	1	2.1	0	0
M0 stage III	1	2.08	0	0
M0 Stage IIIc	3	6.25	0	0
M1a	2	4.17	3	7
M1b	8	16.7	9	21
M1c	33	68.8	31	72
LDH				
ANL	12	25	7	16.3
WNL	30	62.5	32	74.4
N/A	6	12.5	4	9.3
Prior treatment*				
0	19	39.6	12	27.9
1	11	22.9	20	46.5
2	12	25.0	7	16.3
3	4	8.3	1	2.3
4	1	2.1	2	4.7
5	0	0.0	1	2.3
6	1	2.1	0	0
Age	67 (26-86)		59 (36-85)	

* Numbers indicate different treatments that patients received.

Table S2. Patient characteristics by pre-treatment serum ANGPT2 levels

Treatment	Ipilimumab/Ipilimumab plus bevacizumab					PD-1 blockade				
	ANGPT2 (pg/ml)				Fisher's exact p-value	ANGPT2 (pg/ml)				Fisher's exact p-value
	> 3175		≤ 3175			> 3175		≤ 3175		
	N	%	N	%		N	%	N	%	
LDH Class					0.21					0.03
ANL	4	14.8	4	6.3		4	44.4	3	8.8	
Not done	3	11.1	3	4.7		1	11.1	3	8.8	
WNL	20	74.1	57	89.1		4	44.4	28	82.4	
Gender					0.16					0.71
Female	7	25.9	28	43.8		6	66.7	18	52.9	
Male	20	74.1	36	56.3		3	33.3	16	47.1	
M Stage					0.07					0.17
M0 III	2	7.4	4	6.3		-	-	-	-	
M1 stage III	-	-	1	1.6		-	-	-	-	
M1a	4	14.8	1	1.6		-	-	3	8.8	
M1b	2	7.4	13	20.3		-	-	9	26.5	
M1c	19	70.4	45	70.3		9	100	22	64.7	

Table S3. Serum ANGPT2 fold-changes by immune checkpoint therapy

	ANGPT2 fold-change							
	N	Mean	S.D.	Min	25th	Median	75th	Max
Overall	127	1.1	0.5	0.3	0.8	1.0	1.3	4.0
Treatment								
Ipilimumab	42	1.2	0.7	0.3	0.9	1.1	1.4	4.0
Ipilimumab plus bevacizumab	42	0.9	0.4	0.4	0.7	0.8	1.1	3.0
PD-1 blockade	43	1.2	0.3	0.7	1.0	1.1	1.3	2.3

Table S4. ANGPT2, CD68 and CD163 staining of melanoma tissues

Treatment	Patient	ANGPT2 staining (H score)				Macrophage staining (%)			
		Melanoma cells		Endothelial cells		$CD68^+$ cells		$CD163^+$ cells	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post
Ipilimumab	Ipi-P1	0	300	0	150	2.9	4.7	3.1	7.1
	Ipi-P2	0	30	0	120	No tissue	11.1	No tissue	6.6
	Ipi-P3	90	90	60	140	17	16.2	17.3	38.8
	Ipi-P4	0	0	160	60	9.1	3.9	7.4	6.2
Ipilimumab plus bevacizumab	P1	100	0	150	0	29.9	19.8	26.5	23.4
	P28	0	0	120	0	11.5	4.2	18.7	3.6
	P20	90	0	0	10	7.8	40.4	14.8	69.2
	P27	0	0	0	20	9.7	19.1	11.8	3.9
	P4	0	0	20	20	16.1	29.2	44.4	33.9
	P9	100	100	0	0	No tissue	35.8	No tissue	48.6
	P31	0	0	0	0	12.6	12.4	7.2	22

Example 4: Phase Ib study to test the safety and potential synergy of pembrolizumab (anti-PD-1) and AMG386 (angiopoietin-2 (Ang-2) in patients with advanced solid tumors

Trial Design

Described herein is a prospective trial in subjects with solid tumors to evaluate the safety, clinical, and immunological effect of the combination of pembrolizumab (MK-3475) and trebananib (AMG386). The treatment includes an induction phase of pembrolizumab and trebananib for 4 cycles (12 weeks) followed by pembrolizumab alone for 2 years. The study plan is to accrue up to 60 subjects.

This trial is conducted in 2 parts. Part I uses a standard 3+3 dose escalation design in all solid tumors. The goal of Part I is to identify the recommended part 2 (expansion cohort) doses (RP2D) for the combination of pembrolizumab plus trebananib (AMG386). Part II enrolls patients on 4 dose expansion cohorts: melanoma, renal cell carcinoma (RCC), ovarian cancer, and colorectal cancer (CRC) (12 patients on each cohort). All patients on the expansion cohorts are required to undergo pre- and post-treatment biopsies.

Clinical Data Summary

As described herein, data were retrieved from the study database from the date the first patient was enrolled (September 11, 2017) until this report data cut off (June 21, 2018). The study enrolled a total of 22 patients (15 CRC, 6 ovarian cancer, and 1 RCC). Six patients were treated on the dose escalation Part I (1 ovarian and 5 CRC) since no Dose Limiting Toxicity (DLT) was identified. Therefore, the weekly dose 30mg/kg of trebananib was deemed to be the PR2D in combination with pembrolizumab 200mg every 3 weeks.

Out of those 22 patients enrolled on the study to date, 11 patients had restaging scan (10 CRC and 1 ovarian cancer). Out of the 10 CRC patients who had scans one had a partial response (6 months after the first treatment) and 2 had stable disease (3 months after first treatment) while 7 patients had progression of disease. The only ovarian patient who had a restaging scan was found to have progression of disease. There are currently 2 CRC patients in screening.

Safety Data Summary to Date

The most commonly occurring event types are: hypoalbuminemia (N=10; 59%), abdominal distension (N=8; 47%), aspartate aminotransferase increased (N=7; 41%), and proteinuria (N=7; 41%). There were no grade-5 adverse events.

OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. Genbank and NCBI submissions indicated by accession number cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

What is claimed is:

1. A method of treating neoplasia in a subject in need thereof comprising:

identifying a subject with neoplasia or at risk of developing neoplasia;

administering to the subject an effective amount of an angiopoietin-2 (Ang-2) inhibitor; and

administering to the subject an effective amount of a programmed cell death protein 1 (PD-1) inhibitor,

thereby treating neoplasia in the subject.

2. The method of claim 1, wherein the Ang-2 inhibitor comprises a small molecule inhibitor, an antibody, or a peptibody.

3. The method of claim 2, wherein the Ang-2 peptibody comprises trebananib (AMG386).

4. The method of claim 1, wherein the PD-1 inhibitor comprises a small molecule inhibitor, an antibody, or a peptibody.

5. The method of claim 4, wherein the anti-PD-1 antibody comprises pembrolizumab.

6. The method of claim 1, wherein the effective amount of trebananib is 3 mg/kg, 10 mg/kg, 15 mg/kg, or 30 mg/kg.

7. The method of claim 1, wherein the pembrolizumab is administered at a dose of 200 mg every three weeks.

8. The method of claim 1, wherein the effective amount of the pembrolizumab is 2 mg/kg.

9. The method of claim 1, wherein the pembrolizumab is administered every three weeks for twelve weeks.

10. The method of claim 9, wherein the trebananib is administered once per week for twelve weeks.

11. The method of claim 10, wherein the pembrolizumab is administered every three weeks for two years.

12. The method of claim 1, wherein the pembrolizumab and trebananib are administered systemically, intravenously, subcutaneously, intramuscularly, or orally.

13. The method of claim 1, wherein the pembrolizumab and trebananib are administered simultaneously or sequentially.

14. The method of claim 1, wherein the trebananib is administered immediately after the pembrolizumab.

15. The method of claim 1, wherein the neoplasia comprises a solid tumor.

16. The method of claim 1, wherein the neoplasia comprises melanoma, ovarian cancer, kidney cancer (renal cell carcinoma), or colorectal cancer.

17. The method of claim 1, wherein the neoplasia is inhibited by at least 5%.

18. The method of claim 1, wherein clinical benefit in the subject is evaluated by response evaluation criteria in solid tumors (RECIST) or immune response criteria (irRC).

19. The method of claim 1, further comprising obtaining a sample from the subject before and after administration of the Ang-2 inhibitor and the PD-1 inhibitor.

20. The method of claim 19, wherein treatment efficacy is evaluated by analyzing a blood sample or a tumor biopsy from the subject.

21. The method of claim 1, wherein the subject is human.

22. A method of determining whether inhibition of CTLA4 and/or inhibition of PD-1 in a subject with melanoma will result in clinical benefit in the subject comprising:

obtaining a test sample from a subject having or at risk of developing melanoma;

determining the expression level of Ang-2 in the test sample;

comparing the expression level of Ang-2 in the test sample with the expression level of Ang-2 in a reference sample; and

determining whether CTLA4 and PD-1 blockade will inhibit melanoma in the subject if the expression level of the Ang-2 in the test sample is differentially expressed as compared to the level of the Ang-2 in the reference sample.

23. The method of claim 22, wherein the test sample is obtained from the melanoma tissue or from the tumor microenvironment.

24. The method of claim 22, wherein clinical benefit in the subject comprises complete or partial response as defined by response evaluation criteria in solid tumors (RECIST), stable disease as defined by RECIST, or long-term survival in spite of disease progression or response as defined by irRC criteria.

25. The method of claim 22, wherein the test sample is obtained from the melanoma; and

determining that inhibition of CTLA4 and/or PD-1 in a subject with melanoma will not result in clinical benefit in the subject if the expression level of Ang-2 in the test sample is higher than the level of Ang-2 in the reference sample.

26. The method of claim 25, wherein the reference sample is obtained from healthy normal tissue, melanoma that received a clinical benefit from CTLA4 and/or PD-1 inhibition, or melanoma that did not receive a clinical benefit from CTLA4 and PD-1 inhibition.

27. A kit for treatment of cancer comprising pembrolizumab and trebananib and instructions for use.

FIG. 1

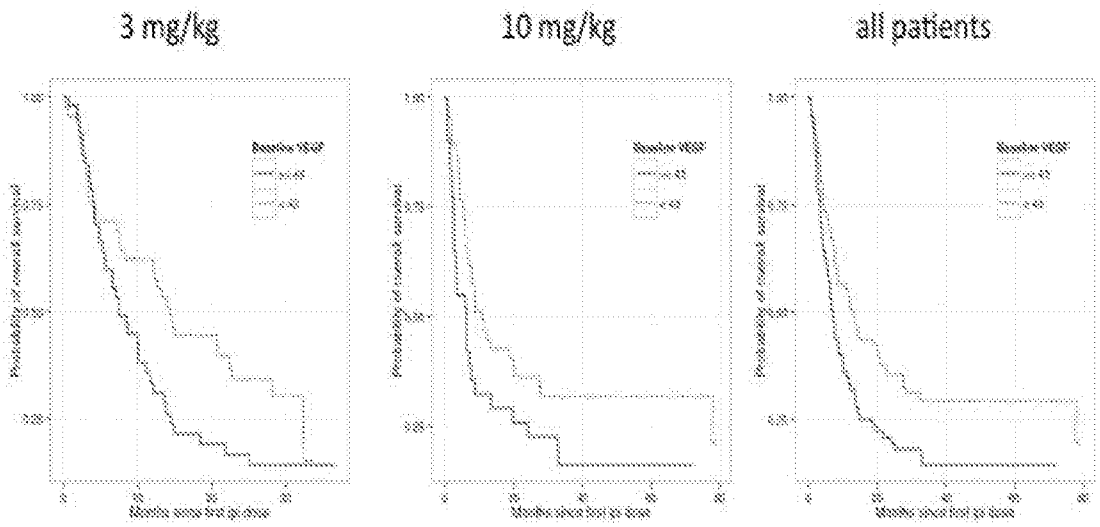


FIG. 2

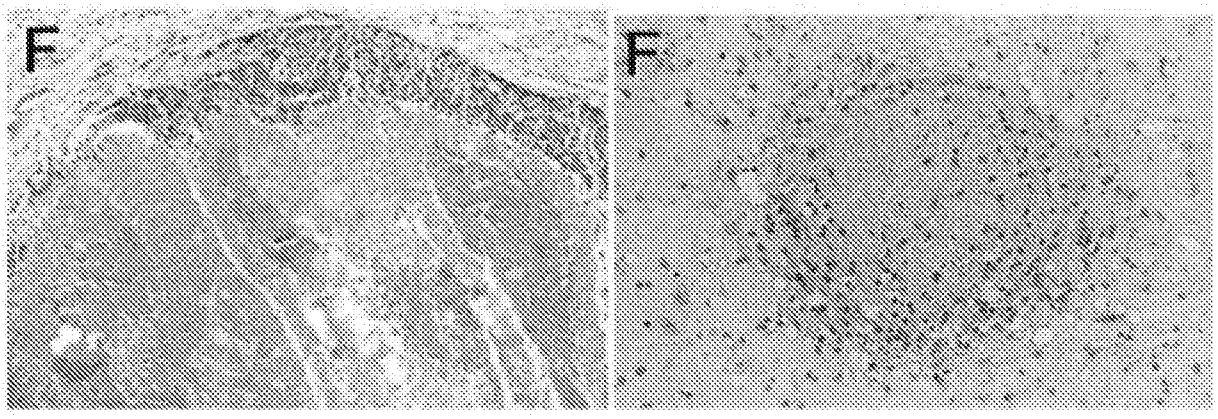


FIG. 3

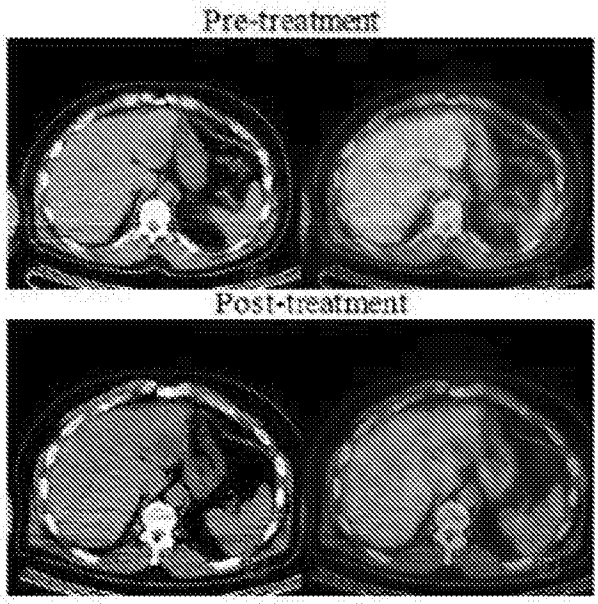

Pre-treatment
Post-treatment

FIG. 4

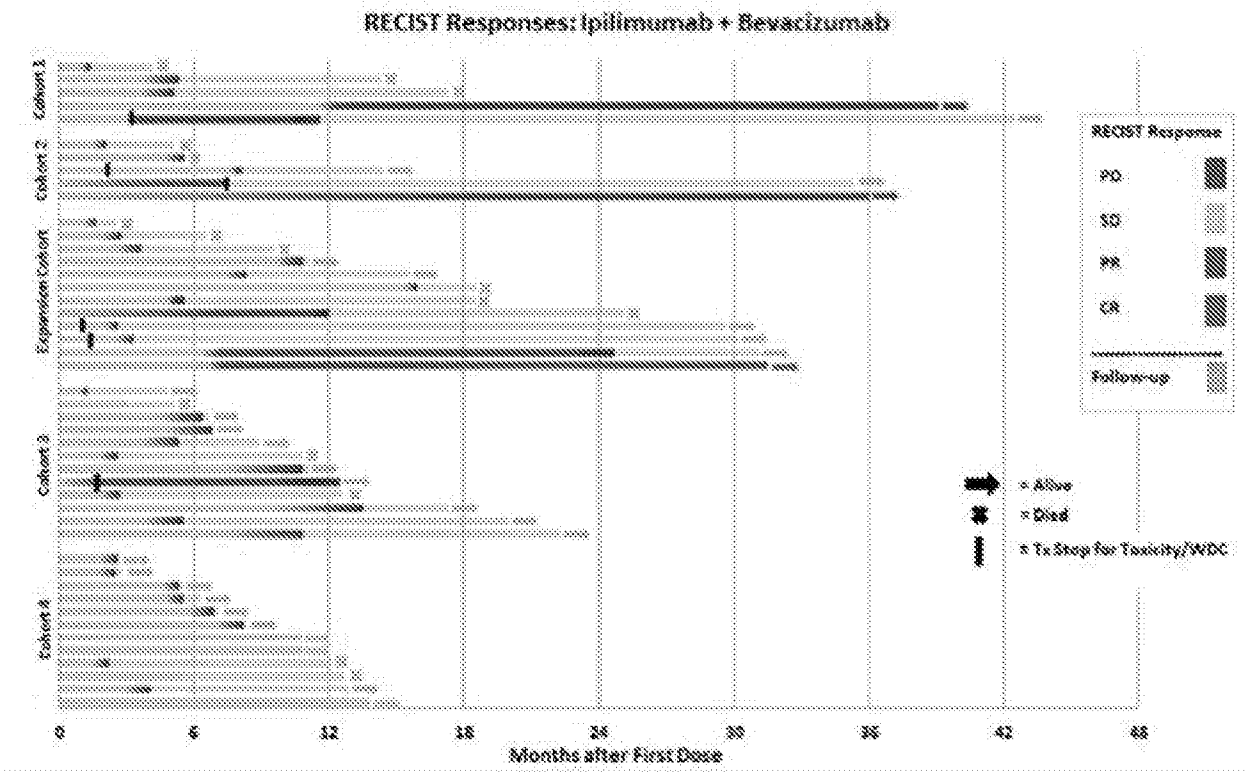

RECIST Responses: Ipilimumab + Bevacizumab
Cohort 1
Cohort 2
Expansion Cohort
Cohort 3
Cohort 4
RECIST Response
PD
SD
PR
CR
Follow-up
= Alive
= Died
= Tx Stop for Toxicity/WDC
0
6
12
18
24
30
36
42
48
Months after First Dose

FIG. 5A

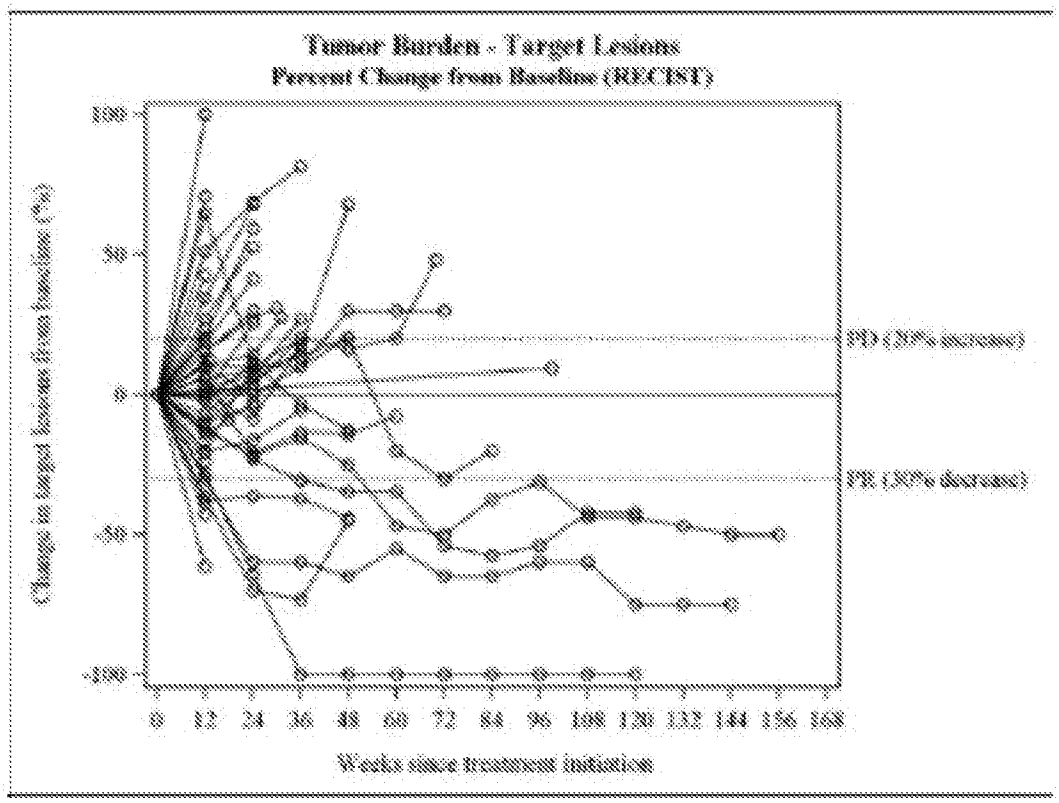

Tumor Burden - Target Lesions
Percent Change from Baseline (RECIST)
Change in target lesions from baseline (%)
100
50
0
-50
-100
PD (20% increase)
PR (30% decrease)
0 12 24 36 48 60 72 84 96 108 120 132 144 156 168
Weeks since treatment initiation

FIG. 5B

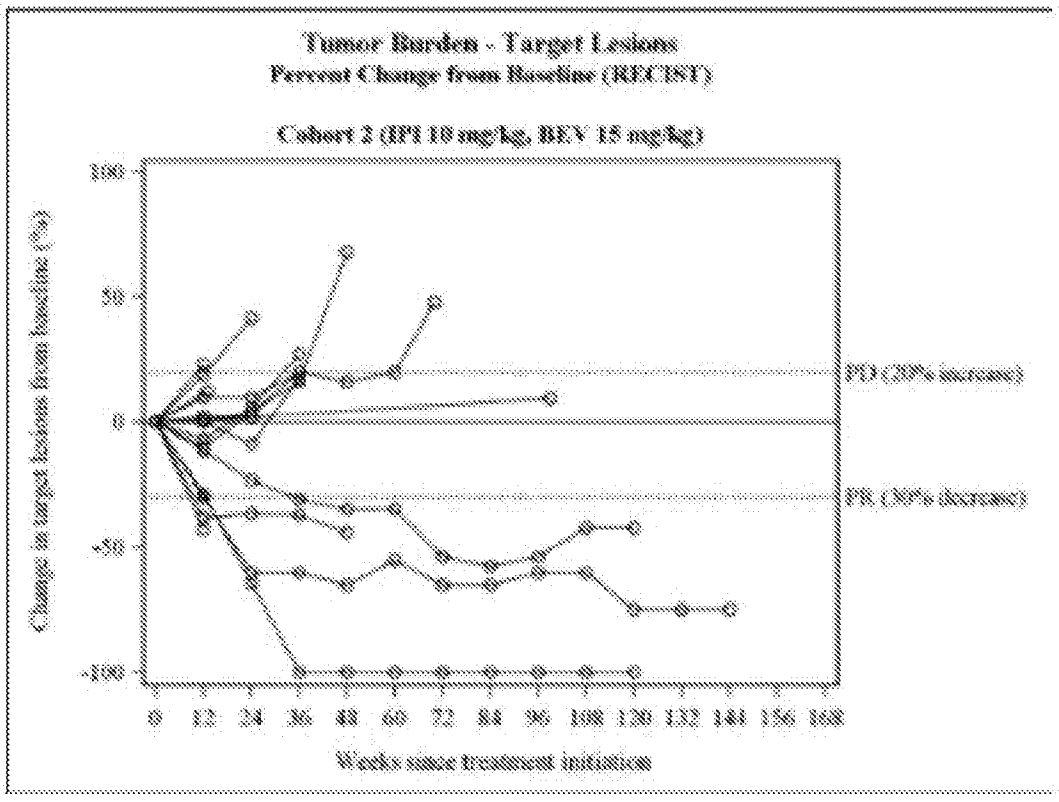

Tumor Burden - Target Lesions
Percent Change from Baseline (RECIST)
Cohort 2 (IPI 10 mg/kg, BEV 15 mg/kg)
Change in target lesions from baseline (%)
100
50
0
-50
-100
PD (20% increase)
PR (30% decrease)
0 12 24 36 48 60 72 84 96 108 120 132 144 156 168
Weeks since treatment initiation

FIG. 6

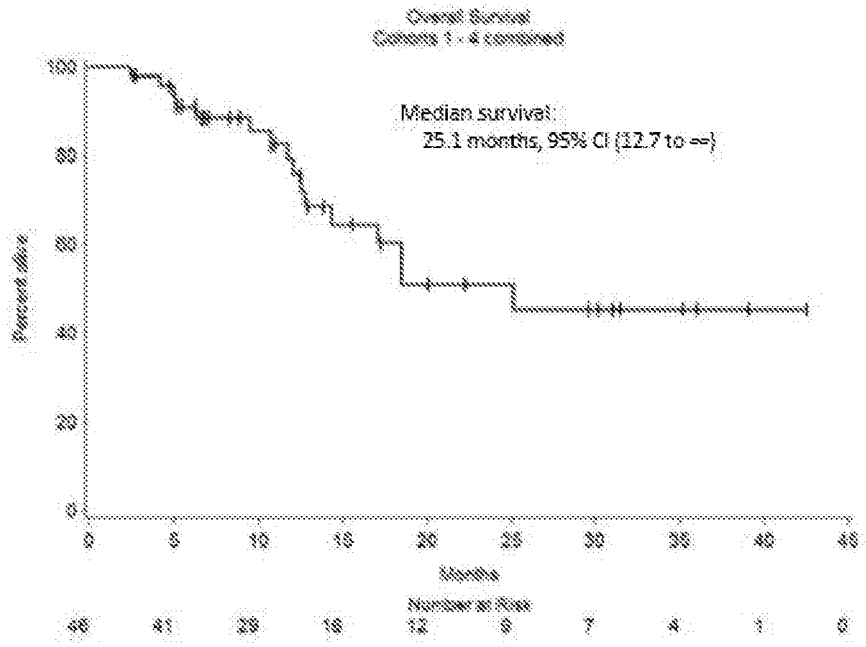

Overall Survival
Cohorts 1 - 4 combined
Median survival:
25.1 months, 95% CI (12.7 to ∞)
Percent Alive
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
Months
Number at Risk
46
41
28
18
12
9
7
4
1
0

FIG. 7

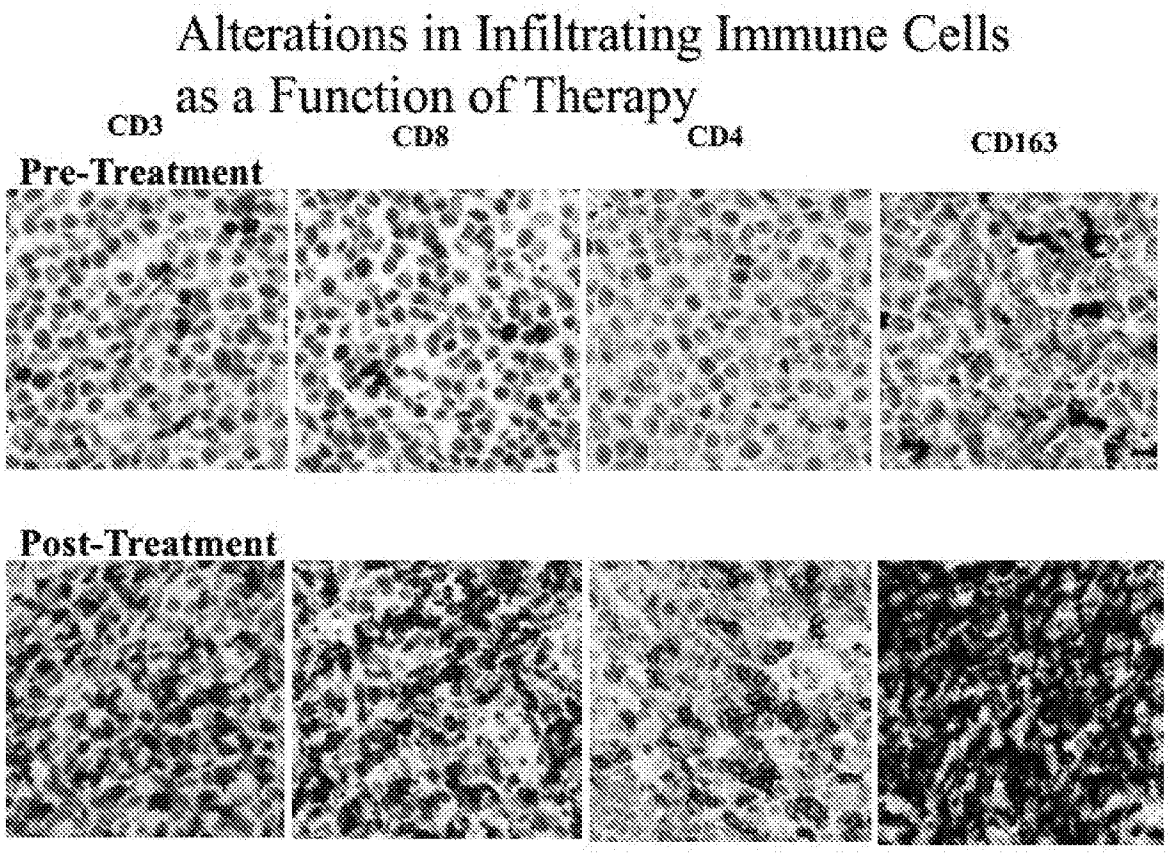

Alterations in Infiltrating Immune Cells
as a Function of Therapy
CD3
CD8
CD4
CD163
Pre-Treatment
Post-Treatment

FIG. 8

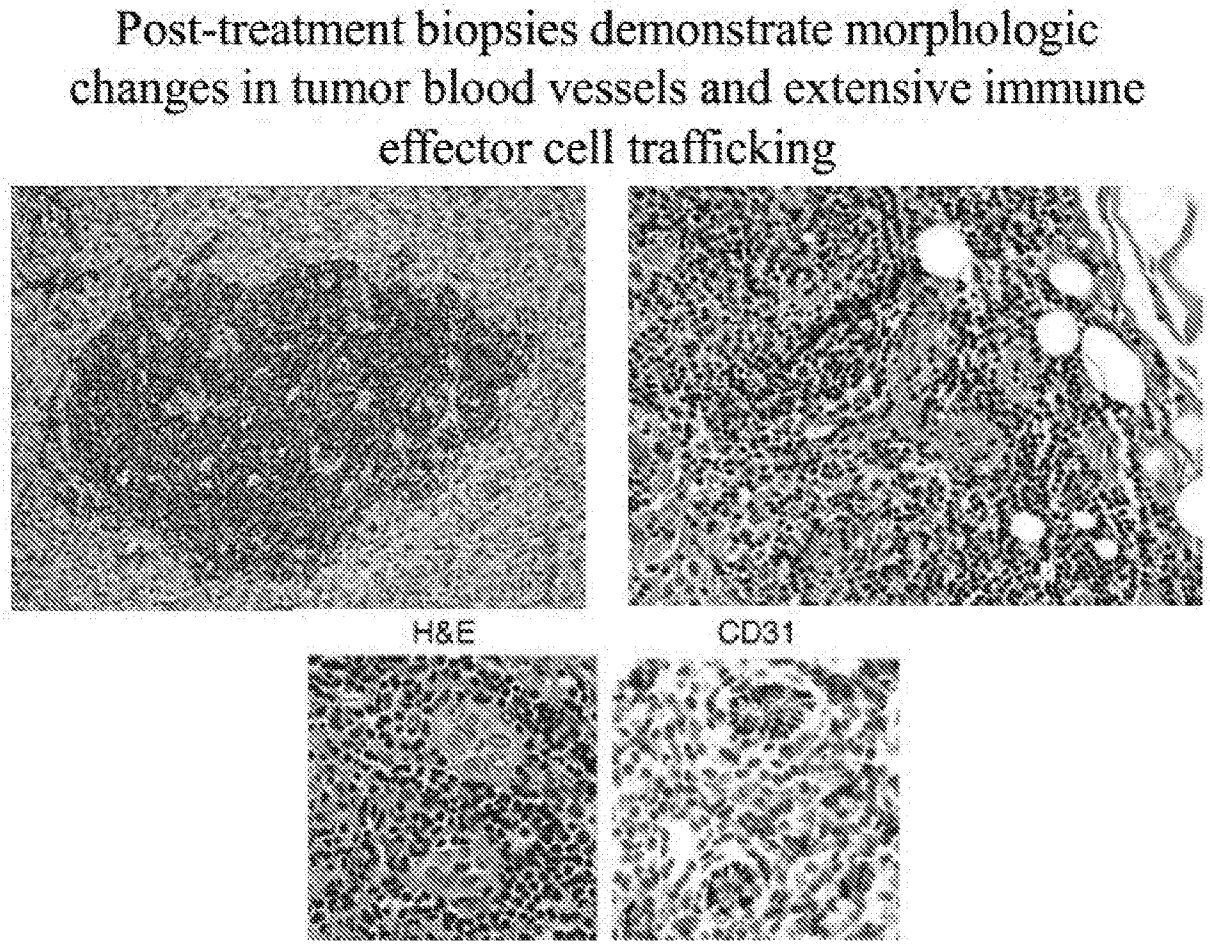

Post-treatment biopsies demonstrate morphologic changes in tumor blood vessels and extensive immune effector cell trafficking
H&E
CD31

FIG. 9

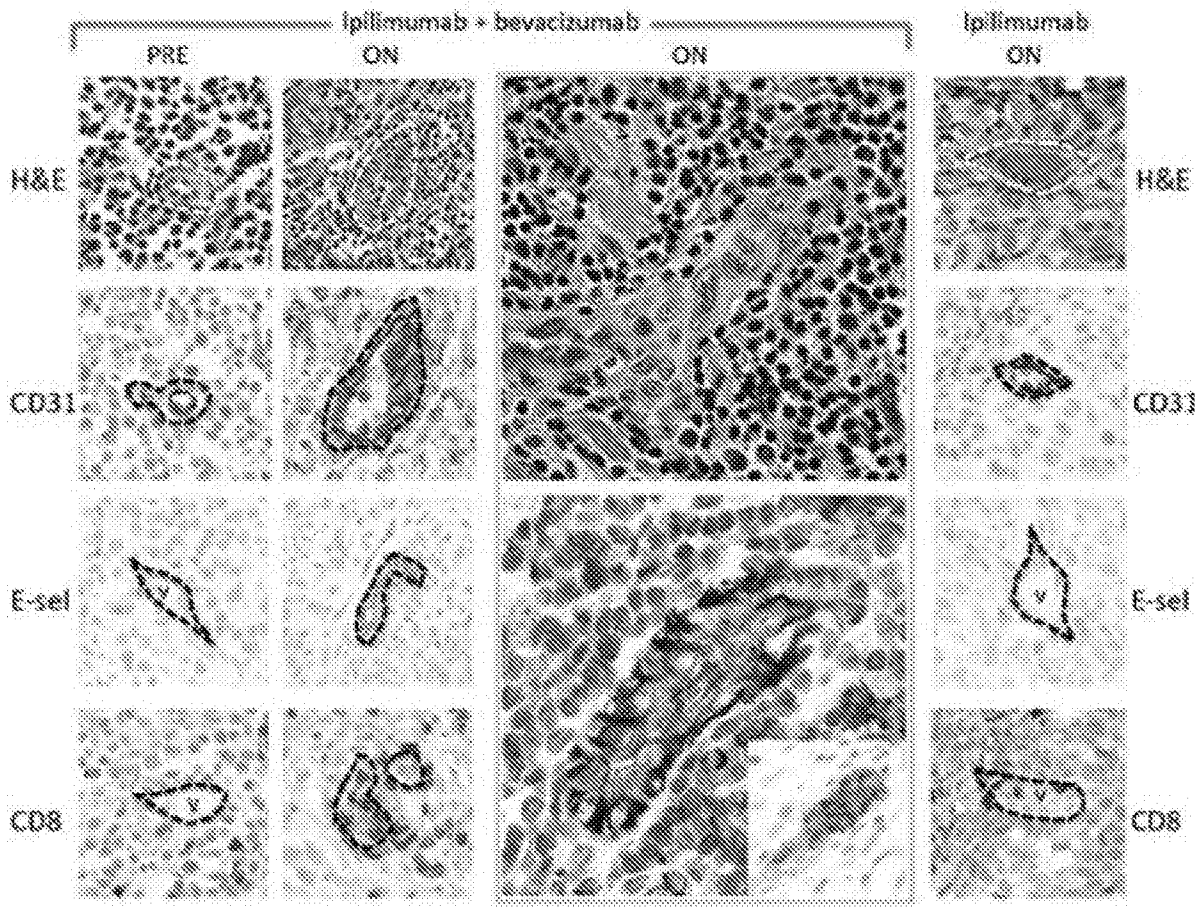

Ipilimumab + bevacizumab
Ipilimumab
PRE
ON
ON
ON
H&E
CD31
E-sel
CD8
H&E
CD31
E-sel
CD8

FIG. 10A

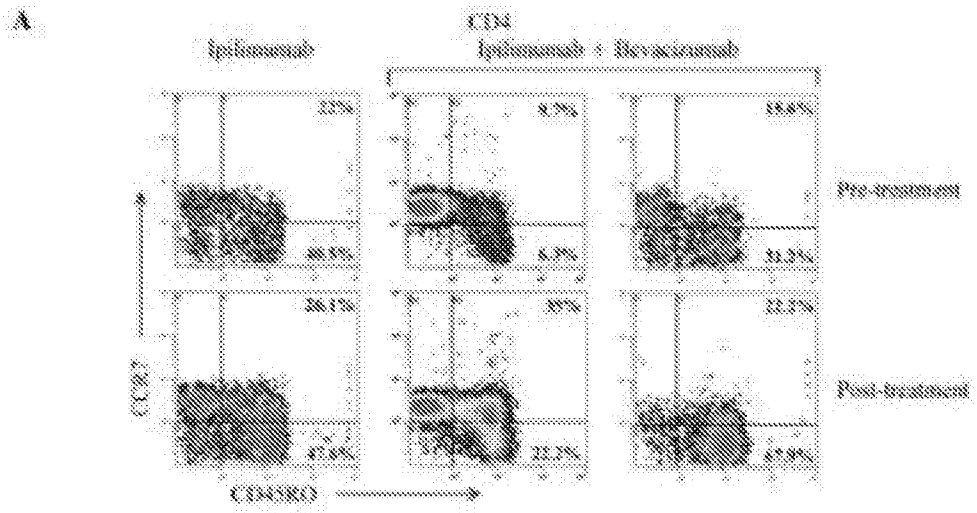

A
CD4
Ipilimumab
Ipilimumab + Bevacizumab
Pre-treatment
Post-treatment
CD45RO

FIG. 10B

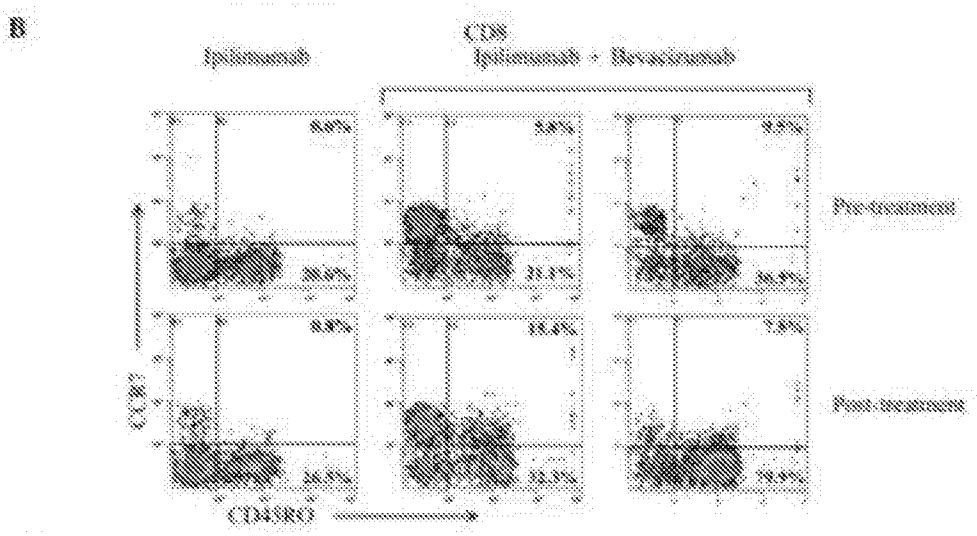


FIG. 10C

C

	Ipilimumab (3 mg/kg) (N=18)	Ipilimumab (3 mg/kg) + Bevacizumab (N=24)	Ipilimumab (10 mg/kg) + Bevacizumab (N=28)
CD4			
CCR7+ CD45RO+	5 (28%)	7 (29%) *	8 (29%) **
CCR7- CD45RO+	13 (72%)	17 (71%) *	20 (71%) *
CD8			
CCR7+ CD45RO+	1 (6%)	10 (42%) *	8 (29%) *
CCR7- CD45RO+	17 (94%)	14 (58%) *	20 (71%) *

FIG. 11

Patient	Anti-ang-1 antibodies	Anti-ang-2 antibodies
K008	5	5
L19	25	25
M34	16	16
OV65	4	4
K027	32	32
M22	3	8
L18	16	16
M28	16	16
M35	16	16
M30	3	3
M9	2	2

FIG. 12

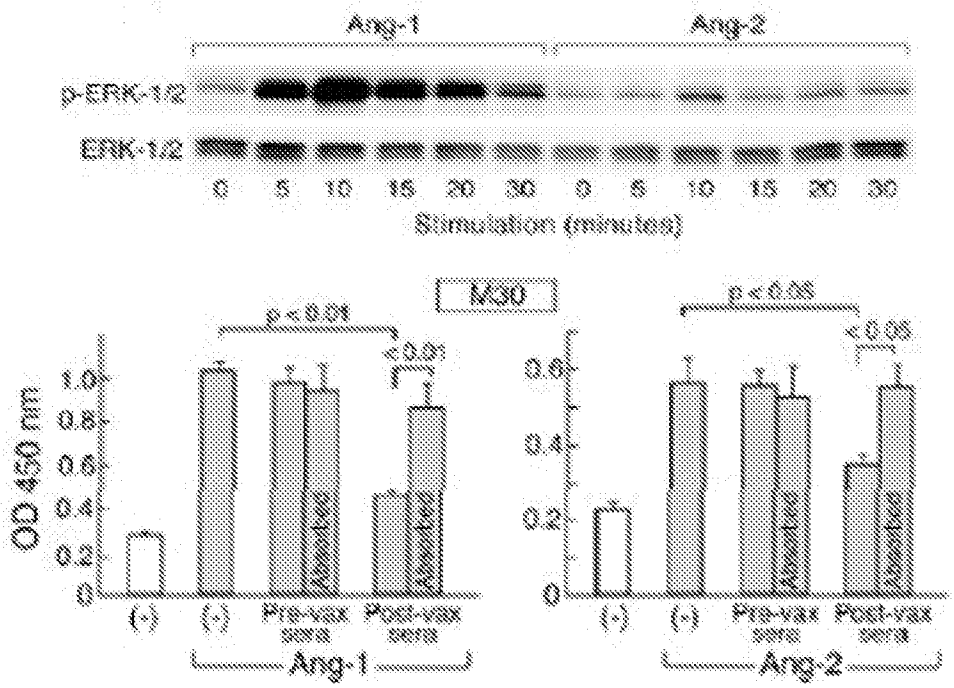

Ang-1
Ang-2
p-ERK-1/2
ERK-1/2
0 5 10 15 20 30 0 5 10 15 20 30
Stimulation (minutes)
M30
p < 0.01
< 0.01
p < 0.05
< 0.05
OD 450 nm
1.0
0.8
0.6
0.4
0.2
0
(-) (-) Pre-vax sera Post-vax sera
Ang-1
Ang-2

FIG. 13

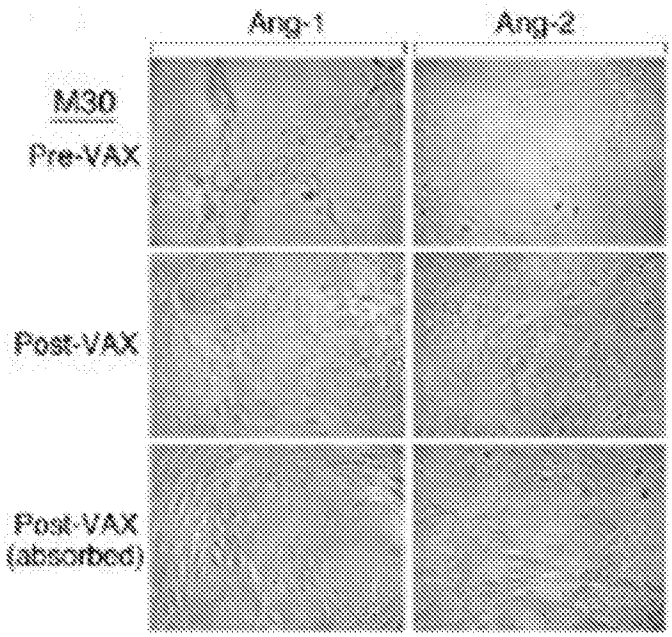

Ang-1
Ang-2
M30
Pre-VAX
Post-VAX
Post-VAX (absorbed)

FIG. 14

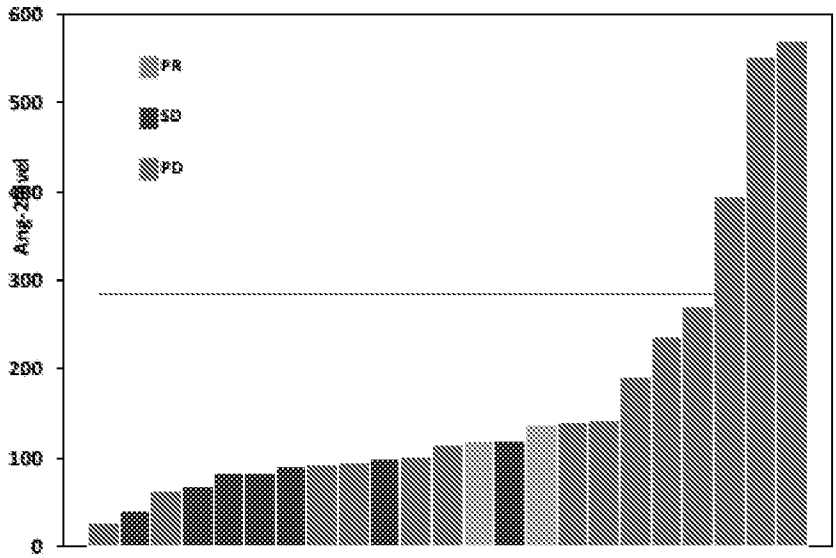


FIG. 15

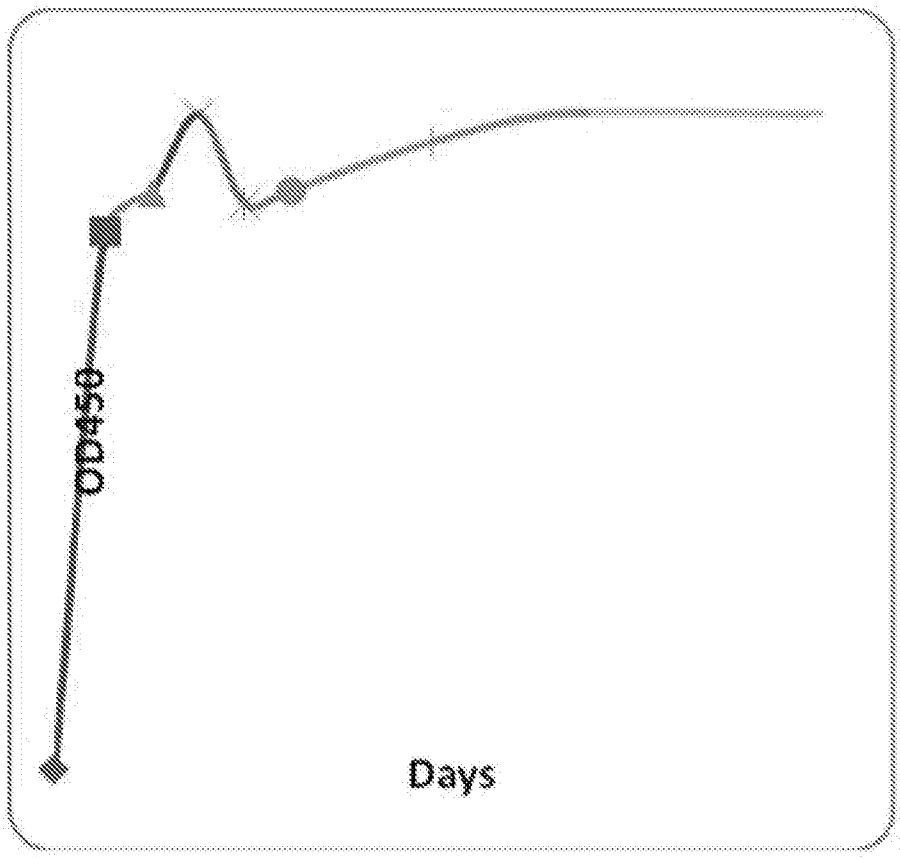


FIG. 16

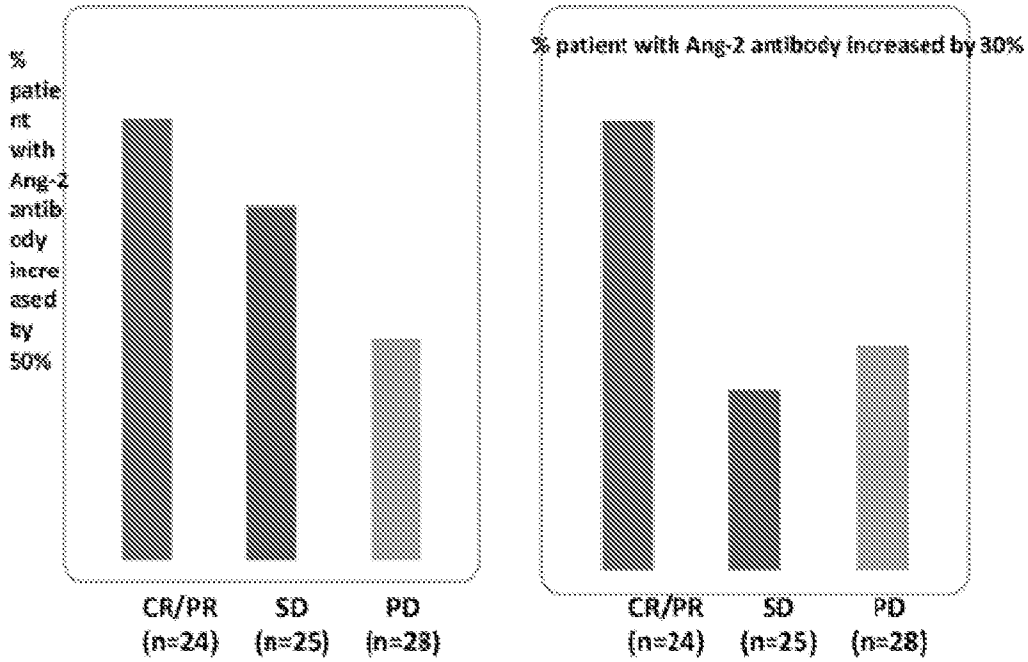

% patient with Ang-2 antibody increased by 50%
CR/PR (n=24)
SD (n=25)
PD (n=28)
% patient with Ang-2 antibody increased by 30%
CR/PR (n=24)
SD (n=25)
PD (n=28)

FIG. 17

a
b
c
d

FIG. 18

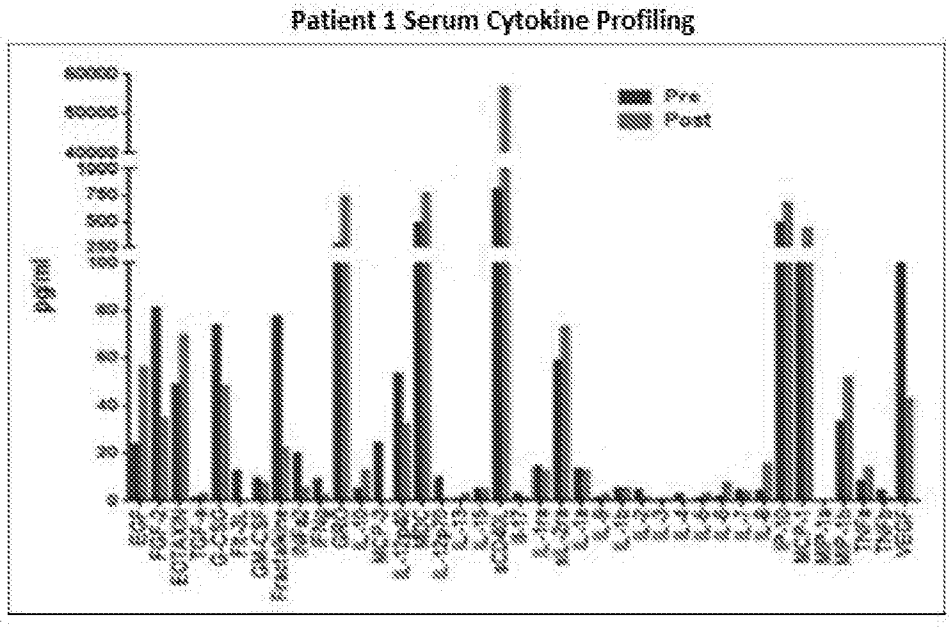

Patient 1 Serum Cytokine Profiling
Pre
Post
pg/ml

FIG. 19

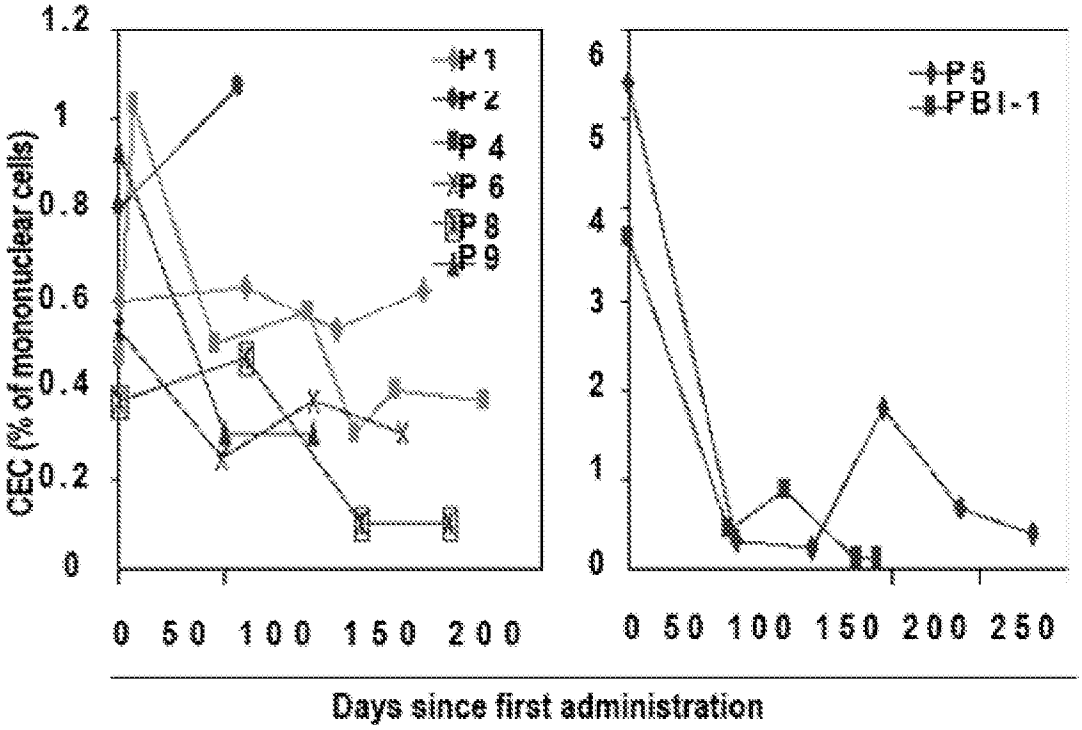

CEC (% of mononuclear cells)
1.2
1
0.8
0.6
0.4
0.2
0
P 1
P 2
P 4
P 6
P 8
P 9
0 50 100 150 200
6
5
4
3
2
1
0
P 5
PBI-1
0 50 100 150 200 250
Days since first administration

FIG. 20

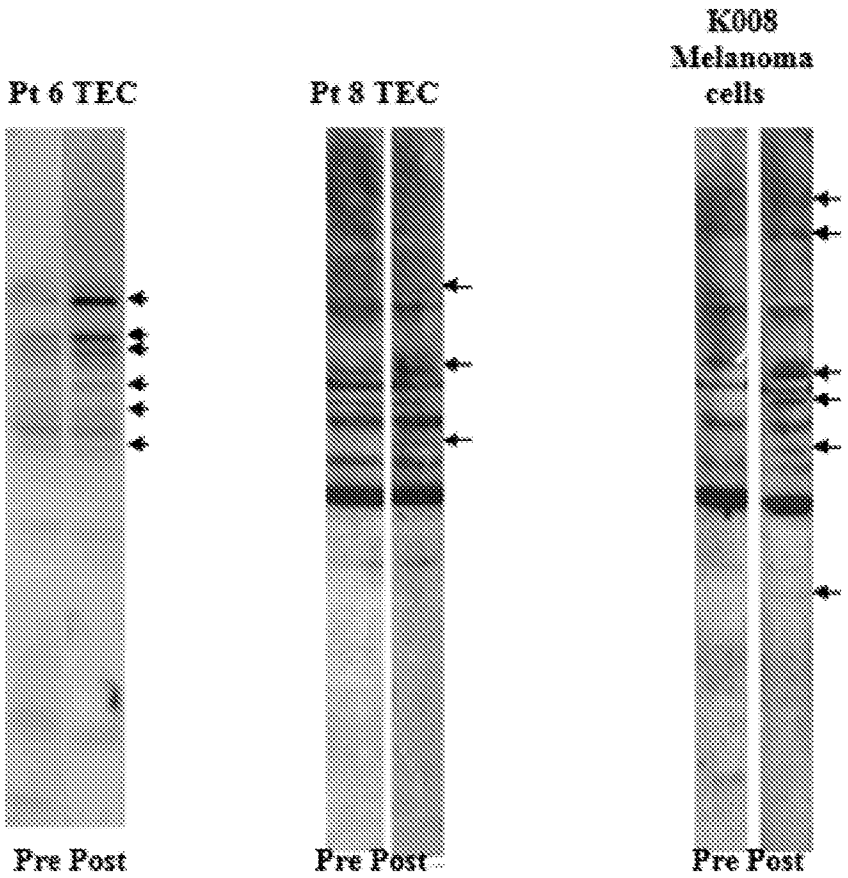

Pt 6 TEC
Pt 8 TEC
K008
Melanoma
cells
Pre Post
Pre Post
Pre Post

FIG. 21

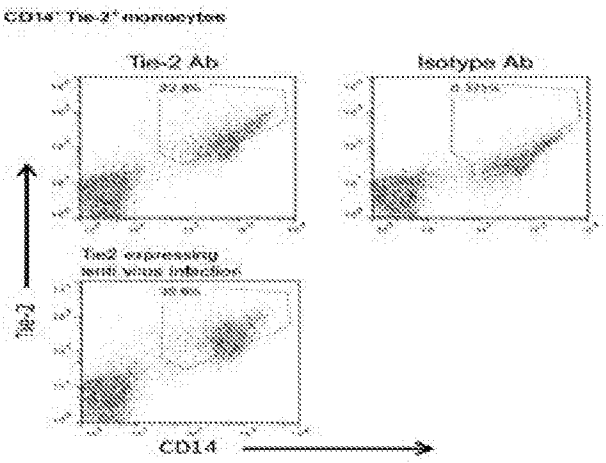

Tie-2 Ab
Isotype Ab
Tie2
CD14

FIG. 22

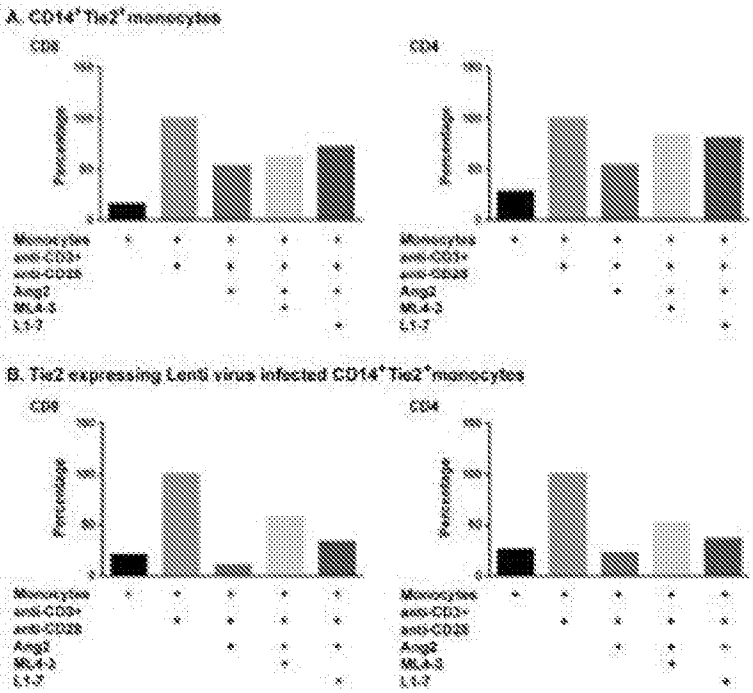


FIG. 23

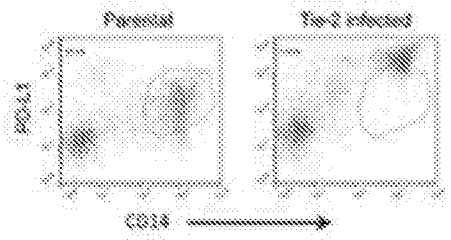


FIG. 24

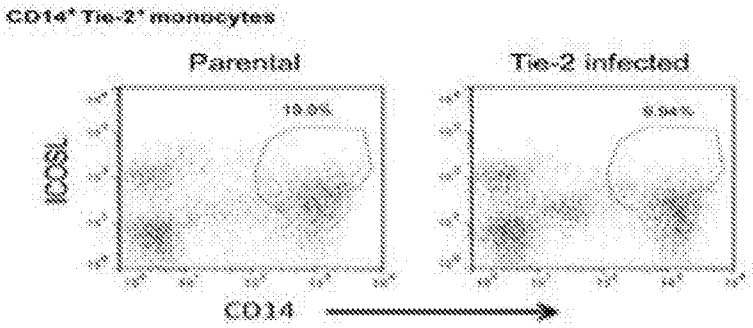


FIG. 25

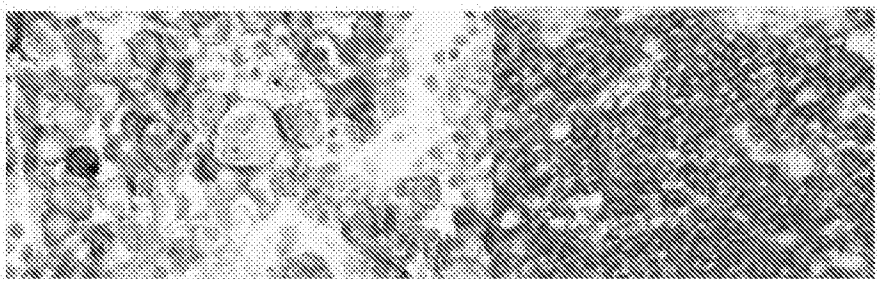


FIG. 26

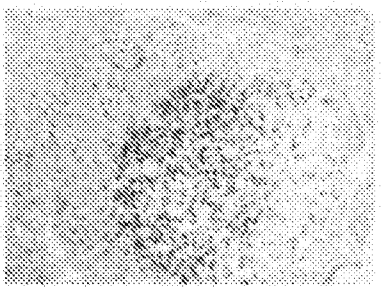


FIG. 27

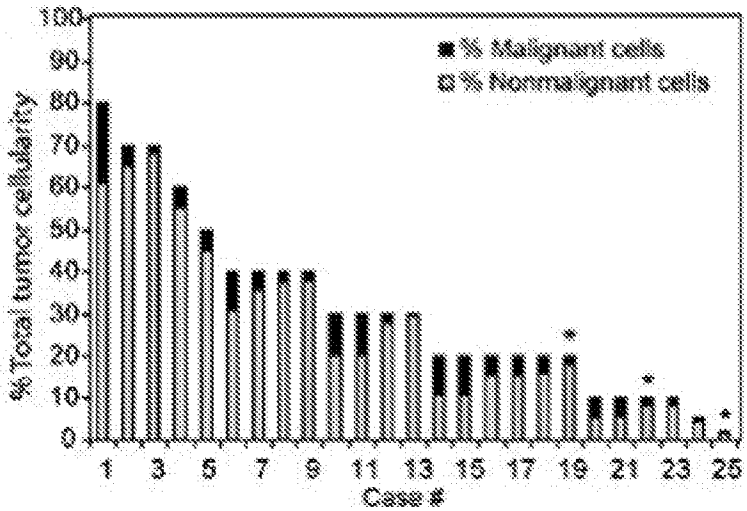


FIG. 28

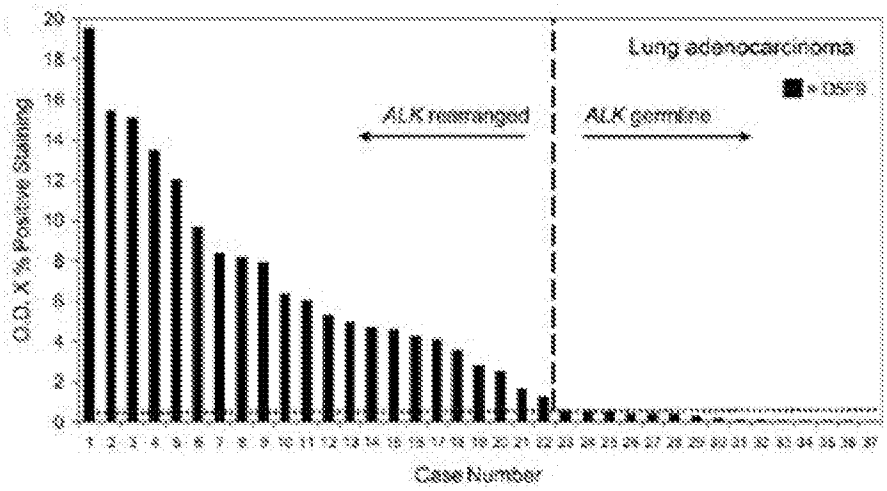

Lung adenocarcinoma
ALK rearranged
ALK germline
Case Number

FIG. 29

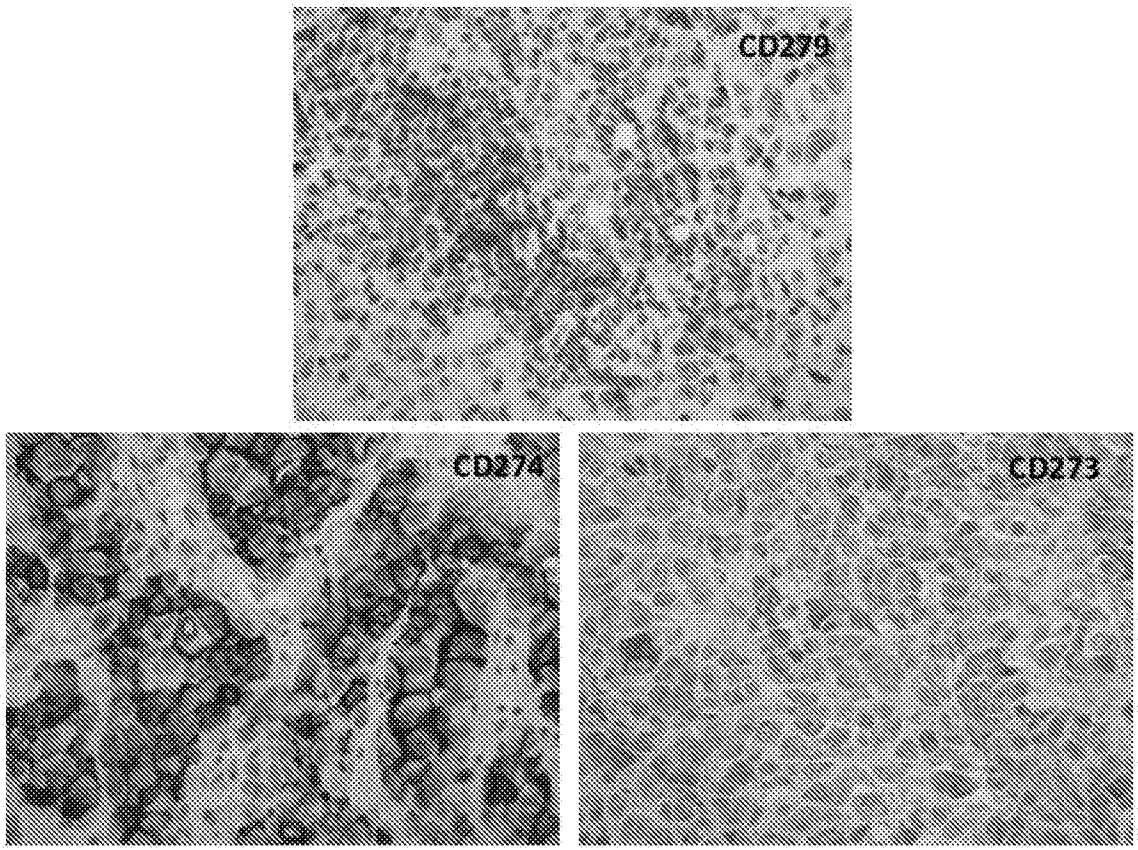

CD279
CD274
CD273

FIG. 30

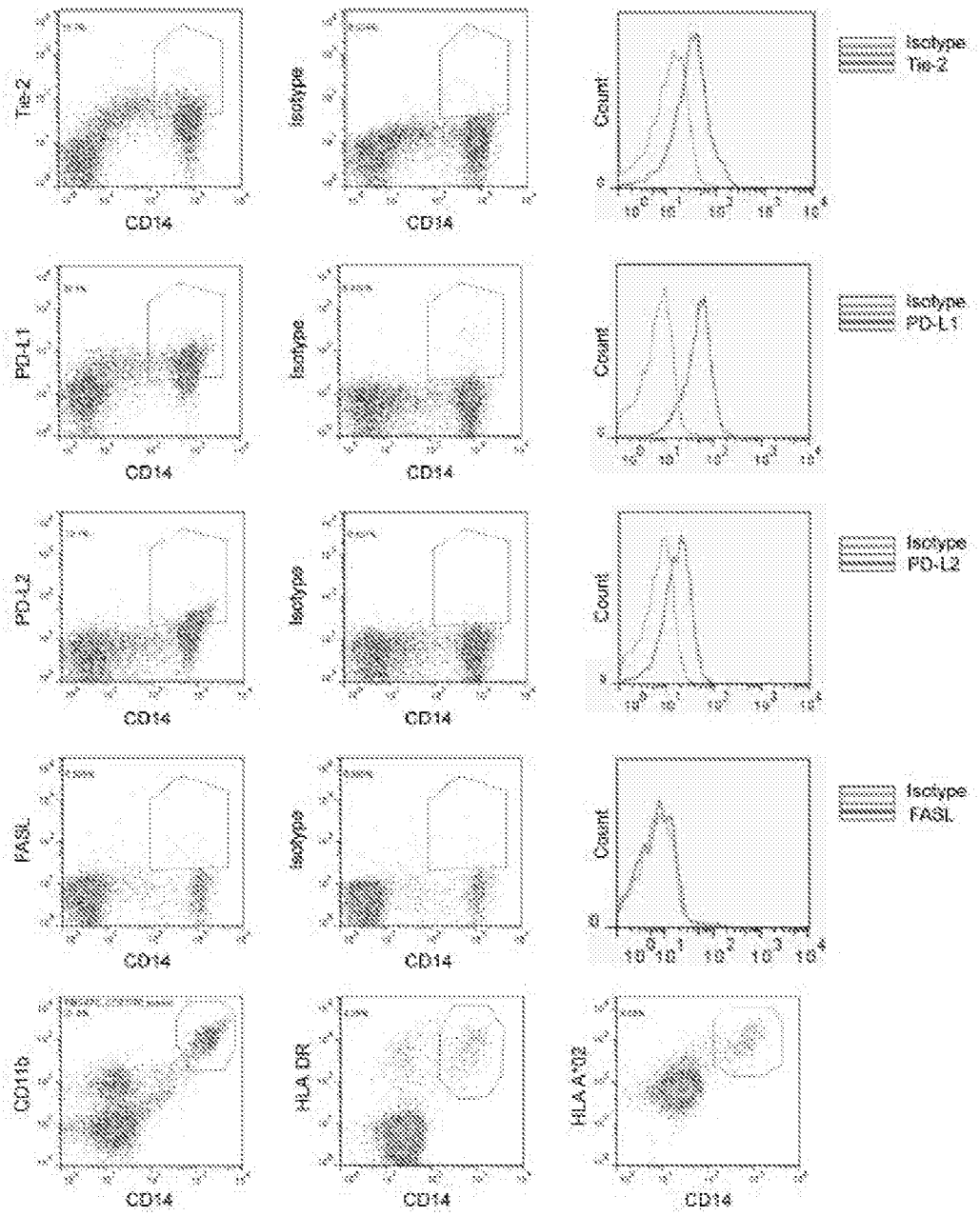

Tie-2
CD14
Isotype
Count
Isotype
Tie-2
PD-L1
Isotype
PD-L1
PD-L2
Isotype
PD-L2
FASL
Isotype
FASL
CD11b
HLA DR
HLA A*02

FIG. 31A

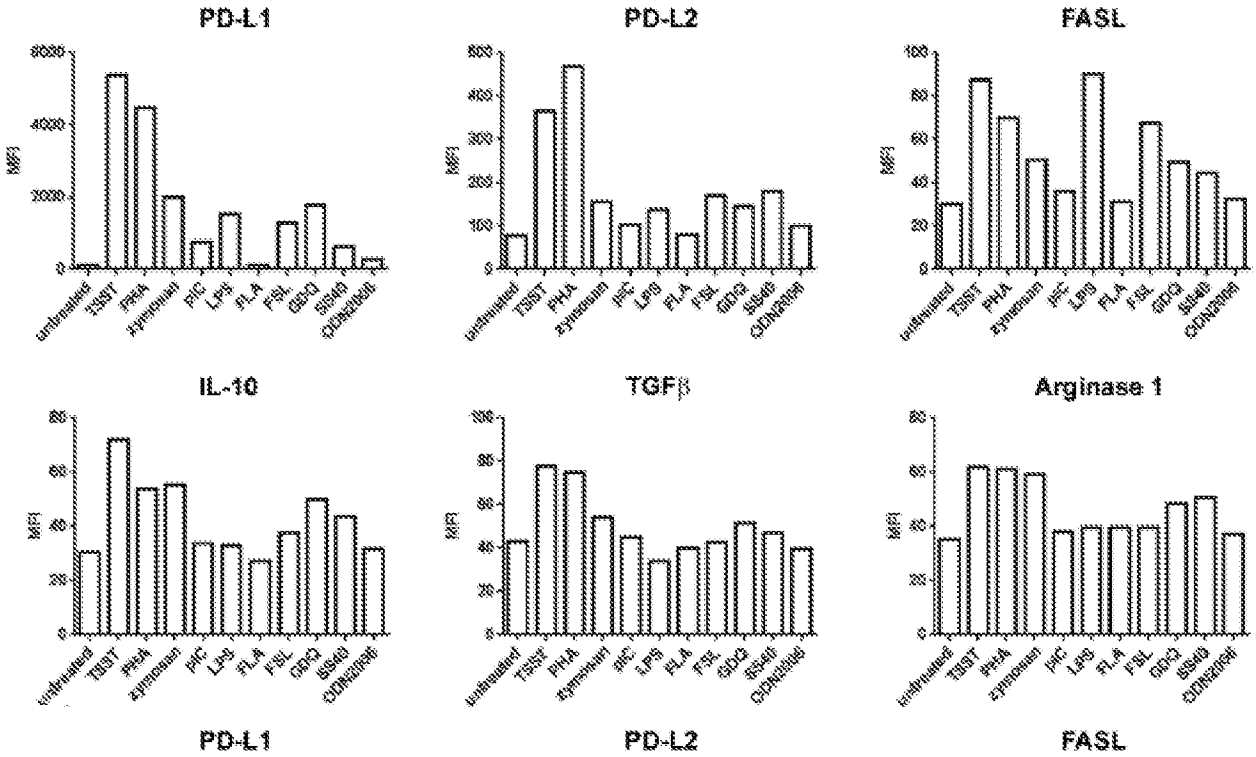


FIG. 31B

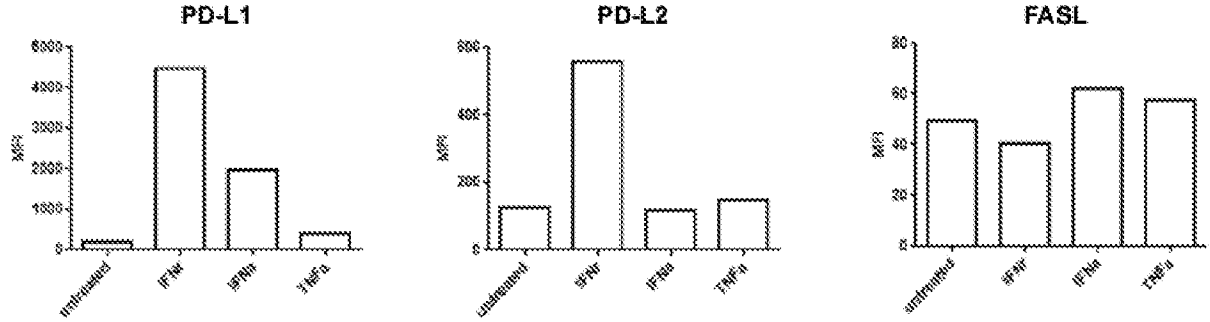


FIG. 31C

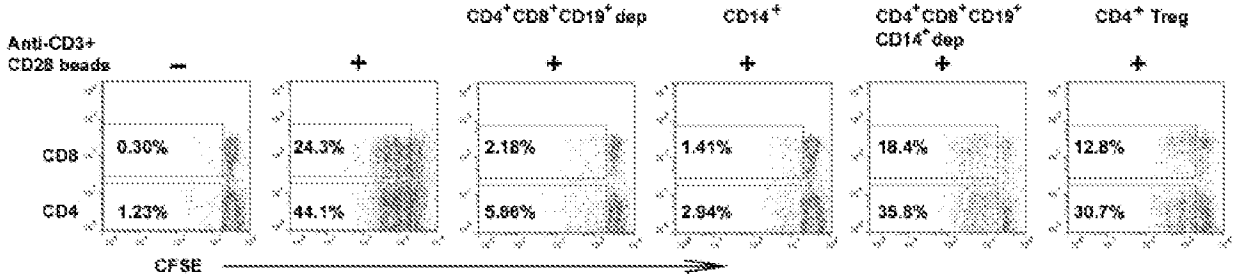


FIG. 32A

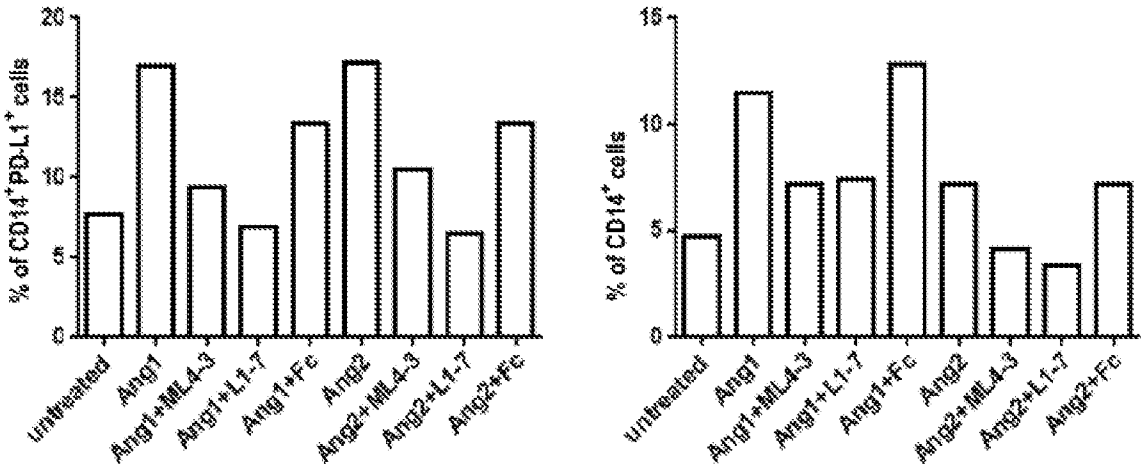


FIG. 32B

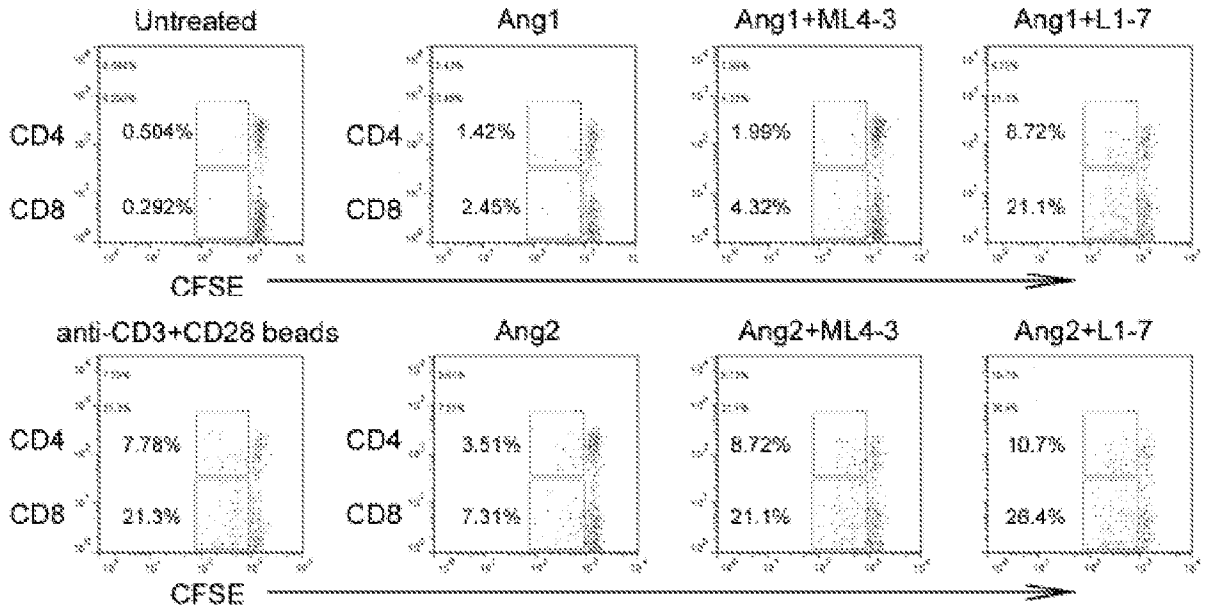


FIG. 32C

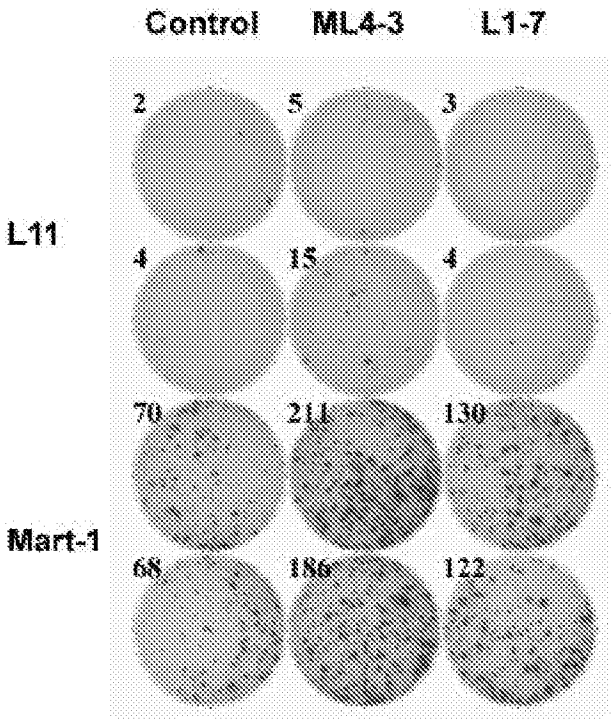


FIG. 33A

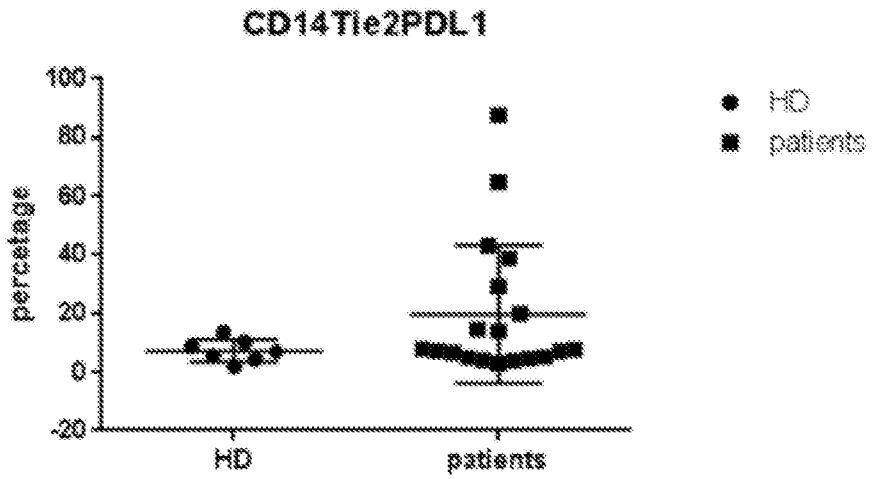


FIG. 33B

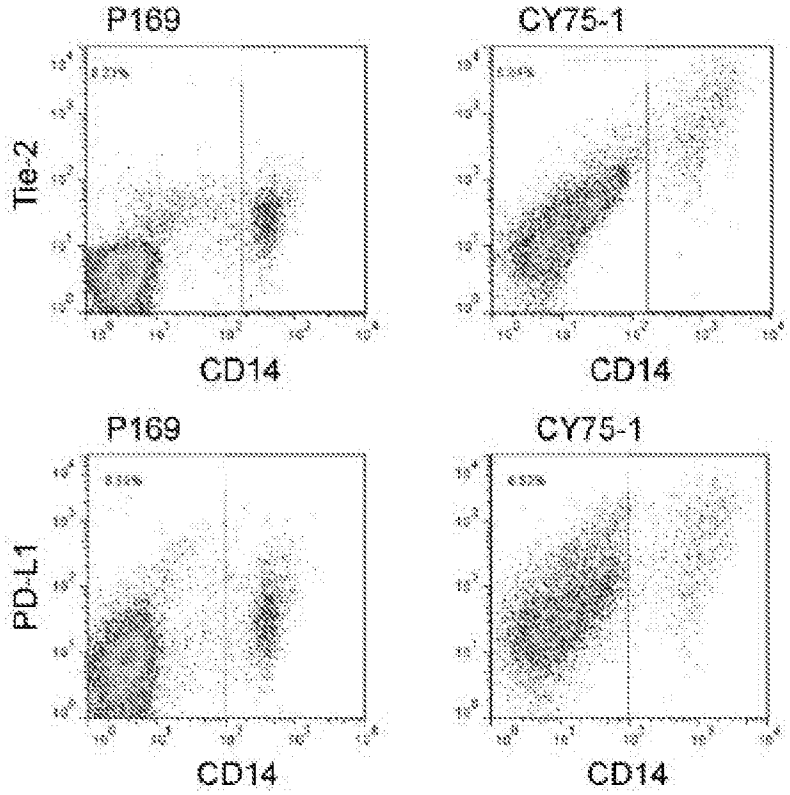


FIG. 34

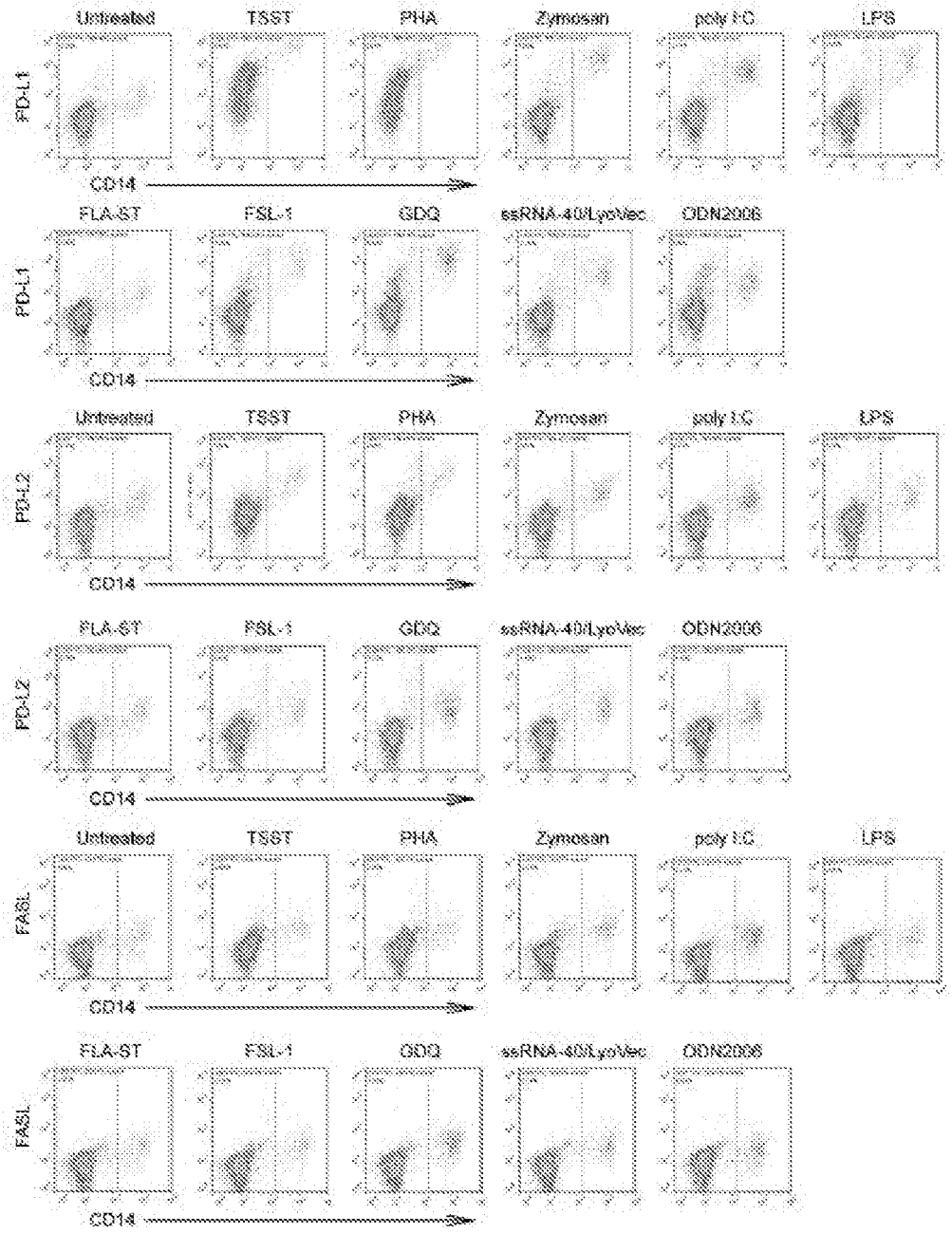

Untreated
TSST
PHA
Zymosan
poly I:C
LPS
PD-L1
CD14
FLA-ST
FSL-1
GDQ
ssRNA-40/LyoVec
ODN2006
PD-L2
FASL

FIG. 35

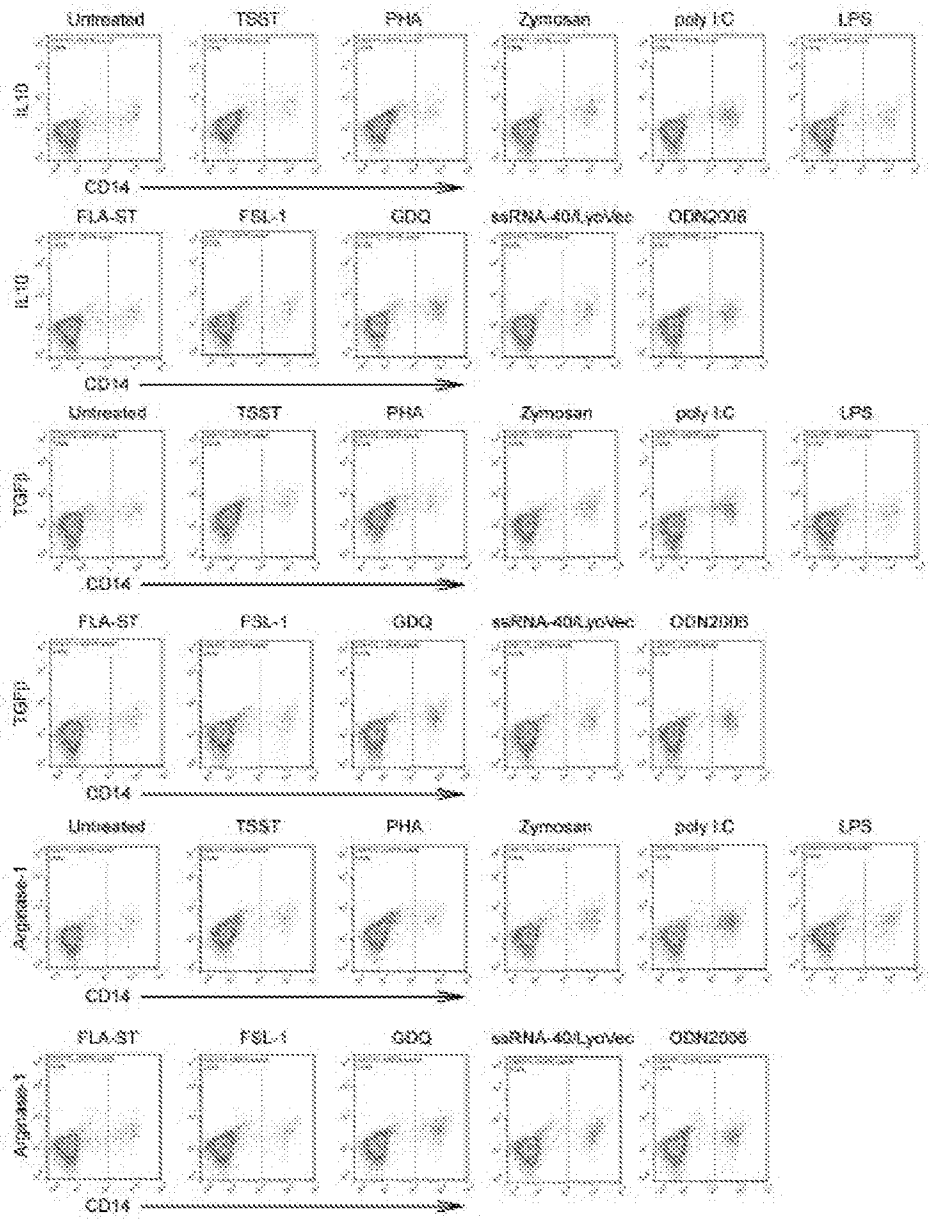

Untreated
TSST
PHA
Zymosan
poly I:C
LPS
IL-10
CD14
FLA-ST
FSL-1
GDQ
ssRNA-40/LyoVec
ODN2006
TGFβ
Arginase-1

FIG. 36

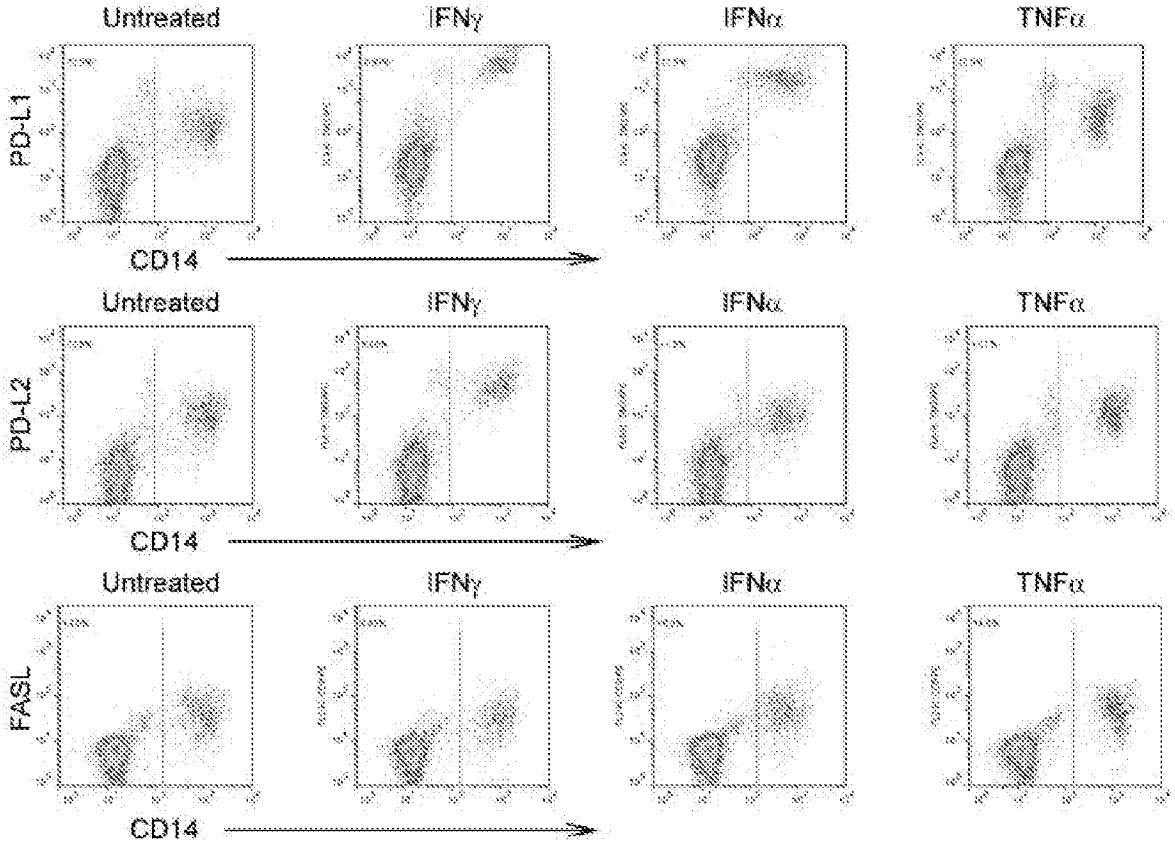

Untreated
IFNγ
IFNα
TNFα
PD-L1
CD14
Untreated
IFNγ
IFNα
TNFα
PD-L2
CD14
Untreated
IFNγ
IFNα
TNFα
FASL
CD14

FIG. 37A

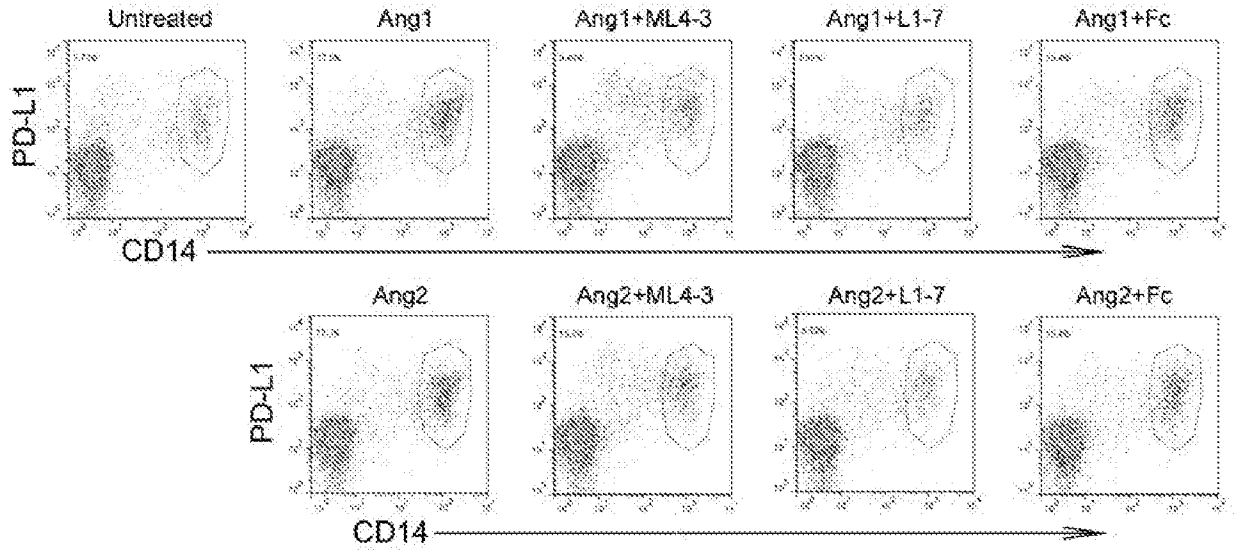


FIG. 37B

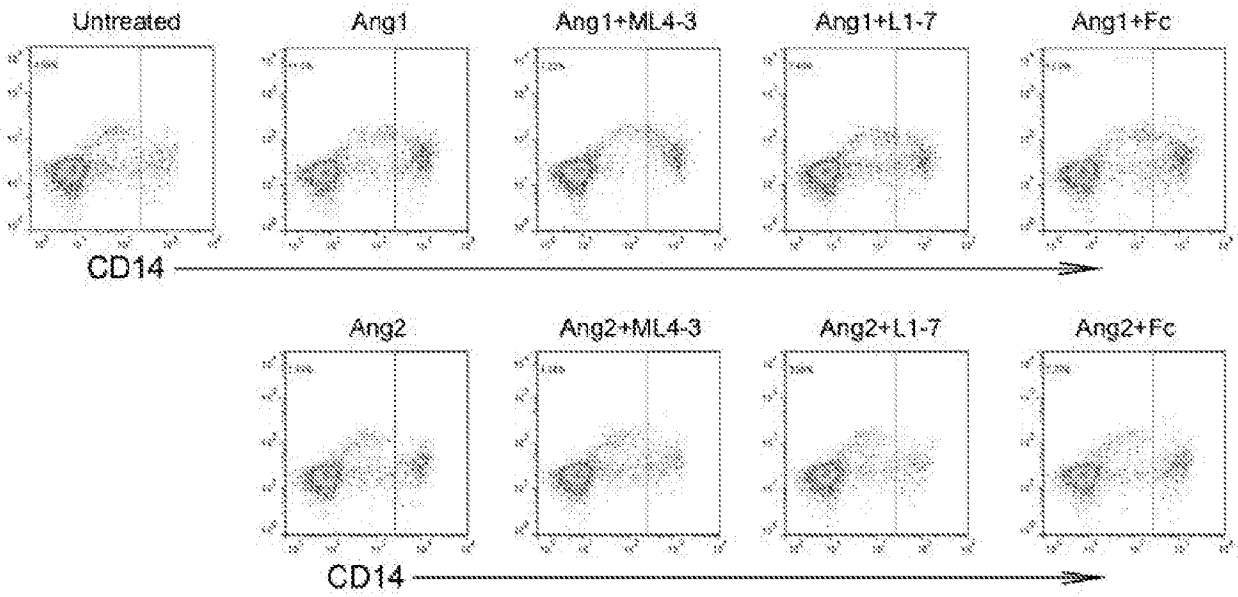
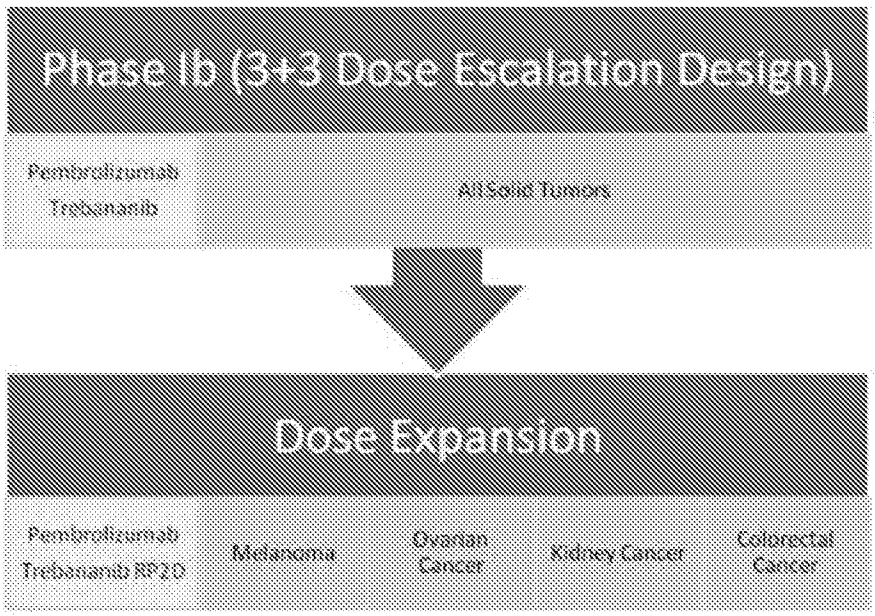


FIG. 38

STUDY SCHEMA



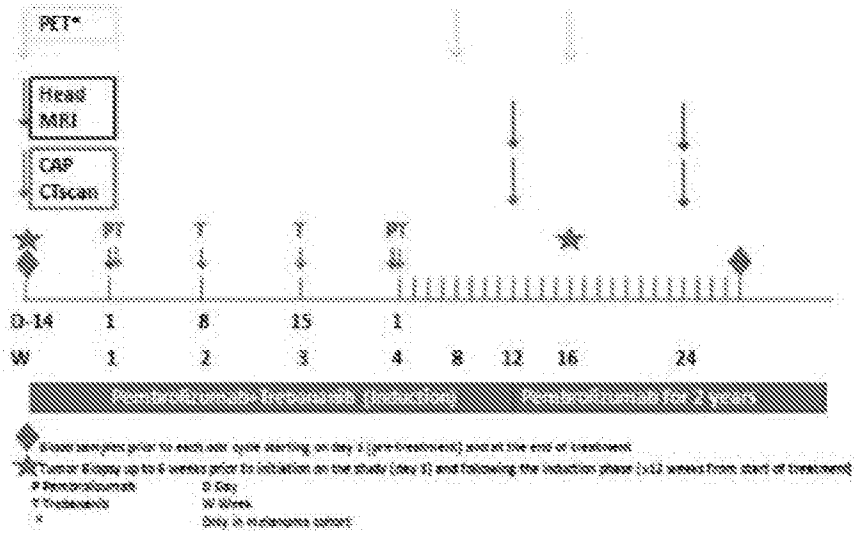


FIG. 39

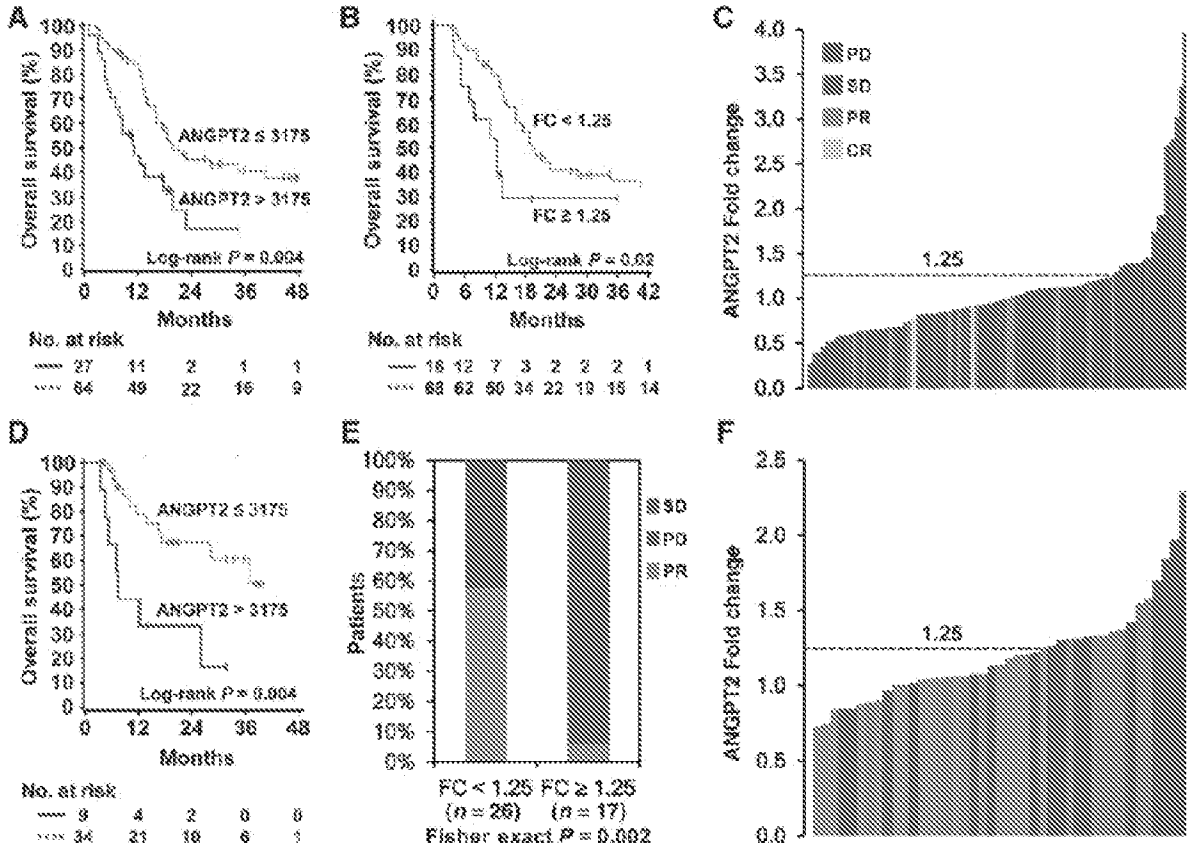

A
Overall survival (%)
ANGPT2 ≤ 3175
ANGPT2 > 3175
Log-rank P = 0.004
Months
No. at risk
B
Overall survival (%)
FC < 1.25
FC ≥ 1.25
Log-rank P = 0.03
Months
No. at risk
C
ANGPT2 Fold change
PD
SD
PR
CR
1.25
D
Overall survival (%)
ANGPT2 ≤ 3175
ANGPT2 > 3175
Log-rank P = 0.004
Months
No. at risk
E
Patients
SD
PD
PR
FC < 1.25 FC ≥ 1.25
(n = 26) (n = 17)
Fisher exact P = 0.002
F
ANGPT2 Fold change
1.25

FIG. 40

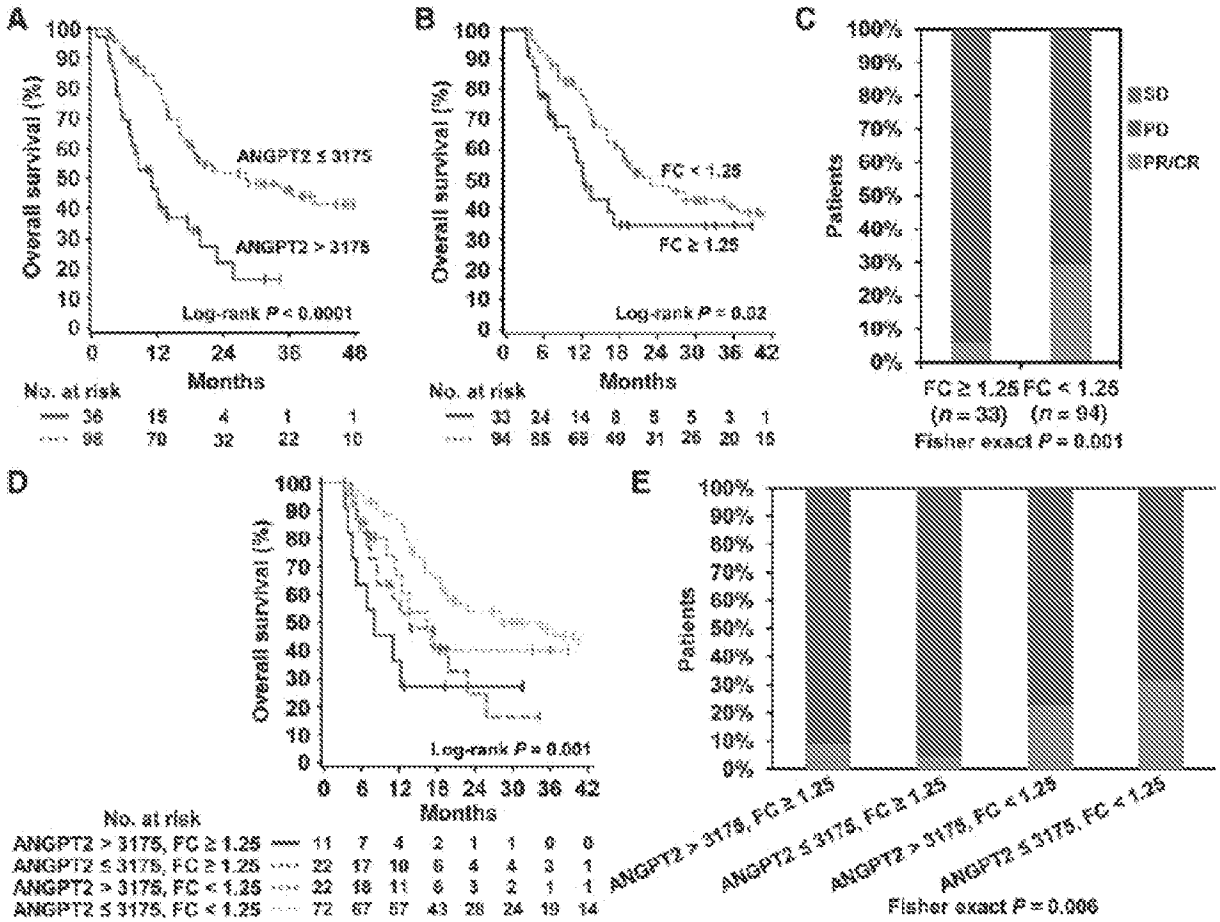

A
Overall survival (%)
ANGPT2 ≤ 3175
ANGPT2 > 3175
Log-rank P < 0.0001
Months
No. at risk
B
Overall survival (%)
FC < 1.25
FC ≥ 1.25
Log-rank P = 0.02
Months
No. at risk
C
Patients
SD
PD
PR/CR
FC ≥ 1.25 (n = 33)
FC < 1.25 (n = 94)
Fisher exact P = 0.001
D
Overall survival (%)
Log-rank P = 0.001
Months
No. at risk
ANGPT2 > 3175, FC ≥ 1.25
ANGPT2 ≤ 3175, FC ≥ 1.25
ANGPT2 > 3175, FC < 1.25
ANGPT2 ≤ 3175, FC < 1.25
E
Patients
ANGPT2 > 3175, FC ≥ 1.25
ANGPT2 ≤ 3175, FC ≥ 1.25
ANGPT2 > 3175, FC < 1.25
ANGPT2 ≤ 3175, FC < 1.25
Fisher exact P = 0.006

FIG. 41

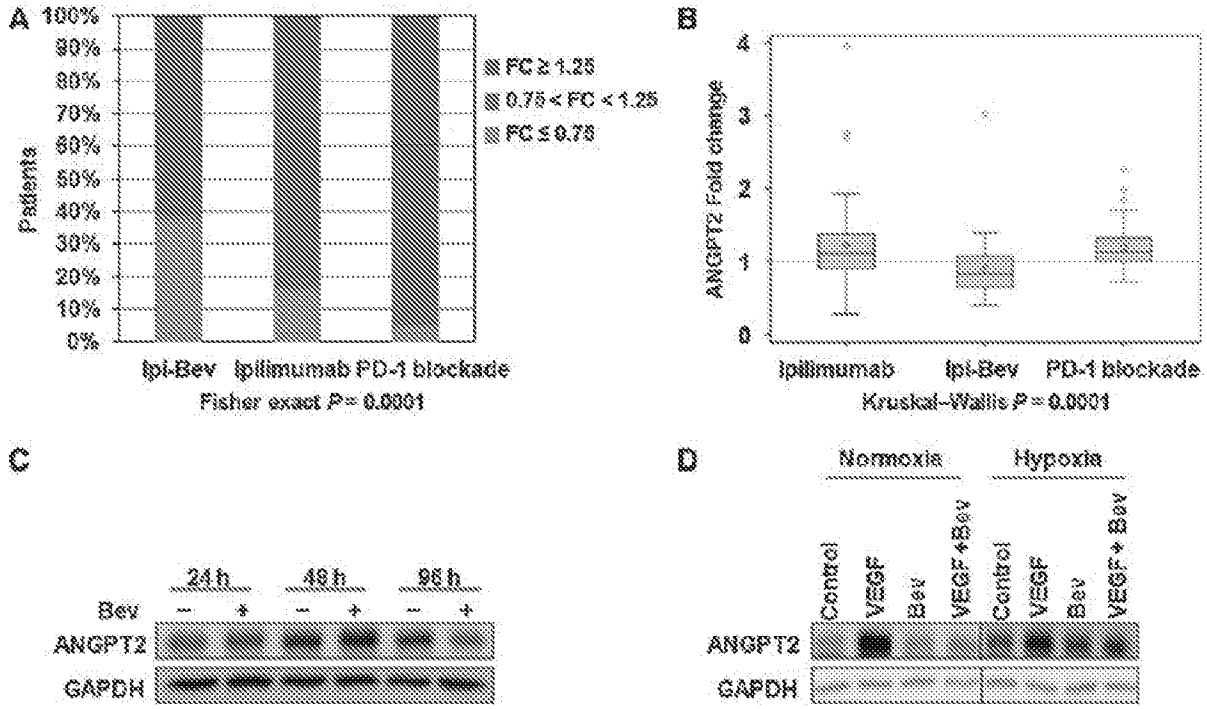

A
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Patients
FC ≥ 1.25
0.75 < FC < 1.25
FC ≤ 0.75
Ipi-Bev
Ipilimumab
PD-1 blockade
Fisher exact P = 0.0001
B
ANGPT2 Fold change
4
3
2
1
0
Ipilimumab
Ipi-Bev
PD-1 blockade
Kruskal–Wallis P = 0.0001
C
24 h
48 h
96 h
Bev
ANGPT2
GAPDH
D
Normoxia
Hypoxia
Control
VEGF
Bev
VEGF + Bev
ANGPT2
GAPDH

FIG. 42

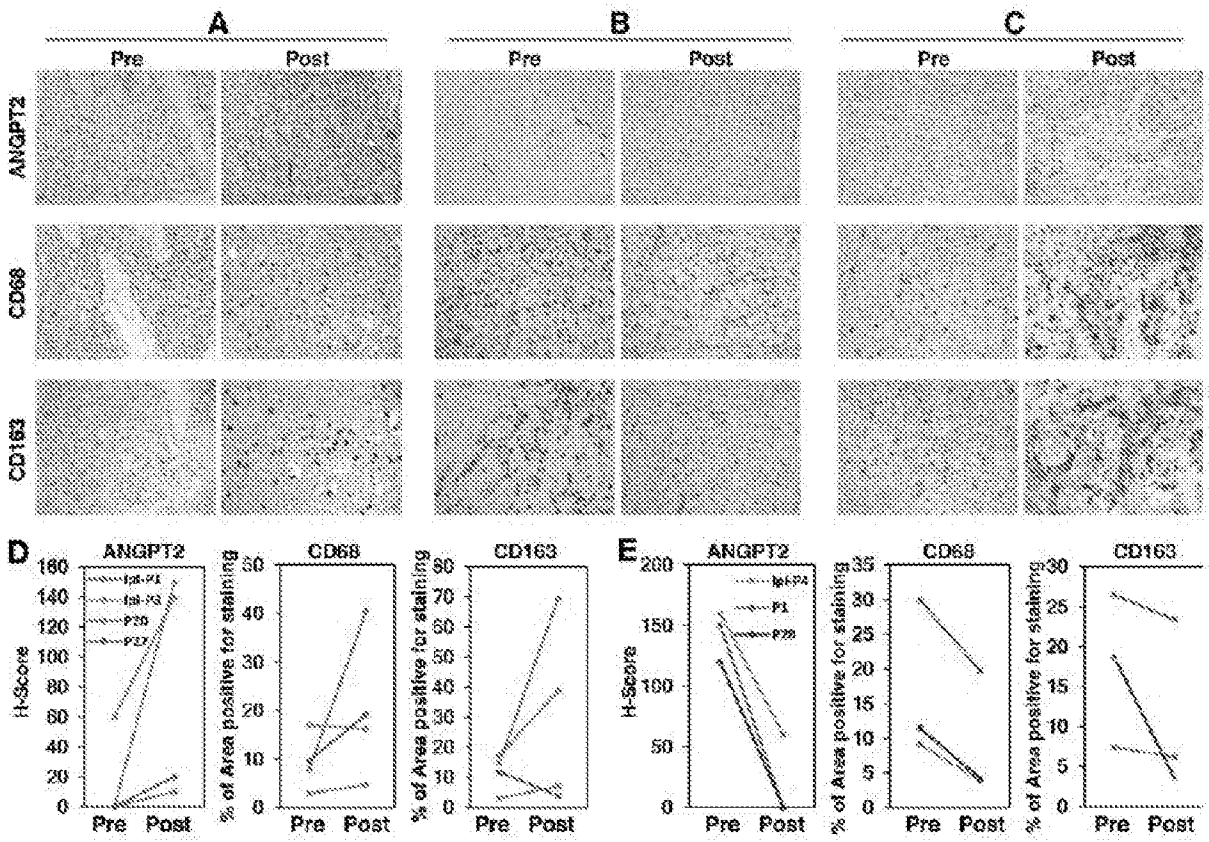

A
B
C
Pre
Post
ANGPT2
CD68
CD163
D
E
H-Score
% of Area positive for staining

FIG. 43

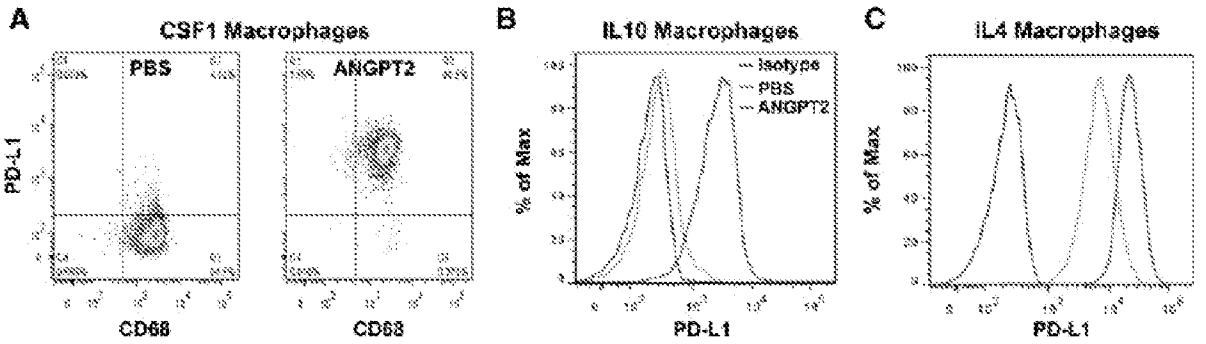

A
CSF1 Macrophages
PBS
ANGPT2
PD-L1
CD68
B
IL10 Macrophages
Isotype
PBS
ANGPT2
% of Max
PD-L1
C
IL4 Macrophages

FIG. 44

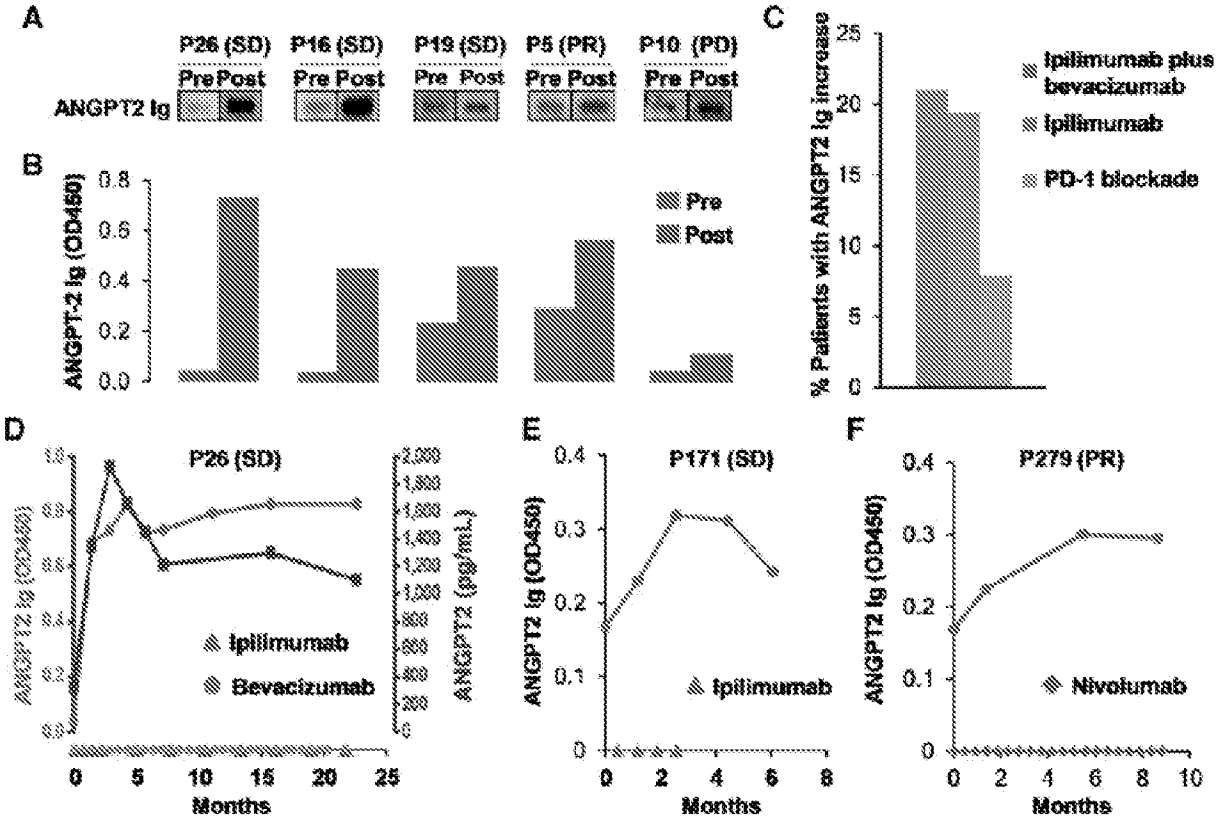

A
P26 (SD)
P16 (SD)
P19 (SD)
P5 (PR)
P10 (PD)
Pre Post
ANGPT2 Ig
B
ANGPT-2 Ig (OD450)
Pre
Post
C
% Patients with ANGPT2 Ig increase
Ipilimumab plus bevacizumab
Ipilimumab
PD-1 blockade
D
P26 (SD)
Ipilimumab
Bevacizumab
ANGPT2 (pg/mL)
Months
E
P171 (SD)
ANGPT2 Ig (OD450)
Ipilimumab
Months
F
P279 (PR)
ANGPT2 Ig (OD450)
Nivolumab
Months

FIG. 45

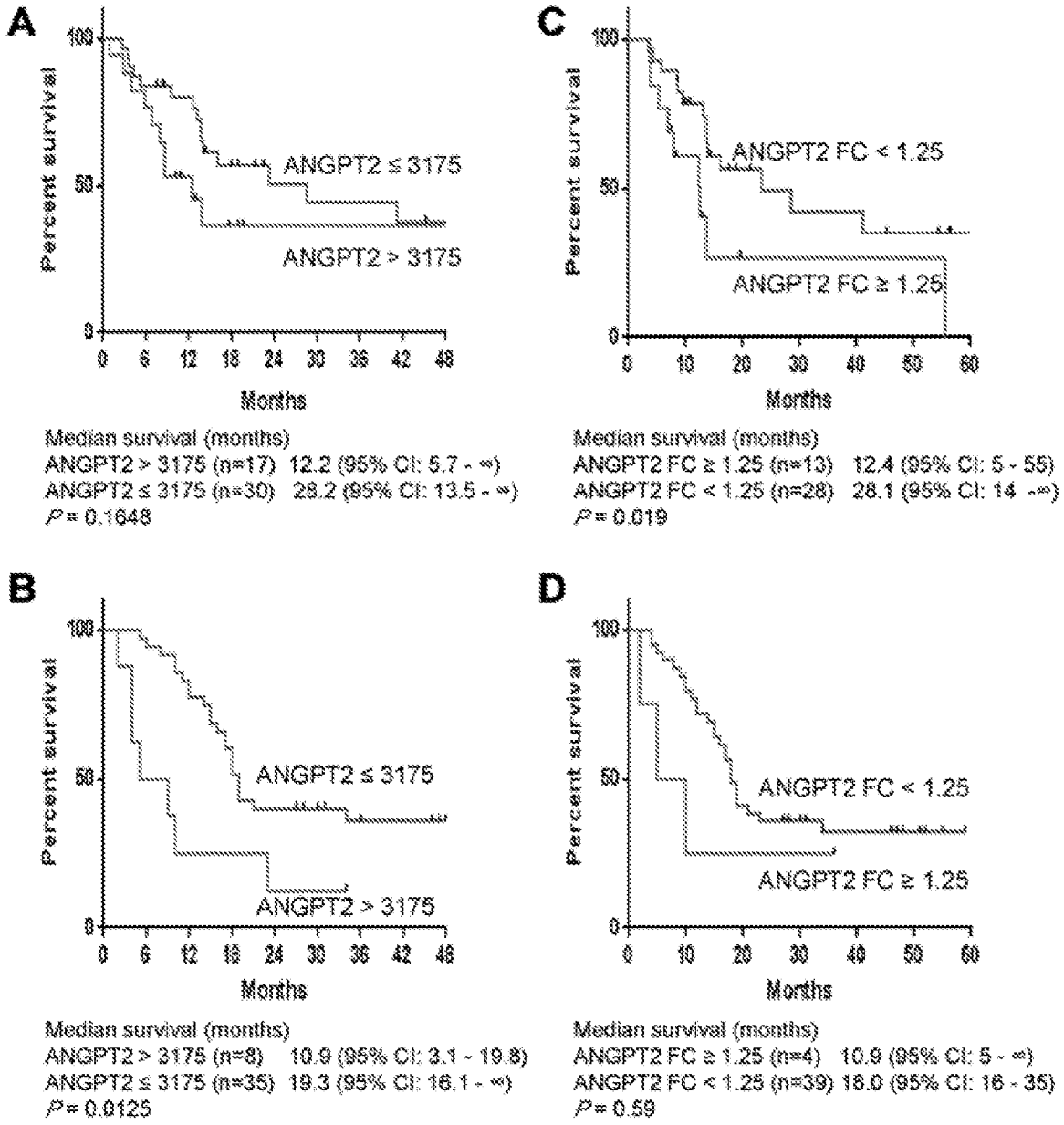

A
Percent survival
100
50
0
ANGPT2 ≤ 3175
ANGPT2 > 3175
0 6 12 18 24 30 36 42 48
Months
Median survival (months)
ANGPT2 > 3175 (n=17) 12.2 (95% CI: 5.7 - ∞)
ANGPT2 ≤ 3175 (n=30) 28.2 (95% CI: 13.5 - ∞)
P = 0.1648
C
Percent survival
100
50
0
ANGPT2 FC < 1.25
ANGPT2 FC ≥ 1.25
0 10 20 30 40 50 60
Months
Median survival (months)
ANGPT2 FC ≥ 1.25 (n=13) 12.4 (95% CI: 5 - 55)
ANGPT2 FC < 1.25 (n=28) 28.1 (95% CI: 14 - ∞)
P = 0.019
B
Percent survival
100
50
0
ANGPT2 ≤ 3175
ANGPT2 > 3175
0 6 12 18 24 30 36 42 48
Months
Median survival (months)
ANGPT2 > 3175 (n=8) 10.9 (95% CI: 3.1 - 19.8)
ANGPT2 ≤ 3175 (n=35) 19.3 (95% CI: 16.1 - ∞)
P = 0.0125
D
Percent survival
100
50
0
ANGPT2 FC < 1.25
ANGPT2 FC ≥ 1.25
0 10 20 30 40 50 60
Months
Median survival (months)
ANGPT2 FC ≥ 1.25 (n=4) 10.9 (95% CI: 5 - ∞)
ANGPT2 FC < 1.25 (n=39) 16.0 (95% CI: 16 - 35)
P = 0.59

FIG. 46

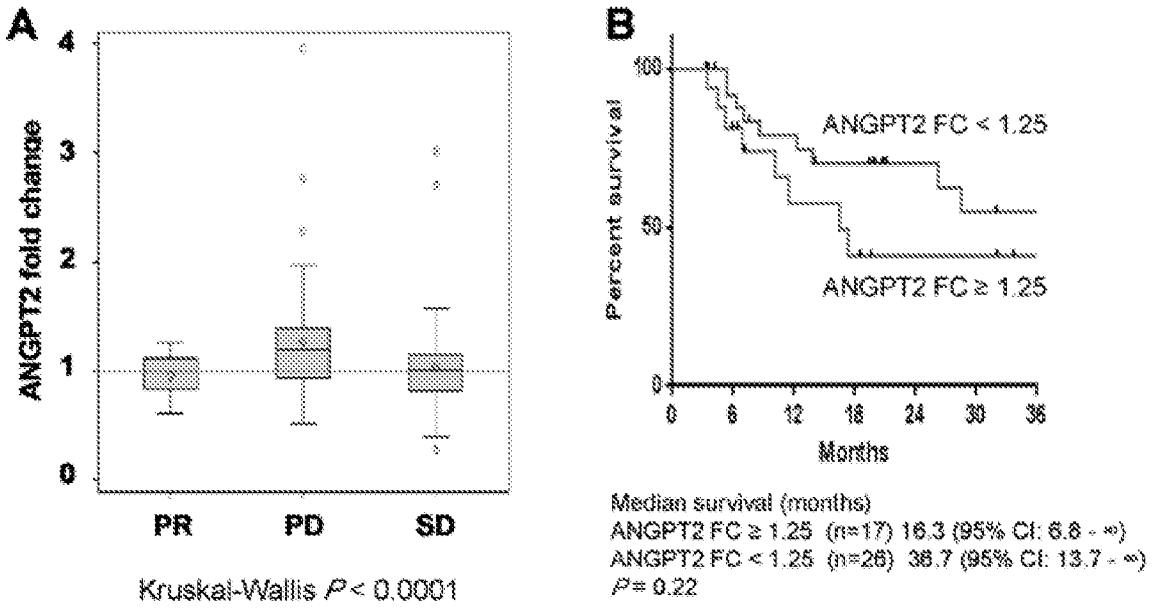

A
ANGPT2 fold change
4
3
2
1
0
PR
PD
SD
Kruskal-Wallis P < 0.0001
B
Percent survival
100
50
0
ANGPT2 FC < 1.25
ANGPT2 FC ≥ 1.25
0
6
12
18
24
30
36
Months
Median survival (months)
ANGPT2 FC ≥ 1.25 (n=17) 16.3 (95% CI: 6.8 - ∞)
ANGPT2 FC < 1.25 (n=26) 38.7 (95% CI: 13.7 - ∞)
P = 0.22

FIG. 47

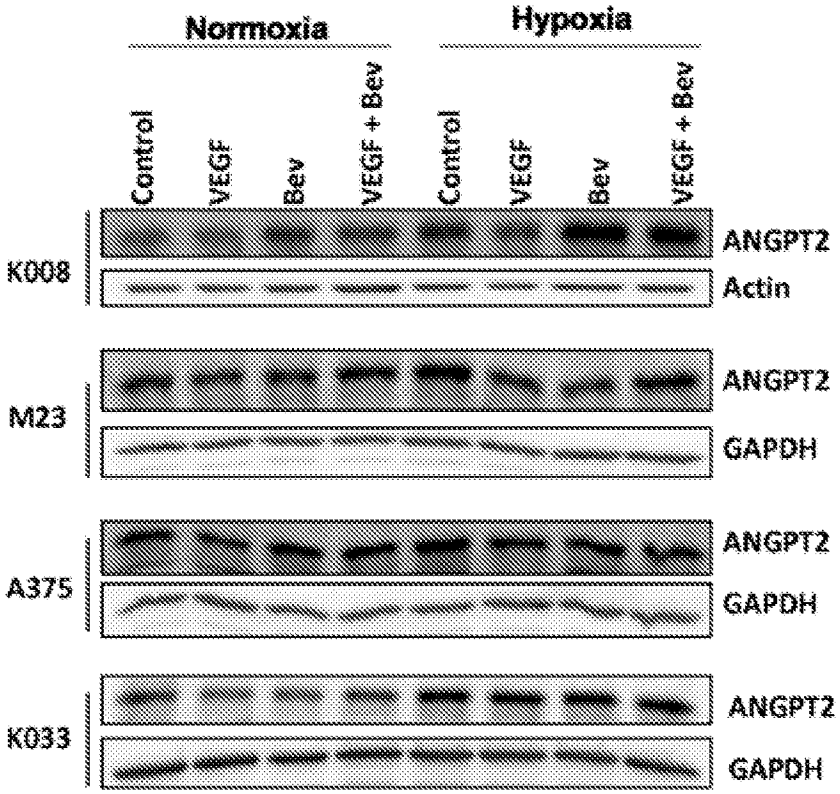

Normoxia
Hypoxia
Control
VEGF
Bev
VEGF + Bev
Control
VEGF
Bev
VEGF + Bev
K008
ANGPT2
Actin
M23
ANGPT2
GAPDH
A375
ANGPT2
GAPDH
K033
ANGPT2
GAPDH

FIG. 48

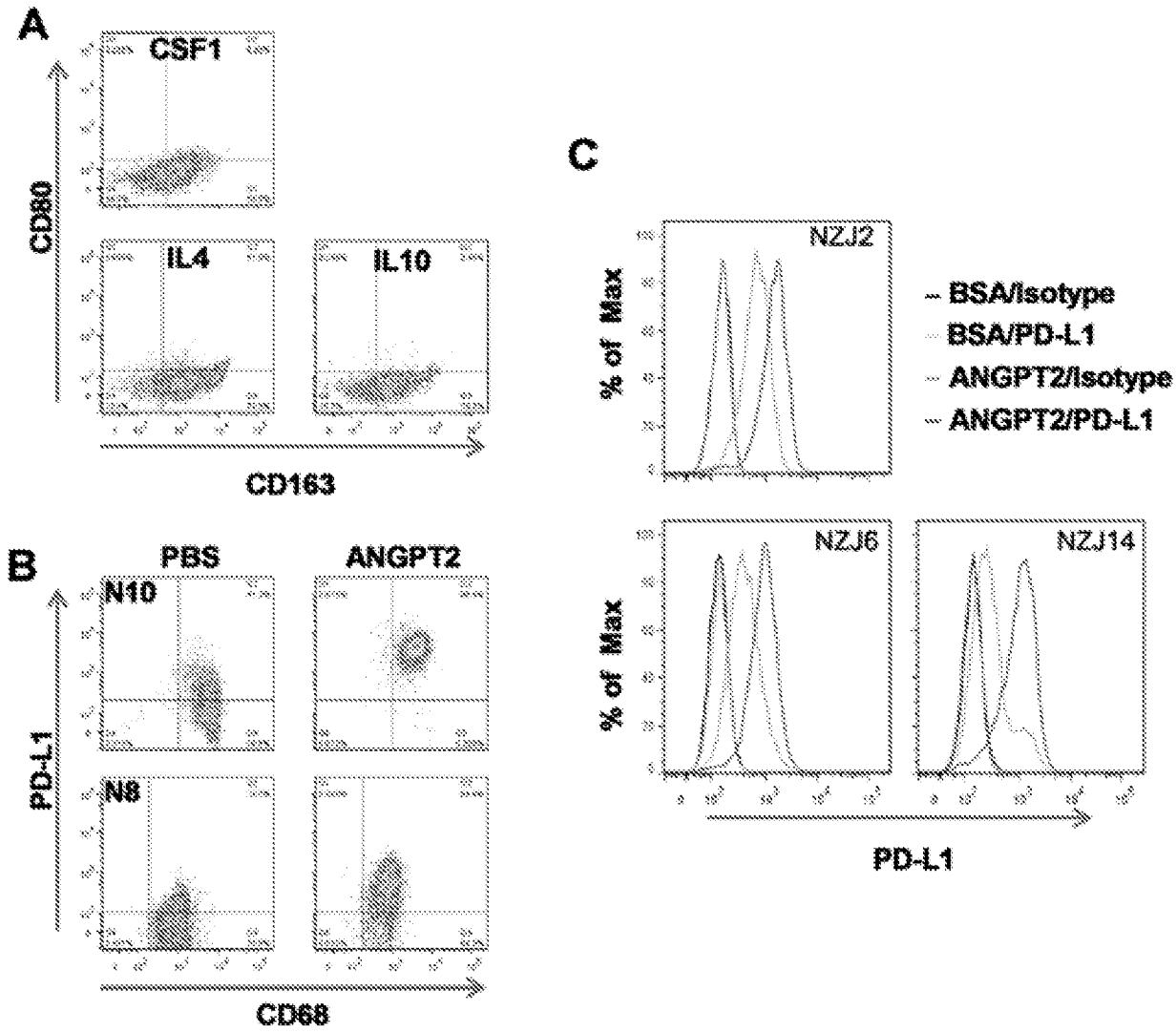

A
CSF1
CD80
IL4
IL10
CD163
B
PBS
ANGPT2
N10
N8
PD-L1
CD68
C
NZJ2
% of Max
BSA/Isotype
BSA/PD-L1
ANGPT2/Isotype
ANGPT2/PD-L1
NZJ6
NZJ14
% of Max
PD-L1

FIG. 49

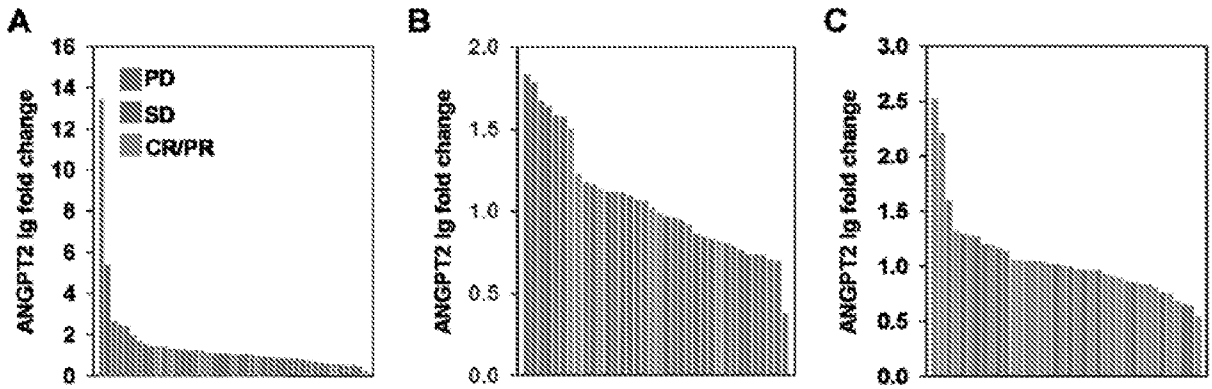
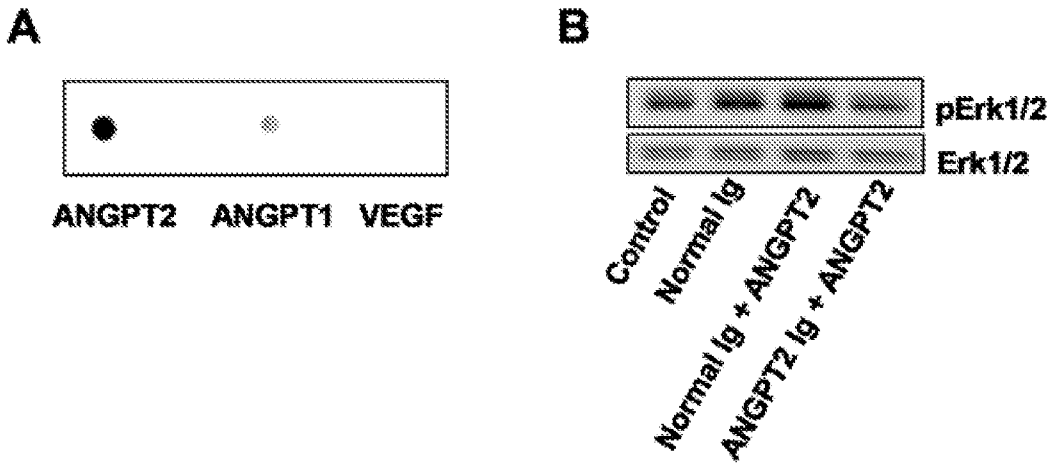

A
B
C
ANGPT2 Ig fold change
PD
SD
CR/PR
0
2
4
6
8
10
12
14
16
0.0
0.5
1.0
1.5
2.0
2.5
3.0

FIG. 50


A
ANGPT2
ANGPT1
VEGF
B
pErk1/2
Erk1/2
Control
Normal Ig
Normal Ig + ANGPT2
ANGPT2 Ig + ANGPT2